Microbiological Spoilage of High-Sugar Products

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Center of Food Safety, University of Georgia, Griffin, GA, USA

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William H. Sperber · Michael P. Doyle
Editors

Compendium of the Microbiological Spoilage of Foods and Beverages

Foreword by R. Bruce Tompkin

Springer

Editors
William H. Sperber
Cargill, Inc.
Corp. Food Safety & Reg. Affairs
5814 Oakview Circle
Minnetonka MN 55345
USA
bill_sperber@cargill.com

Michael P. Doyle
University of Georgia
Center of Food Safety
1109 Experiment Street
Griffin GA 30223
Melton Building
USA
mdoyle@cfs.griffin.peachnet.edu

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Foreword

The increased emphasis on food safety during the past two decades has decreased the emphasis on the loss of food through spoilage, particularly in developed countries where food is more abundant. In these countries spoilage is a commercial issue that affects the profit or loss of producers and manufacturers. In lesser developed countries spoilage continues to be a major concern. The amount of food lost to spoilage is not known. As will be evident in this text, stability and the type of spoilage are influenced by the inherent properties of the food and many other factors.

During the Second World War a major effort was given to developing the technologies needed to ship foods to different regions of the world without spoilage. The food was essential to the military and to populations in countries that could not provide for themselves. Since then, progress has been made in improved product formulations, processing, packaging, and distribution systems. New products have continued to evolve, but for many new perishable foods product stability continues to be a limiting factor. Many new products have failed to reach the marketplace because of spoilage issues.

Disruptions in the food supply are more severely felt by countries that depend on readily available low-cost food. For example, the increased diversion of corn to produce fuel, in combination with other factors, led to higher food prices after 2007 and reduced the ability of international agencies with limited budgets, e.g., the Food and Agriculture Organization, to provide food assistance. In addition, certain countries limited exports to ensure a stable food supply for their populations. This experience demonstrates the dependence of many countries on assistance to bolster their food supply and the significance of barriers to international trade.

The world's population continues to increase. In 1960, 1980, and 2000 the population was estimated to be 3.0, 4.5, and 6.1 billion, respectively. It is projected to reach 6.9 and 9.5 billion by 2010 an 2050, respectively.[1] To provide for the population increase, improvements in food production and protection against spoilage will be required.

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[1] U. S. Census Bureau. (2008). International database. Total midyear population for the world: 1950–2050. Accessed on 24 September 2008. http://www.census.gov/ipc/www/idb/worldpop.html

Food production, processing, and distribution systems generally fall into two categories: large or small scale. Large-scale systems incorporate new technologies more quickly and can lead to innovations that bring products of greater variety and convenience to consumers. This segment of the industry is generally more highly regulated and its suppliers are frequently audited by large corporations. Proper coding and inventory control is essential to minimize product loss due to spoilage. Sell-by or use-by dates are commonly applied to indicate the date the food will be acceptable and to facilitate traceability. Larger companies strive to improve control of their incoming raw materials and processing and packaging conditions to ensure compliance with their code-dating procedures and in some cases further delay spoilage. Products that exceed the sell-by or use-by dates are discarded by retailers. The amount discarded is documented by the retailer and can influence future negotiations between supplier and retailer. Continued spoilage problems can lead retailers and others to discontinue the item.

Manufacturers may apply special procedures that enable them to meet the expected demand for their perishable products at certain holidays. For example, this could involve accumulating and holding certain perishable foods at temperatures closer to freezing. As the holiday approaches, the food is released for shipment to retailers. Success requires knowledge of the product, the impact of lowering storage temperature on microbial growth, and validation that the procedure will be successful. Failure to validate the procedures can lead to significant financial losses during a critical season and temporary loss of consumer confidence.

Another characteristic of large-scale systems is that processing occurs in fewer facilities and the products are shipped longer distances. While this may be economically beneficial for the manufacturer, greater control must be exercised to transfer food from the manufacturer to the ultimate user without spoilage.

Considerable advances have been made in delaying or preventing spoilage. For example, this writer spent about 40% of his time solving spoilage issues associated with raw and cooked perishable meat and poultry products from the mid-1960s to the early 1990s. The collective effect of the improvements, for example, in processing conditions, formulation, packaging, control of temperature, and efforts to control *Listeria* and *Salmonella* reduced this time to well below 5%.

It is of interest that as the quantity of foods produced on a larger scale has increased, there is a desire by some consumers to return to foods produced on a smaller, more local scale. This desire is based on the perception that the foods are fresher, less processed, and more wholesome. It has not been documented, however, whether this approach results in a greater or lesser amount of food lost through spoilage on a worldwide basis.

Smaller scale systems are slower to accept and may even reject new technologies. The smaller businesses generally lack the technical knowledge and support available in larger companies. Thus, it is not surprising that many of the authors are involved with large companies and have collaborated with other experts in preparing this text.

LaGrange, IL, USA R. Bruce Tompkin

Preface

Protection of foods and beverages from microbiological spoilage is essential to assure an adequate food supply for the world's population. Several generations of food microbiologists have labored to understand food spoilage and to develop control procedures for its prevention. Because many of these highly experienced food microbiologists are at or near retirement age, we were motivated to organize this *Compendium* in an effort to document and preserve as much of their accumulated knowledge and wisdom as possible. We are pleased that many expert food microbiologists eagerly agreed to contribute to this effort. To our knowledge, this is the first reference and textbook focused exclusively on the microbiological spoilage of foods and beverages.

We also think that this *Compendium* is necessary now because the resources of the food industry and academia have increasingly become focused on food safety initiatives over the past 30 years. To a significant extent, resources previously available to develop an understanding and the means to control food spoilage have been shifted into food safety programs. The emergence of prominent foodborne pathogens, such as *Escherichia coli* O157:H7, *Listeria monocytogenes,* and *Campylobacter,* combined with increased competition for limited financial resources, has resulted in decreased attention being given to food spoilage research. Global public health issues such as bovine spongiform encephalopathy and avian influenza H5N1, and their potential impacts on the food supply, have further reinforced the shift toward "mission-oriented" research. The increased number of potential microbiological food safety issues affecting the food supply also fueled a substantial increase in the number of food safety regulations and policies, both at the national and the international levels. Moreover, food regulatory actions are almost always related to food safety controls and requirements, thereby commanding a larger share of the food industry's technical resources to assure regulatory compliance.

The shift in emphasis from food quality research toward various types of food safety programs is understandable and necessary. This shift, however, is not as counterproductive for food quality and spoilage research as might first be suspected. The implementation of numerous new food safety control procedures and regulations can also help to reduce food spoilage and protect product quality through its shelf life as they also provide greater assurance of food safety. For example, pasteurization treatments intended to eliminate pathogens in raw milk also significantly

enhance the quality and shelf life of fluid milk. In fact, the unanticipated enhancement of product quality was a very strong selling point in gaining the food industry's acceptance of the hazard analysis and critical control point (HACCP) system of food safety management in the 1970s. Because of the successful development of HACCP, there remains today a very strong link between food quality and food safety control measures.

We are further motivated to develop this Compendium because, ultimately, the control of food spoilage means more than simply providing high quality, convenient, processed foods for consumption in economically developed regions of the world. We must think about feeding people in every region of the world. Food spoilage is a significant threat to food security, our ability to provide an adequate food supply to a large and increasing global human population. Shrinking fossil fuel and water reserves, soil erosion, loss of soil fertility, climate change, and political uncertainty are important factors that collectively threaten food security. If food spoilage and other factors that contribute to the waste of food could be substantially reduced, we would be able to feed more people without increasing primary food production. In the opinion of a former World Health Organization official, "This large increasing world population needs food and we have a moral obligation to utilize all our skills and technologies to increase not only food production but also *to limit food spoilage* (italics added for emphasis)."[1] Together with many of our colleagues, we share Dr. Käferstein's sense of professional responsibility. We anticipate that this *Compendium* will play a role in the global reduction of food spoilage and the accompanying enhancement of food security.

In 1958 professor William C. Frazier first published his widely used textbook, *Food Microbiology*. His comprehensive yet concise explanations of food spoilage and food safety were prominent features in the education of several generations of food microbiologists, including this *Compendium*'s editors. It is our sincerest hope that this *Compendium* will provide similar benefits to future generations of food microbiologists.

Minnetonka, MN, USA — William H. Sperber
Griffin, GA, USA — Michael P. Doyle

[1] Käferstein, F. K. (1990). Food irradiation and its role in improving food safety and the security of food. *Food Control 1,* 211–214.

Contents

Contributors

Margaret Barth Responsible Source, 350 Berkshire Drive, Lake Forest, IL 60045, USA, Margaret.barth@sbcglobal.net

Karen Battista Kraft Foods, 200 DeForest Avenue, East Hanover, NJ 07936, Karen.battista@kraft.com

Frederick Breidt USDA Agricultural Research Service, 322 Shaub Hall, Box 7624, North Carolina State University, Raleigh, NC 27603, USA, fred.breidt@ars.usda.gov

John Cerveny 17 Ridgeview Court, Apt. 7, Madison, WI 53704, USA, jcerveny@itis.com

Frederick K. Cook Malt-O-Meal Company, 701 West 5th St, Northfield MN 55057, fred_cook@malt-o-meal.com

George M. Evancho 19693 Marimar Court, Lewes, DE 19958-3500, USA, george_evancho@verizon.net

Timothy A. Freier Cargill, Inc., 15407 McGinty Road W., Wayzata, MN 55391, USA, tim_freier@cargill.com

Lone Gram National Institute of Aquatic Resources, Technical University of Denmark, Soltofts Plads Bldg 221, DK-2800 Kgs Lyngby, gram@aqua.dtu.dk

Paul A. Hall AIV Microbiology and Food Safety Consultants, LLC, 17 Tournament Drive South, Hawthorn Woods, IL 60047, USA, paul.hall@sbcglobal.net

Thomas R. Hankinson Produce Safety Solutions, Inc., 1120 Newark Road, Toughkenamon, PA 19374, USA, trhank@aol.com

Billie L. Johnson Menu Foods Midwest, 1400 East Logan Ave., Emporia, KS 66801, USA, bjohnson@menufoods.com

Kathleen A. Lawlor PepsiCo, Inc., 100 Stevens Avenue, Valhalla, NY 10595, USA, kathy.lawlor@pepsi.com

Loralyn H. Ledenbach Kraft Foods, Inc., 801 Waukegan Road, Glenview, IL 60025, USA, lharris@kraft.com

Robert T. Marshall University of Missouri, 122 Eckles Hall, Columbia, MO 65211, USA, marshallr@missouri.edu

Joseph D. Meyer Kellogg's, 919 Aldora Lane, Waunakee, WI 53597-3001, USA, joseph.meyer@kellogg.com

Theodora Morille-Hinds Kraft Foods, 555 South Broadway, Tarrytown, NY 10591, USA, tmorille-hinds@kraft.com

Joan M. Pinkas McCormick & Co. Inc., 204 Wight Avenue, Hunt Valley, MD 21031, USA, joan_pinkas@mccormick.com

James D. Schuman PepsiCo, Inc., 617 Main Street, Barrington, IL 60010, USA, jay_schuman@quakeroats.com

Virginia N. Scott U.S. Food and Drug Administration, 5100 Paint Branch Parkway, College Park, MD. 20740, jennyscott@verizon.net

Joseph R. Shebuski Cargill, Inc., 15407 McGinty Road W., Wayzata, MN 55391, USA, joe_shebuski@cargill.com

Peter G. Simpson The Coca-Cola Company, P. O. Box 1734, Atlanta, GA 30301, USA, pesimpson@na.ko.com

William H. Sperber Cargill, Inc., Corp. Food Safety & Reg. Affairs, 5814 Oakview Circle, Minnetonka MN 55345, USA, bill_sperber@cargill.com

Peter J. Taormina John Morrell & Co., 805 E. Kemper Road, Cincinnati, OH 45246, USA, ptaormina@johnmorrell.com

Sterling Thompson The Hershey Company, 1025 Reese Avenue, Hershey, PA 17033, USA, sthompson@hersheys.com

Suzanne Tortorelli Campbell Soup Company, 1 Campbell Place, Camden, NJ 08103, USA, suzanne_tortorelli@campbellsoup.com

Hong Zhuang Agricultural Research Service—USDA, Russell Research Center, 950 College Station Road, Athens, GA 30605, hong.zhuang@ars.usda.gov

Introduction to the Microbiological Spoilage of Foods and Beverages

William H. Sperber

Introduction

Though direct evidence of ancient food-handling practices is difficult to obtain and examine, it seems safe to assume that over the span of several million years, prehistoric humans struggled to maintain an adequate food supply. Their daily food needed to be hunted or harvested and consumed before it spoiled and became unfit to eat. Freshly killed animals, for example, could not have been kept for very long periods of time. Moreover, many early humans were nomadic, continually searching for food. We can imagine that, with an unreliable food supply, their lives must have often been literally "feast or famine." Yet, our ancestors gradually learned by accident, or by trial and error, simple techniques that could extend the storage time of their food (Block, 1991). Their brain capacity was similar to that of modern humans; therefore, some of them were likely early scientists and technologists. They would have learned that primitive cereal grains, nuts and berries, etc. could be stored in covered vessels to keep them dry and safer from mold spoilage. Animal products could be kept in cool places or dried and smoked over a fire, as the controlled use of fire by humans is thought to have begun about 400,000 years ago. Quite likely, naturally desiccated or fermented foods were also noticed and produced routinely to provide a more stable supply of edible food. Along with the development of agricultural practices for crop and animal production, the "simple" food-handling practices developed during the relatively countless millennia of prehistory paved the way for human civilizations.

Less than 10,000 years of recorded history describes the civilizations that provided the numerous advances leading to our modern civilization. Chief among these advances were the development of agricultural and food preservation technologies that permitted large human populations to live permanently in one place and use their surplus time to develop the other technologies we enjoy today, such as writing

W.H. Sperber (✉)
Cargill, Inc., Food Safety & Reg. Affairs, 5814 Oakview Circle, Minnetonka, MN 55345, USA
e-mail: bill_sperber@cargill.com

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this chapter on a laptop computer while sitting in a heated office on a Minnesota winter evening.

Yet, for most of this 10,000-year period, food preservation was accomplished by quite simple, but not completely effective, technologies. These typically involved the use of the techniques that had been put into practice countless years earlier – drying, salting, smoking, fermentation, and cool storage when possible. Only in the past 200 years of our long existence have we humans developed more advanced technologies for advanced food production, preservation, and distribution. Preservation of some foods by canning began in the early nineteenth century. In the middle of that century, Louis Pasteur and the first microbiologists began to understand and control the microbiological causes of disease, foodborne illness, and food spoilage.

Another century elapsed before the emergence of major advances leading to the widespread availability of fresh and processed foods. The most significant advances, after 1945, were the development of reliable mechanical refrigeration systems, logistical systems for the refrigerated transportation and distribution of food, and widely available home refrigerators and freezers. Numerous refinements continue to improve the microbiological quality of our food supply today. Additional refinements will certainly be made in the future.

In the past several decades, we have also made substantial improvements in food production and management systems. National governments and the food industry promulgated and implemented Good Manufacturing Practices (GMPs) in the United States, which are called Good Hygienic Practices (GHPs) in the rest of the world. In particular, those GMPs related to employee practices, sanitary design of food production facilities and equipment, and cleaning and sanitation procedures have improved food quality. Similarly, the HACCP (hazard analysis and critical control point) system, while developed to assure food safety, has also improved food quality. The HACCP system entails three broad and essential functions – product design, process control, and management accountability (Troller, 1993; Mortimore & Wallace, 1998). These topics will be handled in greater detail later in this chapter and in several of the following chapters.

Additional food regulations and industry practices have been implemented to reduce the public health threat posed by particular foodborne pathogens. While this compendium is focused solely on the microbiological spoilage of foods, regulations and practices that are used to improve public health protection against foodborne pathogens will also improve the microbiological quality of food, thereby reducing the incidence of microbiological spoilage and extending the shelf life of foods.

Food Loss Data

Despite the advanced technologies that support our modern civilization, a large proportion of our food supply is nevertheless lost to spoilage or otherwise wasted. The Economic Research Service (ERS) of the United States Department of Agriculture (USDA) has extensively documented the percentage of food losses in the food chain from primary production through consumption (ERS, 2005). This research was done

Table 1 Percent loss of the United States' food supply from primary production through consumption (abstracted from ERS/USDA, Feb. 1, 2005)

	Data for 2003 based on pounds per capita/year				
Commodity	Primary weight	Retail weight	Consumer weight	Consumed weight	Percent total loss
Meat, poultry, and fish					
Red meat	161	112	104	68	58
Poultry	113	71	66	41	64
Fish, shellfish	16	16	11	11	31
Grain and cereal products	194	194	171	136	30
Sweeteners	142	142	126	101	29
Eggs and egg products	253	250	232	197	22
Dairy products					
Fluid milk, yogurt	194	194	171	137	30
Cheese	28.3	28.3	26	22.1	22
Frozen	26.7	26.7	23.5	18.8	30
Dried	3.8	3.8	3.3	2.6	30
Fats and oils	102	102	82	68	33
Fruits					
Fresh	127	121	106	53	58
Dried	10	2.4	2.2	2	80
Canned	17	13.4	12.6	11.3	33
Frozen	3.9	3.5	3.3	3	24
Vegetables					
Fresh	196	181	160	86	56
Frozen	79	39	37	26	67
Canned	101	47	44	40	60
Dried	16.9	2.3	2.2	2	88
Potato chips	17.2	4.3	4.1	3.7	79
Peanuts and tree nuts	9.3	9.3	8.8	7.9	15
Pounds/year	1811	1597	1396	1037	
Pounds/day	4.96	4.38	3.82	2.84	

to support the development of the Food Guide Pyramid (MyPyramid) serving sizes. The percent losses for all food categories during 2003 in the United States are summarized in Table 1. All data are presented as pounds per capita/year. The Primary Weight column refers to the product weight as it leaves the processing plant, for example, boned meat products, trimmed vegetables, etc. The retail weight is the amount of food purchased at retail, the consumer weight is the amount of food available for consumption at home or at food service establishments, and the consumed

weight is the amount of food actually eaten. Food losses can occur from insect or rodent damage, microbiological spoilage, chemical and physical spoilage, losses in transportation, further processing, product discarded at the end of shelf life, and plate waste. According to these data, about five pounds of food are processed each day for each person in the United States. Only about three pounds are consumed, indicating an average food loss for all categories of about 40%. ERS economists feel that the reported data tend to underestimate the actual amount of food losses.

It is not possible to tell from the current data what proportion of the food losses could be attributed to microbiological spoilage. According to ERS economists, this capability may be developed in the near future. Under any circumstances, it would be difficult to know the proportion of microbiological food spoilage with a high degree of precision. The World Health Organization estimated that in developing countries the loss caused by spoilage microorganisms ranges from >10% for cereal grains and legumes to as much as 50% for vegetables and fruits (Käferstein, 1990). The other food commodities fall within this range. Todd (1987) points out that worldwide postharvest food losses are caused more by insects and rodents than by microorganisms. Of course, microorganisms are still important in food losses, with fungi representing the most important group of spoilage microorganisms responsible for food losses.

Microorganisms and Mechanisms Involved in Spoilage

Sources of Contamination

Preharvest Contamination

The sources of microbiological contamination are practically everywhere in the earth's biosphere, in or on plants, animals, soil, and water. Many types of bacteria, such as pseudomonads, lactics, micrococci, and coliforms, grow readily on agricultural and horticultural plants. Many of these and other types of bacteria, particularly the enterics, also colonize animals, both on the skin or hide and in the gastrointestinal tract. The resident bacteria on both plants and animals can be carried along with the raw materials during harvest, slaughter, and processing and remain in the food products derived from these sources (Frazier, 1958).

Soil is an obvious source of contamination, as a diverse community of microorganisms – bacteria, yeasts, molds, actinomycetes, etc. – thrive in most soils and can grow to very large numbers. Direct contamination with soil microorganisms occurs during production and harvesting. Indirect contamination with soil occurs through the deposition of wind-borne dust particles. Wind-borne mold spores, for example, are a very common cause of mold spoilage of foods, as well as human allergies.

Water can serve as a source and a vector of contamination. Pseudomonads, in particular, grow well in surface waters, whereas the enteric bacteria are present in sewage and waters polluted with sewage. Water can serve as a vector of contamination, especially if polluted surface waters are sprayed onto crops for irrigation or used in primary produce processing.

Postharvest Contamination

Many raw materials and foods have a structural integrity that protects most of their mass from microbial contamination (Frazier, 1958). The endosperm of cereal grains is protected from contamination by a tough bran layer. The shells of eggs and nuts protect the interior of these foods. When intact, the skin or the rind that covers fruits and vegetables keeps the interior of the produce largely free from external contamination. Similarly, most animal flesh is sterile in its natural state, being protected by skin or hide. Therefore, most of the microorganisms in the raw materials of our food supply are present only on the exterior of the food or in the gastrointestinal tract in the case of animals. When you think about it, even the gastrointestinal tract is essentially outside of the animal as well. Therefore, living muscle tissues and other interior structures are usually sterile.

The first steps of primary processing violate the natural sterility of the interior parts of our raw food materials. The milling of cereal grains removes most of the exterior microorganisms with the bran, but some of these microbes will be relocated into the otherwise nearly microbe-free endosperm. Trimming, chopping, or crushing of fruits and vegetables will similarly contaminate the interior portions with those microorganisms existing on the exterior. The most prolific possibility of interior contamination exists in animal slaughter operations. The feces of animals contain exceedingly high numbers of microorganisms, $>10^{11}$ cells/g feces. If the gastrointestinal tract is not carefully removed during slaughter, very high contamination of the muscle tissue could occur. In the case of meat production, the first slaughter operations contaminate the surface of the exposed muscles to some extent. Further fabrication (cutting) of the carcass into prime cuts can spread the initial contamination across larger product areas. The grinding of meat will spread exterior contamination essentially throughout the entire muscle mass.

During further processing, additional contamination can occur when workers handle the food. Contamination can occur from unclean hands or gloves and uniforms. Human contamination of foods can also occur when talking, coughing, or sneezing creates aerosols. In-process foods can be further contaminated by cross-contamination with raw materials and by contact with unclean food-handling utensils and processing equipment. There are also several points of waterborne contamination in food-processing plants. The most direct means of potential contamination is the use of water as a food ingredient. If the food plant's water supply is not potable, significant contamination with spoilage microorganisms could occur. A major indirect source of waterborne contamination may exist during cleaning and sanitation operations, since the use of water is essential for most of these operations. The use of high-pressure hoses to clean floors creates aerosols containing bacteria that were present, and likely growing, on the floor or the process equipment. The bacteria-containing aerosols can drift through the air and directly contaminate raw materials and in-process foods if these are not removed or adequately protected before cleaning commences, or they can indirectly contaminate food after they are deposited on the food-processing equipment. Another inadvertent source of water contamination may be presented by condensate that is formed

in refrigeration units and can be spread by the ventilation systems in the food-processing plants.

Ecology of Microbiological Spoilage

The many kinds of microorganisms that can grow on food have evolved biochemical mechanisms to digest components of the food, thereby providing energy sources for their own growth. However, in a given type of food, usually only one or a few types of microorganisms will grow sufficiently well to become the predominant spoilage organisms (Mossel & Ingram, 1955). Parameters, such as pH, water activity, and storage temperature to name a few, exert intensive selective pressures on the original food microflora. The driving forces that guide the selection of predominant spoilage microorganisms will be detailed later in this chapter in sections "Intrinsic Factors to Control Microbiological Spoilage" and "Extrinsic Factors to Control Microbiological Spoilage."

Microorganisms Involved in Spoilage

It is useful to consider the types of microorganisms involved in food spoilage in two ways. The first way is a consideration of laboratory tests and biochemical features that are used to broadly characterize and differentiate microorganisms. The second way is to describe the groups of similar microorganisms that are involved in food spoilage.

Means to Characterize and Differentiate Microorganisms

Morphology. A microscope was the first tool with which early microbiologists could begin to understand microorganisms. The microscope enabled the observation of the size and shape, or morphology, of microbial cells. Bacterial cells usually appear as cylindrical rods or spheres. The bacterial rods, often called bacilli, are typically about 1 micrometer (μm) in diameter and 2–6 μm long. The spherical cells, usually called cocci, are typically about 1 μm in diameter. Yeast cells are larger than bacterial cells. They are elliptical and usually about 3–5 μm long. Molds have two predominant morphological features: individual hyphae, which can collectively form a visible mycelial mat, and sporangia, which contain very high numbers of individual spores, each of which is capable of starting a new mold colony. The hyphae are about 15 μm in diameter and are of indefinite length. The individual spores are about 1 μm in diameter.

Gram stain. The Gram stain is a differential staining technique that permits the microscopic determination of a bacterium as either "Gram positive" or "Gram negative." This procedure consists of four steps – initial staining with crystal violet, fixation with iodine, decolorization with ethanol, and counterstaining with safranin. A fundamental difference in the composition of cell walls of bacteria is responsible for the differential results of the Gram stain. Gram-positive cells are not decolorized

by ethanol and retain the original blue color of crystal violet. Gram-negative cells are decolorized by ethanol and take up the red color of safranin.

Ability to form endospores. Several genera of bacteria are able to form an internal structure, or endospore, that is very heat resistant and capable of surviving in quite adverse environments. Yeast and molds form spores, different from bacterial endospores, that are not significantly more heat resistant than their vegetative cells. However, the fungal spores help the fungi survive in dry environments.

Temperature relationships. The temperature range in which they can grow often characterizes microorganisms. Psychrophiles grow well at cold temperatures, as low as 0°C, and often cannot grow above 20°C. Thermophiles grow best at high temperatures, in the range from 45°C to 70°C. Mesophiles grow best at the intermediate temperatures between 20°C and 45°C. There have been some efforts to further divide the psychrophilic microorganisms by creating a category called "psychrotrophs," but such a consideration is beyond the practical needs of this compendium.

Oxygen relationships. Aerobic microorganisms can grow in the presence of oxygen, while anaerobic microorganisms can grow in the absence of oxygen. The use of the term "obligate," as in "obligate aerobe," means that the microbe requires some level of oxygen for growth. The term "obligate anaerobe" refers to a microbe that cannot grow if any amount of oxygen is present. The term "microaerophilic" refers to microorganisms that can grow best when only a small amount of oxygen is available. Facultative with respect to oxygen, a term that applies to most microorganisms, refers to the ability to grow either with or without the availability of oxygen. Additionally, each food has a chemical oxidation–reduction (O/R) potential that is somewhat analogous to the situation described here for atmospheric oxygen content. Aerobic microorganisms grow best at positive O/R values, while obligate anaerobes require negative O/R values for growth.

Type of metabolism. Metabolically speaking, microorganisms can usually be characterized as having either an oxidative or a fermentative type of respiration for the production of energy. This trait is linked both to the oxygen relationships described above and to the evolutionary stature of the particular microorganism. Fermentative metabolism is a relatively primitive anaerobic process in which carbohydrates are metabolized to organic acids and alcohols. Oxidative metabolism is an advanced aerobic process in which carbohydrates may be completely metabolized to carbon dioxide and water. Microorganisms with this capability are usually oxidase positive, possessing the same intracellular electron transport system that is present in higher life forms, including humans. Microbes lacking this capability are often also catalase negative, lacking this enzyme to degrade peroxides that can be formed in anaerobic metabolism.

Water relations. The addition of solutes to a growth medium diminishes a microorganism's ability to grow as the osmotic pressure of the medium is increased, and the water activity of the medium is reduced. Several terms describe microbes that can accommodate reduced water activities. "Osmophile" generally refers to any organism that grows at increased osmotic pressure, and specifically to yeasts that can grow at very high sugar concentrations. "Halophile" refers to organisms, usually

bacteria, which grow at high salt concentrations, even in saturated sodium chloride solutions. "Xerotrophic" refers to organisms that grow under dry conditions. This term is often applied to molds that grow in relatively dry cereal products. The term "osmotolerant" can be used to describe those microorganisms that are capable of growth in reduced water activity, or "intermediate moisture" foods, generally in the range of water activity from 0.85 to 0.95.

pH relations. Most foodborne microorganisms grow best at relatively neutral pH values, in the range of pH 6.0–8.0. None of these grow at extremely high pH values, but some can grow at pH values as low as 0.5–2.0. Those that can grow at such low pH values are called "acidophiles." Those that cannot grow, but can tolerate low pH values without being killed, are called "acidurics."

Groups of Microorganisms Involved in Spoilage

The nearly countless microbial genera that can be involved in food spoilage are organized here in 11 groups. The first two groups are fungi, and the remaining groups are bacteria. Specific information about the fungi can be found in Pitt and Hocking (1997) and Deak and Beuchat (1996). Information about Gram-negative bacteria can be found in Krieg and Holt (1984) and about Gram-positive bacteria in Sneath, Mair, and Sharpe, (1986).

Molds. Capable of growth across a broad range of temperatures, molds are obligate aerobes with oxidative metabolism. Particular genera are also capable of growth across the range of water activity from 0.62 to nearly 1.0. Molds are the most common food spoilage microorganisms at every step of the food chain from field crops to consumer food products. Remarkably, they are even capable of spoiling bottled mineral water (Criado, Pinto, Badessari, & Cabral, 2005). Representative genera of food spoilage molds are *Penicillium, Aspergillus*, *Rhizopus, Mucor, Geotrichum, Fusarium, Alternaria, Cladosporium, Eurotium*, and *Byssochlamys.*

Yeasts can be described in two broad categories: fermentative and oxidative. Yeasts are generally mesophilic and grow best above water activity values of 0.9. Both molds and yeasts grow at slower rates than bacteria. Spoilage of perishable foods by these microorganisms often indicates that the food has simply been "stored too long."

Fermentative yeasts. The most commonly known spoilage yeasts are facultatively anaerobic fermentative organisms, producing ethanol and carbon dioxide from simple sugars. Some fermentative yeasts are the most osmophilic organisms known, capable of slow growth at water activity 0.60 (Martorell, Fernández-Espinar, & Quereol, 2005). Representative genera include *Saccharomyces* and *Zygosaccharomyces.*

Oxidative yeasts. Less common are the aerobic "film yeasts" which can grow on fermented foods and metabolize organic acids and alcohols. These yeasts seem to occupy an evolutionary middle ground between fermentative yeasts and molds, possessing the morphological characteristics of yeasts and the metabolic characteristics of molds. Representative genera include *Mycoderma, Candida, Pichia,* and *Debaryomyces.*

Pseudomonadaceae. The principal genera in this family of bacteria, *Pseudomonas* and *Xanthomonas,* are Gram-negative rods, nonspore forming, psychrophilic, aerobic, and oxidase positive. They are also completely intolerant of reduced water activity, growing in foods mostly above water activity 0.98. The addition of small amounts of solutes, such as 2% sodium chloride, will substantially restrict their growth. Pseudomonads are primary spoilage microorganisms in fresh meat, poultry, seafood, and eggs.

Neisseriaceae. Like the pseudomonads, the microbes in this family are Gram-negative rods, nonspore forming, aerobic, and catalase positive. The spoilage genera are *Acinetobacter* (oxidase negative) and *Moraxella*(oxidase positive). Some strains of *Acinetobacter* are psychrophilic.

Enterobacteriaceae. This family of Gram-negative rods is facultatively anaerobic, fermentative, mesophilic, nonspore forming, oxidase negative, and catalase positive and is generally incapable of growth below water activity 0.95. All of the 28 genera in this family are commonly called "enteric" bacteria and ferment glucose with the production of acid and gas. A subset of this family, containing about half of the genera, is commonly called "coliform" bacteria, as established by their ability to ferment lactose with the production of acid and gas. Representative spoilage genera include *Escherichia, Erwinia, Enterobacter, Citrobacter, Serratia*, and *Proteus.* Enteric bacteria are often involved in the spoilage of fresh vegetables, meat, poultry, fish, and eggs.

Micrococcaceae. The two principal genera of bacteria in this family are *Micrococcus* and *Staphylococcus*. They are Gram positive, spherical, catalase positive, and mesophilic. *Micrococcus* is oxidative, growing on glucose without the production of acid or gas, while *Staphylococcus* is fermentative, producing both acid and gas from glucose. *Staphylococcus* is osmotolerant. Both the genera are commonly involved in the spoilage of fresh produce and processed meat, poultry, and seafood.

Lactic Acid Bacteria. All members of this group are Gram positive, catalase negative, microaerophilic or facultatively anaerobic, and fermentative. Homofermentative lactics ferment glucose with the production of lactic acid only. Heterofermentative lactics ferment glucose with the production of lactic acid, carbon dioxide, and ethanol or acetic acid. *Lactobacillus* is rod shaped, while *Streptococcus, Lactococcus, Leuconostoc, Enterococcus*, and *Pediococcus* are spherical. The "lactics" are generally mesophilic and grow at water activity values above 0.9. The growth of lactics in meat, vegetable, and dairy products is used to advantage to produce fermented foods such as salami, sauerkraut, and cheese. However, the growth of these bacteria in the same fresh foods, such as luncheon meats, vegetable salads, and fluid milk, constitutes spoilage.

Coryneforms. These microorganisms, of relatively minor importance in food spoilage, are sometimes involved in cheese spoilage. Representative genera are *Corynebacterium* (facultatively anaerobic) and *Brevibacterium* (aerobic). Both are Gram positive and catalase positive. Their sources of contamination are usually soil, animals, or humans.

Spore-forming Bacilli. There are three major genera of bacterial sporeformers important in food spoilage – *Bacillus, Clostridium*, and *Alicyclobacillus*. All

are Gram-positive rods and are generally mesophilic or thermophilic. Because these genera produce heat-resistant endospores, they are the predominant spoilage microorganisms in pasteurized foods in which all vegetative cells have been killed and in improperly sterilized foods.

Bacillus species are aerobic or facultatively anaerobic, catalase positive, and generally not osmotolerant. While most species are mesophilic, individual species cover the entire temperature spectrum for food spoilage. *Bacillus cereus* can spoil pasteurized milk (psychrotrophic), *B. subtilis* can spoil bakery products (mesophilic), and *B. stearothermophilus* can spoil canned foods (thermophilic).

Clostridium species are obligate anaerobes, catalase negative, and not osmotolerant. They are typically involved in the spoilage of foods that have a highly negative O/R potential, such as canned or vacuum-packaged foods. The principal spoilage species are *C. sporogenes* and *C. butyricum* (mesophilic) and *C. thermosaccharolyticum* (thermophilic).

Alicyclobacillus species were discovered in the 1960s and originally classified as *Bacillus* spp. First isolated from acid hot springs in Yellowstone Park, these bacteria typify a significant new ecological grouping of microorganisms called "extremophiles."

Quite unlike all other foodborne bacteria, alicyclobacilli are extreme acidophiles, growing within a pH range of about 2.0–6.0. They are moderate-to-obligate thermophiles, catalase positive, and microaerophilic. Like pseudomonads, the alicyclobacilli cannot tolerate osmotically increased environments, that is, below water activity of 0.98. They have evolved to grow in acid and hot water, and it is these types of foods that they can spoil. The principal spoilage species *A. acidoterrestris* is sometimes involved in the spoilage of pasteurized fruit or vegetable juices that have been improperly cooled or stored at relatively high temperatures, above 30°C.

Microbiological Food Spoilage Mechanisms

The microbiological spoilage of foods occurs because of the biochemical activity of microorganisms as they grow in the food. The consumer is usually alerted to the existence of spoilage by changes in the food's appearance, odor, texture, or taste. While food spoilage may be universally considered to be undesirable, it affords perhaps one protective advantage for consumers. Food spoilage is usually an indicator that a food has been improperly handled or stored too long. Such mishandling could permit the growth of foodborne pathogens that could cause illness or death if the food were to be consumed. Since foodborne pathogens do not typically give an organoleptic indication of their presence, the organoleptic changes caused by spoilage microorganism serve as a warning to the consumer that the food could be unsafe for consumption. It can be argued that spoilage microorganisms routinely protect millions of people from foodborne illness (Frazier, 1964. personal communication).

The protective feature of food spoilage does not always protect the consumer from the threat of foodborne illness, of course. A main reason for this fact is that microbiological spoilage of foods is not organoleptically detectable until a substantial growth of the spoilage organism has occurred. Typically, the threshold level for observation of food spoilage by odor, taste, or sight is not reached until the spoilage microflora exceeds about 10^7 organisms/g of food. A secondary reason for the failure of the spoilage warning signal to protect consumers is the fact that many people, because of ignorance, frugality, or sheer necessity, will consume even obviously spoiled food.

Spoilage characteristics develop in food as microorganisms digest the food to support their growth. The digestion of sugars, complex carbohydrates, proteins, and fats can all produce undesirable effects in the food if the spoilage microorganisms grow to significant levels.

Sugar fermentation with acid production. A number of catabolic pathways are used by bacteria to metabolize pentoses and hexoses for energy production. Lactic acid is the principal product of these pathways. Its production, often by lactic acid bacteria, yields a sour taste in the food. To a limited extent, some enteric bacteria could also cause this type of spoilage. As a matter of practical interest, the production of lactic acid during spoilage usually lowers the pH of the food, thereby providing further protection against the growth of the foodborne pathogens mentioned above, should any be present. Sugar fermentation by bacteria can occur with (heterolactic) or without (homolactic) the production of a gas, typically carbon dioxide.

Sugar fermentation with gas production. The catabolism of hexoses by fermentative yeasts produces ethanol and carbon dioxide. Relatively low pH and high sugar-containing products would support this type of spoilage. The typical yeast spoilage defect in products such as sugar syrups and tomato products in hermetically sealed packages is caused by gas production. In these instances, flexible product containers will expand, while rigid containers may explode. This type of spoilage is one of the few that violates the common observation that high numbers of microorganisms are required to cause spoilage. It has been observed that over several months of storage, yeast spoilage by gas production can occur in products that never exceed a yeast population of about 10^4 cells/g (Sperber, unpublished data). Nongrowing yeast cells can remain metabolically active, producing ethanol and carbon dioxide.

Protein hydrolysis. Many spoilage bacteria produce proteolytic enzymes that hydrolyze proteins in foods such as milk, meat, poultry, and seafood products. Anaerobic proteolysis by *Clostridium* spp. can result in a noxious putrefaction of the food. Pseudomonads can carry the proteolysis one step further by metabolizing amino acids to produce very foul-smelling compounds, such as the aptly named putrescine and cadaverine.

Digestion of complex carbohydrates. Produce spoilage can be caused by bacteria and molds that produce pectinases. These enzymes digest the pectin layer between the plant cell walls, resulting in a soft or mushy texture. One such spoilage organism that is very appropriately named is *Erwinia carotovora* (carrot eating). When accompanied by proteolytic activity, mushy produce will also develop a foul odor.

Amylolytic enzymes produced by molds and several bacteria digest starches to polysaccharides and simple sugars. This activity will destroy the viscosity of products in which starches are used as thickening agents, such as gravies and pie fillings.

Lipolysis. A wide variety of microorganisms, including pseudomonads, molds, and staphylococci, produce lipolytic enzymes that hydrolyze lipids, producing readily oxidizable substrates that have a rancid odor. As pointed out in Chapter "Microbiological Spoilage of Dairy Products," this type of enzyme activity can be used to develop desirable cheese flavors.

Oxidation of organic acids and alcohols. Many molds and oxidative yeasts can grow on acidified foods and metabolize the organic acid. If substantial growth occurs, the pH of the food could be raised to levels high enough to permit the growth of other types of spoilage organisms.

Guaiacol production. Alicyclobacilli can grow in some fruit or vegetable juices, metabolizing vanillin and other precursor molecules to guaiacol, a product with an asphalt-like or phenolic odor.

Surface growth. Most groups of microorganisms can spoil food by growing on the surface. Refrigerated cured meats and cooked products can become slimy or sticky to the touch because of the growth of yeasts, lactic acid bacteria, and some enterics and pseudomonads. This particular spoilage defect is caused simply by the accumulation of very high numbers of microbial cells and not by any specific metabolic activity of the microbes. Similarly, color changes in food can occur because of the surface growth of microorganisms. Examples include the greening of meats, caused by lactic acid bacteria; fluorescence in milk, caused by pseudomonads; and red spots on breadstuffs, caused by *Serratia marcescens.*

Quorum Sensing

Some of the spoilage mechanisms described above do not involve the steady production and secretion of enzymes as the microbial population increases. The phenomenon called quorum sensing has been discovered to be responsible for many of the effects of large microbial populations (Smith, Fratamico, & Novak, 2004). Quorum sensing has been shown to be active in the production of toxins, invasive factors, dental plaque, biofilms, bioluminescence, bacteriocins, and even food spoilage (Cotter, Hill, & Ross, 2005; Rasch et al., 2005; Gram et al., 2002).

Whether the microorganism in question is an animal or a plant pathogen producing a variety of invasive factors or a food spoilage microbe secreting extracellular enzymes, the production and secretion of these compounds does not occur when the microorganism is present at low levels. Rather, the production of invasive and spoilage factors occurs only when the population reaches high numbers. (This author has not found an estimate of the "high number" necessary to activate quorum sensing, but it is intriguing to speculate that it could be approximately 10^7 cells/g or higher, which is the threshold level for the organoleptic detection of spoilage described above.) It is reasoned that low numbers of microbial cells do not produce

the invasive or digestive factors in order to avoid triggering host defense systems. When high numbers of cells are achieved, quorum sensing enables the coordinated release of such factors, with a better chance to overwhelm the host defenses.

Quorum sensing depends upon the synthesis of a biochemical signal molecule, followed by its accumulation in the growth environment and recognition by other cells of the same microbial species. *N*-Acylhomoserine lactones (AHLs) produced by Gram-negative bacteria are the most common quorum-sensing signal examined to date. Numerous different quorum-sensing signals, too complex to be described here, are produced by both Gram-negative and Gram-positive bacteria. Novel means to control microbiological problems ranging from food spoilage to biofilms to human illness would be the development of techniques to interfere with the molecular quorum sensing between bacteria (Gram et al., 2002).

Associations Between Groups of Spoilage Microorganisms

All of the above discussions are written in the typical style as if the individual microorganisms under consideration were growing alone in pure cultures. In reality, of course, the situation is quite different. Whether a microorganism is growing in a food or in a natural environment, it is in a steady ecological struggle to maintain its existence and dominate the ecosystem in which it is growing. Faster growing microorganisms have a distinct advantage over slower growing organisms. In general, bacteria grow faster than yeasts, which grow faster than molds (Frazier, 1958). Yeasts and molds, however, possess growth characteristics that permit them to dominate harsh environments in which bacteria grow very slowly or not at all. The types of ecological interactions between microorganisms have been grouped into three categories – antagonisms, synergisms, and metabiosis.

Antagonisms

Most of the associations between microorganisms are antagonistic, in which each microbe is trying to gain an advantage over a multitude of competitors. Many microorganisms produce organic acids and alcohols that are inhibitory to some of their competitors. Some produce antibiotics or bacteriocins, which possess highly specific antimicrobial activity, often against closely related species. Some microbes can gain a competitive advantage by using or hoarding an essential mineral or vitamin that is needed by its competitors. Some pseudomonads produce siderophores, an iron-chelating compound, thereby preventing the growth of competitors that require iron (Gram et al., 2002).

Synergisms

A synergistic association exists when two or more microorganisms grow together, producing an effect that none of the individual microbes could produce alone. Few genuine synergisms have been documented. It has been known for a long time that

Pseudomonas syncyanea and *Lactococcus lactis* will produce a blue color in milk only when growing together (Frazier, 1958).

Metabiosis

Metabiotic associations are essentially "sequential synergisms," in which the growth of one microorganism produces environmental conditions favorable for the growth of a second microorganism, which in turn can create favorable conditions for a third microorganism, and so on. Raw milk provides an excellent example of extended metabiosis. *Lactococcus lactis* and some coliforms are the first bacteria to grow in raw milk. They produce lactic acid, which creates a favorable environment for aciduric lactobacilli. When the accumulated acidity of the milk stops the growth of lactobacilli, oxidative yeasts and molds begin to grow and oxidize the lactic acid, thereby raising the pH of the milk and permitting the growth of proteolytic bacteria (Frazier, 1958).

The production of sauerkraut is also an excellent example of metabiosis. It will not be described here because it is not considered to be a food spoilage process.

The growth of aerobic, oxidative microorganisms can remove oxygen and reduce the O/R potential of a food, thereby creating anaerobic conditions that favor the growth of vastly different microbes. Even a seemingly simple spoilage pathway can exemplify a metabiotic association. The amino acid arginine can be metabolized by lactic acid bacteria to ornithine, which in turn is metabolized by enteric bacteria to the foul-smelling amine putrescine (Edwards, Dainty, & Hibbard, 1985).

Intrinsic Factors to Control Microbiological Spoilage

There are a number of inherent, or intrinsic, food properties, such as water activity, pH, preservative compounds, and O/R potential, that affect the type and rate of microbial spoilage. Each of these properties, while present in the food at some "natural" level, can be manipulated during food product formulation to better control food quality and safety. The past century of advances in food science and technology have led to a very large increase in the number and quantity of food products available to consumers. It is estimated that in ca. 1900, the US consumers had their choice of about 100 different kinds of food. Nearly a century later, about 12,000 kinds of food were available (Todd, 1987).

The increasing complexity of food products has forced greater emphasis on the ability of food processors to better manage food quality and safety. Product design is an essential feature of the HACCP system of food safety. It is during this product design phase of research and development activities that validated control measures must be tested and incorporated into the food formulation (Sperber, 1999; Mortimore & Wallace, 1998).

To a large extent, the intrinsic properties of foods determine the expected shelf life or perishability of foods. Several terms to describe this relationship are commonly used, albeit imprecisely defined (Frazier, 1958):

Perishable. Most fresh foods, such as milk and dairy products, meats, poultry, seafood, and produce, have shelf lives ranging from several days to about 3 weeks.

Semiperishable. Some fresh foods such as whole vegetables, fruits, and cheeses may be stored without spoilage for about 6 months under proper storage conditions (refer to section "Extrinsic Factors to Control Microbiological Spoilage" for proper storage conditions).

Nonperishable or shelf stable. Many natural and processed foods have an indefinite shelf life. They can be stored without microbiological spoilage for periods of several years or longer. Examples of shelf-stable foods are dry beans and nuts, flour, sugar, canned fruits and vegetables, mayonnaise and salad dressings, and peanut butter.

The following intrinsic factors are important in the control of microbiological spoilage of foods and beverages. While each of the factors is known to exert its individual effect on spoilage microbes, the food processor must be aware that combinations of the intrinsic factors interact in all foods. Thus, moderate reductions of water activity and pH, along with moderate usage of chemical preservatives, can accomplish the same antimicrobial effect as major alterations of any single intrinsic factor. Moreover, the intrinsic factors interact with the extrinsic factors for food preservation described in section "Extrinsic Factors to Control Microbiological Spoilage" of this chapter.

Water Activity

The determination of the water activity (a_w) value of a food has replaced the percent moisture determination as the most accurate means to determine the growth potential of microorganisms. Some of the water present in foods is chemically bound by hydrogen bonds, by the constituent food molecules, and by added solutes. The a_w value indicates the proportion of the food's moisture that is physically available for microbial growth.

The a_w can be determined manometrically by dividing the vapor pressure of the food by the vapor pressure of water. It can be estimated mathematically for individual solutes by dividing the moles of water by the moles of water plus the moles of solute. It is most easily determined by measuring the food's equilibrium relative humidity (ERH) and dividing it by 100 (Scott, 1957). This last procedure is most commonly used today, as practical instrumentation for rapid a_w determinations has been commercialized. Since ERH values can range from 0 to 100%, a_w values will range from 0 to 1.0.

The influence of a solute on water activity varies inversely with its molecular or ionic size. Therefore, smaller molecules or ions will be more effective than larger molecules in reducing water activity in food formulations. Sodium chloride (ionic weight = 29.25) is theoretically 11.7 times more effective than sucrose (molecular weight = 342) on an equal weight basis in reducing water activity.

Table 2 Minimum water activity (a_w) values to support the growth of representative spoilage microorganisms (derived from Christian, 2000; Deak & Beuchat, 1996; Sperber, 1983)

Microorganism	Minimum a_w for growth
Alicyclobacilli	0.97
Pseudomonads	0.97
Enteric bacteria	0.95
Lactic acid bacteria	0.92
Saccharomyces cerevisiae	0.92
Spoilage yeasts	0.90
Bacillus subtilis	0.90
Spoilage molds	0.84
Xerotrophic molds	0.62
Osmophilic yeasts	0.60

Microorganisms vary greatly in their ability to grow in foods with increased osmotic pressure or reduced water activity values (Table 2). The alicyclobacilli and pseudomonads are hardly osmotolerant, while some species of molds and yeasts are the most osmotolerant organisms known. The type of spoilage organism likely to spoil a particular food can be estimated by the determination of the food's water activity (Table 3).

Table 3 Water activity values of foods (derived from Christian, 2000; Sperber, 1983)

Food	Water activity
Fresh meat, poultry, and seafood	0.99
Bread	0.94
Mayonnaise	0.90
Icing, frosting	0.80
Dried fruit	0.65–0.75
Pancake syrup	0.70
Wheat flour, freshly milled	0.65
Wheat flour	0.60
Dry pasta, spices, milk	0.40–0.60

Water moves freely across the cytoplasmic membranes of microbial cells. When a microbial cell is subjected to high external osmotic pressure, the cell will be dehydrated, resulting in its inability to grow or even in its death. The more osmotolerant microbes indicated in Table 2 cope with the increased external osmotic pressure by greatly increasing their internal concentration of small solute molecules or ions (Csonka, 1989; Sperber, 1983). Enteric bacteria accumulate potassium ions, enabling their growth at a_w 0.95. Further accumulation of potassium ions beyond this point, however, is toxic to the cells, so a_w 0.95 represents the lower limit for the growth of enteric bacteria. The more osmotolerant microbes accumulate "compatible solutes" that do not readily poison the cells as they accumulate to higher concentrations. The most common compatible solutes are proline and glycerol. Therefore, when a food or a growth medium is osmotically adjusted with glycerol rather than

Table 4 Influence of solute type on the minimum water activity to support the growth of spoilage microorganisms (derived from Christian, 2000; Deak & Beuchat, 1996; Sperber, 1983)

	Water activity achieved by		
Microorganism	Sodium chloride	Glycerol	Sucrose
Pseudomonas fluorescens	0.957	0.940	–
Clostridium sporogenes	0.945	0.935	–
Bacillus megaterium	0.94	0.92	–
Lactobacillus helveticus	0.963	0.928	–
Streptococcus thermophilus	0.965	0.94	–
Saccharomyces cerevisiae	0.92	–	0.89
Candida dulciaminis	0.86	–	0.81

sodium chloride, bacteria are able to grow at lower minimum a values (Table 4). Similarly, yeasts are able to grow at reduced a_w values when sucrose, rather than sodium chloride, is used as the solute.

pH

The pH value of foods is another important intrinsic value that determines what types of microorganisms can spoil a food. pH is expressed as

$$\mathrm{pH} = -\log_{10}[\mathrm{H}^+] = \log_{10} 1/[\mathrm{H}^+]$$

where $[H^+]$ is the hydrogen ion concentration.
Since pH is a logarithmic function, doubling or halving the $[H^+]$ will alter a substrate's pH value by 0.3 units ($\log_{10}2 = 0.3$). This means that as the acidity of a system increases, the pH value will decrease. The pH of pure water is 7.0. Values <7.0 are acidic, while values >7.0 are alkaline or basic.

Some microorganisms have developed elaborate acid tolerance responses to cope with reduced pH environments. In general, many kinds of foodborne spoilage microorganisms can grow collectively over most of the pH range, from 0.5 to 11.0 (Table 5). Most foodborne bacteria can grow in the pH range of 4.5–9.0. Most foods range in pH from slightly acidic to strongly acidic (Table 6).

Table 5 Microbial pH range for growth (derived from Jay, 2000; Sperber, unpublished data)

Microorganism	Minimum pH	Maximum pH
Molds	0.5	11.0
Yeasts	1.5	8.5
Alicyclobacilli	2.0	6.0
Lactic acid bacteria	3.5	9.0
Enteric bacteria	4.5	9.0

Table 6 Food pH values (derived from Lund & Eklund, 2000; Sperber, unpublished data)

Food	Typical pH value
Carbonated beverages	2.0
Vinegar	3.0
Apple juice	3.1
Orange juice	3.6
Tomato juice	4.2
Cheddar cheese	5.2
Ground beef	6.2
Milk	6.4
Peas, sweet corn, honeydew melons	6.5
Fresh fish	6.7
Surface-ripened cheeses	>7.0
Hominy	8.5
Nixtamalized corn	10.0

Table 7 Influence of acidulant on the minimum pH for growth of *Salmonellae* (derived from Chung & Goepfert, 1970)

Acidulant	Minimum pH for growth
Hydrochloric	4.05
Citric	4.05
Malic	4.30
Lactic	4.40
Acetic	5.40
Propionic	5.50

The ability of microbes to grow at lower pH values varies greatly with the type of acid that is used to establish the pH (Table 7). The reader should note that it seems obvious that some organic acids, such as acetic and propionic acids in Table 7, have an inhibitory effect that exceeds the inhibition that would be expected solely by pH reduction. Such enhanced inhibitory effects are a large part of the basis for the use of chemical preservatives, as discussed below.

Chemical Preservatives

Chemical Properties of Organic Acids

In addition to the microbiostatic action of their pH effect, organic acids exert various internal metabolic effects. Only undissociated acids, however, can enter the microbial cell by migrating through the cytoplasmic membrane. Therefore, the preservative activity of organic acids is dramatically affected by the pH of the food.

The acid's dissociation constant (pK_a) is the pH value at which the acid is 50% dissociated. Therefore, the proportion of undissociated acid is inversely related to

the food or the growth medium's pH value. The proportion of undissociated acid can be calculated by the Henderson–Hasselbalch equation (Lund & Eklund, 2000)

$$\mathrm{pH} = \mathrm{p}K^{\mathrm{a}} + \log_{10}[\mathrm{A}^-]/[\mathrm{HA}]$$

where [HA] is the concentration of undissociated acid and $[A^-]$ is the concentration of dissociated acid (or anion concentration).

When $[A^-] = [HA]$, $pH = pK_a$ and the acid is 50% dissociated, that is, 50% of the added compound is effective as a preservative.

The proportion of fat and water in the food further affects the antimicrobial activity of organic acids. Most organic acids are preferentially fat soluble, as expressed by their partition coefficients (PC, sometimes called distribution coefficient), which are defined as follows:

$$\mathrm{PC} = \text{solubility of compound in fat phase/solubility of compound in water phase}$$

The relatively low pK_a values and the relatively high PC values of common food preservatives limit their effectiveness in foods that are greater than pH 5.5 or contain fat (Table 8).

Table 8 Chemical properties and typical usage levels of common food preservatives (abstracted from Raczek, 2005)

Compound	pK_a[a]	PC[b]	Usage level (%)
Sorbic acid	4.76	3.0	0.1–0.3
Propionic acid	4.87	0.17	0.2–0.8
Benzoic acid	4.20	6.1	0.1
Methyl paraben[c]	8.47	6.0	0.1
Propyl paraben	8.47	88.0	0.05

[a] Dissociation constant
[b] Partition coefficient
[c] Parahydroxybenzoic acid

Chemical Food Preservatives

Some of the commonly used preservatives occur naturally, especially in acidic foods. Therefore, it is possible that they have been used as food preservatives since antiquity. A wide range of chemical preservatives are described below. Many uses of these preservatives will be further explained in the following chapters on specific food categories. Many of this chapter's references treat some aspects of the chemical preservation of foods. Lund & Eklund (2000) and Foegeding & Busta (1991) organized particularly comprehensive reviews.

Sorbic acid. Naturally occurring in European mountain ash berries, sorbic acid is relatively insoluble in water (<0.2%). Most food applications use the sodium or potassium salts of sorbic acid, as these are about 50% soluble in water. The sorbates, particularly effective in preventing mold and yeast growth, can also effectively

prevent bacterial growth, more so than other compounds in this category. Acquired resistance to sorbates by some species of penicillia has been noted in cheeses (Marth, Capp, Hasenzahl, Jackson, & Hussong, 1966).

Propionic acid. This compound is produced in Swiss cheese by propionibacteria. It is commonly used in foods in the calcium salt form. It is moderately effective against molds, with little activity against yeasts or bacteria. Therefore, it is widely used to prevent mold spoilage in yeast-leavened baked goods. It is the only preservative in this category to have a PC <1.0; therefore, it could be more effective than the other preservatives in fat-containing foods.

Benzoic acid. Normally used in the sodium salt form, benzoate is found in significant concentration in cranberries. It possesses some antibacterial activity, is moderately active against molds, and is very effective against yeasts. Therefore, it is widely used to prevent yeast spoilage in hermetically sealed acidic foods that are not subject to mold spoilage because of the absence of oxygen. Pitt (1974) detected benzoate-resistant yeasts in a variety of foods, including carbonated beverages and canned tomato products.

Methyl and propyl parabens. Formed by synthetic additions to benzoic acid, parabens are widely used to preserve cosmetic and pharmaceutical products but have limited use in foods because of their flavor and high PC values. Both methyl and propyl parabens are quite effective against molds, are moderately effective against yeasts, and have limited activity against bacteria. The parabens are the only commonly used food preservative with a pK_a value (8.47) above neutrality. Methyl paraben is used in foods more often than propyl paraben because of its considerably lower PC value − 6.0 vs. 88.0.

Carbon dioxide. A feedback respiratory inhibitor, carbon dioxide inhibits the growth of obligately aerobic microorganisms such as molds and pseudomonads. It is typically used as a component of headspace gases in packaged bakery and meat products.

The inhibitory effect of carbon dioxide can be enhanced by the addition of small amounts of ethanol to the headspace of bread products (Vora & Sidhu, 1987). When used in carbonated beverages, the principal antimicrobial effect of carbon dioxide is quite likely exerted by its soluble form, carbonic acid (Pitt, 1982, personal communication).

Sodium nitrite. The principal effective component of meat-curing salts, sodium nitrite, is widely used in many meat, seafood, and poultry products including hams, wieners, smoked fish, and luncheon meats. It has many effects, such as stabilizing color, adding flavor, and inhibiting the germination and outgrowth of bacterial spores. This last effect is most important because it reduces the risk of botulism from cured meats as well as inhibits the growth of spoilage microorganisms, including bacterial sporeformers.

Nisin. Nisin is a bacteriocin produced by *L. lactis.* It has regulatory approval in the United States to be used in cheese spreads and liquid eggs to prevent the germination and growth of bacterial sporeformers, particularly *C. botulinum.*

Potassium lactate and sodium diacetate. In recent years, these salts have been used in combination to inhibit the growth of *Listeria monocytogenes* in refrigerated

meat and poultry, especially deli meats. Their effect on spoilage microflora is under investigation; however, a priori, they should inhibit microbes, such as the lactic acid bacteria, that possess growth characteristics similar to those of *Listeria* spp.

Sodium bisulfite. This is the most interesting preservative because of its long use and complex chemistry involving six sulfur-containing compounds. Early civilizations burned elemental sulfur in barrels to preserve wine, without knowing they were using the antimicrobial properties of sulfur dioxide (Block, 1991). When burned in the presence of oxygen, sulfur (S_2) forms the gaseous sulfur dioxide (SO_2). When dissolved in water, SO_2 forms sulfurous acid (H_2SO_3), which because of its low pK_a value (1.8) quickly dissociates to bisulfite anions (HSO_3^-). A dry form of this family of compounds, sodium metabisulfite ($H_2S_2O_5$), also ionizes to the bisulfite form in solution. As the pH of the solution is increased toward neutrality, the bisulfite ions further dissociate ($pK_a = 7.0$) to sulfite ions (SO_3^{2-}) (Foegeding & Busta, 1991). Sodium bisulfite is commonly used to prevent yeast spoilage of wine and dried fruits and as a processing aid to minimize microbial growth during the production of products such as dried potatoes.

Dimethyldicarbonate. This compound, which dissolves in water to form ethanol and carbon dioxide, has been used occasionally as a preservative in beverages. Its use has been limited because of periodic questions about its carcinogenicity.

Spices and essential oils. A wide variety of plants and herbs produce essential oils that exhibit antimicrobial properties. In particular, antifungal properties have been observed (López-Malo, Alzamora, & Palou, 2005).

The chemical preservatives described here are somewhat limited in their antimicrobial activities. Moreover, because of potential adverse effects upon the organoleptic or functional properties of a food, it is usually not practical to increase the concentration of chemical preservatives in order to achieve the desired level of microbial inhibition. It is often practical, however, to use more than one chemical preservative and preservative factor to achieve this goal. Known as the "hurdle effect," the simultaneous use of multiple preservative factors can interact additively or synergistically to protect a food from spoilage during its intended shelf life (Leistner & Gould, 2002; Guynot, Ramos, Sanchis, & Marín, 2005). Moreover, the hurdle effect can be substantially enhanced by even relatively minor reductions in pH and water activity values of a food, as well as extrinsic factors discussed in the following section. Specific applications of chemical preservatives are included in the following chapters on specific product categories.

Oxidation–Reduction Potential

The oxidation–reduction potential of a food is often referred to as the O/R potential or the Eh of the food. The food Eh is changed by the oxidation or the reduction of its constituent compounds. As the compounds are oxidized, electrons are lost (or "free"); as the compounds are reduced, electrons are recaptured. The electron motive force can be directly measured as the Eh. It is expressed in a range with

a maximum Eh value of +816 mV (highly oxidized) and a minimum Eh value of −421 mV (highly reduced). Many factors influence the Eh of a food, including the original Eh of a particular food, its ability to resist ("buffer" or "poise") changes in Eh, and the availability of atmospheric oxygen to the food (Jay, 2000).

Therefore, positive Eh values favor the growth of aerobic microorganisms because these can grow in the presence of oxygen and often use oxidative metabolic processes (which would tend to further increase the food Eh). Negative Eh values favor the growth of anaerobic microorganisms, whose growth and use of fermentative metabolic pathways will further reduce the food's Eh value. It is a mistake to assume that the availability of oxygen to a particular food will prevent the growth of anaerobic microorganisms. The interior of fresh or canned vegetables, for example, will remain poised at negative Eh values even though the exterior of the product is directly exposed to oxygen (Sperber, 1982).

Extrinsic Factors to Control Microbiological Spoilage

As mentioned above, food processors must be aware of the many interactions that exist between the extrinsic factors described below, as well as the interactions between these extrinsic factors with the intrinsic factors described above.

Thermal Processes

Thermal Destruction of Microorganisms

In the thermal killing of unicellular organisms, the surviving population decreases logarithmically ($\log_{10}$) as the heating time increases linearly. This first-order relationship is quantified by D and z values (Joslyn, 1991). The D value is the amount of time, expressed in minutes, at a given temperature required to reduce a population by 90%. The z value is the amount of change in temperature, expressed as °C, that will shift the D value in either direction by 90%. For example, if a microorganism heated at 110°C has a $D_{110°C}$ value of 10 min and a z value of 10°C, its $D_{120°C}$ value would be 1.0 min if the heating occurred at 120°C, and the $D_{100°C}$ value would be 100 min if it occurred at 100°C. These particular D and z values are typical for many bacterial spores.

Thermal process values for several representative bacteria are presented in Table 9.

Some bacteria have evolved mechanisms to cope with adverse environmental changes. *Bacillus subtilis* produces a set of stress proteins that can be induced by exposure to mild heat or mild salt concentrations. Induction of these stress proteins enables *B. subtilis* to survive exposure to higher temperatures and salt concentrations (Völker, Mach, Schmid, & Hecker, 1992).

Table 9 Thermal *D* and *z* values for representative species of vegetative bacteria and bacterial spores (derived from Farkas, 2001)

Microorganism	*D* (min)	°C	*z*
	Vegetative bacteria		
Escherichia coli	4	55	–
Pseudomonas fluorescens	1–2	55	–
Salmonella senftenberg (775 W)	10.8	60	6.0
Staphylococcus aureus	7.8	60	4.5
	Bacterial spores		
Bacillus stearothermophilus	4–4.5	121	7
Clostridium sporogenes	0.1–1.5	121	9–13
Clostridium botulinum, types A and B	0.1–0.2	121	10
Bacillus coagulans	0.01–0.1	121	–

Sterilization

Sterilization processes are used to produce foods that from a microbiological standpoint will have an indefinite shelf life as long as the integrity of the packaging material prevents contamination of the food. Most commonly, these foods are filled into metal, glass, or plastic containers that are hermetically sealed and processed under steam pressure at temperatures of 121°C or higher. A series of incidents in the early 1970s in which contamination of canned foods with botulinum toxin types A or B led to product recalls and several illnesses and deaths prompted the US Food and Drug Administration (FDA) to develop regulations for low-acid and acidified canned foods in 1974 (CFR, 2008a; 2008b). While these regulations were developed to assure food safety, their enforcement also provides greater assurance of product quality.

The low-acid canned food regulation (CFR, 2008a) requires a "12D botulinum cook." Therefore, canned foods processed at 121°C would require at least 2.4 min at this temperature, since the $D_{121°C}$ value is about 0.2 min (Table 9). In practice, these canned foods receive more than a "bot cook" in order to inactivate other mesophilic sporeformers that are more heat resistant than *C. botulinum* and could spoil the product if any spores survived the sterilization process. *Bacillus stearothermophilus* and similar microbes are thermophilic sporeformers whose spores have a $D_{121°C}$ value of 4 min or greater, that is, they are about 20 times as heat resistant as *C. botulinum.* Sterilization processes adequate to kill the thermophilic sporeformers cause serious degradation of product quality. Such intense processes, however, have proven to be unnecessary because the thermophilic sporeformers cannot grow during the distribution and storage of these products at ambient temperatures that support the growth of mesophilic microbes. This phenomenon has led to the concept of "commercial sterility," in which a food is not sterile in the strictest sense because it may contain viable thermophilic spores. However, because these spores can only germinate and grow above temperatures of about 45°C, the food remains sterile in a practical sense.

The second canned food regulation (CFR, 2008b) governs the processing of acidified foods. A food stored in a hermetically sealed container that has a pH <4.6 or an a_w <0.85 is protected from the growth of *C. botulinum* and spore-forming spoilage bacteria, and does not require the commercial sterilization process described above. Acidified foods require a heat treatment sufficient to kill the nonspore-forming bacteria and fungi that could otherwise spoil the product.

Sterile foods can also be produced by aseptic processes in which the food is sterilized at ultrahigh temperatures (UHT), ~150°C. UHT-sterilized food is packaged aseptically into packaging material that itself has been sterilized by superheated steam, flame sterilization, high-dose ultraviolet irradiation, or chemical sterilants, particularly hydrogen peroxide (von Bockelmann, 1991).

Pasteurization

In its broader application in the food industry, the term "pasteurization" refers to most cooking processes that occur at 100°C or lower. Such processes kill all microorganisms except bacterial spores. More narrowly, the term pasteurization can be used to refer to specific regulated products. Since 1924, for example, the FDA has enforced regulations for the pasteurization of dairy products (FDA, 2005). An important feature of pasteurization processes is that many combinations of time and temperature can provide equivalent lethal processes. As the process temperature is increased, the holding time can be decreased (Table 10). The Pasteurized Milk Ordinance was enacted to protect the public health because "It has been demonstrated that the time−temperature combinations specified by this Ordinance, if applied to every particle of milk, will devitalize all milkborne pathogens" (FDA, 2005, p. 71). Of course, pasteurization to prevent foodborne illness also provides dairy products of higher quality and a longer shelf life because of the simultaneous reduction in spoilage microflora.

Table 10 Combinations of time and temperature required for the pasteurization of milk in the United States (Examples from FDA, 2005)

Time (s)	Temperature (°C)
1800	63
15	72
1.0	89
0.1	94
0.01	100

Hot-fill processes. Foods that have high acidity such as fruit juices or a reduced water activity such as sugar syrups are easily stabilized against microbiological spoilage by cooking or pasteurization. The heating step kills all vegetative spoilage microorganisms and the acidity or the reduced water activity prevents the growth of surviving bacterial spores. Many of these products are produced commercially simply by packaging the product into its container while it is still hot, generally >70°C, from its cooking or pasteurization process. The residual heat is sufficient to

kill yeasts, molds, and vegetative bacteria. In some applications, the containers need to be inverted for several minutes in order to pasteurize the top of the container.

Atmospheric steam pasteurization in container. This process is analogous to hot-filled processes, except that the heat is applied to the product after it is hermetically sealed in its consumer package. Heat, applied for several minutes by infrared bulbs or microwave or conventional ovens, generates steam that pasteurizes the product's surface. Upon cooling, the condensed steam, originally generated from the product's interior, is reabsorbed by the product. This process, particularly applicable to baked goods, produces products that have an indefinite microbiological shelf life (Bouyer, 1970; Richardson & Hans, 1978). The process is also applicable to other perishable foods such as meat and poultry products in which surface spoilage can limit shelf life and other types of surface pasteurization are impractical.

Nonthermal Processes

A wide variety of nonthermal processes (NTPs) have been extensively researched and developed for the preservation of food. A principal advantage of NTP is that they typically do not degrade the texture, color, odor, flavor, or nutritional properties of a food to the same extent as thermal processes. Nevertheless, in contrast to thermal processes, NTP usually have limited commercial applications because of their higher cost, lesser efficacy for microbial reductions, difficulty in application for high-volume commercial production, and/or regulatory restrictions.

Ionizing Irradiation

Ionizing irradiation is any electromagnetic radiation that has sufficient energy to ionize the molecules it contacts. Gamma, beta, cosmic, and X-rays can disrupt microorganisms by a direct hit on an essential molecule such as DNA or by producing free radicals that will denature and inactivate essential molecules. Because it produces no appreciable increase in product temperature, ionizing irradiation is referred to as "cold sterilization" or "cold pasteurization" (Silverman, 1991). The most common sources of ionizing irradiation for commercial use are cobalt^{60} or high-energy electron beams.

The unit of radiation energy is the rad. One rad equals 100 ergs/g. One gray (Gy) equals 100 rads. The dose ranges of irradiation used in food processing are expressed in kilograys (kGy). One kGy equals 10^5 rads or 10^7 ergs/g. The ionizing irradiation *D* values for a wide range of microorganisms are presented in Table 11. Considerably higher doses are required to inactivate bacterial spores compared to vegetative cells. High doses are required to inactivate small targets such as viruses or enzymes, whereas low doses will inactivate large targets such as parasitic worms. Vegetative microbes are inactivated by an intermediate irradiation dose. Regulatory approvals have been granted for use of ionizing irradiation with a variety of fruits and vegetables, and meat and poultry products.

Table 11 Ionizing irradiation *D* values of representative organisms (derived from Silverman, 1991)

Organism	*D* (kGy)
Clostridium botulinum	3.3
Enterococcus faecium	2.8
Bacillus subtilis	0.6
Saccharomyces cerevisiae	0.5
Salmonella serovar Typhimurium	0.2
Pseudomonas spp.	0.06
Saccharomyces cerevisiae	0.5
Aspergillus niger	0.5
Foot and mouth virus	13
Complete inactivation of:	
Enzymes	20–100
Insects	1–5
Trichinella spiralis	0.2–0.5

High Hydrostatic Pressure

Liquid or moist solid packaged foods can be pasteurized or sterilized by submersion in water and subjected to very high pressures, ranging from 50 to 1000 megapascals (MPa) for periods ranging from several seconds to minutes. One MPa is equivalent to 10 atmospheres of pressure. The very high pressure disrupts noncovalent bonds in microorganisms without altering the texture or other organoleptic properties of the food. Typically, the pressure will disrupt the hydrogen bonds in nucleic acids, thereby altering DNA and ribosomes (Jay, 2000; Ross, Griffiths, Mittal, & Deeth, 2003).

High hydrostatic pressure treatment is not effective on dry foods. The first commercial applications of this technology were for fruit juices and high-value perishable refrigerated foods. More recently it has been used in some meat and poultry products to inactivate *L. monocytogenes.*This particular application for food safety consideration also kills spoilage microorganisms, yielding a product of higher quality and longer shelf life.

Pulsed Electric Fields

A liquid food can be pasteurized by passage of a high-voltage electric field (up to 80 kV/cm) that is pulsed at microsecond intervals. This treatment disrupts the microbial cell membrane, eventually killing the cell by lysis (Jay, 2000; Ross et al. 2003). Pulsed electric fields have been applied to a number of liquid foods, including fruit juices, milk, and eggs.

High-Intensity Ultrasound

This procedure is applied to liquid foods by an ultrasonic probe (Ross, et al., 2003). The ultrasonic waves produce rapid pressure fluctuations at the macromolecular

or organelle level. The resulting cavitation effect disrupts microbial cell walls and membranes, thereby killing the microbe. When used in combination with thermal treatments, ultrasound can reduce the heat resistance of bacterial spores.

Ultraviolet Irradiation

Ultraviolet (UV) light used in food applications in the near-visible light range of approximately 220–300 nm inactivates cells primarily by cross-linking adjacent thymine molecules on DNA strands (Schechmeister, 1991). The UV light is applied by high-intensity bulbs and can be used to disinfect liquid streams that flow over the bulbs. Large arrays of UV bulbs are sometimes used to treat municipal water supplies. The food industry uses smaller arrays to treat recycled flume water. UV installations in ventilation systems can be used to disinfect the air supplied to food production areas. The effectiveness of UV light in killing microorganisms is limited by particulates in liquids or air and by its low penetrating ability.

Filtration

Filters with an effective pore size of 0.22–0.45 μm can be used to produce sterile liquids, ranging from water to liquid sweeteners, and sterile air (Levy & Leahy, 1991). In food production, the intake air can be filtered to minimize surface contamination of foods during processing and packaging.

Sterilizing Gases

Ethylene oxide and propylene oxide were formerly used to reduce the microbial population of dry foods, for example, the reduction of mold spores in cocoa powder, nuts, and dried fruits (Parisi & Young, 1991). Ethylene oxide has largely been banned from food applications because of its mutagenicity and carcinogenicity. Propylene oxide is seldom used today because of its comparative ineffectiveness in reducing microbial populations. In addition to these two gases, gaseous formaldehyde, β-propiolactone, and chlorine dioxide can be used to disinfect rooms and packaging materials. Chlorine dioxide and ozone can be used to disinfect water and other liquids.

Refrigeration

The widespread proliferation of refrigerated and frozen distribution and storage systems throughout the developed and developing world has enabled global food commerce and provided consumers with a greater variety of foods available throughout the year. A large proportion of our diet consists of refrigerated and frozen foods because of this practical ability to extend the shelf life of perishable foods.

Food Preservation by Refrigerated Storage

Temperature has a direct influence on the growth rate of microorganisms because the rate of biochemical reactions, that is, microbial metabolism varies directly with increases or decreases in temperature. For this reason, the optimal growth rate of a particular microorganism occurs close to maximum temperature at which it can grow. As the temperature is decreased, the growth rate slows and the growth stops at temperatures below the minimum growth temperature (Herbert & Sutherland, 2000).

Refrigeration temperatures also retard microbial growth by affecting internal cellular changes. The composition and permeability of the cell membrane can change, and the structural integrity or the catalytic activity of vital enzymes can be impaired. Some psychrophilic bacteria have evolved metabolic enzymes that function well at refrigeration temperature. Many psychrophilic bacteria are obligate aerobes. Because oxygen is more soluble in water as temperature decreases, the increased oxygen content of refrigerated foods that are exposed to the atmosphere enhances the growth rate of these bacteria.

Some microorganisms are able to survive exposure to low temperatures by the production of cold-shock proteins. A thermophilic strain of *S. thermophilus* with an optimum growth temperature of 42°C produces inducible cold-shock proteins when exposed to 20°C, enabling it to survive freeze-thaw cycles 1,000-fold better than noninduced cells (Wouters, Rombouts, de Vos, Kuipers, & Abee, 1999).

When produced under good sanitary conditions resulting in a "normal" microbiological load, the shelf life of many refrigerated, perishable foods ranges from several weeks to several months. If held considerably longer than the intended shelf life, it is virtually certain that such a refrigerated food will spoil because of microbial growth, even when good refrigeration temperatures, <5°C, are used. Given the existence of billions of refrigeration units worldwide for the storage of foods, it is almost certain that a significant proportion of these will not maintain proper temperature control, thereby hastening the microbiological spoilage of the food.

Food Preservation by Frozen Storage

Commercially produced frozen foods are usually stored and distributed at −18°C, a temperature that will prevent microbial growth indefinitely. However, microbial growth and spoilage is possible in foods that are stored at somewhat higher temperatures. Food solutes prevent some water in food from freezing as its temperature is reduced below 0°C. Bacterial growth has been reported in meat frozen at −3°C. Mold growth has been reported in foods stored at −8°C (Lund, 2000).

While the formation of ice crystals during freezing can damage or kill some cells of a microbial population, it will not destroy the entire population. In fact, freezing is a principal means of preserving microbial cultures. In general, Gram-negative bacteria are more sensitive to freezing than are Gram-positive bacteria. Generally, Gram-negative bacterial cell numbers are reduced by 10- to 100-fold during freezing. Freezing has little effect on bacterial spores, yeasts, and molds.

Vegetables are blanched with steam or hot water before freezing in order to inactivate enzymes that could degrade product quality during frozen storage. Product temperatures of about 90°C are usually reached during blanching, substantially reducing the original microbial population. Spoilage microbes that survive freezing can grow in thawed meat and produce products more quickly than they can grow in the fresh products because of the release of nutrients when meat or plant cells burst during freezing.

Many specific examples of the effects of refrigeration and freezing on microbiological spoilage are presented in the following chapters.

Vacuum and Modified Atmosphere Packaging

Many food products are packaged in containers that have a headspace filled with air at atmospheric pressure. In such products, growth of aerobic spoilage microorganisms can be inhibited or prevented either by the removal of headspace oxygen or by the addition of inhibitory gases. In either case, it is important that the packaging material be hermetically sealed to prevent the ingress of oxygen or the egress of the inhibitory gases.

Vacuum Packaging

In theory, the total removal of all oxygen from a package will prevent the growth of spoilage microorganisms such as molds. The complete removal of oxygen, however, is almost impossible to accomplish, and some molds can grow at oxygen levels as low as 0.4%. Some commercial applications have been developed for vacuum-packaged foods in which small packets of reduced iron or iron oxide are used to scavenge residual headspace oxygen (Smith, Ooraikul, Koersen, Jackson, & Lawrence, 1986).

A similar phenomenon can occur in fat-containing foods that are hermetically sealed, with or without the application of vacuum, and the headspace/product ratio is very small. Refrigerated ground beef is often spoiled by the growth of aerobic psychrophiles.

If ground beef is packaged in an oxygen-impermeable film, the residual oxygen is chemically combined with fat, thereby preventing the growth of psychrophiles and product spoilage. In this particular case, the preferential selection of the growth of lactic acid bacteria further stabilizes the ground beef against the growth of other microbes (Frazier, 1958). Similarly, mayonnaise and salad dressings are packaged in containers with very little headspace. The residual oxygen rapidly combines with the product oil, thereby preventing the growth of molds and aerobic yeasts (Sperber, unpublished data).

Modified Atmosphere Packaging

Carbon dioxide gas is used to actively inhibit the growth of aerobic microbes, in contrast to the passive removal of oxygen by vacuum packaging. Carbon dioxide

inhibits the growth of aerobic microbes by feedback inhibition of cellular respiration. Because of the active inhibition, it is not necessary to remove all headspace oxygen. This is a most important consideration, because some headspace oxygen is sometimes necessary to preserve other organoleptic properties, such as product color.

The CO_2 gas can be directly injected into the product headspace during packaging. More commonly, it is introduced in a controlled mixture of gases that are used to backflush a headspace after vacuum treatment. Gas mixtures containing 2–5% O_2, 8–10% CO_2, and the remainder being N_2 are commonly used to extend the shelf life of fresh fruits and vegetables (Farkas, 2007; Jay, 2000). As with most other preservative factors, there is an interaction between the temperature and the effective concentration of CO_2 to prevent spoilage. Sperber (unpublished data) found that a mixture of 70% CO_2/30% N_2 prevented the molding of baked goods at ambient temperature, whereas a mixture of 30% CO_2/70% N_2 was sufficient to prevent molding at 5°C.

More recently, the carefully controlled application of headspace carbon monoxide in combination with carbon dioxide and nitrogen has been developed to inhibit the microbiological spoilage and preserve the color of packaged fresh meats (Nissen, Alvseike, Bredhlt, Holck, & Nesbakken, 2000).

Food Plant Sanitation

The topic of food plant sanitation deserves inclusion as an extrinsic factor to control the microbiological spoilage of food and beverages. In this author's extensive experience in the food-processing industry, it was frequently determined that inadequate cleaning and sanitation of food-processing equipment caused spoilage episodes, some of very large scope. Often, the inability to conduct adequate cleaning and sanitation procedures was caused by the poor sanitary design of the equipment. Therefore, it should be widely acknowledged that poor sanitary design and inadequate cleaning and sanitation procedures are extrinsic factors that favor the buildup of spoilage microbes in the processing equipment. This is a sobering consideration when one considers that the microbes are being selected and enriched to grow in a substrate that is identical to the food that requires protection against the growth of the spoilage microorganisms.

Numerous incidents of microbial spoilage of raw bakery dough products have been caused by poor sanitary design of dough-handling equipment. Some of the equipment could never be completely cleaned and sanitized; thus, it served as a constant source of contamination with spoilage microorganisms. Other incidents of spoilage were caused simply because dough mixers were cleaned infrequently.

Most food processors would not suspect that mechanical blast freezers, which operate at about −40°C, could also serve as a source of microbial, and possibly allergen, contamination. Because the blast freezers are operated at a very high air velocity, small bits of food debris from the product being frozen are blown around

the freezer and accumulate on the floor and the conveying apparatus. Blast freezers are shut down periodically, usually on weekends, to remove the food debris and to clean and sanitize the freezer interior. Typically, the freezer is allowed to equilibrate to ambient temperature for 1 day or more before the cleaning procedure begins. During this time, extensive growth of spoilage (and pathogenic) microorganisms can occur. Because of the large amount of food debris and the complexity of the conveying apparatus, it is usually difficult to remove all of the debris. Therefore, the effectiveness of sanitation procedures would be reduced. Surviving microorganisms contaminate new food products when the blast freezer is restarted. This situation could lead to spoilage problems in the food supply chain. Blast frozen raw materials such as diced meat or blanched vegetables could be contaminated during freezing and cause microbiological spoilage later when they are used as ingredients in perishable refrigerated products such as salads.

Food processors can address this situation directly by collaborating with their cleaning and sanitation chemicals suppliers and by following the advice in reference books such as *Sanitation in Food Processing, Second ed.* (Troller, 1993).

Similarly, adherence to good manufacturing practices in food-processing plants will minimize the contamination of food and food materials with spoilage microorganisms.

Detection of Microbiological Spoilage

There are three principal methods for the detection of microbiological spoilage – organoleptic, microbiological, and chemical.

Organoleptic Methods for Spoilage Detection

Odor

The growth of spoilage microorganisms in foods often produces volatile metabolites that are detectable by their odor. Particularly, foul-smelling amines are often produced during proteolysis (Edwards et al., 1985). Fruity and alcoholic odors can be produced by sugar catabolism. The human nose contains 5×10^7 odor receptors/cm^2, making it a very sensitive detector for many volatile compounds. Even though instrumental procedures are available for the detection of many volatile compounds, the simple "sniff" test is still a very important procedure for the detection of spoilage in fresh meats, poultry, and fish (Ólafsdóttir & Fleurence, 1998).

Visual

Food spoilage can often be detected by visual examination. Ropy baked goods can be detected by a stringy, mucilaginous appearance when the crumb is pulled apart (Fisher & Halton, 1928). Fermentation of high sugar products can be detected by the

visible bubbling of the product from gas produced or by expansion of the hermetically sealed packages. The surface growth of spoilage microorganisms is visible as slime or isolated colonies that are sometimes pigmented. Mold spoilage is visible on the surface of products as fuzzy white colonies that turn various colors as spores are produced. When mold spoilage occurs in the interior of a product, it is not visible until the spores are produced.

Taste

Yeast spoilage of condiments and syrups by alcohol production results in a medicinal taste. In fruit juices, yeast spoilage produces a buttery taste caused by diacetyl.

A phenolic-tasting compound, guiaicol, is produced when *Alicyclobacillus* grows in fruit juices. Sour tastes are produced in dairy, meat, and vegetable products after extensive acid production by lactic acid bacteria.

Microbiological Methods for Spoilage Detection

Except for fermented foods such as cheese and sausage, foods that are obviously spoiled usually have microbial counts $>10^7$ cells/g. Therefore, there is no need to perform quantitative microbiological analyses on obviously spoiled foods, except for the occasional isolation of the spoilage microorganism for research and development activities (see section "Product Challenge Testing"). It is, however, sometimes useful to perform quantitative microbiological analyses for the assessment of incipient spoilage before it becomes organoleptically detectable. Such assessments are often performed during research and development of food products in order to establish and optimize a product's shelf life and, as part of quality assurance programs during commercial production, to verify that the anticipated shelf life can be attained.

Simple, conventional microbiological testing procedures such as total aerobic, yeast, and mold counts are sufficient for this purpose. Sophisticated procedures such as immunological or polymerase chain reaction analyses are not useful for this procedure unless reagents are developed for the detection of a very specific spoilage microorganism (Gram & Dalgaard, 2002).

Chemical Methods for Spoilage Detection

The detection of spoilage by acid production can be performed by pH measurements. The amount of acid produced can be determined by titration.

Gas chromatography can be used to detect volatile, short-chain alcohols, carbonyls, amines, aldehydes, and aromatic compounds produced during the spoilage of fish (Ólafsdóttir & Fleurence, 1998). Some automated applications, commonly called "electronic nose technology," have been developed for the early

detection of spoilage metabolites. The volatile mold metabolites in bread were detected in 1–2 days by "e-nose" technology, whereas 1–2 weeks were necessary for the visual detection of mold growth (Keshri, Voysey, & Magan, 2002). A similar system was effective in the early detection of mold growth in stored wheat grain (Costello, Ewen, Gunson, Ratcliffe, Sivanand, & Spencer-Phillips, 2003).

Fourier transform infrared spectroscopy was successfully used to distinguish spoilage strains, that is, guaiacol-producing strains, from nonspoilage strains of *Alicyclobacillus* spp. (Lin et al., 2005).

Development of Spoilage Control Measures

Most food companies expend considerable resources during product and process development in order to assure food safety. A similar expenditure of resources is usually required to assure product quality. This brief discussion of spoilage control measures has been organized to assist those who are not completely experienced in the identification and control of spoilage in a particular food product. It addresses the microbiological spoilage of perishable foods. While chemical and physical spoilage of foods are not covered here, these topics are covered in several of the references.

Isolation of Microorganisms in Spoilage Incidents

Some microbiological spoilage incidents, particularly of new food products, are not anticipated during the product's development and first appear during its commercial distribution. It is then the responsibility of the food microbiologist to determine the cause of spoilage and correct the product's formulation or processing parameters so that future spoilage incidents are avoided. Often the problem is difficult to resolve and considerable resources, including time, are required. This function is often performed under crisis conditions involving product recalls, closed production facilities, and angry managers.

Many years ago, a new shelf-stable salad dressing in glass jars was introduced into a large test market. Within weeks, reports of exploding jars were received from the marketplace. Soon the reports were so numerous that the manufacturer ordered a complete market recall. It was quickly determined that the salad dressing had been spoiled by fermentative yeasts. The expectation of a quick and easy resolution rapidly disappeared when fresh product inoculated with the spoilage yeast did not spoil. Considerable time elapsed before the production operator who had formulated the first commercial runs of salad dressing was interviewed. Starch paste, one component of the salad dressing, was used directly out of the drums in which it was received. The operator noticed that one drum of starch paste smelled "funny." In the interest of frugality, instead of disposing of this drum, the operator used a small portion in each batch over a period of several weeks, thereby uniformly contaminating all the product that had been shipped into the test market. The harried

microbiologist was then able to quickly determine the mode of spoilage. Only after "preconditioning" and growth in the starch paste could the fermentative yeast grow in the harsher environment of the salad dressing (Lawrence, 1970).

The determination that a particular microorganism is responsible for food spoilage can be performed in the manner of Koch's postulates that were developed for the determination of the microorganisms responsible for illness in humans and animals.

1. Isolate microorganisms in pure culture from the spoiled food. This step can be expanded if necessary to include analysis of the microflora of the ingredients used in the food and of samples taken from the processing equipment and the plant environment. Conventional aerobic plate count procedures can be used both to quantify the microbial count and to pick isolated colonies for purification and identification.
2. Inoculate pure cultures of the presumptive spoilage microbe(s) into fresh food and store under conditions that support spoilage.
3. Isolate and identify spoilage microorganisms from spoiled samples in step 2.
4. Preferably, the spoilage microorganisms should be identified by microscopic, biochemical, and/or immunological tests, and preserved for research efforts to improve spoilage control measures.

It is important that appropriate controls be included in this analysis. In particular, uninoculated control samples must be included in step 2, above, to assure that spoilage is not caused in the inoculated samples by a previously undetected microorganism.

In a major investigation of the cause of spoilage of a refrigerated product, several thousand pure cultures of lactic acid bacteria, representing several dozen genera of bacteria, were isolated from product, ingredient, and environmental samples. Each isolate was purified and identified. Several cultures of each genus were tested for the ability to spoil the product. It was proven that only two species of lactic acid bacteria could cause product spoilage. Cultures of these spoilage bacteria were preserved by frozen storage for further investigations. All nonspoilage cultural isolates were discarded (Sperber, unpublished data).

The microbiology laboratory should maintain a culture collection of representative spoilage microorganisms for use in research and development activities.

Product Challenge Testing

Inoculation of Test Samples

During product research and development, it is advisable to conduct challenge tests in which various product formulations are inoculated with representative spoilage microorganisms. It is preferable to use cultures of spoilage microbes that have been recently isolated from spoiled containers of similar products, rather than cultures that have been carried in the laboratory for many years or obtained from a reference

culture collection. The latter may be "laboratory attenuated" and less capable of growth in food products than cultures recently obtained from spoiled foods.

It is preferable to use more than one strain of each microbial species that is used in challenge testing, as strains of the same species may vary in their ability to grow at the product's pH, water activity, storage temperature, etc. The combined inoculum should provide a relatively low level of microbes in the test samples, on the order of 10^2–10^3 microbes/g. This controlled low level of inoculum is higher than that which would usually occur during normal production so that it provides a sufficient challenge to the food. It is also low enough to avoid the creation of microenvironments in which clusters of spoilage microorganisms could overwhelm the preservative factors present in the food and grow to levels capable of spoiling the product.

The inoculated samples may be stored at several temperatures and examined periodically for spoilage. Product samples should be stored for a period at least as long as the anticipated shelf life or until products are spoiled. If desired, conventional aerobic plate counts can be performed to detect growth of the spoilage microorganism before the spoilage is organoleptically detectable.

Accelerated Shelf Life Testing (ASLT)

It is often impractical to store a product for its entire anticipated shelf life to determine whether or not it will spoil. This is an important consideration for quality assurance testing in plant laboratories, when an early prediction of shelf life for a given production lot may be required. It is desirable to store such products under "abuse" conditions that will hasten spoilage in the laboratory. This practice, known as ASLT, has been very thoroughly developed (Labuza & Schmidl, 1985) and is widely used in research and quality laboratories. Time and temperature are the principal factors that are changed in ASLT. As temperature is increased, spoilage will occur during a shorter period of time. Considerable research and testing must be done before equivalent ASLT storage conditions can be established. An example of well-established equivalent ASLT conditions is extensively used for a refrigerated food category (Table 12).

Table 12 Example of equivalent storage conditions that permit accelerated shelf life testing of a perishable refrigerated food (Sperber, unpublished data)

Storage time (days)	Storage temperature (°C)
2	25
4	20
10	15
28	7
90	4

Predictive Microbiology

The effective research and development of a food product typically requires the testing of dozens of product formulations and processing conditions, and the analysis of thousands of samples to establish the product's microbiological safety and stability. This voluminous research can be substantially reduced by the application of new statistical techniques and the use of computers for data analysis. A number of systems, collectively referred to as "predictive microbiology," have made it possible to conduct research very effectively while greatly reducing the numbers of formulations and samples that must be tested (Buchanan, 1993; Ross & McMeekin, 1994).

Predictive microbiology can be used to model the lag time and growth rate of microorganisms (Zwietering, de Koos, Hasenack, de Wit, & van 't Riet, 1991). It was used to successfully estimate the shelf life of cottage cheese stored at various temperatures (Schmidt & Bouma, 1992) and to predict fungal growth rates in bakery products as a function of pH, potassium sorbate concentration, and water activity (Guynot, Marin, Sanchis, & Ramos, 2005). An interesting application of a "Weibull hazard analysis" allows the prediction of the probability of spoilage during shelf life (Thiemig, Buhr, & Wolf, 1998).

The same techniques that are used to predict microbial growth and spoilage can also be used to predict microbial inactivation. The inactivation of spoilage microorganisms during food processing will improve product quality and reduce product spoilage during shelf life.

Temperature Monitoring During Commercial Distribution

The large and growing quantity of refrigerated foods being distributed and consumed around the world has led to increased demands for temperature control during distribution. Most food distributors and retailers monitor and document product temperatures, often as part of HACCP plan. As mentioned earlier, the existence of billions of refrigeration units means that, inevitably, some will not maintain adequate refrigeration temperatures. Such abuse can lead to product spoilage and potential food safety issues.

Packaging technology has advanced to the point that time–temperature indicators (TTIs) can be placed on individual cases or packages of food (Taoukis & Labuza, 1989). Some of the TTIs change color when a maximum temperature is exceeded, whereas others are calibrated to integrate time and temperature exposure, changing color when an equivalent time and temperature combination, analogous to the ASLT conditions described above, has been reached.

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Microbiological Spoilage of Dairy Products

Loralyn H. Ledenbach and Robert T. Marshall

Introduction

The wide array of available dairy foods challenges the microbiologist, engineer, and technologist to find the best ways to prevent the entry of microorganisms, destroy those that do get in along with their enzymes, and prevent the growth and activities of those that escape processing treatments. Troublesome spoilage microorganisms include aerobic psychrotrophic Gram-negative bacteria, yeasts, molds, heterofermentative lactobacilli, and spore-forming bacteria. Psychrotrophic bacteria can produce large amounts of extracellular hydrolytic enzymes, and the extent of recontamination of pasteurized fluid milk products with these bacteria is a major determinant of their shelf life. Fungal spoilage of dairy foods is manifested by the presence of a wide variety of metabolic by-products, causing off-odors and flavors, in addition to visible changes in color or texture. Coliforms, yeasts, heterofermentative lactic acid bacteria, and spore-forming bacteria can all cause gassing defects in cheeses. The rate of spoilage of many dairy foods is slowed by the application of one or more of the following treatments: reducing the pH by fermenting the lactose to lactic acid; adding acids or other approved preservatives; introducing a desirable microflora that restricts the growth of undesirable microorganisms; adding sugar or salt to reduce the water activity (a_w); removing water; packaging to limit available oxygen; and freezing. The type of spoilage microorganisms differs widely among dairy foods because of the selective effects of practices followed in production, formulation, processing, packaging, storage, distribution, and handling.

Types of Dairy Foods

The global dairy industry is impressive by large. In 2005, world milk production was estimated at 644 million tons, of which 541 million tons was cows' milk. The

L.H. Ledenbach (✉)
Kraft Foods, Inc., 801 Waukegan Road, Glenview, IL 60025, USA
e-mail: lharris@kraft.com

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leading producers of milk were the European Union at 142 million tons, India at 88 million tons, the United States at 80 million tons (20.9 billion gallons), and Russia at 31 million tons. Cheese production amounted to 8.6 million tons in Western Europe and 4.8 million tons in the United States (Anonymous, 2007; Kutzemeier, 2006). The vast array of products made from milk worldwide leads to an equally impressive array of spoilage microorganisms. A survey of dairy product consumption revealed that 6% of US consumers would eat more dairy products if they stayed fresher longer (Lempert, 2004). Products range from those that are readily spoiled by microorganisms to those that are shelf stable for many months, and the spoilage rate can be influenced by factors such as moisture content, pH, processing parameters, and temperature of storage. A short summary of the types of dairy products and typical spoilage microorganisms associated with them is shown in Table 1.

Table 1 Dairy products and typical types of spoilage microorganisms or microbial activity

Food	Spoilage microorganism or microbial activity
Raw milk	A wide variety of different microbes
Pasteurized milk	Psychrotrophs, sporeformers, microbial enzymatic degradation
Concentrated milk	Spore-forming bacteria, osmophilic fungi
Dried milk	Microbial enzymatic degradation
Butter	Psychrotrophs, enzymatic degradation
Cultured buttermilk, sour cream	Psychrotrophs, coliforms, yeasts, lactic acid bacteria
Cottage cheese	Psychrotrophs, coliforms, yeasts, molds, microbial enzymatic degradation
Yogurt, yogurt-based drinks	Yeasts
Other fermented dairy foods	Fungi, coliforms
Cream cheese, processed cheese	Fungi, spore-forming bacteria
Soft, fresh cheeses	Psychrotrophs, coliforms, fungi, lactic acid bacteria, microbial enzymatic degradation
Ripened cheeses	Fungi, lactic acid bacteria, spore-forming bacteria, microbial enzymatic degradation

Types of Spoilage Microorganisms

Psychrotrophs

Psychrotrophic microorganisms represent a substantial percentage of the bacteria in raw milk, with pseudomonads and related aerobic, Gram-negative, rod-shaped bacteria being the predominant groups. Typically, 65–70% of the psychrotrophs isolated from raw milk are *Pseudomonas* species (García, Sanz, Garcia-Collia, & Ordonez, et al., 1989; Griffiths, Phillips, & Muir, 1987). Important characteristics of pseudomonads are their abilities to grow at low temperatures (3–7°C) and to hydrolyze and use large molecules of proteins and lipids for growth. Other important psychrotrophs associated with raw milk include members of the genera *Bacillus, Micrococcus, Aerococcus,* and *Lactococcus* and of the family Enterobacteriaceae.

Pseudomonads can reduce the diacetyl content of buttermilk and sour cream (Wang & Frank, 1981), thereby leading to a "green" or yogurt-like flavor from an imbalance of the diacetyl to acetaldehyde ratio. For cottage cheese, the typical pH is marginally favorable for the growth of Gram-negative psychrotrophic bacteria (Cousin, 1982), with the pH of cottage cheese curd ranging from 4.5 to 4.7 and the pH of creamed curd being within the more favorable pH range of 5.0–5.3. The usual salt content of cottage cheese is insufficient to limit the growth of contaminating bacteria; therefore, psychrotrophs are the bacteria that normally limit the shelf life of cottage cheese. When in raw milk at cell numbers of greater than 10^6 CFU/ml, psychrotrophs can decrease the yield and quality of cheese curd (Aylward, O'Leary, & Langlois, 1980; Fairbairn & Law, 1986; Mohamed & Bassette, 1979; Nelson & Marshall, 1979).

Coliforms

Like psychrotrophs, coliforms can also reduce the diacetyl content of buttermilk and sour cream (Wang & Frank, 1981), subsequently producing a yogurt-like flavor. In cheese production, slow lactic acid production by starter cultures favors the growth and production of gas by coliform bacteria, with coliforms having short generation times under such conditions. In soft, mold-ripened cheeses, the pH increases during ripening, which increases the growth potential of coliform bacteria (Frank, 2001).

Lactic Acid Bacteria

Excessive viscosity can occur in buttermilk and sour cream from the growth of encapsulated, slime-producing lactococci. In addition, diacetyl can be reduced by diacetyl reductase produced in these products by lactococci growing at 7°C (Hogarty & Frank, 1982), resulting in a yogurt-like flavor.

Heterofermentative lactic acid bacteria such as lactobacilli and *Leuconostoc* can develop off-flavors and gas in ripened cheeses. These microbes metabolize lactose, subsequently producing lactate, acetate, ethanol, and CO_2 in approximately equimolar concentrations (Hutkins, 2001). Their growth is favored over that of homofermentative starter culture bacteria when ripening occurs at 15°C rather than 8°C (Cromie, Giles, & Dulley, 1987). When the homofermentative lactic acid bacteria fail to metabolize all of the fermentable sugar in a cheese, the heterofermentative bacteria that are often present complete the fermentation, producing gas and off-flavors, provided their populations are 10^6 CFU/g (Johnson, 2001). Residual galactose in cheese is an example of a substrate that many heterofermentative bacteria can metabolize and produce gas. Additionally, facultative lactobacilli can cometabolize citric and lactic acids and produce CO_2 (Fryer, Sharpe, & Reiter, 1970; Laleye, Simard, Lee, Holley, & Giroux, 1987). Catabolism of amino acids in cheese by nonstarter culture, naturally occurring lactobacilli, propionibacteria, and

Lactococcus lactis subsp. *lactis* can produce small amounts of gas in cheeses (Martley & Crow, 1993). Cracks in cheeses can occur when excess gas is produced by certain strains of *Streptococcus thermophilus* and *Lactobacillus helveticus* that form CO_2 and 4-aminobutyric acid by decarboxylation of glutamic acid (Zoon & Allersma, 1996).

Metabolism of tyrosine by certain lactobacilli causes a pink to brown discoloration in ripened cheeses. This reaction is dependent on the presence of oxygen at the cheese surface (Shannon, Olson, & Deibel, 1977). The racemic mixture of L(+) and D(−)-lactic acids that forms a white crystalline material on surfaces of Cheddar and Colby cheeses is produced by the combined growth of starter culture lactococci and nonstarter culture lactic acid producers. The latter racemize the L(+) form of the acid to the L(−) form, which form crystals (Johnson, 2001).

Fungi

Yeasts can grow well at the low pH of cultured products such as in buttermilk and sour cream and can produce off-flavors described as fermented or yeasty. Additionally, yeasts can metabolize diacetyl in these products (Wang & Frank, 1981), thereby leading to a yogurt-like flavor. Contamination of cottage cheese with the common yeast *Geotrichum candidum* often results in a decrease of diacetyl content. *Geotrichum candidum* reduced by 52–56% diacetyl concentrations in low-fat cottage cheese after 15–19 days of storage at 4–7°C (Antinone & Ledford, 1993).

Yeasts are a major cause of spoilage of yogurt and fermented milks in which the low pH provides a selective environment for their growth (Fleet, 1990; Rohm, Eliskasses, & Bräuer, 1992). Yogurts produced under conditions of good manufacturing practices should contain no more than 10 yeast cells and should have a shelf life of 3–4 weeks at 5°C. However, yogurts having initial counts of >100 CFU/g tend to spoil quickly. Yeasty and fermented off-flavors and gassy appearance are often detected when yeasts grow to 10^5–10^6 CFU/g. Giudici, Masini, and Caggia (1996) studied the role of galactose in the spoilage of yogurt by yeasts and concluded that galactose, which results from lactose hydrolysis by the lactic starter cultures, was fermented by galactose-positive strains of yeasts such as *Saccharomyces cerevisiae* and *Hansenula anomala*.

The low pH and the nutritional profile of most cheeses are favorable for the growth of spoilage yeasts. Surface moisture, often containing lactic acid, peptides, and amino acids, favors rapid growth. Many yeasts produce alcohol and CO_2, resulting in cheese that tastes yeasty (Horwood, Stark, & Hull, 1987). Packages of cheese packed under vacuum or in modified atmospheres can bulge as a result of the large amount of CO_2 produced by yeast (Vivier, Rivemale, Reverbel, Ratomahenina, & Galzy, 1994). Lipolysis produces short-chain fatty acids that combine with ethanol to form fruity esters. Some proteolytic yeast strains produce sulfides, resulting in an egg odor. Common contaminating yeasts of cheeses include *Candida*

spp., *Kluyveromyces marxianus*, *Geotrichum candidum*, *Debaryomyces hansenii*, and *Pichia* spp. (Johnson, 2001).

Molds can grow well on the surfaces of cheeses when oxygen is present, with the low pH being selective for them. In packaged cheeses, mold growth is limited by oxygen availability, but some molds can grow under low oxygen tension. Molds commonly found growing in vacuum-packaged cheeses include *Penicillium* spp. and *Cladosporium* spp. (Hocking & Faedo, 1992). *Penicillium* is the mold genus most frequently occurring on cheeses. A serious problem with mold spoilage of sorbate-containing cheeses is the degradation of sorbic acid and potassium sorbate to *trans*-1,3-pentadiene, causing an off-odor and flavor described as "kerosene." Several fungal species, including *Penicillium roqueforti*, are capable of metabolizing this compound from sorbates. Marth, Capp, Hasenzahl, Jackson, and Hussong (1966), who was the first group to study this problem, determined that cheese-spoilage isolates of *Penicillium* spp. were resistant to up to 7,100 ppm of potassium sorbate. Later, Sensidoni, Rondinini, Peressini, Maifreni, and Bortolomeazzi (1994) isolated from Crescenza and Provolone cheeses sorbate-resistant strains of *Paecilomyces variotii* and *D. hansenii* (a yeast) that produced *trans*-1,3-pentadiene, causing off-flavors in those products.

Cream cheeses are susceptible to spoilage by heat-resistant molds such as *Byssochlamys nivea* (Pitt & Hocking, 1999). *Byssochlamys nivea* is capable of growing in reduced oxygen atmospheres, including in atmospheres containing 20, 40, and 60% carbon dioxide with less than 0.5% oxygen (Taniwaki, 1995). Once this mold is present in the milk supply, it can be difficult to eliminate during normal processing of cream cheese. Engel and Teuber (1991) studied the heat resistance of various strains of *B. nivea* ascospores in milk and cream and determined a *D*-value of 1.3–2.4 s at 92°C, depending on the strain. They calculated that in a worst-case scenario of 50 ascospores of the most heat-resistant strain per liter of milk, a process of 24 s at 92°C would result in a 1% spoilage rate in packages of cream cheese.

Spore-Forming Bacteria

Raw milk is the usual source of spore-forming bacteria in finished dairy products. Their numbers before pasteurization seldom exceed 5,000/ml (Mikolajcik & Simon, 1978); however, they can also contaminate milk after processing (Griffiths & Phillips, 1990). The most common spore-forming bacteria found in dairy products are *Bacillus licheniformis, B. cereus, B. subtilis, B. mycoides,*and *B. megaterium.*In one study, psychrotrophic *B. cereus* was isolated in more than 80% of raw milks sampled (Meer, Baker, Bodyfelt, & Griffiths, 1991). The heat of pasteurization activates (heat shock) many of the surviving spores so that they are primed to germinate at a favorable growth temperature (Cromie, Schmidt, & Dommett, 1989). Coagulation of the casein of milk by chymosin-like proteases produced by many of these bacilli occurs at a relatively high pH (Choudhery & Mikolajcik, 1971). Cromie

et al. (1989) reported that lactose-fermenting *B. circulans* was the dominant spoilage microbe in aseptically packaged pasteurized milk. *Bacillus stearothermophilus* can survive ultra-high-temperature treatment of milk (Muir, 1989). This bacterium produces acid but no gas, hence causing the "flat sour" defect in canned milk products (Kalogridou-Vassiliadou, 1992).

If extensive proteolysis occurs during aging of ripened cheeses, the release of amino acids and concomitant increase in pH favors the growth of clostridia, especially *Clostridium tyrobutyricum,* and the production of gas and butyric acid (Klijn, Nieuwendorf, Hoolwerf, van der Waals, & Weerkamp, 1995). Spores are concentrated in cheese curd, so as few as one spore per milliliter of milk can cause gassiness in some cheeses (Myhara & Skura, 1990). Spore numbers of more than 25/ml were required to produce this defect in large wheels of rindless Swiss cheese (Dasgupta & Hull, 1989). Cheeses most often affected, e.g., Swiss, Emmental, Gouda, and Edam, have a relatively high pH and moisture content, and low salt content. An example of gassing caused by *C. tyrobutyricum* in Swiss cheese is shown in Fig. 1.

Fig. 1 Gassy Swiss cheese caused by *Clostridium tyrobutyricum*. L. H. Ledenbach photo

Occasionally, gassy defects of process cheeses are also caused by *C. butyricum* or *C. sporogenes.* These spores are not completely inactivated by the normal cooking treatment of process cheeses. Therefore, they may germinate and produce gas unless their numbers are low, the pH is not higher than 5.8, the salt concentration is at least 6% of the serum, and the cheese is held at 20°C or lower (Kosikowski & Mistry, 1997). The products of fermentation in these cheeses are butyric and acetic acids, carbon dioxide, and hydrogen. A summary of known causes of gassiness in cheese products is shown in Table 2.

Thermoduric and thermophilic spore-forming bacteria are the common causes of spoilage of concentrated milks. They survive pasteurization and the extended high temperatures of evaporative removal of moisture to increase the milk solid content to 25.5–45%. When these foods are contaminated, the survivors are heat-resistant *Bacillus* spp. (Kalogridou-Vassiliadou, 1992).

Table 2 Causes of gassiness in different types of cheese

Organism	Cheese affected	Time to defect
Coliforms	Raw milk pasta filata cheese	Early blowing
Yeasts	Raw milk Domiati (Egyptian), Camembert, blue-veined, Feta	Early blowing
Lactobacillus fermentum	Provolone, mozzarella	Late blowing
Heterofermentative Lactobacilli	Cheddar, Gouda, Saint Paulin, Oka	Late blowing
Propionibacteria	Sbrinz (Argentinean)	Late blowing
Clostridium tyrobutyricum	Gouda, Emmental, Swiss, Cheddar, Grana	Late blowing
Eubacterium sp.	Cheddar	Late blowing

Sources: Bottazzi and Corradini (1987); Dennien (1980); El-Shibiny, Tawfik, Sharaf, and El-Khamy (1988); Font de Valdez, Savoy de Giori, Ruiz Holgado, and de Oliver (1984); Johnson (2001); Klijn et al. (1995); Laleye et al. (1987); Myhr et al. (1982); Melilli et al. (2004); Roostita & Fleet (1996); Vivier et al. (1994)

Other Microorganisms

Eubacterium sp., a facultative anaerobe that is able to grow at pH 5.0–5.5 in the presence of 9.5% salt (Myhr, Irvine, & Arora, 1982), can cause gassiness in Cheddar cheese. An unusual white-spot defect caused by a thermoduric *Enterococcus faecalis* subsp. *liquefaciens* has occurred in Swiss cheese. This bacterium is inhibitory to propionibacteria and *Lactobacillus fermentum*, resulting in poor eye development and lack of flavor in the cheese as well (Nath & Kostak, 1985).

Enzymatic Degradation

An indirect cause of dairy product spoilage is microbial enzymes, such as proteases, phospholipases, and lipases, some of which may remain active in the food after the enzyme-producing microbes have been destroyed. Populations of psychrotrophs ranging from 10^6 to 10^7 CFU/ml can produce sufficient amounts of extracellular enzymes to cause defects in milk that are detectable by sensory tests (Fairbairn & Law, 1987). Adams, Barach, and Speck (1975) reported that 70–90% of raw milk samples tested contained psychrotrophic bacteria capable of producing proteinases that were active after heating at 149°C (300°F) for 10 s. Others have verified this observation (Griffiths, Phillips, & Muir, 1981).

Extracellular proteases can affect the quality of milk products in various ways, but largely by producing bitter peptides. Thermally resistant proteases have caused spoilage of ultra-high-temperature (UHT) milk (Shah, 1994; Sørhaug & Stepaniak, 1991). In addition, phospholipases can be heat stable. Experimentally, phospholipase production in raw milk can result in the development of bitter off-flavors due to the release of fatty acids by milk's natural lipase (Fox, Chrisope, &

Marshall, 1976; Chrisope & Marshall, 1976). Heat-stable bacterial lipases have been associated with the development of rancid flavors in UHT milk (Adams & Brawley, 1981). *Pseudomonas fluorescens* is the most common producer of lipases in milk and milk products, but lipases can also be produced by Gram-negative psychrotrophic bacteria. Products that may be affected by residual lipases include UHT milk, butter, some cheeses, and dry whole milk. The release of short-chain fatty acids, C4 through C8, results in the occurrence of rancid flavors and odors, whereas the release of long-chain fatty acids results in a soapy flavor. Oxidation of free unsaturated fatty acids to aldehydes and ketones results in an oxidized flavor (Deeth & Fitz-Gerald, 1983), and fruity off-flavor results from lipolysis of short-chain fatty acids by *Pseudomonas fragi* followed by esterification with alcohols (Reddy, Bills, Lindsey, & Libbey, 1968).

Lipase tends to partition into cream instead of the nonfat milk portion when cream is separated from milk (Downey, 1980; Stead, 1986). The large concentration of fat globules and the activation of lipase caused by some disruption of the fat globule membrane increase the probability of enzyme–substrate interactions. In the production of butter, lipolysis can cause excessive foaming during churning of cream (Deeth & Fitz-Gerald, 1983), hence increasing the time of churning. Rancidity of butter may result from the activity of lipase in the raw milk or the residual heat-stable microbial lipase in the finished butter. Although short-chain fatty acids from rancid cream, being water-soluble, are partially lost in the buttermilk and wash water during manufacture (Stead, 1986), microbial lipases remaining in the butter can hydrolyze the fat even during frozen storage (Nashif & Nelson, 1953). Low pH limits the rate of lipase activity, but in some cheeses, e.g., Brie and Camembert, the pH rises to near neutrality as ripening progresses, making them especially susceptible to lipolysis (Dumont, Delespaul, Miquot, & Adda, 1977). For Cheddar cheese, however, a high concentration of lipase is needed to create the desired flavor (Law, Sharpe, & Chapman, 1976). Products such as whole milk powder may be affected by residual heat-resistant bacterial lipases. Residual lipases in nonfat dry milk and dry whey products can hydrolyze fats in products into which they are added as ingredients (Stead, 1986).

Sources of Spoilage Microorganisms

Contamination of Raw Milk

The highly nutritious nature of dairy products makes them especially good media for the growth of microorganisms. Milk contains abundant water and nutrients and has a nearly neutral pH. The major sugar, lactose, is not utilized by many types of bacteria, and the proteins and lipids must be broken down by enzymes to allow sustained microbial growth. In order to understand the source of many of the spoilage microflora of dairy products, it is best to discuss how milk can first become contaminated, via the conditions of production and processing.

The mammary glands of many very young cows yield no bacteria in aseptically collected milk samples, but as numbers of milkings increase, so do the chances of isolating bacteria in milk drawn aseptically from the teats. The stresses placed on the cow's teats and mammary glands by the very large amounts of milk produced and the actions of the milking machine cause teat canals to become more open and teat ends to become misshapen as time passes (Fig. 2). These stresses may open the teat canal for the entry of bacteria capable of infecting the glands.

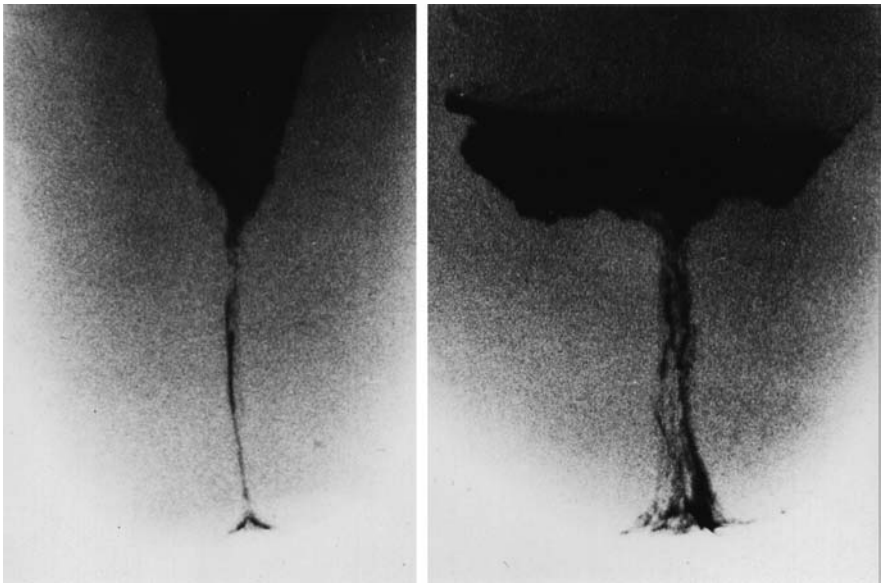

Fig. 2 X-ray photographs showing an increase in the diameter of the teat canal of the same teat of a milking cow between the first lactation (*left*) and a later lactation (*right*). Courtesy Dr. J. S. McDonald, National Animal Disease Laboratory, U. S. Department of Agriculture, Ames, Iowa

Environmental contaminants represent a significant percentage of spoilage microflora. They are ubiquitous in the environment from which they contaminate the cow, equipment, water, and milkers' hands. Since milking machines exert about 38 cm (15 in.) of vacuum on the teats during milking, and since air often leaks into the system, bacteria on the surfaces of the cow or in water retained from premilking preparation can be drawn into the milk. Also, when inflation clusters drop to the floor, they pick up microorganisms that can be drawn into the milk. The pumping or agitation of milk supplies the oxygen needed by aerobes for growth and breaks chains and clumps of bacteria. Single cells, having less competition than those in colonies, have the opportunity for more rapid multiplication. Bacteria recontaminating pasteurized milk originate primarily from water and air in the filling equipment or immediate surroundings and can be resident for prolonged periods of time (Eneroth, Ahrne, & Molin, 2000). In a study performed in Norway and Sweden, Ternstrom, Lindberg, and Molin (1993) investigated nine dairy plants and found that five taxa of psychrotrophic *Pseudomonas* spp. were involved in the

spoilage of raw and pasteurized milk and that the same strains were recovered from both the raw and pasteurized milk, suggesting that recontamination originated from the raw milk. Additionally, the investigators found that *Bacillus* spp. (mainly *B. cereus* and *B. polymyxa*) were responsible for spoilage in 77% of the samples that had been spoiled by Gram-positive bacteria. The spoilage *Bacillus* spp. grew fermentatively, and most were able to denitrify the milk, which has implications for cheeses that contain added nitrate/nitrites for protection against clostridia. Sporeforming bacteria are abundant in dust, dairy feed concentrates, and forages; therefore, they are often present on the skin and hair of cattle from which they can enter milk. The presence of sporeformers such as *C. butyricum* in milk has been traced to contaminated silage (Dasgupta & Hull, 1989).

Contamination of Dairy Products

Washed curd types of cheeses are especially susceptible to growth of coliforms (Frank, Marth, & Olson, 1978), so great care must be taken to monitor the quality of water used in these processes. A high incidence of contamination of brine-salted cheeses by yeasts results from their presence in the brines (Kaminarides & Lakos, 1992). Many mold species are particularly well adapted to the cheese-making environment and can be difficult to eradicate from a production facility. Fungi causing a "thread mold" defect in Cheddar cheeses (Hocking & Faedo, 1992) were found in the cheese factory environment, on cheese-making equipment, in air, and in curd and whey. In a study of cheese-making facilities in Denmark, *Penicillium commune* persisted in the cheese coating and unpacking areas over a 7-year period (Lund, Bech Nielsen, & Skouboe, 2003). Ascospores of *B. nivea* and other heat-resistant species shown to be able to survive pasteurization, such as *Talaromyces avellaneus*, *Neosartorya fischeri* var. *spinosa*, and *Eupenicillium brefeldianum*, have also been found in raw milk (Pitt & Hocking, 1999).

A major cause of failure of processing and packaging systems is the development of biofilms on equipment surfaces. These communities of microorganisms develop when nutrients and water remain on surfaces between times of cleaning and reuse. Bacteria in biofilms (sessile form) are more resistant to chemical sanitizers than are the same bacteria in suspension (planktonic form) (Mosteller & Bishop, 1993). Chemical sanitizers may be rendered ineffective by biofilms leaving viable bacteria to be dislodged into the milk product (Frank & Koffi, 1990).

Factors Affecting Spoilage

Spoilage of Fluid Milk Products

The shelf life of pasteurized milk can be affected by large numbers of somatic cells in raw milk. Increased somatic cell numbers are positively correlated with

concentrations of plasmin, a heat-stable protease, and of lipoprotein lipase in freshly produced milk (Barbano, Ma, & Santos, 2005). Activities of these enzymes can supplement those of bacterial hydrolases, hence shortening the time to spoilage. The major determinants of quantities of these enzymes in the milk supply are the initial cell numbers of psychrotrophic bacteria, their generation times, their abilities to produce specific enzymes, and the time and temperature at which the milk is stored before processing. Several conditions must exist for lipolyzed flavor to develop from residual lipases in processed dairy foods, that is, large numbers ($>10^6$ CFU/ml) of lipase producers (Stead, 1986), stability of the enzyme to the thermal process, long-term storage and favorable conditions of temperature, pH, and water activity.

Spoilage of Cheeses

Factors that determine the rates of spoilage of cheeses are water activity, pH, salt to moisture ratio, temperature, characteristics of the lactic starter culture, types and viability of contaminating microorganisms, and characteristics and quantities of residual enzymes. With so many variables to affect deteriorative reactions, it is no surprise that cheeses vary widely in spoilage characteristics. Soft or unripened cheeses, which generally have the highest pH values, along with the lowest salt to moisture ratios, spoil most quickly. In contrast, aged, ripened cheeses retain their desirable eating qualities for long periods because of their comparatively low pH, low water activity, and low redox potential.

For fresh, raw milk pasta filata cheeses, Melilli et al. (2004) determined that low initial salt and higher brining temperature (18°C) allowed for greater growth of coliforms, which caused gas formation in the cheese. Factors affecting the growth of the spoilage microorganisms, *Enterobacter agglomerans* and *Pseudomonas* spp. in cottage cheese, were higher pH and storage temperature of the cheese (Brocklehurst & Lund, 1988). Some of the spoilage microorganisms were able to grow at relatively low pH values (4.6–4.7) when incubated at 7°C and were able to grow at pH 3.6 when grown in media at 20°C. Rate of salt penetration into brined cheeses, types of starter cultures used, initial load of spores in the milk used for production, pH of the cheese, and ripening temperature affect the rate of butyric acid fermentation and gas production by *C. tyrobutyricum* (Stadhouders, 1990c). Fungal growth in packaged cheeses was found to be most significantly affected by the concentration of CO_2 in the package and the water activity of the cheese (Nielsen & Haasum, 1997). Cheddar cheese exhibiting yeast spoilage had a high moisture level (39.1%) and a low salt in the moisture-phase value (3.95%) (Horwood et al., 1987). Roostita and Fleet (1996) determined that the properties of yeasts that affected the spoilage rate of Camembert and blue-veined cheeses were the abilities to ferment/assimilate lactose, produce extracellular lipolytic and proteolytic enzymes, utilize lactic and citric acid, and grow at 10°C.

Prevention and Control Measures

Prevention of Spoilage in Milk

In the early days of development of the commercial dairy industry, milk was produced under much less sanitary conditions than are used today, and cooling was slow and inadequate to restrict bacterial growth. Developments during the first half of the twentieth century created significant reductions in the rate of spoilage of raw milk and cream, by making it possible for every-other-day pickup of milk from farms and shipments of raw milk over long distances with minimal increases in bacterial cell numbers. Rapid cooling and quick use of raw milk are accepted as best practices and can affect the spoilage ability of *Pseudomonas* spp. present in milk. Pseudomonads that had been incubated in raw milk for 3 days at 7°C (44.6°F) had greater growth rates and greater proteolytic and lipolytic activity than those isolated directly from the milk shortly after milking (Jaspe, Oviedo, Fernandez, Palacios, & Sanjose,1995).

As the quality of raw milk improved, so did that of pasteurized milk. Heating of milk to 62.8°C (145°F) for 30 min or to 71.7°C (161°F) for 15 s kills the pathogenic bacteria likely to be of significance in milk as well as most of the spoilage bacteria. However, processors learned that long shelf life of pasteurized fluid milk products requires a higher temperature treatment as well as prevention of contamination between the pasteurizer and the sealed package. In particular, it is imperative that filling equipment be sanitary and that the air in contact with the filler, the milk, and the containers be practically sterile. Whereas in the early to mid-twentieth century, milk was delivered daily to homes because of its short shelf life, today's fluid milk products are generally expected to remain acceptable for 14–21 days. Pasteurization standards for several countries are listed in Table 3.

A shelf life of 21 days and beyond can be attained with fluid milk products that have been heated sufficiently to kill virtually all of the vegetative bacterial cells and protected from recontamination. Ultra-pasteurized milk products, heated at or above 138°C for at least 2 s, that have been packaged aseptically can have several weeks of shelf life when stored refrigerated. Ultra-high-temperature (UHT) treatment destroys most spores in milk, but *B. stearothermophilus* can survive. Aseptic processing, as defined in the Grade A Pasteurized Milk Ordinance (2003), means that the product has been subjected to sufficient heat processing to render it commercially sterile and that it has been packaged in a hermetically sealed container. These dairy foods are stable at room temperature.

The addition of carbon dioxide to milk and milk products reduces the rates of growth of many bacteria (Dixon & Kell, 1989). King and Mabbitt (1982) demonstrated improved keeping quality of raw milk by the addition of CO_2. Loss and Hotchkiss (2002) found lowered survivor rates of both *P. fluorescens* and the spores of *B. cereus* during heating of milk containing up to 36 mM CO_2. McCarney, Mullen, and Rowe (1995) determined that carbonation may be a desirable treatment for cheese milk when on the day of collection populations of psychrotrophic

Table 3 Dairy product heat treatment standards in different countries

Treatment	Temperature	Time
United States[a]		
Pasteurization of milk	63°C/145°F	30 min*
	72°C/161°F	15 s*
Ultra-pasteurization of milk	138°C/280°F	2 s
Ultra-high temperature (UHT)-treated milk	140–150°C/ 284–302°F	Few seconds
*If fat content >10% or contains sweeteners, increase the temperature by 3°C/5°F		
Product	Temperature	Time
Australia[b]		
Pasteurization of milk and liquid milk products (includes milk used for production of cream/cream products, fermented milks, yogurt, dried, condensed, and evaporated milks, butter, and ice cream)	72°C/162°F	15 s
Pasteurization of milk for cheese production	72°C/162°F	15 s
	62°C/144°F	15 s*
*__and__ cheese is stored at ≥2°C/36°F for 90 days prior to sale __or__ curd is heated to ≥48°C/119°F and moisture is ≤36% after storage at ≥10°C/50°F for ≥6 months prior to sale		
European Union[c]		
Raw milk and raw milk for production of dairy products	Milk is not heated beyond 40°C/104°F	
Thermized milk and thermized milk for production of dairy products	57–68°C/135–155°F	≥15 s
Pasteurization of milk	71.7°C/161.1°F	15 s
UHT-treated milk	>135°C/275°F	>1 s

[a]Source: USPHS/FDA Pasteurized Milk Ordinance, 2003
[b]Source: Australia Food Code Standard 1.6.2, 2001
[c]Source: EU Council Directive 92/46/EEC, 1992

bacteria are approximately 10^5 CFU/ml. Rajagopal, Werner, and Hotchkiss (2005) demonstrated that treatment with CO_2 at a pressure of 689 kPa and temperature of 6.1°C produced a substantial decrease in bacterial counts, resulting in milk that was within the grade A raw milk limits for up to 8 days of storage. A disadvantage can be that an acidic flavor note may be produced in a CO_2-treated milk product. When CO_2 is dissolved in milk, the pH decreases (Ma, Barbano, Hotchkiss, Murphy, & Lynch, 2001) and does not return to the original pH value following the removal of CO_2 before pasteurization (Ruas-Madiedo, Bascaran, Brana, Bada-Gancedo, & Reyes-Gavilan, 1998).

High hydrostatic pressure treatments of milk are effective in killing vegetative bacterial cells, but spores are mostly refractory to this treatment (McClements, Patterson, & Linton, 2001). The phase of growth of the bacteria and the temper-

ature of incubation are significant variables affecting the sensitivities of bacterial cells to high pressures. Cells in the stationary phase are more resistant than those in the exponential phase of growth. Survivor curves have shown resistant tailing populations (McClements et al., 2001; Metrick, Hoover, & Farkas, 1989). Other alternative treatments for the pasteurization of milk, such as ohmic heating, microwave heating, UV radiation, electron beam irradiation, pulsed electric fields, infrared processing, and high voltage arc discharge, may have the potential to be used alone or in combination with other treatments. However, all pasteurization processes need to be validated through the combined use of process authorities, challenge studies, and predictive modeling, and must be verified to ensure that critical processing limits are achieved (NACMCF, 2006).

Prevention of Spoilage in Cultured Dairy Products

Cultured products such as buttermilk and sour cream depend on a combination of lactic acid producers, the lactococci, and the leuconostocs (diacetyl producers), to produce the desired flavor profile. Imbalance of the culture, improper temperature or ripening time, infection of the culture with bacteriophage, presence of inhibitors, and/or microbial contamination can lead to an unsatisfactory product. A buttery flavor note is produced by *Leuconostoc mesenteroides* subsp. *cremoris*. This bacterium converts acetaldehyde to diacetyl, thus reducing the "green" or yogurt-like flavor (Lindsey & Day, 1965). A diacetyl to acetaldehyde ratio of 4:1 is desirable, whereas the green flavor is present when the ratio is 3:1 or less. Proteolysis by the lactococci is necessary to afford growth of the *Leuconostoc* culture, and citrate is needed as substrate for diacetyl production.

Although cooking of the curd destroys virtually all bacteria capable of spoiling cottage cheese, washing and handling of the curd after cooking can introduce substantial numbers of spoilage microorganisms. It is desirable to acidify alkaline waters for washing cottage cheese curd to prevent solubilization of surfaces of the curd. However, more pseudomonads can be adsorbed onto cottage cheese curd from wash water when adjusted to pH 5 (40–45%) rather than adjusted to pH 7 (20–30%) (Wellmeyer & Marshall, 1972). Flushing packages of cottage cheese or sour cream with CO_2 or N_2 suppressed the growth of psychrotrophic bacteria, yeasts, and molds for up to 112 days, but a slight bitterness can occur in cottage cheese after 73 days of storage (Kosikowski & Brown, 1973).

Cheesemakers can use the addition of high numbers of lactic acid bacteria to raw milk during storage to reduce the rate of growth of psychrotrophic microbes. For fresh, raw milk, brined cheeses, gassing defects can be reduced by presalting the curd prior to brining and reducing the brine temperature to <12°C (Melilli et al., 2004). Pasteurization will eliminate the risk from most psychrotrophic microbes, coliforms, leuconostocs, and many lactobacilli, so cheeses made from pasteurized milk have a low risk of gassiness produced by these microorganisms. Most bacterial cells, including spores, can be removed from milk by centrifugation at

about 9,000*g*. The process, known as bactofugation, removes about 3% of the milk, called bactofugate. Kosikowski and Mistry (1997) invented and patented a process for recovering this bactofugate which is heated at 135°C for 3–4 s, then added back to the cheese milk. The process can reduce the population of butyric acid-producing spores by 98% (Daamen, van den Berg, & Stadhouders, 1986). Spore-forming bacterial growth and subsequent gas production in aged, ripened cheeses can be minimized with a salt to moisture content of ≥3.0% (Stadhouders, 1990c). Other potential inhibitors of butyric acid fermentation and gas production in cheese are the addition of nitrate (Stadhouders, 1990b), addition of lysozyme (Lodi, 1990), cold storage of cheese prior to ripening, direct salt addition to the cheese curd, addition of hydrogen peroxide, or use of starter cultures that form nisin or other antimicrobials (Stadhouders, 1990a).

The most popular mold inhibitors used on cheeses are sorbates and natamycin. Sorbates tend to diffuse into the cheese, thereby modifying flavor and decreasing their concentration, whereas very little natamycin diffuses (de Ruig & van den Berg, 1985). Electron beam irradiation, studied by Blank, Shamsuzzaman, and Sohal (1992) for mold decontamination of Cheddar cheese, can reduce initial populations of *Aspergillus ochraceus* and *Penicillium cyclopium* by 90% with average doses of 0.21 and 0.42 kGy, respectively. Since nearly all mold spores are killed by pasteurization (Doyle & Marth, 1975), practices that limit recontamination and growth, although difficult, are vital in prevention of moldy cheeses. Modified atmosphere packaging (MAP) of cheeses can retard or prevent the growth of molds, and optimum MAP conditions for different types of cheeses were described by Nielsen and Haasum (1997). For processed cheeses containing no active lactic acid starter bacteria, low O_2 and high CO_2 atmospheres were optimum; for cheeses containing active starter cultures, atmospheres containing low O_2 and controlled CO_2 using a permeable film provided the best results. For mold-ripened cheeses requiring the activity of the fungi to maintain good quality, normal O_2 and high, but controlled, CO_2 atmospheres were best. In Italian soft cheeses such as Stracchino, vacuum packaging decreased the growth of yeasts, resulting in a shelf life extension of >28 days (Sarais, Piussi, Aquili, & Stecchini, 1996).

Processing times and temperatures used in the manufacture of cream cheese and pasteurized process cheese are able to eliminate most spoilage microorganisms from these products. However, the benefit of the presence of competitive microflora is also lost. It is very important to limit the potential for recontamination, as products that do not contain antimycotics can readily support the growth of yeasts and molds. Sorbates can be added; however, their use in cream cheese is limited to amounts that will not affect the delicate flavor.

Prevention of Spoilage in Other Dairy Products

The high salt concentration in the serum-in-lipid emulsion of butter limits the growth of contaminating bacteria to the small amount of nutrients trapped within

the droplets that contain the microbes. However, psychrotrophic bacteria can grow and produce lipases in refrigerated salted butter if the moisture and salt are not evenly distributed (Deeth & Fitz-Gerald, 1983). When used in the bulk form, concentrated (condensed) milk must be kept refrigerated until used. It can be preserved by addition of about 44% sucrose and/or glucose to lower the water activity below that at which viable spores will germinate (a_w 0.95) (Jay, 1996). Lactose, which constitutes about 53% of the nonfat milk solids, contributes to the lowered water activity. When canned as evaporated milk or sweetened condensed milk, these products are commercially sterilized in the cans, and spoilage seldom occurs. Microbial growth and enzyme activity are prevented by freezing. Therefore, microbial degradation of frozen desserts occurs only in the ingredients used or in the mixes prior to freezing.

Methods for Detection and Isolation

It has been a long-standing practice to use microbiological standards for indicator microorganisms as a predictor of the safety and quality of dairy products, and many countries have regulations or guidelines for these microbes (Table 4). While these tests can be useful as a general indication of the cleanliness of the dairy processing operation, they may not necessarily correlate with the shelf life of the products. Boor, Carey, Murphy, and Zadoks (2005) reported results of audits of pasteurized milk quality collected from 23 plants in New York State over a 10-year period. On an annual basis, the percentage of samples that met the Grade A Pasteurized Milk Ordinance Standard Plate Count limit of 20,000 CFU/ml after 14 days of storage at 6.1°C ranged from 12 to 32%. Tests for coliform bacteria were positive for 5–15% of the samples on initial testing and increased up to 34% after 14 days of storage. Sensory tests on the 14th day of storage revealed that 33–59% of the milks were still acceptable. After about 17 days of storage, the dominant spoilage bacteria belonged to the spore-forming genera *Paenibacillus* (39%) and *Bacillus*(32%) and to heat-tolerant *Microbacterium lacticum* (14%).

As an outgrowth of the efforts in the early twentieth century to improve the safety and quality of milk products, the American Public Health Association standardized the methods for detection of spoilage indicators and published them in the Standard Methods for the Examination of Dairy Products (Marshall, 2001). Recommended methods for various microorganisms are listed in Table 5. Common tests in use today for the prediction of shelf life of fluid milk products use a preliminary incubation or keeping quality step followed by standard microbiological testing. These methods are designed to determine low levels of thermoduric Gram-negative bacteria, such as psychrotrophic coliforms and pseudomonads, that have survived pasteurization and are most likely to grow under typical storage conditions. The recommended methods have the disadvantage of taking several days to complete. There is a vast array of rapid test methods available for use (Entis et al., 2003) in dairy product testing. The preferred method for assaying for specific spoilage

Table 4 Regulatory standards for indicator organisms in different countries

Product	Test	Limits			
		n	c	m	M
United States[a]					
Grade A raw milk and milk products for further processing	Standard plate count (SPC)	100,000/ml max individual bulk tank 300,000/ml max commingled milk			
	Somatic cell count (SCC)	750,000/ml max individual bulk tank (1,000,000/ml max. goat's milk)			
Grade A pasteurized milk and milk products	SPC	20,000/ml max.			
	Coliforms	10/ml max. (100/ml max. bulk)			
Grade A aseptically packaged dairy products		No growth			
Grade A nonfat dry milk	SPC	30,000/gm max.			
	Coliforms	10/gm max.			
Grade A condensed whey and whey products, dry whey and whey products, dry buttermilk and buttermilk products	Coliforms	10/gm max.			
European Union[b]					
Raw cow's milk for production of heat-treated drinking milk, fermented milk, junket, jellied or flavored milk and cream	Aerobic plate count (APC)	100,000/ml max.			
	SCC	400,000/ml max.			
Raw cow's milk for manufacture of milk-based products other than above	APC	400,000/ml max.			
	SCC	500,000/ml max.			
Raw buffalo's milk for manufacture of milk-based products	APC	1,000,000/ml max.			
	SCC	500,000/ml max.			
Raw buffalo's milk for "product with raw milk" not involving further heat treatment	APC	500,000/ml max.			
	SCC	400,000/ml max.			

(Continued)

Table 4 (Continued)

Product	Test	Limits			
		n	c	m	M
Raw goat and sheep's milk for manufacture of milk-based products not involving heat treatment	APC	1,000,000/ml max.			
Raw goat and sheep's milk for "products with raw milk" not involving heat treatment	APC	500,000/ml max.			
Raw cow's milk for drinking	APC	50,000/ml max.			
Pasteurized milk	APC at 21°C (after incubation for 5 days at 6°C)	5	2	50,000	500,000
	Enterobacteriaceae	5	2	0	5
Liquid milk-based products	Enterobacteriaceae	5	2	0	5
Butter made from pasteurized milk or cream	Enterobacteriaceae	5	2	0	10
Soft cheese made from heat-treated milk	Enterobacteriaceae	5	2	10,000	100,000
Powdered milk-based products	Enterobacteriaceae	5	2	10	100
Liquid heat-treated unfermented milk-based products	APC at 21°C (after incubation for 5 days at 6°C)	5	2	50,000	100,000
Frozen milk-based products, including ice cream	APC	5	2	100,000	500,000
	Enterobacteriaceae	5	2	10	100
UHT milk	APC at 30°C (after incubation for 30 days at 30°C)				10/0.1 ml max.

Table 4 (Continued)

Australia/New Zealand[c]					
Unpasteurized milk	SPC	5	1	25,000	250,000
	Coliforms	5	1	100	1,000
	E. coli	5	1	3	9
Pasteurized milk, cream	SPC	5	1	50,000	100,000
	Coliforms	5	1	1	10
	Psychrotrophs	5	1	10	100
Butter from unpasteurized milk and/or unpasteurized milk products	SPC	5	0	500,000	
	Coliforms	5	1	10	100
	E. coli	5	1	3	9
Pasteurized butter – salted and unsalted	SPC	5	1	50,000	100,000
	Coliforms	5	1	10	100
	Psychrotrophs	5	1	10	100
Yogurt and other fermented milk products	Coliforms	5	2	10	100
	E. coli	5	0	0	
All cheeses	*E. coli*	5	1	10	100
Ice cream and edible ices	SPC	5	2	10,000	50,000
	Coliforms	5	2	10	100
	E. coli	5	0	0	
Dried milk powder	SPC	5	2	50,000	200,000
	Coliforms	5	2	10	100
UHT/Sterilized milk		Commercially sterile			

[a]Source: USPHS/FDA Pasteurized Milk Ordinance, 2003
[b]Source: Australia/New Zealand Food Standards Code-Microbiological Limits for Foods-Standard 1.6.1, 2001
[c]Source: EU Council Directive 92/46/EEC, 1992

Table 5 Recommended methods for testing of dairy products (Entis, et al., 2003; Richter & Vedamuthu, 2003)

Product	Property	Method – Reference
Raw milk	General quality	Direct microscopic count – SMEDP[a]
		Direct microscopic somatic cell count – SMEDP
		Electronic somatic cell count – SMEDP
	Shelf life	Preliminary incubation – SMEDP
	Microorganism counts	Standard plate count – SMEDP
		Thermoduric count – SMEDP
		Coliform count – SMEDP
		Psychrotrophic count – SMEDP
Pasteurized milk	Shelf life	Preliminary incubation – SMEDP
		Mosley keeping quality – SMEDP
	Microorganism counts	Standard plate count – SMEDP
		Coliform count – SMEDP
		Psychrotrophic count – SMEDP
Dried milk products	Microorganism counts	Standard plate count – SMEDP
		Coliform count – SMEDP
		Direct microscopic clump count – SMEDP
		Thermoduric count – SMEDP
		Psychrotrophic count – SMEDP
		Yeast and mold count – SMEDP
Butter products	Microorganism count	Standard plate count – SMEDP
		Coliform count – SMEDP
		Lipolytic count – SMEDP
		Proteolytic count – SMEDP
		Psychrotrophic count – SMEDP
		Yeast and mold count – SMEDP
Frozen dairy products	Microorganism counts	Standard plate count – SMEDP
		Coliform count – SMEDP
		Thermoduric Count – SMEDP
		Yeast and mold count – SMEDP
Concentrated milk products	Microorganism counts	Standard plate count – SMEDP
		Coliform count – SMEDP
		Thermoduric count – SMEDP
		Thermophilic count – SMEDP
		Yeast and mold count – SMEDP
Cheeses, yogurt, fermented milk products	Microorganism counts	Coliform count – SMEDP
		Yeast and mold count – SMEDP
		Psychrotrophic count – SMEDP

[a]Standard Methods for the Examination of Dairy Products, 2001

microorganisms can often depend on the product characteristics, such as amount of competing microflora, pH, and water activity.

Fungi can be particularly troublesome, because they can adapt to the environment of the food and can be difficult to detect on conventional plating media within the standard incubation times. In yogurts, yeasts often grow slowly in conventional laboratory plating methods, but as few as 10 CFU/ml were detectable after 16 h of incubation by PCR amplification of the conserved region of their 18S rRNA (García et al., 2004). Several investigators (Ingham and Ryu, 1995; Vlaemynck, 1994; Beuchat, Nail, Brackett, & Fox, 1990) have made comparisons of a number of alternative yeast and mold detection methods in shredded cheese, hard and soft cheeses, cottage cheese, yogurt, and sour cream, and found that, while results for all of the methods were statistically similar, price, speed, and convenience of use are often overarching considerations when users choose a method. Rapid genomic subtyping methods, such as RAPD, RFLP, and AFLP, can be used to determine the sources of fungal contamination in a manufacturing environment (Lund et al., 2003).

Laleye et al. (1987) compared four plating media for recovery of spoilage lactococci from gassing cheeses and determined that MRS agar and APT agar gave the best results. For detection of *C. tyrobutyricum* in gassing cheeses, the classical method of most-probable-number testing in RCM-lactate or BBMB-lactate medium followed by confirmation on LATA or DRCM medium, and gas chromatographic analysis of volatile and nonvolatile organic acid by-products was determined to be both lengthy and difficult to perform (Bergere & Sivela, 1990). Herman et al. (1995) and Lopez-Enriquez, Rodriguez-Lazaro, and Hernandez (2007) have developed PCR-based detection methods that are reported to detect less than one spore of *C. tyrobutyricum* per milliliter of milk. Cocolin, Innocente, Biasutti, and Comi (2004) developed a PCR-denaturing gradient gel electrophoresis method that could detect 10^4 CFU of *Clostridium* spp. per milliliter in gassing cheeses.

Conclusion

While the introduction of pasteurization has helped to ensure the safety of dairy products, progress has been slower in preventing the microbial spoilage of cheese and dairy products. Worldwide standardized pasteurization practices would be an effective first step in eliminating or reducing the levels of many spoilage microorganisms. However, preventing postprocess contamination by spoilage microorganisms and retarding the growth of surviving organisms remain a challenge. Novel technologies and preservatives are needed to prevent the growth of spoilage microorganisms and extend the shelf life of dairy products. Limited applicability of current approved antimycotics such as sorbic acid and natamycin provides a major opportunity to expand the arsenal of preservatives available for today's dairy processor. In addition, studies to determine the interaction of current preservative technologies against spoilage microorganisms are also needed. Improved methods for detecting spoilage microbes, especially the slow-growing psychrotrophs and fungi, could assist in finding the niche environments in processing facilities that lead to

postprocess contamination. The next century will bring many challenges to the dairy processor, but maintaining the quality and shelf life of this highly nutritious food should not be one of them.

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Microbiological Spoilage of Meat and Poultry Products

John Cerveny, Joseph D. Meyer, and Paul A. Hall

Introduction

Humankind has consumed animal protein since the dawn of its existence. The archaeological record shows evidence of animal protein consumption as early as 12,500 BC (Mann, 2005). Raw meat and poultry are highly perishable commodities subject to various types of spoilage depending on handling and storage conditions. Because of this high potential for spoilage, the historical record reveals that early civilizations used techniques such as salting, smoking, and drying to preserve meat (Mack, 2001; Bailey, 1986). Today, more than ever, because of the globalization of the food supply, and increasing demands from exacting consumers, the control of meat and poultry spoilage is essential.

Today's consumers enjoy a variety of meat and poultry products. Each form or variety of meat and poultry has a specific range of shelf life primarily governed by its spoilage microflora.

Microbiological spoilage of meat and poultry is primarily due to the activity of psychrotrophic microbes that produce off-flavors, off-odors, and undesirable appearance at refrigeration temperatures. General factors that can mitigate against bacterial spoilage include (Bailey, 1986)

(1) use of good sanitation practices during slaughter and processing to limit initial contamination;
(2) destroying or removing spoilage microorganisms;
(3) reducing the rate of growth of spoilage microorganisms by maintaining low temperature during processing, transportation, and storage; and

J. Cerveny (Retired) (✉)
Madison, WI 53704, USA
e-mail: jcerveny@itis.com

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(4) knowing time–temperature response limitations for maintaining quality and turning product over within these limitations.

Efforts to control microbiological spoilage of meat and poultry generally focus on the effective application of these four factors. Failure to effectively control spoilage has major economic consequences. The USDA's Economic Research Service estimated that in 1995, approximately 7.6 billion lbs of meat and poultry were wasted at the retailer, foodservice, and consumer levels (Kantor, Lipton, Manchester, & Oliveira, 1997). A significant portion of this loss is due to microbial spoilage. The economic impact of these losses is enormous. The USDA estimates that if 5% of this food loss could be prevented, that quantity would represent the equivalent of a day's meat and poultry consumption for approximately 320,000 people.

Microbiological Spoilage of Meat Products

A number of animal species are used for red meat production, including cattle, swine, sheep, goat, deer, buffalo, camel, and horse. Meats are subject to spoilage by a wide range of microorganisms, including Gram-positive and Gram-negative bacteria, yeasts, and molds (Jay, Loessner, & Golden, 2005). There are a number of factors that influence the composition of the meat spoilage microflora:

- Pre-slaughter husbandry practices (e.g., free range vs. intensive rearing)
- Age of the animal at the time of slaughter
- Sanitary handling during slaughter, evisceration, and processing
- Temperature controls during slaughter, processing, and distribution
- Preservation controls
- Type of packaging
- Consumer handling and storage

All of these factors play a role in governing the microbiological shelf life of meat products.

Red meat products can be categorized as follows:

- Fresh, refrigerated raw meat products
- Perishable salted and cured meat products
- Cured and uncured ready-to-eat meat products
- Canned meat products
- Vacuum and modified atmosphere-packaged meat products
- Fermented and dried meat products
- Bacon
- Organic meat products
- Variety meat products (offal)

The potential for microbiological spoilage varies greatly with the category of meat product. Spoilage potential can also vary within a meat category as formulations are varied. Careful consideration must be given to the specific formulation parameters when establishing product shelf life, spoilage potential, or troubleshooting a problem.

Fresh, Refrigerated Raw Meat Products

This category of products can be subdivided into two subcategories:

- Raw, intact meat products
- Raw, ground/minced meat products

The microbiology of raw, intact meats at the time of slaughter is greatly influenced by the associated slaughtering and handling practices, as well as the microbiological conditions of the animal at the time of slaughter (ICMSF, 1998a). Because animals and poultry are exposed to soil, water, insects and birds, and fecal material, the carcasses are contaminated with a variety of microorganisms, including both spoilage and pathogenic microorganisms, during the slaughter and cutting procedures. The initial microflora on red meat at the time of slaughter is primarily composed of mesophilic microbes that cannot compete upon chilling and processing/storage at refrigerated temperatures. During chilling and refrigeration, there is a shift in the composition of the initial microflora to psychrotrophic microorganisms. The initial microbial counts in red meat range from 10^2 to 10^5 CFU/cm^2, with only about 10% of the microbial population able to continue growth upon refrigeration (Garcia-Lopez, Prieto, & Otero, 1998). Desiccation of carcasses causes the surface water activity (a_w) to decrease during the first 4 hours to a limit below which pseudomonads and others cannot grow. However, during the first 24 hours, diffusion of water from the interior counterbalances this effect. Yeasts, such as *Candida* spp., *Cryptococcus* spp., and *Rhodotorula* spp., and many mold species also contaminate the surface of fresh meat (Garcia-Lopez et al., 1998).

As the temperature of the refrigerated carcass equilibrates, psychrotrophic microorganisms begin to predominate. The predominant microflora of red meat that is stored aerobically under refrigeration is composed of *Pseudomonas* spp., *Moraxella* spp., *Psychrobacter* spp., and *Acinetobacter* spp. Gram-negative psychrotrophic members of the family Enterobacteriaceae are frequently present on refrigerated red meat and are especially prevalent on pork and lamb (ICMSF, 1998a).

Spoilage of intact cuts of meat under refrigeration occurs primarily on the surface resulting in slime formation, off-odor development, with or without gas production. The shelf life of these products is influenced by a number of factors:

- Water activity (a_w)
- Initial number of psychrotrophs present on the meat surface
- Inherent pH of the meat surface
- Storage temperature
- Oxygen availability
- Nutritional content

Gram-positive microorganisms, including *Micrococcus* spp., *Staphylococcus* spp., *Bacillus* spp., *Lactobacillus* spp., the coryneform group, and *Brochothrix* spp., are also present in a significant percentage of raw, intact meat.

Procedures such as effective carcass decontamination, proper evisceration procedures, and rapid chilling procedures can greatly reduce the initial microflora and extend product shelf life.

The shelf life of raw, refrigerated ground/minced meat and similar products is directly influenced by the microbiological quality of the intact meat from which it is made. Fresh ground/minced meat tends to have a short shelf life because the quality of the raw ingredients is usually lower (i.e., has higher number of contaminating microorganisms), and is recontaminated through the grinding/handling process. The primary spoilage flora of ground/minced meat is mainly composed of pseudomonads and psychrotrophic Enterobacteriaceae. These bacteria are responsible for spoilage of fresh meat because they are good competitors, have the ability to metabolize the meat constituents, and grow well in the 5.5–7.0 pH range (Doyle, Beuchat, & Montville, 2007). Lactic acid bacteria, clostridia, yeasts, and molds are found in fresh ground/minced meat products. Maintaining adequate temperature control throughout the distribution chain is essential to minimize the growth of spoilage microorganisms. Mincing and grinding of meat at the retail location can introduce more spoilage microorganisms if proper equipment hygiene and handling measures are not followed. Proper controls throughout the distribution chain are critical to the quality and shelf life of all refrigerated, raw meat products.

Perishable Salted and Cured Meat Products

Perishable raw salted and salted-cured products such as fresh pork sausage, Polish sausage, Italian sausage, bratwurst, chorizo, uncooked hams, bacon, and corned beef are common in the USA and elsewhere. The type of packaging used for these products is a major determinant of shelf life. Fresh sausage bulk-packaged into trays or sold in edible casings has a very short shelf life, typically 7–21 days. The predominant spoilage microflora for these products is the psychrotrophic pseudomonads, molds, and yeasts. On the other hand, fresh sausage products sold in oxygen-impermeable casings generally have a longer shelf life (about 4 weeks). The predominant spoilage microflora in these products is the lactic acid bacteria.

In raw, salted-cured products, such as corned beef, uncooked hams, and bacon, psychrotrophic lactic acid bacteria, enterococci, micrococci, and yeasts eventually

predominate as the primary spoilage microflora because of their resistance to curing salts.

Cured and Uncured Ready-to-Eat Products

Cooked, ready-to-eat (RTE) meat products may be either cured or uncured. Historically, salting was used to preserve meat. It was noted that occasionally the meat developed a characteristic pink color. This color formation was related to the contamination of the salt with nitrate. A German scientist determined that the nitrate was reduced to nitrite by bacterial action. We now know that the nitrite is responsible for the development of the characteristic pink color (Cassens, 1994). The USDA regulates the level of nitrate or nitrite added to meat products (USDA, 2001a). Since the USDA permits nitrite to be added to the meat formulation, the need for nitrate has been eliminated. Cured meats are formulated from intact muscle and cuts, from pieces of meat that have been pressed, or from fully comminuted meat that has been extruded into casings. The curing agent, nitrite at approximately 125–156 mg/kg, is added to the meat before cooking by brine injection, by soaking in brine, or through emulsion blending (ICMSF, 1998a). RTE cured meats are usually cooked to 65–75°C, a temperature sufficient to destroy most vegetative microorganisms, but not thermoduric enterococci and bacterial sporeformers. The latter microorganisms, however, do not cause spoilage in cured meat products. Subsequent handling, packaging, and preparation of these uncured products can lead to surface contamination and high microbial counts.

Additional agents can be added to cured meats, including sugar, phosphate, ascorbate, spices, antioxidants, citrate, sodium lactate, sodium diacetate, liquid natural smoke, and nonmeat extenders. All of these components may influence the potential spoilage and shelf life of the products. For example, combinations of potassium lactate and sodium diacetate, used to inhibit growth of the pathogen *Listeria monocytogenes* in these products, can also inhibit growth of spoilage microorganisms (Seman, Borger, Meyer, Hall, & Milkowski, 2002). Glass et al., (2002) and Seman et al. (2002) determined that the lactate and diacetate combination is more effective in cured meat products than in uncured products. Because of the nature of these uncured products, they will eventually be spoiled by a wide variety of microorganisms, including pseudomonads, lactic acid bacteria, enterococci, psychrotrophic Gram-negative bacteria, yeasts, and molds.

The product formulation, processing, packaging, and storage temperature will greatly influence the type of spoilage that occurs. With the exception of bacon, RTE cured meats undergo a cooking step that destroys the vegetative microflora of the raw formulation. Cured RTE meat products include hot dogs, bologna, ham, and many types of luncheon meats. The spoilage of cured meat-packaged aerobically is caused, in most instances, by the growth of yeasts and molds. When the same cured products are packaged anaerobically in impermeable films, spoilage is caused by the growth of lactic acid bacteria, which include *Enterococcus,*

Lactococcus, Lactobacillus, Leuconostoc, Pediococcus, and *Weissella* (Seman et al., 2002). Microorganisms from the genus *Brochothrix* are also responsible for spoilage in cured meat. By definition, the lactic acid bacteria produce lactic acid from hexoses. The lactic acid bacteria are categorized in two groups based on the end products from glucose fermentation (Jay et al., 2005). *Pediococcus*, *Streptococcus*, *Lactococcus*, and some strains of *Lactobacillus* are considered to be "homofermentative." They convert almost all of the glucose to lactic acid. "Heterofermentative" lactics, which include *Leuconostoc*, *Carnobacterium*, and some lactobacilli, convert glucose to carbon dioxide, ethanol, and lactic acid. Spoilage defects in RTE products include turbidity of the, free juice in the package, slime formation, gas production, and discoloration. A strain of *Streptococcus* causes yellow discoloration in cooked cured meat held under aerobic and anaerobic conditions (Whiteley & Dsouza, 1989). The growth of lactobacilli has little effect on the organoleptic properties of vacuum-packaged meat products (Labadie, 1999).

Uncured, cooked RTE products are also subject to surface recontamination after cooking. Formulation, storage temperature, and packaging conditions will govern the type of spoilage that occurs. Yeasts and molds will spoil uncured products within 2–3 weeks, before the contaminating bacteria can grow to levels that become visually apparent. However, in oxygen-impermeable packaging, the spoilage microflora at 5°C or less is predominantly lactic acid bacteria (Seman, et al., 2002). It is common to give RTE meat products a post-packaging heat treatment to eliminate *L. monocytogenes* because of the potential for recontamination. This heat treatment also reduces the spoilage microflora, thereby extending the product's refrigerated shelf life.

Psychrotrophic, nonpathogenic, anaerobic clostridia can cause spoilage under normal refrigerated conditions in some types of cooked, uncured products, such as roast beef, cooked pork, and cooked turkey (Kalinowski & Tompkin, 1999; Meyer, Cerveny, & Luchansky, 2003). The source of these clostridia can be the raw meat from which the cooked, uncured product is produced. Anaerobic spores can survive cooking processes and eventually germinate and grow during prolonged storage.

Brine chill systems can be a major site of contamination with spoilage microorganisms for both cured and uncured RTE products. Other sources include peelers, slicers, conveyors, and personnel who handle the products. Strict hygienic standards and sanitation procedures should be used to eliminate niches for these spoilage microorganisms (as well as pathogens) to effectively minimize recontamination as much as possible. Cross-contamination potential from other products should also be considered. For example, ropy slime-producing lactic acid bacteria that cause spoilage in fermented sausage has been shown to cause spoilage in cooked product processed in the same area (Makela, 1992). The addition of herbs, spices, and other ingredients after cooking can also be a significant source of spoilage microorganisms if these ingredients are not properly handled.

Canned Meat Products

Typical uncured, canned meat products include meat-containing stews, soups, gravies, sauces, chili, pasta-containing products, sloppy joe products, roast beef and gravy, and other similar products. Because these products are canned, they receive a heat process sufficient to kill spores of *Clostridium botulinum* ($F_o = 2.5$). In practice, they receive a greater heat treatment to kill spore-forming bacteria having greater heat resistance than *C. botulinum*($F_o = 4-6$). Spoilage of these types of products from putrefactive anaerobes such as *Clostridium sporogenes* can occur if the product is under-processed or if the container is compromised after retorting (e.g., pinholes, faulty seams, and leakers). "Leaker" spoilage is generally characterized by the presence of mixed microbial cultures, sometimes with non-spore-forming vegetative cells present. However, the use of chlorine in cooling water has occasionally resulted in the isolation of only spore-forming bacteria from leaking containers, because vegetative microbial cells in the cooling water are killed by the chlorine.

Vacuum and Modified Atmosphere-Packaged Products

Vacuum packaging and modified atmosphere packaging (MAP) are two common methods for extending the refrigerated shelf life of meat products. In vacuum-packaged meat, the residual oxygen is usually reduced to less than 1% (Dainty, 1992). Most packaging films used for vacuum-packaged meat have some degree of oxygen permeability, so absolute anaerobic conditions are not achieved. Vacuum packaging and MAP can have dramatic impacts on the microflora of red meat. During storage, aerobic microorganisms, such as pseudomonads and molds, are replaced by slower growing, facultatively anaerobic microorganisms, such as lactic acid bacteria. The latter metabolize glucose to produce mixed organic acids giving the meat a sour, cheesy odor and taste. Proteolysis and lipolysis are rare in vacuum-packaged products because of the limited ability of lactic acid bacteria to produce the requisite enzymes. Rarely, psychrotrophic Enterobacteriaceae have been reported to cause spoilage of vacuum-packaged beef, pork, and lamb (Garcia-Lopez et al., 1998). High initial contamination levels, film permeability, elevated storage temperatures, and other factors are thought to contribute to these occurrences.

In MAP, the product package is flushed with a mixture of gases to extend the shelf life. Carbon dioxide is usually the chief active component that causes microbial inhibition. Carbon dioxide is often used in combination with nitrogen and oxygen to achieve an organoleptically acceptable product. Generally, CO_2 concentrations range from 10 to 40% with the balance being O_2 or N_2. Oxygen has been traditionally used in the gas mixture for MAP red meat to mitigate the off-color appearance associated with MAP red meat packed under anaerobic conditions. Higher CO_2 concentrations produce a greater inhibition of the spoilage microflora. However, since O_2 is used to stabilize red meat color, the shelf life of meat with O_2 is not as long

as that obtained with anaerobic MAP red meat. To address this challenge, meat scientists in Norway pioneered the incorporation of low levels of carbon monoxide (CO) into MAP gas mixture to stabilize the color of red meat. The low levels of CO (0.1–2.0%) form the stable carboxymyoglobin pigment in the red meat tissue, which has a red appearance that is virtually indistinguishable from the less stable oxymyoglobin, which is formed in the presence of O_2. Over time, oxymyoglobin is converted to metmyoglobin which imparts the characteristic brown color to the meat. Both the FDA and USDA have approved the use of CO MAP packaging as safe for use with red meat. However, these approvals are not without controversy as some consumer groups question the safety of the technology (European Commission, 2001). Similar to vacuum-packaged meat products, Enterobacteriaceae and *Aeromonas* spp. have occasionally been reported to spoil MAP-packaged products depending on a variety of factors, including initial cell numbers, film permeability, pH, and storage temperature (Garcia-Lopez et al., 1998).

Fermented and Dried Meat Products

The stability of fermented sausages is due to a low pH, low a_w, or a combination of the two. Many fermented sausages are produced by mixing ground meat with fat, sodium chloride (2.5–3.0%), sodium nitrite and/or nitrate, sugar (0.4–0.7%), spices, and starter cultures. The sausage mixture is stuffed into moisture-permeable casings. After fermentation at 20–40°C for variable periods of time, the sausages are dried and smoked, if required. Dry fermented sausages lose 25–50% of their initial moisture and semidry sausages lose up to 15% of their moisture (ICMSF, 1998a). Generally, the added sugar is fermented by homofermentative lactic acid bacteria to lactic acid and the final pH is in the range of 4.6–5.5. Most, but not all manufacturers of fermented sausage rely on commercial starter cultures to convert carbohydrates to lactic acid. A few producers still rely on the indigenous lactic acid bacteria in the batter for this conversion. The presence of leuconostocs and heterofermentative lactic acid bacteria is undesirable because of their ability to produce gas and mixtures of organic acids resulting in organoleptic defects.

Control of temperature and time to reach the target pH of 5.3 or below is critical to prevent the growth and toxin production by *Staphylococcus aureus* (American Meat Institute, 1982). Often, starter culture bacteria such as *Pediococcus acidilactici*, *Lactobacillus sake*, and *Lactobacillus curvatus* are used to ensure proper acid production, final pH, and flavor development. Bacterial spoilage of fermented meat products can occur during fermentation if the reduction of pH and moisture content do not occur as needed. Spoilage by indigenous bacteria can occur if a starter culture is not used or if the starter culture does not function properly. Yeasts such as *Debaryomyces* spp. have been known to develop in the aerobic layers of fermented meats and wild-type molds can also grow on meat surfaces if not MAP or vacuum-packaged. It is common for European-style sausage producers to use starter cultures that contain, for the development of unique lipolytic and

proteolytic flavors, desirable molds such as Penicillia and Aspergilli (Ayres, Lillard, & Leistner, 1967). Because semidried fermented sausages like Lebanon bologna, Thuringer, and summer sausages are not dried; they have higher moisture contents, ~50%, than dried sausages and require heating after fermentation.

Dried meats are microbiologically stable at ambient temperature because of their low a_w. In the USA, beef jerky is sold as a heat-treated, shelf-stable, ready-to-eat meat product. The USDA has established a moisture to protein ratio of 0.75 for beef jerky. The a_w of jerky products is 0.86 or less. Molds and yeasts can cause spoilage of jerky products during shelf life if the product acquires moisture from the environment. Vacuum packaging or maintaining the a_w at a level of 0.70 or below can prevent spoilage by these microorganisms. Beef jerky is heat-treated not only to dry the product, but also to destroy *Escherichia coli* O157:H7. Research has revealed that the fat content and the time and temperature at which the strips are dried, impact directly on the viability of *E. coli* O157:H7 (Faith, et al., 1998).

The cure for dried-cured hams is applied as a dry salt, by pumping with pickle, covering with a pickle, or a combination of these treatments (ICMFS, 1998a). Initial curing and ripening is done at <5°C. and the final a_w is less than 0.90. The predominant surface microfloras during curing are micrococci and nontoxigenic strains of staphylococci. Both *Penicillium* spp. and *Aspergillus* spp. can grow on the ham surface during curing. Dried-cured hams are shelf stable. Those made in Europe can be made only with a salt cure applied on the surface; neither nitrite nor nitrate is added. This category of products is described as shelf-stable salted and salted-cured products (Tompkin, McNamara, & Acuff, 2001). In the USA, shelf-stable raw salted and salted cured products such as salted pork, dry-cured bacon, and country-cured hams are popular. Salted pork, bacon, and hams are made by coating and recoating the meat in salt at intervals and storing at 10°C for a designated period of time. At the end of the salting period, the products are dried at ambient temperature, rubbed with a thin layer of salt and spices, and then placed into distribution for sale. Country-cured (dry-cured) hams and bacon contain nitrite/nitrate with lower salt concentrations.

RTE shelf-stable dried bacon and ham are not heated during processing. These products are dried at ambient temperature for 35–140 days before being distributed as shelf-stable products. Spoilage of these products rarely occurs because of the processing procedures they received described above. Deep tissue spoilage near the bone in hams, known as "bone taint," can occur if proper equilibration of curing salt levels does not occur (Tompkin, et al., 2001). Mold growth can occur on the surface of these products, especially under humid conditions.

Bacon

Bacon is a unique cured meat product. It is not considered to be an RTE product, although it is heated to 53–55°C for flavor and color development. The bacon bellies are also smoked during a 10–12-hour process. Bacon must be cooked by the end user

because its low final processing temperature is not sufficient to ensure inactivation of microbial pathogens. The USDA Food Safety and Inspection Service requires that bacon be made with 120-ppm nitrite and 550-ppm ascorbate or erythorbate (USDA, 2001a). The use of nitrate is prohibited. When bacon is packaged in aerobic film, spoilage is caused by a variety of molds and yeasts after 2–3 weeks at refrigeration temperature (Jay et al., 2005). Even though the bacon surface contains a variety of bacteria, their growth is minimal within the first 2–3 weeks of storage when surface mold and yeast colonies become visible. When bacon is packaged anaerobically, the spoilage microflora is primarily lactic acid bacteria, micrococci, and staphylococci. Packaged in this manner, the product's shelf life is extended to 10 or more weeks. Although molds and film yeasts are a part of the naturally occurring microflora, they are unable to grow in an anaerobic environment. Slime production is common in anaerobically packaged bacon when sucrose is included in the formulation.

Organic Meat Products

Meat curing was used in ancient times for preservation (Jay et al., 2005). Meat curing is also designed for improved color and flavor development. Meat curing ingredients include salt, nitrite, nitrate, ascorbate or erythorbate, phosphate, carbohydrate, and spices. The USDA regulations define the composition of certain organic meat products such as ham, hot dogs, bologna, and bacon. USDA regulations and label requirements indicate that organic products cannot include sodium nitrite, nitrate, or chemical preservatives (Sebranek & Bacus, 2007). However, both nitrate and nitrite can be added indirectly to these products in the form of a vegetable-based ingredient such as celery juice or celery powder, which have a high level of naturally occurring nitrate. A bacterial starter culture is also added to the formulation and after a short incubation period the nitrate is reduced to nitrite, which is needed for developing the characteristic cure color. Two starter culture bacteria used for this reduction of celery powder are *Staphylococcus xylosus* and *S. carnosus* (Sebranek & Bacus, 2007). The word "uncured" and the phrase "no nitrate or nitrite added" must appear on the label. Meat receiving treatment combinations containing vegetable juice powder and the starter culture *S. carnosus* was comparable to a sodium nitrite-treated control for color, lipid oxidation, and cured meat pigmented sensory measurements (Sindler, Cordway, Sebranek, Love, & Han, 2007). The spoilage microflora of organic meat products is not different than that which is associated with meat products that have nitrite added directly to the batter.

Variety Meat Products (Offal)

Edible offal consists mainly of the internal organs of an animal, including liver, kidney, heart, brains, and tongue. High bacterial counts are common in variety meats if they have not been chilled properly. The definition of variety meats used for human

consumption varies among countries. Pseudomonads tend to predominate during refrigerated storage, whereas enteric bacteria tend to predominate at slightly elevated refrigerated temperatures. The predominant microflora of pork livers stored at 5°C for 7 days was found to consist of *Pseudomonas* spp., *Alcaligenes* spp., *Escherichia* spp., lactic streptococci, and *Brochothrix thermosphacta* (Garcia-Lopez et al., 1998). Lactic acid bacteria and streptococci can be predominant in spoiled comminuted or diced offal, such as kidneys and livers, because of their relatively high carbohydrate content. The pH tends to decline over time with spoilage, eventually favoring these microbes. However, in whole organ meats, such as liver, surface conditions favor the development of psychrotrophic Gram-negative bacteria because of their ability to grow more rapidly than the Gram-positive lactic acid spoilage bacteria (AMI, 1982). Vacuum-packaging of edible offal shifts the predominant spoilage microflora to Enterobacteriaceae, *Flavobacterium* spp., *Alteromonas* spp., *Moraxella* spp., *Acinetobacter* spp., and *Shewanella putrefaciens* (ICMSF, 1998a). When offal is used in a sausage formulation, the sausage must be labeled with the phrase "byproducts" or "variety meats" (USDA, 2001b).

Microbiological Spoilage of Poultry and Poultry Products

The consumption of poultry and poultry products has increased markedly during the past decade. Per capita poultry consumption in the USA has risen to 105.0 lbs retail weight in 2006 (47.4% of total per capita meat consumption) (USDA, 2007). Growing demand in poultry consumption has led to the evolution of intensive broiler flock production and mechanized slaughter practices, which influence the microbiology of the raw products.

Microbiological Profiles of Refrigerated Raw Poultry Products

Each step in the processing of raw poultry influences the level of spoilage microflora on the product (Fig. 1). Some steps lead to a significant increase in the microbial load, whereas others lead to a significant decrease. In general, whole poultry carcasses have lower microbial populations than cut-up poultry. One study of six poultry-processing operations revealed initial mean surface counts of 3.30 log CFU/cm^2, increasing to 3.81 log CFU/cm^2 after cutting-up, and to 4.08 log CFU/cm^2 after packaging (May, 1962). Poultry carcasses receive several washing steps during the slaughter process. In an effort to reduce the microbial load on poultry carcasses, a number of antimicrobial sanitizing treatments have been incorporated into the processing line. A variety of organic acids, including acetic, lactic, citric, and succinic, are used in washing, spraying, and rinsing carcasses to reduce microbial populations (Keener, Bashor, Curtis, Sheldon, & Kathariou, 2004). Other antimicrobial treatments that are used depending on the processor include chlorine, chlorine dioxide, trisodium phosphate, and acidified sodium chlorite (Table 1).

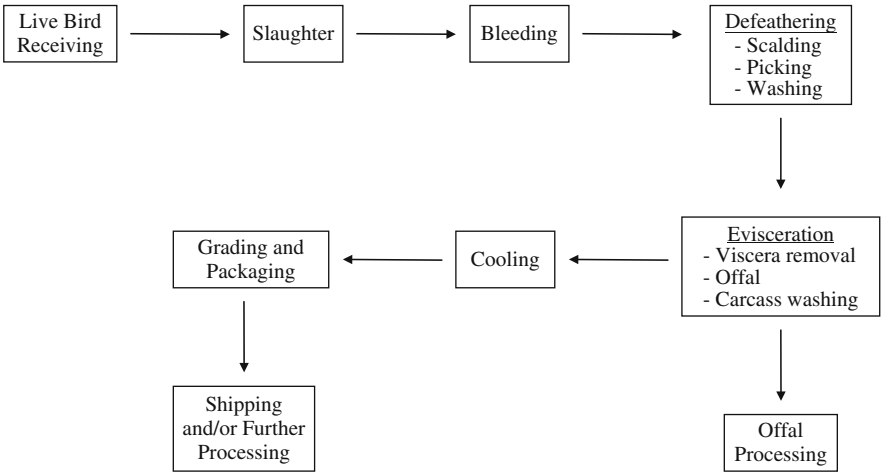

Fig. 1 Generic process flow for poultry operations

Table 1 Proportions of psychrotrophic spoilage microorganisms isolated from chicken carcasses ($n = 5920$)[a]

Type of microorganisms	Percentage of population
Pseudomonads	30.5
Acinetobacter spp.	22.7
Flavobacterium spp.	13.9
Corynebacterium	12.7
Yeasts, enterics, and others	20.2

[a]Lahellec, Menrier, and Bennejean (1975)

More than 50 strains of spoilage bacteria representing 25 genera have been reported as part of the initial microflora in raw chicken (ICMSF, 1998b; Cox, Russell, & Bailey, 1998). At refrigerated temperatures, pseudomonads represent the largest genus represented. Cell numbers of nonpigmented pseudomonads increase substantially during spoilage, and storage conditions greatly influence the cell numbers of pigmented pseudomonads. MAP storage dramatically changes the predominant spoilage microflora compared to aerobic storage, as it prevents the growth of obligately aerobic microorganisms (Table 2).

Temperature has a dramatic affect on the development of spoilage microflora of raw poultry. The rate of microbial spoilage of broiler carcasses is twice as fast at 10°C than at 5°C, and three times more rapid at 15°C (Cox, et al., 1998). The shelf life of eviscerated, cut-up poultry is 2–3 days at 10.6°C, 6–8 days at 4.4°C, and 15–18 days at 0°C, respectively (Ayres, Ogilvy, & Stewart, 1950).

The hallmarks of raw poultry spoilage are the formation of off-odors and the development of surface slime. Initially, the spoilage microflora will metabolize glucose, and then shift to substrates such as amino acids as the glucose is depleted. In

Table 2 Predominant microorganisms in spoiled refrigerated poultry meat packaged under aerobic or MAP conditions[a]

Aerobic package	Modified atmosphere package
Pseudomonads	*Lactobacillus* spp.
Acinetobacter spp.	*Carnobacterium* spp.
Moraxella spp.	*Brochothrix* spp.
Psychrobacter spp.	*Shewanella* spp.
Candida spp.	Enteric bacteria
Yarrowia spp.	

[a]Barnes and Impey (1968)

particular, amino acid metabolism contributes to off-odor production. Slime formation generally follows the production of undesirable odors. During storage, small translucent drop-like colonies will appear on the tissue surface, eventually coalescing into a uniform slime layer. In general, off-odors are detected when counts reach 10^7–10^8 CFU/cm^2 of tissue surface and slime formation occurs at levels >10^8 CFY/cm^2 (Ayres et al., 1950).

Fungi typically play a minor role in poultry spoilage. However, when antibiotics are used to control bacterial growth, fungi can become major causes of spoilage. The principal spoilage yeasts found on raw poultry are *Candida* spp., *Rhodotorula* spp., and *Torulopsis* spp. (Jay et al., 2005).

Uneviscerated poultry (sometimes known as "New York dressed" poultry) is especially prone to pronounced temperature-dependent spoilage. "Visceral taint" characterized by a sour odor can occur (Jay et al., 2005). Mesophilic microorganisms in the intestines can also produce hydrogen sulfide that can eventually diffuse into muscle tissue and combine with oxygen and heme pigments to form a green discoloration of the meat.

Further Processed Poultry

Whole eviscerated poultry carcasses are often further processed, for example, by cutting into pieces, mechanical deboning, grinding, comminuting, and batter coating. Subsequent handling of these carcasses during further processing leads to substantially higher microbial counts. One study revealed that aerobic plate counts of chicken carcasses increased sixfold in processing plant cutting areas and about eightfold at the retail store level (May, 1962). It is important for processors to focus on sanitation and good manufacturing practices (GMPs) during handling of these products to minimize cross-contamination.

Mechanically deboned poultry meat is recovered from chicken or turkey parts, such as carcasses, necks, and backs, that are left over after the cut-up operations. Mechanically deboned poultry is finely comminuted and tends to be contaminated with high levels of psychrotrophic spoilage microorganisms (10^5-10^6CFU/g) and coliforms (10^2-10^3CFU/g) (Mulder & Dorresteijn, 1975). Good sanitation

and hygiene measures are needed to prevent buildup and proliferation of spoilage microorganisms on processing equipment.

Cooked RTE Cured and Uncured Poultry Products

The sources of spoilage microorganisms in cooked poultry products originate primarily in ready-to-eat environment of the processing facility where the products are sliced and/or packaged. It is important that cooked products be handled in a separate room and not be exposed to raw poultry or to cross-contamination from raw product areas, particularly by air currents or employee traffic. Other materials used in the RTE room, such as batter coatings and spices, can also contribute spoilage microorganisms. Spices can contain $\geq 10^6$ bacterial spores/g (ICMSF, 1998c). Most cooking procedures are sufficient to kill vegetative microbes but bacterial spores will survive.

RTE poultry products can be cured or uncured. As in the production of red meat products, a number of different curing agents may be used for cured poultry products, including salt, sugar, nitrite, nitrate, ascorbic acid, polyphosphate, and spices. The muscle or carcass may be immersed in brine composed of curing agents or injected with such brine. Parts or whole bird carcasses can also be smoked under a variety of conditions to add flavor (ICMSF, 1998b). Both the curing and smoking of poultry products can dramatically change the microflora of these products. The shelf life of these products depends on the degree of cooking, composition of the cure, smoke protocol, storage temperature, and type of packaging. It is always advisable when developing these types of products to conduct proper shelf-life studies for both quality and safety purposes (Labuza & Szybist, 2001). Poultry meats can also be produced with other preservatives such as potassium lactate/sodium diacetate and sold as sliced, prepackaged RTE luncheon meats or as loaf products to be sliced and packaged in the delicatessen area. The shelf life of these products depends on the type of packaging, the type of preservation system, and the potential for recontamination. Luncheon meats sliced and packaged at the delicatessen counter generally have a shorter shelf life than presliced luncheon meats. Appropriate research and sensory evaluations should be conducted during product development to determine the expected consumer-acceptable product shelf life.

Cooked poultry products, in general, have low aerobic bacterial counts; however, a combination of factors influence the product shelf life, including the temperature and time of cooking, the level of post-processing contamination, and the temperature and time of storage (ICMSF, 1998c). Aerobic bacterial counts of barbecued chicken can increase substantially after only a few hours when stored at improper holding temperatures (Seligman & Frank-Bluhm, 1974).

In canning operations, poultry may be cooked and packed into cans prior to retorting or the raw poultry may be packed into cans with broth and then retorted. A competent process authority must validate the commercial retort processes. Properly retorted products achieve “commercial sterility” conditions in which vegetative microorganisms are killed and mesophilic bacterial spores that can grow

during ambient storage are inactivated. Spoilage of canned poultry products can occur because of under-processing or, more commonly, because of can seal defects or pinhole leaks. Procedures have been developed for verification of the commercial sterility of canned foods and for identifying the causes of spoilage of canned products (Downes & Ito, 2001).

Microbiological Testing Methods for Meat and Poultry Products

There are a variety of sampling and testing methods that can be used for meat and poultry products. The choice of sampling plan and testing method is dependent on the type of product and the reason for conducting the testing. Microbiological procedures can be used to conduct shelf-life determinations and verifications, spoilage investigations, regulatory compliance determinations, sanitation effectiveness evaluations, and preservation system efficacy determinations.

Carcass samples can be sampled using the excision technique, sponging technique, whole bird rinse technique (in the case of poultry), or the skin scraping technique (ICMSF, 1998b). Ground meat products can be sampled using a variety of compositing techniques. These products can also be analyzed by the extract release volume method to estimate the shelf life of ground meat. Cooked meat products can be sampled using core sample techniques, rinse sample techniques, and sterile compositing techniques. A variety of microbiological tests for nonpathogenic spoilage microorganisms can include aerobic plate counts (mesophilic and/or psychrotrophic), anaerobic plate counts, and lactic acid bacteria, enterococci, coliform, yeast, and mold counts. (Tompkin et al., 2001).

When sausage is not fermented properly, *S. aureus* can grow and produce enterotoxin in the product. However, enterotoxin production occurs only near the surface of the sausage, not in the core of the sausage (Barber & Deibel, 1972). As a result of this observation, the USDA (1998) developed a sampling procedure in which only a thin layer of surface material, not a core sample, is tested for the presence of enterotoxin.

Molecular genetic techniques can provide invaluable information about the microbiology of meat and poultry products. These techniques have been used to trace the specific source of spoilage microorganisms and to determine the changes in spoilage microflora under different packaging conditions (Björkroth, Vandamme, & Korkeala, 1998; Ercolini, Russo, Torrieri, Masi, & Villani, 2006). Classical microbiological techniques can be paired with molecular genetic methods to provide powerful discriminating ability in the identification of beef spoilage microorganisms (Ercolini, et al., 2006). An experienced food microbiologist or process authority should be consulted when developing a sampling and testing scheme and to assist in the interpretation of data obtained from meat and poultry products.

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Microbiological Spoilage of Fish and Seafood Products

Lone Gram

Introduction

Fish and seafood products are some of the most important protein sources in human nutrition. At the same time, these products are perishable and, if left unpreserved, spoil rapidly. Some fish products are heavily cured (salted, dried) and shelf stable at ambient temperature. An increasing number of fish products are preserved by low levels of salt, cooling, packaging in modified atmosphere, and/or addition of low levels of preservatives. The microflora of these products is often complex; however, spoilage is mostly caused by microbial action.

Production of fish has increased over many years and the increase in the last two decades has mostly been due to a dramatic growth in the aquaculture sector (Fig. 1). Catches from wild fish populations have stagnated at approximately 90–100 million metric tons and today (2005), 40% of the fish used for human consumption are aquaculture-reared species. Also, our processing of fish has changed. The increase in fish production has mostly been utilized as "fresh fish," whereas cured and canned products have become, proportionally, less popular (Fig. 2).

The raw materials for seafood products are bivalve mollusks, crustaceans, cephalopods, or finfish. These organisms have very different lifestyle and different gross composition, which influence subsequent spoilage patterns. Bivalve mollusks such as oyster accumulate glycogen (Martino & da Cruz, 2004), whereas other fish raw materials are virtually devoid of carbohydrates. Therefore, postmortem pH does not decrease to the same extent as pH in red meats and *Shewanella* species, which are quite sensitive to low pH can therefore grow and spoil chilled finfish similar to their involvement in spoilage of high-pH meat (Borch, KantMuermans, & Blixt, 1996). Crustacea have a high content of free amino acids, some of which (e.g., arginine and glycine) contribute to the characteristic crustacean flavor (Finne, 1992). Many cephalopods contain ammonia (NH_4^+) in quite high concentration

L. Gram (✉)
National Institute of Aquatic Resources, Technical University of Denmark, Soltofts Plads bldg 221, DK-2800 Kgs Lyngby, Denmark
e-mail: gram@aqua.dtu.dk

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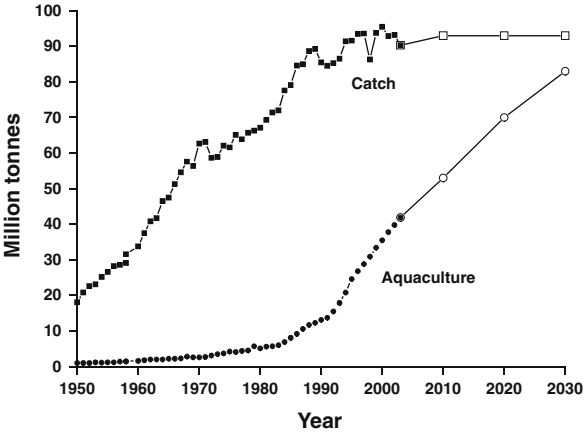

Fig. 1 Global fish production from wild fish catches and from aquaculture (FAO, 2004)

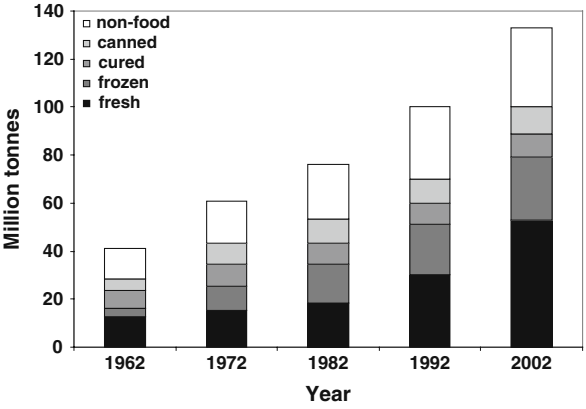

Fig. 2 Utilization of fish catches (FAO, 2004)

(100–300 mM) (Seibel, Goffredi, Thuesen, Childress, & Robison, 2004). Seafood raw materials are, in general, very rich in nonprotein nitrogen (NPN) such as amino acids and trimethylamine oxide, and bacterial metabolism of these compounds is often the cause of spoilage.

Spoilage Concepts

Spoilage of a food product is, in this chapter, a term used to describe that the product is no longer edible based on a sensory assessment. Safety issues are not included in the spoilage or shelf-life considerations. The sensory rejection may be caused by discoloration, physical changes, textural changes, slime or gas formation, or the development of off-flavors and off-odors. Spoilage may arise due to a number of chemical or microbiological changes. Lipid hydrolysis and oxidation are very

common causes of spoilage in many fatty fish species and rejection of small uneviscerated species such as anchovies may be caused by "belly burst" in which the enzymes and microorganisms of the digestive tract cause massive gas development (Careche, Garcia, & Borderias, 2002). Protein denaturation and development of "card box" flavor due to changes in the protein and lipid fraction are common causes of spoilage of frozen fish products. However, microbial growth and metabolism is the major cause of spoilage of fresh, lightly preserved, and semi-preserved fish products. Microbial spoilage involves growth of bacteria, yeast, or fungi to high numbers and products of their metabolism give rise to the sensory impressions perceived as spoilage. Visible slime may appear as a result of the formation of extracellular polysaccharides from carbohydrate substrates (Lyhs, Koort, Lundstrom, & Bjorkroth, 2004). Fungal growth on, for example, dried fish is also a very visible spoilage form (Chakrabarti & Varma, 2000) as is the red discoloration that often accompanies spoilage of heavily salted fish (Prasad & Panduranga Rao, 1994). Off-odors and off-flavors arise when low-molecular-weight compounds are degraded by microorganisms. Typical spoilage compounds of seafood products include ammonia from deamination of amino acids, sulfides formed from sulfur-containing amino acids (Herbert & Shewan, 1975), trimethylamine resulting from bacterial reduction of trimethylamine oxide, and esters that may arise from degradation of phospholipids (Table 1).

The microflora on newly caught or produced product is a function of the microorganisms present on the live animal and the microorganisms contaminating during processing. Each seafood processing operation has its own unique microflora reflecting the raw materials and the preservation parameters used (Bagge-Ravn, Yin, Hjelm, Christiansen, Johansen, & Gram, 2003). Only some of the microorganisms will be able to tolerate the product-specific conditions (temperature, packaging, pH, water activity) and proliferate during storage. Those successful will form the spoilage microflora (or microbiota), which simply are the microorganisms present on the product at the point of sensory rejection. Only some of the species present are able to produce the off-odors and off-flavors, which are typical of the spoiling product, and this spoilage potential is typically determined by inoculating pure cultures of bacteria in sterile food systems (Chinivasagam, Bremner, Wood, & Nottingham, 1998; Dalgaard, 1995b; Gram, Trolle, & Huss, 1987; Herbert, Hendrie, Gibson, & Shewan, 1971; Joffraud, Leroi, & Chevalier, 1998; Miller, Scanlan, Lee, & Libbey, 1973). Subsequently, it must be determined if the microorganisms with spoilage potential are capable of producing the amounts of compounds associated with the spoiling product under relevant conditions of storage. This assesses the so-called spoilage activity of the microorganisms. For instance, *Vibrio* spp., *Aeromonas* spp., *Shewanella* spp., and *Photobacterium phosphoreum* are capable of reducing trimethylamine oxide (TMAO) to trimethylamine (TMA). The latter, fishy smelling compound, is typical of spoiling gadoid fish species. Gadoid species are those fish belonging to the Gadidae family, which includes cod, haddock, whiting, and pollock.

Shewanella species can grow under aerobic storage to 10^8–10^9 cfu/g and produce the amounts of TMA found in aerobically, iced-stored fish. When the fish is packed

Table 1 Examples of substrates, spoilage products, and spoilage bacteria from different seafood products (modified from Gram, 2005)

Sensory impression	Spoilage substrate	Spoilage product	Food product	Specific organism	Reference
Slime	Sugars	Extracellular polysaccharide (dextran)	Acetic acid herring preserve	*Leuconostoc gelidum* *Leuconostoc gasicomitatum*	Lyhs et al. (2004)
Gas	Sugars, Protein	CO_2	Preserved herring	*Lactobacillus alimentarius* Yeast	Lyhs, Lahtinen, et al. (2001)
Fishy off-odor	Trimethylamine oxide	Trimethylamine	Several fish products	*Shewanella baltica* *Photobacterium phosphoreum* *Aeromonas* spp. *Vibrio* spp. Enterobacteriaceae	Dalgaard et al. (1993) Gram et al. (1990) Vogel et al. (2005)
Musty off-odor			Salted fish, fresh fish	*Pseudomonas* *Psychrobacter*	Bjorkevoll, Olsen, and Skjerdal (2003) Castell, Greenough, and Jenkin (1995)
Ammonia	Amino acids	Ammonia, NH_3	Several fish products	Enterobacteriaceae, lactic acid bacteria *P. phosphoreum*	Jorgensen, Huss, et al. (2000)
Sulfide	Cysteine	Hydrogen sulfide, H_2S	Several fish products	*S. baltica* Enterobacteriaceae *Lactobacillus saké,* *Lactobacillus curvatus*	Chai et al. (1968) Hansen (1995) Joffraud, Leroi, Roy, & Berdague (2001) Kadota and Ishida (1972) Segal and Starkey (1969)
Sulfhydryl off-odors	Methionine	Dimethyldisulfide, $(CH_3)_2S_2$	Several fish products	*Pseudomonas* Enterobacteriaceae	Chinivasagam, Bremner, et al. (1998) Kadota and Ishida (1972) Segal and Starkey (1969)

in CO_2 atmosphere, large amounts of TMA are detected; however, counts of *Shewanella* remain below 10^6 cfu/g. Although they have the spoilage potential, they do not possess sufficient spoilage activity. Instead, CO_2-tolerant *P. phosphoreum* grow and produce the TMA formed in packed fish (Dalgaard, 1995b; Dalgaard, Gram, & Huss, 1993).

In some products, the specific spoilage microorganisms are one single group or species, for example, *Shewanella baltica* in iced cod (Chai, Chen, Rosen, & Levin, 1968; Vogel, Venkateswaran, Satomi, & Gram, 2005), *P. phosphoreum* in CO_2-packed chilled fish (Dalgaard, Mejlholm, Christiansen, & Huss, 1997), or *Lactobacillus alimentarius* in some marinated herring (Lyhs, Korkeala, Vandamme, & Bjorkroth, 2001). The spoilage patterns of other products may be much more complex and spoilage is brought about by a combination of several microorganisms. Typically, these as single cultures do not give rise to the product spoilage off-odors but only when cocultured (Jorgensen, Huss, & Dalgaard, 2000; Mejlholm, Boknaes, & Dalgaard, 2005).

Fish Substrates of Spoilage

Fish muscle is rich in nonprotein nitrogen and the amino acids, nucleotides, and trimethylamine oxide serve as microbial substrates or electron acceptors. The products of microbial metabolites result in the spoilage of the products (Table 1). Trimethylamine oxide is a small odorless compound which accumulates in finfish, elasmobranches, cephalopods, and some bivalves (Sadok, Uglow, & El-Abed, 2003; Seibel & Walsh, 2002). In finfish, it is mostly found in marine gadoid fish species but can also be formed in other species. It was believed for many years that the compound was not produced in freshwater fish species; however, it has been detected in both Nile perch and tilapia (Anthoni, Borresen, Christophersen, Gram, & Nielsen, 1990). The role of TMAO in fish is not known but it is generally believed that it acts as a compatible solute balancing the effects of salt levels in the marine environment, deep pressure, or high concentrations of urea found in elasmobranches (Seibel & Walsh, 2002; Yancey, Clark, Hand, Bowlus, & Somero, 1982), where it stabilizes protein folding (Yancey, 2005). Several bacterial species may use TMAO as an electron acceptor in an anaerobic respiration and the reduced compound, trimethylamine (TMA), is the most prominent compound giving rise to "fishy" odor. TMAO/TMA is a redox couple and its presence in fish ensures a positive redox potential of approximately 200 mV as opposed to red meat where the Eh is negative. The Eh decreases to negative values when TMAO is reduced to TMA (Huss & Larsen, 1980; Yancey, 2005).

The ability to use TMAO as an electron acceptor gives obligately respiratory microorganisms an advantage when oxygen becomes limited. Ringo, Stenberg, and Strom (1984) suggested that the complete Krebs' cycle was used when *Shewanella* respired using TMAO and that amino acids such as cysteine were used as substrates, resulting in the parallel formation of hydrogen sulfide (H_2S). More recent studies

have determined that the metabolism is slightly more complex and that TMAO reduction involves only part of the TCA cycle (Scott & Nealson, 1994).

Sulfurous odors arise from bacterial degradation of L-cysteine and L-methionine. The volatiles consist of an array of compounds including hydrogen sulfide, methyl mercaptan, and dimethyl sulfide (Herbert & Shewan, 1975). Some sulfurous off-odors give rise to more fruity or onion-like off-odors. The fruity off-odors also consist of esters and are typically formed from degradation of monoamine–monocarboxylic amino acids (Castell & Greenough, 1959) but also low-molecular-weight fatty acids can act as substrates for bacterial production of fruity off-odors (Reddy, Bills, & Lindsay, 1969).

Bacterial decarboxylation of amino acids gives rise to the formation of the so-called biogenic amines (Table 2), some of which are off-odorous. These compounds have mainly been of interest due to their role in food safety being the cause of histamine poisoning; however, they have also been used as indicators of spoilage in a number of products.

Table 2 Amino acids and biogenic amines formed by bacterial degradation

Amino acid	Biogenic amine	Reaction
Histidine	Histamine	Decarboxylase
Tyrosine	Tyramine	Decarboxylase
Lysine	Cadaverine	Decarboxylase
Arginine	Agmatine	Decarboxylase
L-Phenylalanine	β-Phenylethylamine	Decarboxylase
Ornithine	Putrescine	Decarboxylase
	Spermidine	Spermidine synthase
	Spermine	Spermine synthase

Seafoods are products rich in protein and it is often stated that proteolytic bacteria are important for the spoilage process. However, the pool of free amino acids is more than sufficient to support bacterial growth and acts as substrates for the off-odors and off-flavors formed. In a series of classical experiments, Lerke and coworkers (Adams, Farber, & Lerke, 1964; Lerke, Adams, & Farber, 1963; Lerke, Adams, & Farber, 1965; Lerke, Farber, & Adams, 1967) inoculated spoilage bacteria into fish juice and separated it into a high-molecular-weight fraction (protein) and a low–molecular-weight fraction (amino acids and TMAO). In the LMW fraction, off-odors and spoilage compounds identical to the spoiling fish developed, whereas no off-odors were formed in the HMW fraction (Table 3).

The odor, flavor, and color of fish are influenced by its feed; however, little is known about the influence of fish feed on subsequent spoilage patterns. This is becoming an issue as an increasing amount of fish raw material is produced in aquaculture. Replacing 50% of fish meal in a typical trout diet with vegetable protein had no influence on subsequent growth of bacteria on iced fillets or on spoilage rates (Ozogul, Ahmad, Hole, Ozogul, & Deguara, 2006).

Table 3 Spoilage of low-molecular-weight and high-molecular-weight fractions of fish muscle press juice inoculated with a fish spoilage bacterium (modified from Lerke et al., 1967)

Sample	Day	Volatile reducing substances (microequivalents of reduction/5 ml)	Total volatile nitrogen (mg/100 ml)	Trimethylamine nitrogen (mg/100 ml)	Log (cfu/ml)
Whole	0	7.5	0	0	3.3
juice	1	7.5	0	0	7.35
	2	36	5	3.3	8.5
	2.5	–	6	3.2	8.9
Nonprotein	0	5	0	0	3.3
fraction	1	4.5	0	0	6.8
	2	16	2.8	2.5	8.6
	2.5	36	5.5	4.7	8.6
Protein	0	5	0	0	3.3
fraction	1	5	0	0	6.3
	2	4	0	0	7.8
	2.5	4	0	0	8.1

Taxonomy of an Important Fish Spoilage Bacterium

The understanding of the bacteriology of seafood spoilage was brought about by studies in a 20-year period from 1950 to 1970 by Castell (Castell & Anderson, 1948), by Lerke's group (Adams et al., 1964; Lerke et al., 1963, 1965, 1967), and by the microbiology team at Torry Research Station led by James Shewan (Castell & Anderson, 1948; Shaw & Shewan, 1968; Shewan, 1977). It was discovered, relatively early, that the "total bacterial count" was not a parameter that could indicate spoilage or remaining shelf life of fresh fish (Castell, Anderson, & Pivnick, 1948; Huss, Dalsgaard, Hansen, Ladefoged, Pedersen, & Zittan, 1974). However, the spoilage off-odors and off-flavors were produced by very specific bacteria, and the most noticeable were the fishy and sulfidy odors of spoiling gadoid fish species produced by Gram-negative, psychrotrophic bacteria capable of producing hydrogen sulfide and reducing trimethylamine oxide. The specific microorganisms associated with chill storage fish spoilage were originally grouped as *Achromobacter*, which was a compilation of Gram-negative, nonfermentative, rod-shaped bacteria, several of which today have been reclassified as *Acinetobacter*, *Moraxella, Psychrobacter*, and *Shewanella*. In 1941, Long and Hammer (Long & Hammer, 1941) reclassified the bacterium as *Pseudomonas* and due to its role in fish spoilage, the species became *Pseudomonas putrefaciens*. The research team at Torry Research Station classified pseudomonads into four groups (I, II, III, IV) (Shewan, Hobbs, & Hodgkiss, 1960) and the fish spoilage bacterium belonged to group IV. This bacterium was moved to the *Alteromonas* genera because of different GC contents between *Ps. putrefaciens* (43–53%) and the majority of pseudomonads (58–72%). Molecular approaches once again led to the bacterium's name being changed. Colwell and coworkers (Macdonell & Colwell, 1985) sequenced the 5S rRNA gene

of several marine bacteria and determined that the bacterium belonged to a completely new genus named *Shewanella.* Recently, results of 16S rRNA gene sequence analyses of genera from this group led to a proposal for a new family Shewanellaceae (Ivanova, Flavier, & Christen, 2004), which contains about 30 *Shewanella* spp. The number of new *Shewanella* species is constantly increasing. In 1998, Ziemke, Hofle, Lalucat, and Rossello-Mora (1998) characterized a large number of *Shewanella* isolates from the Baltic Sea using DNA–DNA hybridization, 16S rRNA gene sequencing, and phenotypic testing. They concluded that strains classified as *Shewanella putrefaciens* were a diverse group and belonged to two different species, one being *S. putrefaciens* and a new one being *S. baltica*. Recent studies (Vogel et al., 2005) have revealed that the H_2S-producing bacteria that develop during iced storage of cod and plaice are indeed *S. baltica* strains. Although it is not possible to know whether bacterial isolates from former studies are *S. baltica* or *S. putrefaciens* or even some of the new psychrotrophic *Shewanella* species such as *S. hafniensis* or *S. morhaue* (Satomi, Vogel, Gram, & Venkataraman, 2006; Shewan et al., 1960), one must assume that several of these probably today would be classified as *S. baltica*. Recent studies of low-temperature-stored garfish and tuna have also identified the H_2S-producing bacteria of the spoilage microflora as *S. baltica* (Dalgaard, Madsen, Samieian, & Emborg, 2006; Emborg, Laursen, & Dalgaard, 2005).

Microbiology of Freshly Caught Fish

The muscles of healthy fish are sterile and microorganisms reside at the surfaces such as skin, gills, and gastrointestinal tract of finfish. The level of microorganisms vary depending on the area of catch; however, the skin typically contains 10^4 cfu/cm^2, the gills 10^6 cfu/g, and the digestive tract up to 10^8 cfu/g (Austin, 2002). The level of microorganisms in the digestive tract may vary from 10^4 to 10^9 cfu/g (Spanggaard et al., 2000). Most of the microbiota are culturable under standard laboratory conditions; however, the digestive tract may contain high levels of anaerobic microorganisms (Huber, Spanggaard, Appel, Rossen, Nielsen, et al., 2004) requiring special culture conditions.

A wide array of different bacterial species can be found on fish; however, the dominant microbes are genera or species typical of the aquatic environment, including pseudomonads, coryneforms, and bacteria belonging to the *Acinetobacter/Moraxella* group (Table 4). In some studies of tropical fish species, Gram-positive bacteria dominate the microbiota; however, the bacterial flora mostly consists of Gram-negative species. The microbiota on the skin surface is often dominated by aerobic, nonfermentative microorganisms, whereas a higher proportion of fermentative bacteria are present on the gills and in the gastrointestinal tract (Spanggaard et al., 2001). The bacteria that subsequently become important in spoilage of fresh finfish are present only in small proportions. Vogel et al. (2005) determined that the number of H_2S-producing bacteria varied between 10^1 and 10^3 and constituted between 0.1 and 10% of the total bacterial count. After 3 weeks of storage, the counts had increased to 10^8–10^9 and H_2S-producing bacteria constituted

Table 4 Bacterial genera associated with raw finfish and crustaceans, and their percentage of the total microflora (modified from ICMSF, 2005)

	Percentage Composition			
	Marine		Freshwater	
Group or species	Temperate	Tropical	Temperate	Tropical
Pseudomonas	3–32	2–22	0–26	0–6
Vibrionaceae	1–29	0–28	0–7	0–2
Acinetobacter–Moraxella	11–56	9–30	0–47	10–43
Flavobacterium–Cytophaga	2–22	4–25	–	0–11
Other Gram-negative bacteria	0–21	0–12	0–55	0–30
Coryneforms	3–81	0–43	0–15	0–5
Gram-positive cocci	1–23	3–51	0–45	30–50
Bacillus	0–4	0–2	0–5	0–40
Others	0–8	0–22	0–34	–

2–30% of the viable count. In the warm summer months, several of the H_2S-producing bacteria were mesophilic *Shewanella algae,* which is a human pathogen. These bacteria quickly disappeared upon iced storage and the psychrotrophic H_2S-producing bacteria subsequently dominated the spoilage bacterial community.

Product Categories and Spoilage

The principal spoilage microorganisms and microbial spoilage processes are described in this chapter. The seafood products are grouped in categories having somewhat similar microbial ecology – and hence, similar spoilage processes. Table 5 provides examples of typical shelf lives of some seafood products.

Raw, Fresh Seafood

Bivalve Mollusks

As mentioned, oysters have a relatively high content of glycogen and spoilage is characterized by the growth of lactic acid bacteria, the formation of lactic acid, and a concurrent decrease in pH. The pH of fresh oysters is approximately 6 and spoiled oysters have pH values of 4.9–5.3 (Aaraas et al., 2004; Cook, 1991). Other studies have noted an increase in bacteria such as *Ps. putrefaciens* (Brown & McMeekin, 1977) or *Pseudoalteromonas* (Romero, Gonzalez, & Espejo, 2002) during spoilage and this is more likely to cause development of amine compounds. It is often stated that bacteria will not grow when mollusks are stored live; however, Lorca, Pierson, Flick, and Hackney (2001) determined that counts of halotolerant bacteria (such as *Vibrio* spp.) increased from approximately 10^5 to 10^8 cfu/g over a 10-day storage period. The increase was seen at storage temperatures of 7, 13, and 21°C.

Table 5 Typical shelf lives of some seafood products – shelf lives are a function of sensory acceptability and do not consider safety aspects

Category	Subcategory	Product example	Storage conditions	Typical shelf life
Raw fresh seafood	Mollusks	Live oysters	Live, 10°C	3–6 days
	Crustaceans	Shrimp	In ice (0°C)	10–16 days
	Cephalopods	Squid	In ice (0°C)	8–12 days
	Finfish	Cod	In ice (0°C)	12–16 days
Packed fresh fish		Cod	CO_2 packed, at 2°C	11–12 days
Cured seafood	Salted, cold-smoked fish	Cold-smoked salmon	Vacuum packed, 5°C	3–8 weeks
	Other lightly preserved products	Brined shrimp	Contain sorbate, benzoate, packed, 5°C	1–2 months
	Semipreserved products	Marinated herring	Acetic acid brine, preservatives, 5°C	3–6 months
	Heavily salted fish	Tropical salted fish	Aerobic	
Heated seafood products	Hot-smoked fish	Hot-smoked mackerel	Ambient temperature, aerobic, vacuum-packed, 5°C	2–6 days 1–2 months
	Sous vide-cooked products	Cod	Heated, vacuum packed, 3°C	1–2 weeks
	Fully canned products	Tuna		1–2 years
Miscellaneous	Frozen fish	Cod	–20°C	1–12 months
	Surimi	White fish	5°C	5 days
			Double washed, –18°C	12 months

Crustaceans

Fresh shrimp are often stored in ice for a few days to facilitate loosening of the shell, known as ripening (Hoegh, 1989). This process is autolytic and partly caused by the increase in pH which loosens the shrimp shell proteins that have a low p*I* (Hoegh, 1989). The content of ammonia increases steadily during iced storage to 1.5 g/kg after 7–8 days of storage. This is primarily a result of autolytic proteases and is accompanied by a concurrent increase in free amino acids (Hoegh, 1989).

Psychrotrophic bacteria increase during storage (Cann, 1973). The spoilage flora may contain a large proportion of *Moraxella* or *Acinetobacter* (Surendran, Mahadeva Iyer, & Gopakumar, 1985; Vanderzant, Cobb, Thompson, & Parker, 1973), but other studies have revealed that pseudomonads and alteromonads dominate (Shamshad, Kher, Riaz, Zuberi, & Qadri, 1990). *Pseudomonas fragi* and *Shewanella putrefaciens*have been identified as the primary spoilage agents of chill-stored shrimp, with *Ps. fragi* spoiling iced-stored shrimp and *S. putrefaciens* being the dominant microorganism in shrimp stored in ice slurry (Chinivasagam, Bremner, Thrower, & Nottingham, 1996). Naturally, spoiling prawns are characterized by amines, sulfides, and esters and both *Ps. fragi* and *S. putrefaciens* produced sulfides, whereas amines were mostly formed by *S. putrefaciens* and the spoilage esters mainly by *Ps. fragi* (Chinivasagam, Bremner, et al., 1998).

Indole has been suggested as a spoilage indicator for shrimp and prawn; however, this is not a universal compound in all species. Indole is produced by bacteria that degrade tryptophan. Levels may, in some species, increase to above 100 μg/100 g and some regulatory agencies use 250 μg/100 g as the spoilage limit. Indole production is mainly an issue of high-temperature storage and is likely caused by members of Enterobacteriaceae; however, indole is not formed in all species (Mendes, Goncalves, Pestana, & Pestana, 2005), not even at elevated temperatures in severely spoiled product (Table 6). Sensory rejection of crustacea may also be caused by the formation of black spots (melanosis). Melanin is formed from tyrosine and it has been suggested that this is a bacterial process as several spoilage pseudomonads can oxidize tyrosine (Chinivasagam, Bremner, & Reeves, 1998). This process is normally prevented or controlled by dipping the shrimp in sulfites or other reducing components.

Cephalopods

The shelf life of iced-stored squid is slightly shorter than that of most finfish and the product is rejected in 8–12 days (Albanese, Cinquanta, Lanorte, & Di Matteo, 2005; Paarup et al., 2002; Vaz-Pires & Barbosa, 2004). Spoilage is characterized by ammoniacal off-odors and the rapid onset of ammonia production at relatively low cell densities suggested that spoilage is mainly autolytic and not caused by bacterial growth (Vaz-Pires & Barbosa, 2004). Paarup et al. (2002) determined that the spoilage microflora was dominated by *Pseudoalteromonas* that reached 10^7 cfu/g. This bacterium may contribute to late ammonia formation. Trimethylamine also

Table 6 Indole formation in shrimp/prawns (ICMSF, 2005)

Species	Temperature (°C)	Time (days)	Indole (μg/100 g)	Reference
Penaeus merguiensis	0–4	9–13	4	Shamshad et al. (1990)
Penaeus setiferus or *Penaeus duorarum*	0–4 12–22	8–13 1–2	10–15 >100	Chang, Cheuk, Nickelson, Martin, and Finne (1983)
Pandalus platycens	0	14–21	30–40	Layrisse and Matches (1984)
Pandalus jordani	0	10	65	Matches (1982)
	11	3	130	
	22	2	623	
Pandalus borealis	0	10	4	Solberg and Nesbakken (1981)
	22	1	1	

develops during iced storage (Paarup et al., 2002) but it is not known which microbes are responsible for its production.

Finfish

Storage of finfish at ambient temperature leads to rapid growth of mesophilic Gram-negative bacteria belonging to Vibrionaceae or Enterobacteriaceae (Len, 1987; Liston, 1992). These bacteria reduce TMAO to TMA and produce several sulfides and shelf life is short, typically less than 24 h. Cooling, mostly in flaked or crushed ice, is the most common and most effective method for preservation of fresh finfish. The initial biochemical changes in the fish muscle are autolytic and related to the breakdown of ATP. These changes, however, do not have a major impact on sensory quality. Lipid hydrolysis and oxidation may proceed in fatty fish species and contribute to the development of unpleasant off-flavors and off-odors. However, the most offensive off-odors and flavors leading to spoilage at a low temperature are a consequence of bacterial action. As temperature decreases, the microbiota of the product changes and at 0–2°C, the spoilage microflora which reach 10^8–10^9 cfu/g after 2–4 weeks is mostly dominated by pseudomonads and shewanellae (Fig. 3, Table 7) even though other psychrotrophic microbes sometimes also grow. Some studies have reported the growth of the Gram-positive bacterium *Brochothrix thermosphacta* in iced finfish (Grigorakis, Alexis, Gialamas, & Nikolopoulou, 2004; Lalitha et al., 2005) but their numbers are two orders of magnitude lower than numbers of *Shewanella* (Koutsoumanis & Nychas, 1999). *Photobacterium phosphoreum* may also grow during aerobic low-temperature storage of some fish species, and together with shewanellae and pseudomonads constitute the spoilage microflora (Dalgaard et al., 2006). The specific importance of each of these three groups in the actual spoilage process has not been described.

The dominance of pseudomonads and shewanellae has been seen in warm tropical freshwater fish and in fish caught in cold, marine waters (Gram, Wedell-Neergaard, & Huss, 1990; Koutsoumanis & Nychas, 1999). Growth of these psychrotrophic bacteria in marine fish is accompanied by the production of TMA

Table 7 Changes in the composition of the microflora during storage of a marine (mackerel, *Rastrelliger faughni*Matsui) and a freshwater (*Tilapia aurea*) fish species in ice (Acuff, Izat, & Finne, 1984; Barile, Milla, Reilly, & Villadsen, 1985a, 1985b)

Species/group	Percentage composition of the microflora at different times during iced storage									
	Mackerel					Tilapia				
Storage time	0	1	5	10	15	0	3	9	15	19
cfu/g	10^4	10^5	5×10^5	5×10^7	10^9	2×10^4	2×10^2	10^3	5×10^6	6×10^7
Pseudomonas	30	25	38	58	53	16	27	62	82	71
Shewanella			38	14	42					
Acinetobacter/Moraxella	10	8				19	11	6	16	15
Flavobacterium						13	1	7		
Vibrionaceae						16	1	6		
Enterobacteriaceae	10		6				4			
Bacillus	40	42	18	14		1	13		1	6
Micrococcus						26	22	3		2
Coryneform	10	17				4	22	16	1	6
Unknown/other		8		14	5	5				

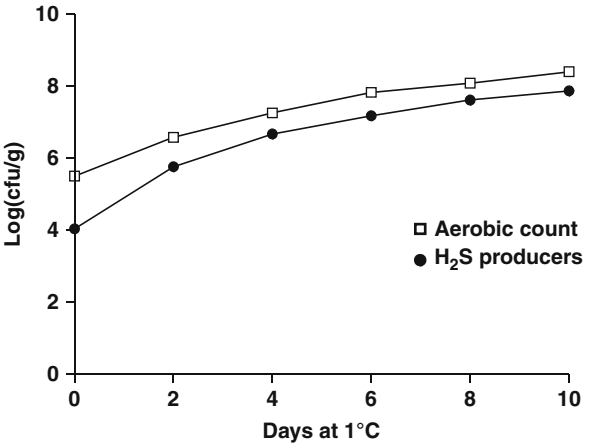

Fig. 3 Changes in total bacterial count and in H_2S-producing bacteria during storage of haddock fillets at 1°C. At day 0 and 10, H_2S-producing bacteria account for 3 and 29% of the total bacterial count, respectively (Chai et al.,1968)

caused by *Shewanella* metabolism. *Pseudomonas* spp. dominate the spoilage of iced freshwater finfish (Chytiri, Chouliara, Savvaidis, & Kontominas, 2004; Gelman, Glatman, Drabkin, & Harpaz, 2001; Gram, 1989) and the spoilage off-odors are typically more fruity or onion-like. Remaining shelf life of iced gadoid species can be predicted based on numbers of H_2S-producing bacteria because their numbers correlate closely with sensory rejection (Fig. 4; Jorgensen, Gibson, & Huss, 1988).

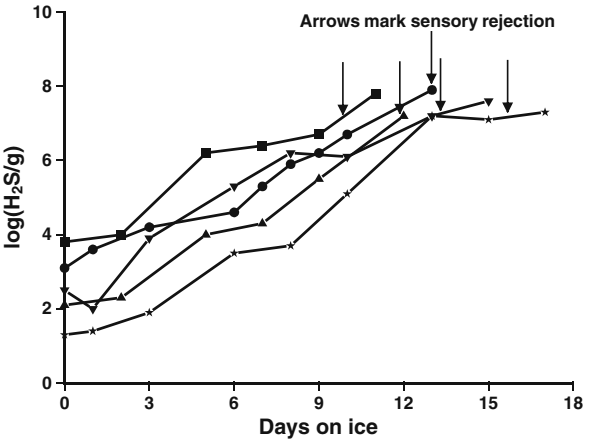

Fig. 4 Growth of H_2S-producing bacteria in iced cod and the rejection based on sensory evaluation (Jorgensen et al., 1988)

Temperature is the most important factor influencing shelf life of fresh finfish. Shelf life of iced cod is approximately 14–16 days (Jorgensen et al., 1988), whereas increasing the temperature to 5°C shortens the shelf life to 6–7 days.

Packed, Fresh Fish

Spoilage of aerobic chill-stored fish is mainly caused by the growth of pseudomonads and shewanellae which are respiratory bacteria and it could be expected that removal of the oxygen-containing atmosphere either by vacuum packing or by CO_2 packaging would result in extension of shelf life. However, *Shewanella* species are capable of anaerobic respiration using TMAO as an electron acceptor (Jorgensen & Huss, 1989; Pitt & Hocking, 1999; Ringo et al., 1984; Scott & Nealson, 1994) and the CO_2-tolerant marine bacterium *P. phosphoreum* also grows well in vacuum-packed fish at low temperatures (Dalgaard et al., 1993). Therefore, vacuum packing does not result in a marked extension of the sensory shelf life of many marine fish species. Freshwater fish and some tropical species do not carry these two spoilage bacteria in high amounts and vacuum packing is likely to select for a microflora dominated by lactic acid bacteria (Hussain, Ehlermann, & Diehl, 1976; Pedersen & Snabe, 1995); however, little is known about the effect on shelf life. Concern has been raised that vacuum packing would increase the risk of botulism because *Clostridium botulinum* type E would be able to grow and produce toxin if temperatures were above 3°C. However, no case of botulism has ever been attributed to fresh, packed fish. First, no toxin was detected in a survey of 1,100 commercial packages of vacuum-packed fresh fish (Lilly & Kautter, 1990) and second, type E toxin is heat labile and would likely be inactivated in cooked product (Ohye & Scott, 1957).

One would assume that packaging in a CO_2-containing atmosphere would cause a dramatic extension of chilled shelf life because CO_2 has a marked inhibitory effect on respiratory bacteria such as shewanellae and pseudomonads. Red meat typically spoils due to the growth and metabolism of pseudomonads and packaging in CO_2 extends chilled shelf life from days to months, with the microflora becoming dominated by lactic acid bacteria (Borch et al., 1996). The shelf life of marine fish species packaged in a CO_2 atmosphere is only marginally extended as compared to nonpackaged product and in gadoid species, significant amounts of trimethylamine are produced (Dalgaard et al., 1993; Debevere & Boskou, 1996; Ruiz-Capillas, Saavedra, & Moral, 2003). This is due to the growth and metabolism of the CO_2-tolerant marine bacteria *P. phosphoreum* (Dalgaard et al., 1993; Debevere & Boskou, 1996; Emborg, Laursen, Rathjen, & Dalgaard, 2002) and remaining shelf life of CO_2-packaged cod can be modeled based on numbers of *P. phosphoreum* (Dalgaard, 1995a). *Photobacterium phosphoreum* grows to 10^7–10^8 cfu/g during low-temperature storage of packed fish and the amounts of TMA produced far exceeds the amounts formed during spoilage of nonpacked fish. This is due to very high production of TMA per cell of *P. phosphoreum* (Dalgaard, 1995b).

CO_2 packaging of fish that do not contain *P. phosphoreum* extends shelf life to some degree. *Photobacterium phosphoreum* is sensitive to freezing and a freezing step before CO_2 packaging eliminates the bacteria and extends shelf life of the packaged product (Dalgaard, Munoz, & Mejlholm, 1998; Emborg et al., 2002). Also, the shelf life of freshwater fish or tropical water fish that do not naturally harbor *P. phosphoreum* may be extended by packaging either under vacuum (Merivirta,

Koort, Kivisaari, Korkeala, & Bjorkroth, 2005) or in a CO_2 atmosphere (Gimenez & Dalgaard, 2004; Reddy, Villanueva, & Kautter, 1995). The elimination of *P. phosphoreum* from packaged fish often leads to a dominance by lactic acid bacteria and *B. thermosphacta* which can reach 10^7–10^8 cfu/g (Emborg et al., 2002; Lannelongue, Finne, Hanna, Nickelson, & Vanderzant, 1982), which is similar to patterns observed for stored, packaged red meats (Borch et al., 1996; Dainty & Mackey, 1992). A bacterium belonging to Enterobacteriaceae has also been detected as a major part of the spoilage microflora in CO_2-packaged tuna fish (Emborg et al., 2005). This bacterium and *P. phosphoreum* are both capable of forming large amounts of histamine at low temperatures (2°C) (Emborg et al., 2005, 2002). The production of biogenic amines is in several fish species believed to be a consequence of elevated temperatures that allow mesophilic *Morganella morganii* to grow and decarboxylate amino acids (Lehane & Olley, 2000). Amine production is of special interest in scombroid fish species which are rich in histidine, the precursor of histamine. *Photobacterium phosphoreum* may produce histamine (van Spreekens, 1987) at refrigeration temperatures but the finding of other *M. morganii*-like psychrotolerant histamine producers is novel. Histamine production in Sri Lankan tuna fish packed in CO_2 and N_2 and stored at 2°C began at day 3 and increased to more than 1,000 ppm on day 9 (Emborg et al., 2005). In garfish stored aerobically at 0°C, only low concentrations of histamine were detected and only after sensory rejection; however, at 5°C, more than 1,000 ppm were formed both in aerobically stored and CO_2-packaged garfish (Dalgaard et al., 2006). Histamine formation parallels TMA formation and, hence, is a similar spoilage indicator.

Cured Seafood

Curing of fish refers to a wide variety of preservation principles combining salting, acidification, fermentation, and addition of preservatives such as sorbate, benzoate, lactate, and acetate. Although the proportion of the fish catch that is used for cured products is decreasing (Fig. 2), still at least 10 million tons are preserved in this manner. As the degree of preservation is "increased" compared to fresh fish, there is a change in the spoilage microflora toward fermentative Gram-negative bacteria, lactic acid bacteria, and yeast (Gram, 2005).

Salted, Cold-Smoked Fish

Especially salmon, but also to some extent trout, cod, and halibut are processed as lightly salted, cold-smoked products. The salt concentration is typically between 3 and 6% (as water-phase salt) and the cold-smoking process never increases above 28–30°C. Fish proteins denature at higher temperatures and the fish would obtain a "cooked" appearance as in hot-smoked products. The process may also rely on a combination of drying and addition of liquid smoke (Siskos, Zotos, & Taylor, 2005). Cold-smoked products are typically packaged under vacuum and retail distributed at refrigeration temperature. Often the products are held under frozen storage before

display at retail. If products are stored aerobically, spoilage occurs more rapidly and visible colony growth of pseudomonads and yeast cells can be seen. Packaging of salmon and other fatty fish species also serves the purpose of eliminating oxidation of the lipid fraction as rancidity otherwise becomes a major off-odor.

The shelf life of vacuum-packaged, cold-smoked fish varies between species and factories and depends to some extent on the degree of drying, smoking, and the amount of salt added (Leroi & Joffraud, 2000). The sensory shelf life varies between 3 and 9 weeks (Jorgensen, Dalgaard, & Huss, 2000); however, the longer shelf lives may not be acceptable from a safety perspective as, for instance, *Listeria monocytogenes* and sometimes *C. botulinum* may be able to grow in this product (Gram, 2001a, 2001b). Recently, consumption of a packed cold-smoked salmon product 3 days after "use-by-date" was linked to a case of botulism (Dressler, 2005). The total bacterial count is reduced slightly by the cold-smoking process and is between 10^1 and 10^4 cfu/g in the freshly produced product (Dondero, Cisternas, Carvajal, & Simpson, 2004; Hansen, Drewes Rontved, & Huss, 1998; Leroi, Joffraud, Chevalier, & Cardinal, 1998).

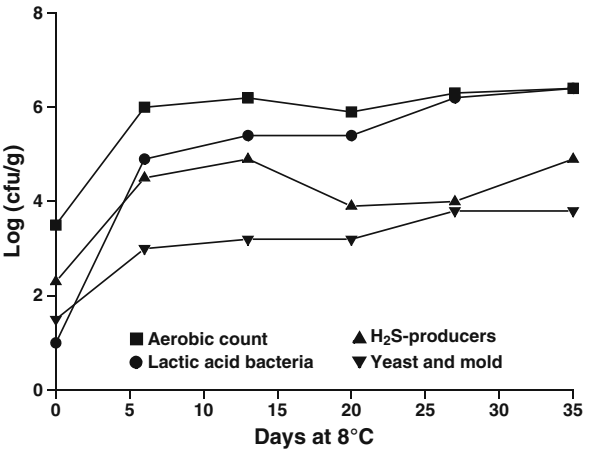

Fig. 5 Changes in aerobic count, lactic acid bacteria, H_2S-producing bacteria, and yeasts and molds in cold-smoked salmon stored under vacuum at 8°C (Leroi et al., 1998)

The total bacterial count of vacuum-packaged, cold-smoked fish increases during refrigerated storage (4–5°) and the spoilage microflora is often dominated by lactic acid bacteria or a combination of lactic acid bacteria and fermentative Gram-negative bacteria (Enterobacteriaceae or *P. phosphoreum*) (Hansen et al., 1998; Leisner, Millan, Huss, & Larsen, 1994; Leroi et al., 1998; Lyhs, Bjorkroth, Hyytia, & Korkeala, 1998). Lactic acid bacteria counts typically increase to 10^6–10^8 cfu/g during a few weeks of refrigerated storage (Fig. 5) (Jorgensen, Dalgaard, et al., 2000a; Leroi & Joffraud, 2000). The spoilage pattern of cold-smoked fish is complex and no single bacterial species has been identified as being responsible for the spoilage. Although autolytic changes may cause some textural changes, the actual spoilage is of bacterial origin (Hansen, Gill, Rontved, & Huss, 1996). Several types of lactic acid bacteria may become dominant in cold-smoked fish; some studies revealed that

the microflora is dominated by carnobacteria (Paludan-Muller, Dalgaard, Huss, & Gram, 1998) and in other trials different *Lactobacillus* species are dominant. Based on phenotypic characteristics, the lactobacilli have been identified as *Lb. curvatus, Lb. sakei*, and *Lb. plantarum,* with each of three processing plants having their own composition of the flora (Hansen & Huss, 1998). The odor profile of spoiled cold-smoked fish varies and a range of potentially odorous volatile compounds are produced during spoilage, including alcohols, aldehydes, esters ketones, and phenols (Jorgensen, Huss, & Dalgaard, 2001). Only some compounds were produced in quantities exceeding an odor threshold and trimethylamine and 3-methylbutanal were both believed to contribute to the spoilage odor profile (Jorgensen et al., 2001). Several of the bacteria that can be isolated from the spoilage microflora of cold-smoked fish produce spoilage off-odors either as pure cultures or as mixed cultures (Joffraud et al., 1998; Stohr, Joffraud, Cardinal, & Leroi, 2001). However, it has not been possible to correlate these odor profiles directly to the profile of the spoiling product.

Cold-smoked fish products are high-value delicatessen products which are traded globally, and an objective chemical "spoilage index" would be valuable in quality and shelf-life determinations (Leroi, Joffraud, Chevalier, & Cardinal, 2001). It has been demonstrated that the production of biogenic amines may be indicative of spoilage, although the amines themselves apparently do not contribute to the spoilage profile (Jorgensen, Huss, et al., 2000). Several different combinations of biogenic amines can be found at the point of spoilage (Table 8) (Jorgensen, Dalgaard, et al., 2000) and some are likely to be the result of metabolism by mixed bacterial communities. For instance, most spoilage bacteria produce only low concentrations (max 10 μg/10 g) of putrescine when grown as single cultures (Jorgensen, Huss, et al., 2000). The spoiling product is characterized by much higher putrescine concentrations and cocultures of lactic acid bacteria and Gram-negative bacteria give rise to the concentrations equivalent of the spoiling product. A similar metabiosis is seen in spoilage of vacuum-packed meat (Dainty, Edwards, Hibbard, & Ramantanis, 1986; Edwards, Dainty, & Hibbard, 1985). Putrescine is formed by ornithine decarboxylation which is common in several Gram-negative bacteria. The pool of ornithine may be replenished by lactic acid bacteria that use arginine deiminase to convert arginine to citrulline and ornithine carbamoyltransferase to convert citrulline to ornithine (Jorgensen, Huss, et al., 2000).

Other Lightly Preserved Seafood Products

A number of other delicatessen seafood products are similar to the cold-smoked fish in preservation profile and, hence, in spoilage microbiology. For instance, cooked shrimp may be packaged in modified atmosphere and distributed at refrigeration temperature (Mejlholm et al., 2005) or brined before packaging (Dalgaard et al., 2003). These products contain 2–3% NaCl (water-phase salt) and are preserved with either lactic acid or a combination of citric, sorbic, and benzoic acid. The shelf life is 2–3 weeks and the spoilage microflora is dominated by lactic acid bacteria accompanied by *B. thermosphacta* that grow to 10^7–10^8 cfu/g (Mejlholm et al., 2005).

Table 8 Production of biogenic amines by pure and mixed cultures of bacteria isolated from spoiled cold-smoked salmon (Jorgensen, Dalgaard, et al., 2000; Jorgensen, Huss, et al., 2000)

Species/group	No of strains or batches	Biogenic amines (μg/g)				
		Agmatine	Cadavarine	Histamine	Putrescine	Tyramine
Photobacterium phosphoreum	3	200	400	180	9	90
Aeromonas	1	<	50	<	<	<
Serratia liquefaciens	2	<	400	<	9	<
Enterobacter	2	13	5	<	10	<
Hafnia	1	<	180	<	5	<
Lactobacillus curvatus (I)	5	<	<	<	6	0–200
Lactobacillus curvatus (IV)	2	<	5	<	6	0–100
Lactobacillus sakei	3	<	<	<	5	<
Carnobacterium divergens	2	<	<	<	4	95
S. liquefaciens or *Hafnia* + *C. divergens* or *Lb. sakei*					25–80	
C. divergens + Gram-negative bacteria						80–130
Spoiling product (I)	6	90–270	150–350	100–240	3–35	80–140
Spoiling product (II)	3	2–30	100–135	3–50	8–40	130–180
Spoiling product (III)	2	2–25	180–300	10–15	190–380	225–335
Spoiling product (IV)	1	20	35	20	30	200

Single bacterial cultures did not produce the odor profile typical of the spoiling product, but a coculture of *Carnobacterium maltaromaticum* and *B. thermosphacta* produced the typical "wet dog" off-odors.

The Scandinavian speciality "gravad" fish is also a lightly preserved product similar to cold-smoked fish. Fillets are sprinkled with salt, sugar, herbs (typically dill), and spices and left under slight pressure for 1–2 days at refrigeration temperature. The product is stored aerobically or vacuum packaged (Leisner et al., 1994; Lyhs, Bjorkroth, & Korkeala, 1999; Lyhs, Lahtinen, et al., 2001). The aerobic count increases in vacuum-packed product to approximately 10^8 cfu/g over 2–3 weeks and the microflora is dominated by lactic acid bacteria and also H_2S-producing bacteria, which may increase to 10^6 cfu/g (Lyhs, Lahtinen, et al., 2001). The dominant lactic acid bacteria are *Lactobacillus sakei*, *Lb. curvatus*, and *Carnobacterium piscicola* (now *C. maltaromaticum*) (Lyhs, Korkeala, & Bjorkroth, 2002).

Barrel-salted lumpfish roe (as described below) is used in a desalted type of product in which the salt concentration is adjusted to 4% and pH is reduced with lactic acid to approximately 5.5 (Basby, Jeppesen, & Huss, 1998a). This product is packed in airtight containers and distributed at refrigeration temperature. Spoilage which manifests itself with off-odors characterized as sulfidy, sour, or rotten is caused by bacterial growth (Basby, Jeppesen, & Huss, 1998b). Lactic acid bacteria increased to 10^7–10^8 cfu/g and Enterobacteriaceae were detected at levels between 10^5 and 10^6 cfu/g. When assessing spoilage potential, only *M. morganii* strains produced off-odors in a heat-treated roe (Basby, Jeppesen, & Huss, 1998c). Although not tested, it is likely that spoilage in this product is brought about by an interaction between the lactobacilli and the Gram-negative bacteria present.

Semipreserved Seafood Products

Several fish products are preserved by NaCl, acid, and preservatives like benzoic acid, sorbic acid, lactate, acetate, and/or nitrate. Some of the products are based on raw fish such as Scandinavian marinated herrings, the German rollmops, or the anchovies of Southern Europe. Other types of products such as brined shrimp are based on cooked raw materials.

The raw materials for marinated herring are either barrel-salted herring stored for 6–12 months or fillets that are acid-brined for 2–3 weeks. Subsequently, the fish is drained and covered in a brine (or marinade) with salt, acetic acid, sugar, and preservatives. The products are left to ripen and stored at 5–10°C. Often these products are microbiologically stable; however, when spoilage occurs, it is typically due to the growth of acetic acid-tolerant lactic acid bacteria (Lyhs et al., 2004; Lyhs, Korkeala, et al., 2001) or yeasts (Somners, 1975). Spoilage can be characterized by gas formation produced by heterofermentative lactobacilli such as *Lb. alimentarius* (Lyhs, Korkeala, et al., 2001). Spoilage may also be manifest by slime or ropiness which is caused by the growth of *Leuconostoc* species (Lyhs et al., 2004) or halophilic Gram-negative rod-shaped bacteria (Magnusson & Moeller, 1985). pH is an important parameter in preventing the growth of spoilage lactobacilli and yeasts (Fig. 6).

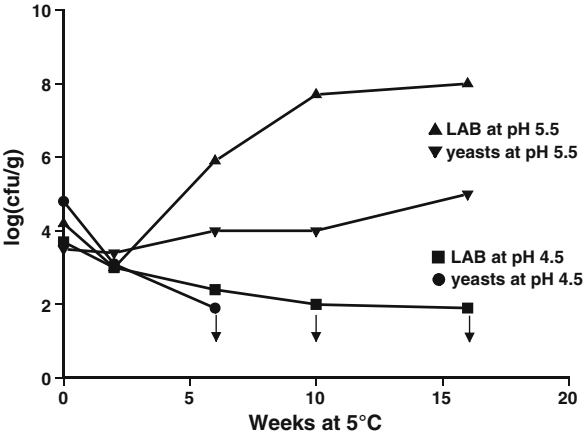

Fig. 6 Growth of lactic acid bacteria and yeasts in brine of marinated herring is dependent on pH (Jessen, 1987)

Ripened anchovies are prepared by salting partially gutted (or nongutted) fresh anchovies. By tradition, the product is packaged in cans but these are not heat sterilized and must be held at refrigeration temperature. Microbial counts decrease during the ripening process to less than 10^4 cfu/g (Pons-Sanchez-Cascado, Veciana-Nogues, & Vidal-Carou, 2003). Biogenic amines increase during the ripening process but do not reach hazardous levels under controlled salt and storage conditions (Pons-Sanchez-Cascado et al., 2003). Total volatile bases increase during ripening (Pons-Sanchez-Cascado, Veciana-Nogues, Bover-Cid, Marine-Font, & Vidal-Carou, 2005) but this is believed, as the herring ripen, to be caused by enzymes from the gut and is not a consequence of bacterial action. The microflora rapidly becomes dominated by halophilic bacteria but their counts remain relatively low (10^4–10^5 cfu/g) (Perrez Villarreal & Pozo, 1992).

Similar to the herring and anchovy products, some types of fish roe are also barrel salted (at 15–25% NaCl) for months before further processing to caviar products. Caviar is produced from a number of fish species of which the most famous is sturgeon, but also a number of other species such as cod and lumpfish are used (Bledsoe, Bledsoe, & Rasco, 2003). Some of these products retain high levels of salt during subsequent marketing but others are desalted before marketing. Yet others are processed directly to lower levels of salt, i. e., 5–8% NaCl (water phase salt) (Bledsoe et al., 2003). Very little is known about spoilage of these products and microbiological studies have mainly addressed food safety issues related to potential for growth of *C. botulinum* and *L. monocytogenes*.

Heavily Salted Fish

Some fish, including gadoid species, are sometimes preserved exclusively by heavy salting. This process eliminates all of the spoilage bacteria described in the products above and the product is shelf stable at ambient temperature for a long time.

Table 9 Fungal spoilage of heavily salted fish (data from Pitt & Hocking, 1999)

Geographical region	Genera of filamentous fungi	Species of filamentous fungi
Temperate climate	*Wallemia*	*W. sebi*
Subtropical climate	*Hortaea*	*H. werneckii*
Tropical	*Aspergillus*	*A. flavus, A. niger*
		A. clavatus, A. penicilliodes, A. wentii
		A. fumigatus,*A. restrictus*
	Penicillium	*P. chrysogenum, P. citrinum*
		P. thomi, P. chalybeum
	Polypaecilum	*P. pisce*
	Eurotium	*E. rubrum, E. amstelodami, E. repens*
	Basipetospora	*B. halophila*
	Cladosporium	*C. cladosporioides*
	Scopulariopsis	*S. brevicaulis*

However, the use of poor-quality salt can lead to a very characteristic type of spoilage called pinking. This is due to the growth of red-pigmented halophilic bacteria (Lamprecht, 1988; Prasad & Panduranga Rao, 1994; Prasad & Seenayya, 2000) that are strongly proteolytic causing a softening of the muscle and rotten off-odors. Pink is a traditional term for visible growth of extremely halophilic Gram-negative bacteria, such as *Halobacterium salinarium*, that belong to the family Halobacteriaceae. Most are nonmotile and obligate aerobes. Also, especially in tropical countries, this type of product may spoil due to fungal growth visible on the salted fillets or fish (Santoso, Gandjar, Sari, & Sembiring, 1999). Several different filamentous fungi have been isolated from spoiled, salted fish (Pitt & Hocking, 1999). Fungal spoilage of salted fish from temperate or subtropical climates differs from that of tropical salted fish with respect to genera and species (Pitt & Hocking, 1999) (Table 9). The spoilage described as "dun" is the visible appearance of brown colonies (1–2 mm in diameter). This is a caused by the growth of the fungus *Wallemia sebi*, an obligate aerobe.

Heated Seafood Products

Seafood raw materials are heated or cooked and result in a number of products such as hot-smoked fish, pasteurized crab meat, sous vide-cooked fillets, and fully canned products. Typically, the temperatures used are sufficient to result in substantial reduction of any microorganism present and it is either postprocess contamination that introduces spoilage microorganisms or spores surviving heat treatment that give rise to vegetative cells with spoilage potential.

Hot-Smoked Fish

The hot-smoking process is very similar to the cold smoking described above in that the fish are typically salted to 3–6% NaCl (water phase salt). However, the smoking process typically takes place at higher temperatures (50–80°C) which results in "cooking" of the fish flesh. Several species, such as herring and mackerel, are hot smoked but recently salmon has also become popular as a hot-smoked product. Hot smoking is also a common preservation technology in many developing countries. The hot-smoking process of mackerel can involve a drying step (30°C) and heating step at 50 and 80°C. The entire process usually requires 3 h. Larger and heavier fish require longer smoking (cooking) time.

Lightly salted, hot-smoked fish can be stored aerobically and bacterial growth limits shelf life of the product (Karnop, 1980). Also, fungal growth is quite common and is a shelf-life-limiting factor (Efiuvwevwere & Ajiboye, 1996). The fungi isolated are typically identified as *Aspergillus* or *Penicillium* species (Lilabati, Vishwanath, & Shymkesho Singh, 1999). If the product is vacuum packaged immediately after smoking under hygienic conditions, there is virtually no change in microbial levels during subsequent storage. The product becomes dry and somewhat tasteless, but microbial spoilage is not apparent.

Sous Vide-Cooked Fish

The "sous vide" (under vacuum) technology involves heating of the product typically at less-than-boiling temperature (65–75°C) followed by storage of the product at refrigeration temperature. The technique has been developed to improve sensory quality of several types of cooked food. Due to risk from *C. botulinum*, e.g., survival of spores and subsequent growth and toxin production, it has been recommended that sous vide fish products be heated to at least 90°C for 10 min. To control *L. monocytogenes*, heating to an internal temperature of 70°C for 2 min must be done (ACMSF, 1992). Several types of bacteria grow during subsequent chill storage and length of lag phase and extent of growth depend on the severity of the preceding heat treatment; however, sensory rejection does not appear to be a consequence of bacterial metabolism (Gonzalez-Fandos, Villarino-Rodriguez, Garcia-Linares, Garcia-Arias, & Garcia-Fernandez, 2005) and shelf lives range from 3 to 5 weeks (Gonzalez-Fandos et al., 2005; Nyati, 2000). Sporadically, Ben Embarek (1994) found that sous vide-cooked cod developed very strong putrid off-odors after storage at 5°C. Spore-forming, Gram-positive bacteria were isolated from these samples; however, their identity (*Bacillus* or *Clostridium*) was never determined (Ben Embarek, 1994).

Crab products are important especially to the US market. Crabs are harvested around the world. Some species are dissected before cooking, whereas others are cooked as whole animals. Subsequently the meat is removed from the shell (Cockey & Chai, 1991). Although cooking leaves the flesh sterile, subsequent picking and handling recontaminates the product. Spoilage of canned crab has, as with spoilage of sous vide cod, been caused by sporeformers (Cockey & Chai, 1991).

Fully Canned Products

Some species of fish are often processed and distributed as fully canned products. This is typical of tuna, mackerel, and salmon. These products are shelf stable if correctly processed and microbiological spoilage is not an issue. The use of raw materials of poor quality may result in the presence of biogenic amines in the canned products. The amines are heat stable and will not be inactivated even during canning (Luten et al., 1992)

Miscellaneous

Frozen Fish

Almost 20% of the global fish production is preserved by freezing (Fig. 2) and storage at −18 to −20°C. Growth of bacteria is stopped at these temperatures and sensory rejection is caused by nonmicrobial changes in the protein and lipid fractions of the fish flesh. Spoilage bacteria may grow in the raw fish if stored above 0°C before freezing and this may be reflected in the quality of the frozen product.

Filamentous fungi may grow in these products if stored at elevated freezing temperatures (−10 to −5°C). Many bacteria may, to some extent, survive frozen storage and if the product is thawed, grow and cause a normal type of spoilage. As mentioned above, *P. phosphoreum* is very sensitive to freezing and a freezing step before thawing and storage in CO_2 atmosphere may increase the shelf life of CO_2-packaged fish at refrigeration temperature quite substantially (Boknes, Osterberg, Nielsen, & Dalgaard, 2000; Dalgaard et al., 2006; Emborg et al., 2002).

Surimi

Surimi is a product prepared from either deboned meat or fillets. It consists mainly of muscle protein fibers that have been washed several times. Surimi is also prepared by mechanical deboning but is reduced essentially to muscle protein fibers by repeated washing. Some studies have reported that the microflora on surimi is similar to the microflora of fresh fish and consists of *Moraxella*, pseudomonads, and *Corynebacterium* (Himelbloom, Brown, & Lee, 1991), with counts of approximately 10^6 cfu/g (Matches, Raghubber, Yoon, & Martin, 1987). Bacteria grow very well in surimi and surimi-based products; however, these are mostly stored frozen, hence bacterial growth is not a problem (Elliott, 1987).

Dried Fish

The water activity of fully dried or salted and dried fish is so low that no bacteria can grow. However, fungal growth is a major problem, specifically in developing countries and spoilage is caused by *Aspergillus* and *Penicillium* species (Chakrabarti & Varma, 2000), but a range of other fungi may also be isolated from these products (Santoso et al., 1999). *Aspergillus niger, Aspergillus flavus*, and *Penicillium*spp. are

among the dominant fungi in salted, dried fish (Chakrabarti & Varma, 2000) and *A. niger* also appear to dominate in dried fish (Atapattu & Samarajeewa, 1990). Not only does visible fungal growth spoil the product per se, but filamentous fungi may also produce mycotoxins, hence constituting a health risk. Aflatoxin has been detected in smoked freshwater fish from Africa (Jonsyn & Lahai, 1992).

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Microbiological Spoilage of Eggs and Egg Products

Joseph R. Shebuski and Timothy A. Freier

Introduction

Chicken eggs are the eggs most commonly consumed by humans. The US per capita consumption was 255 eggs in 2005. Approximately 77 billion eggs were produced in the USA in 2005 (American Egg Board, 2005). Of these about 30% were further processed in some manner and the remainder were consumed as whole shell eggs. The greatest increase in production and consumption of eggs, however, is in the developing countries. China is now the number one producer of eggs, with the USA second, and India third. In fact, developing countries currently have >67% of the global egg production share (Clark, 2007). Only a small percentage of eggs are exported because shell eggs are relatively difficult to transport.

Eggs were not created as a food for human consumption. Rather, eggs provide a wonderfully evolved package for the purpose of ensuring the successful maturation of an embryo into a chick. While our handling of eggs can greatly influence the spoilage of this food, there are many factors involved in mitigating microbial spoilage already integrated into the egg.

This chapter is primarily focused on the spoilage concerns of chicken eggs. While the spoilage of other types of eggs may be similar, differences exist; hence, the information in this chapter may not entirely extrapolate to eggs of all species.

This chapter will address the types of microbial spoilage that may occur in a variety of different egg products – shell eggs, liquid eggs, and processed eggs (cooked, frozen, dried, and baked). The microbes primarily responsible for the spoilage of shell eggs and liquid eggs are typically Gram-negative microorganisms. Microbial spoilage of processed eggs is not commonly a problem unless these products undergo some form of abuse. In the case of cooked or frozen eggs, this would involve a loss of temperature control. However, the spoilage of dried or baked eggs would typically involve the introduction of water into these products. In each case,

J.R. Shebuski (✉)
Cargill, Inc., 15407 McGinty Road W., Wayzata, MN 55345, USA
e-mail: joe_shebuski@cargill.com

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the spoilage microorganisms could be either bacteria or fungi, depending upon the severity of the abuse.

Shell Eggs

Sources and Types of Spoilage Microorganisms

A shell egg at the time of oviposition should be essentially free from microbial contamination. (Transovarian transmission of *Salmonella enteritidis* into the egg yolk has been found to occur, but this does not likely relate to egg spoilage.) However, this condition is quickly changed once the exterior of the egg comes in contact with the nesting material where the egg is deposited. Dust, soil, and feces are the primary sources of contaminating microorganisms. Gram-positive microorganisms dominate the microbial contaminants present, but low numbers of Gram-negative bacteria can also be found (Board & Tranter, 1995). The relatively few Gram-negative microorganisms are primarily responsible for egg spoilage. The types of spoilage or rots are sometimes characterized by the color of the spoiled eggs (Table 1). For example, black rots are associated with the presence of species of *Proteus* and *Aerobacter*. *Serratia* species are associated with red rot and certain species of *Psuedomonas* with green and pink rots (Florian & Trussell, 1957; Board & Tranter, 1995).

Table 1 Bacteria associated with various types of egg spoilage or rot

Spoilage bacteria	Type of spoilage or rot (color)
Proteus spp.	Black
Aeromonas liquefaciens	Black
Serratia marcescens	Red
Enterobacter spp.	Custard
Pseudomonas maltophilia	Green
Pseudomonas fluorescens	Pink
Flavobacterium cytophaga	Yellow
Other *Enterobacter* and *Alcaligenes* spp.	Colorless

In the USA, washing of eggs and prompt and proper refrigeration shortly after the eggs are laid have greatly reduced the incidence of these types of spoilage. The European Community's Egg Marketing Regulations require that eggs be labeled to indicate that consumers keep the eggs refrigerated after purchase. They also must mark the eggs or egg packaging with a "best before" date (Department for Environmental Food and Rural Affairs, 2005). However, in some parts of the world these types of spoilage problems may still be a concern where egg washing and proper refrigeration are not practiced. Identification of microbial species isolated from unwashed shell eggs at egg processing facilities included the following: *Aeromonas, Cedecea, Chryseomonas, Citrobacter, Enterobacter, Erwinia, Escherichia, Hafnia, Klebsiella, Kluyvera, Leclercia, Listonella, Morganella, Proteus, Providencia, Pseudomonas, Rahnella, Salmonella, Serratia, Sphingobacterium, Vibrio,*

Xanthomonas (Musgrove, Northcutt, Jones Cox, & Harrison, 2008). Many of these microorganisms will be removed from the surface of the egg during washing and processing; however, a few will persist. Given the proper conditions, some may overcome the natural defenses of the egg and produce the type of spoilage or rots described previously.

Methods for Detection

Most of the microorganisms associated with egg spoilage can be detected and isolated using routine and standard methodology. The egg products methodology is described in the *Compendium of Methods for the Microbiological Examination of Foods* by Ricke, Birkhold, and Gest (2001).

Egg Structure

The egg structure and composition influence microbial growth and are responsible for defending the egg against spoilage microorganisms. The egg structure is depicted in Fig. 1.

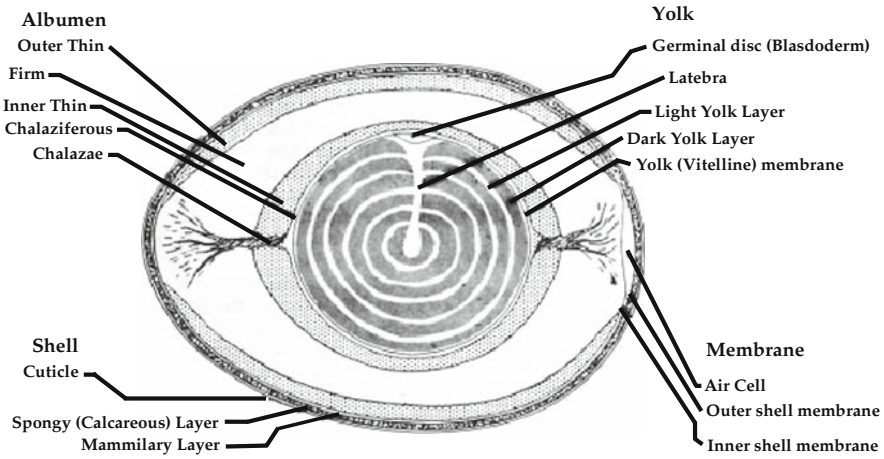

Fig. 1 The parts of an egg (AMS, 2000)

Cuticle

Moving inward from the outside of the egg, the first and outermost component encountered is a thin layer of primarily proteinaceous material known as the cuticle, which coats the eggshell. This material is deposited on the shell just before the egg is laid (Fields, 1979). It enters the pores of the shell where it dries to form a barrier to slow the entry of water from outside the egg and the loss of water from

inside the egg. The cuticle is also the initial line of defense against the penetration of microorganisms into the egg. However, in some cases, the cuticle may not fill all of the pores of the shell and bacterial entry may be possible through the unprotected pores.

Shell

The next component of the egg is the shell. It is the most important physical barrier to preventing microbial entry. A microscopic view of the cuticle, the shell, and a pore running through the depth of the eggshell is shown in Fig. 2.

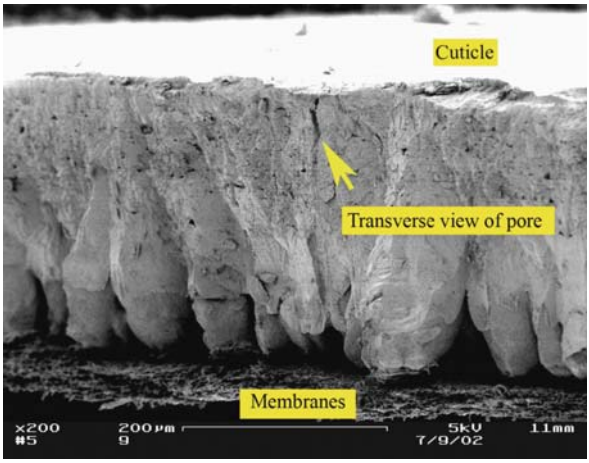

Fig. 2 Photomicrograph of the eggshell (Courtesy of Musgrove, Northcutt, Jones, Cox, & Harrison, 2008)

Cracking or abrasive damage to this barrier through processing will enhance the possibility for microbial penetration and may lead to rapid spoilage of the egg. Aside from direct damage to the shell, several additional factors related to the shell have also been identified impacting spoilage. The eggshell has in excess of 17,000 pores that permit the diffusion of gases – the entry of oxygen and the release of carbon dioxide and water vapor. This diffusion is critical to the survival and development of the embryo (Board & Tranter, 1995). A photomicrograph of the openings of the pores on the egg surface is shown in Fig. 3.

Shells having fewer pores and consequently a higher specific gravity are more resistant to microbial invasion (Kraft, McNally, & Brant, 1958). The number of pores is reported to increase with the age of the flock (Rahn, Christensen, & Edens, 1981); hence, eggs from older flocks may experience a higher level of internal contamination than those from younger flocks. In addition to the number of pores in the shell, two other factors are also involved in making the egg more susceptible to microbial invasion, including the age of the egg and the condition of the laying hen. Older eggs are more likely to be invaded by microorganisms (Fromm & Monroe, 1960). Stress on the laying hen may result in oviduct damage and subsequent structural defects in the shell (Nascimento & Solomon, 1991). Other factors may

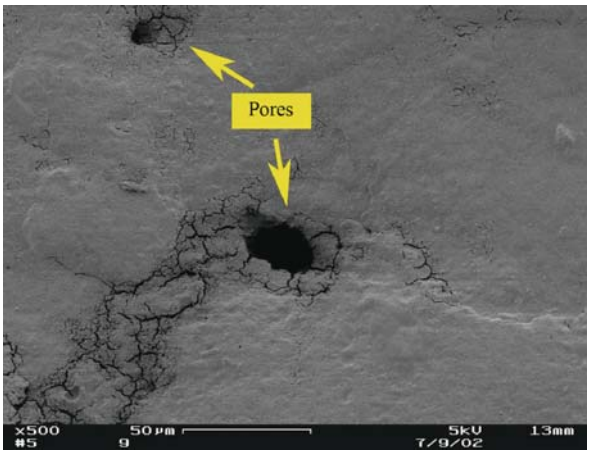

Fig. 3 Photomicrograph of the pore openings on the surface of the eggshell (Courtesy of Musgrove, et al., 2008)

also have an impact on the penetration of the shell by microorganisms. *Salmonella enterica* serovar Enteritidis was highly variable in its ability to penetrate eggshells. Penetration was not correlated to the genetic strain of the laying hen or the type of housing system; however, there was a trend toward higher penetration when hens were fed a corncob mix diet versus standard feed. There was also more frequent microbial penetration into eggs lacking cuticle spots and eggshells having lower dynamic stiffness values (Messens et al., 2007).

Membranes

Just beneath the surface of the shell are the inner and outer shell membranes. How these two membranes function to provide an antimicrobial defense to the egg is not completely understood (Board & Tranter, 1995). However, each membrane is composed of anastomosing fibers and it is suggested this fibrous nature of the membranes act as microbial filters. The inner membrane is believed to provide the greatest resistance to microorganisms, but neither membrane is capable of completely preventing the entrance of bacteria. At best it is believed that the presence of these membranes may delay the entrance of motile bacteria for a brief period of time.

Albumen

The albumen is beneath the shell membranes and is important in providing three antimicrobial defenses to the egg. The first defense is the viscosity of the albumen components, which provide conditions in which bacteria cannot move easily. The second defense is the alkaline pH of the albumen that initially ranges from 7.6 to 7.8 but quickly increases over a period of a few days due to the loss of carbon dioxide during storage, resulting in a pH of 9.1–9.6. This is a pH range that is not conducive

to the growth of most microorganisms. The third defense is the presence of a number of proteins and glycoproteins that possess antimicrobial activity. Among those that have been characterized are lysozyme c, ovoalbumin, ovoconalbumin, ovoglobulin, ovomucoid, and ovomucin (Chen, Thesmar, & Kerr, 2005). Lysozyme is a potent inhibitor of Gram-positive microorganisms and, to a much lesser extent, Gram-negative microorganisms. Ovoconalbumin is a strong chelator capable of removing metal ions (Fe^{2+}, Cu^{2+}, and Zn^{2+}), thereby making them unavailable for microbial growth. Ovomucoid inhibits trypsin, but this phenomenon does not affect Gram-negative bacteria.

Vitelline Membrane

The final recognized antimicrobial barrier prior to the yolk is the vitelline membrane. It surrounds the yolk and consists of two principal layers. The outer layer contains lysozyme, an ovomucin complex, and vitelline membrane protein. The inner layer is a network of anastomosing fibers and a slightly porous ground substance (Chen et al., 2005).

Yolk

If spoilage microorganisms are able to breach the many outer defenses of the egg, there is evidence that the yolk itself may contain some antimicrobial lipoproteins (Brady, Gaines, Fenelon, McPartlin, & O'Farrelly, 2002). However, if spoilage microorganisms reach the yolk, they will likely be able to take advantage of the yolk's rich nutrient pool present and subsequently spoil the egg. The antimicrobial components of the egg are summarized in Table 2.

Table 2 Summary of the antimicrobial components of the egg

Egg component	Antimicrobial function
Cuticle	Initial exterior layer of defense, physical barrier to microbial penetration
Shell	Most critical physical barrier to microbial penetration
Shell membranes (inner and outer)	Marginal physical barrier to penetration composed of anastomosing fibers
Albumen	(1) Viscous material, which slows microbial movement, (2) alkaline pH not suitable for microbial growth, (3) presence of antimicrobial proteins (e.g., lysozyme, a bactericidal enzyme against Gram-positive bacteria)
Vitelline membrane	Contains lysozyme and other potentially antimicrobial proteins and anastomosing fibers which slow microbial movement

Egg Handling

No single naturally occurring antimicrobial defense of the egg by itself is likely to prevent spoilage. However, when acting in synergy, these defenses can provide a substantial barrier to microbial invasion and spoilage. Proper handling and processing of eggs can further support this barrier. When eggs are stored at ambient temperatures, changes occur which can influence spoilage. The increase in albumen pH results in a thinning of the albumen and a reduction in viscosity. A breakdown of the membrane proteins also occurs. This occurs for the vitelline membrane as well (Chen et al., 2005). Under commercial storage conditions (at ambient temperature), there is a 10- to 20-day lag between shell penetration and the presence of large numbers of microorganisms in the albumen (Board & Tranter, 1995). However, if the eggs are properly cleaned and appropriate holding temperatures are maintained, the eggs should have a much longer shelf life. Commercial egg processing significantly reduces the cell numbers of aerobic bacteria, *Enterobacteriaceae*, *E. coli*, and yeast and molds (Musgrove, Jones, Northcutt, Harrison, & Cox, 2005). Another study, which focused only on *Salmonella*, addressed the concern that washing can lead to increased contamination and microbial penetration of the egg. Results indicated if strictly controlled best practice conditions for washing were followed, *Salmonella* would not become an internal contaminant of the egg and a 5-log reduction in *Salmonella* counts on the egg surface could be achieved (Hutchinson et al., 2004). Regarding storage temperature, it has been determined that storage at 4°C can postpone the egg-aging process by preserving the antimicrobial properties of the albumen and the integrity of the vitelline membrane (Chen et al., 2005). A study of commercially processed eggs revealed that those stored beyond the "best if used by date" were rarely contaminated internally (Jones, Musgrove, & Northcutt, 2004).

Spoilage of individual shell eggs was a common occurrence before the many improvements that have been made in modern egg production and distribution. Several decades ago, it was a common practice in the home to crack eggs into a small bowl and sniff the egg before adding it to the batter. This step is no longer necessary with the current shell egg controls. However, one problem that remains is that there is typically no external visual appearance change accompanying the spoilage of shell eggs. This can be an issue for the many consumers that remove eggs from the date-marked container and place them into another holder in the refrigerator and do not practice strict first-in-first-out stock rotation. This problem has been overcome, in part, with a recent United States Department of Agriculture (USDA) authorized method for permanently laser-etching shell eggs with a use-by date and lot code (Banasiak, 2005).

Various types of oils, proteins, or other polymers have been coated onto the shell with the intention of extending shelf life by slowing the chemical changes within the egg and preventing the penetration of microorganisms. Several novel technologies have been developed to optimize the safety and shelf life of shell eggs. When eggs are coated with soy protein isolate, whey protein isolate, carboxylmethyl cellulose, or wheat gluten, they become more puncture resistant and have reduced post-wash blue lake dye penetration that correlates to reduced bacterial penetration (Xie et al., 2002).

Chitosan coating also has the potential to extend the shelf life of shell eggs. This coating can extend the shelf life of shell eggs by approximately 2 weeks when chemical quality attributes are considered (No, Prinyawiwatkul, & Meyers, 2005). Although microbial shelf life has not been examined, chitosan may work especially well for this use, as it has intrinsic antimicrobial properties (No, Park, Lee, & Meyers, 2002).

Shell Egg Pasteurization

Recently, a process for pasteurizing shell eggs that minimally impacts their sensory characteristics has been patented and applied (Cox, Cox, & Cox, 1999). This process was developed to kill internalized *Salmonella*, thus providing a safe alternative for uncooked or minimally cooked egg dishes. The main technological hurdle was the application of a very even, consistent, and controlled heat process to the egg, providing a uniform heat treatment to kill pathogens, yet not cooking or affecting the functional properties of the shell egg. This technique was developed to meet the USDA time–temperature requirements for the pasteurization of liquid whole eggs at the center of the egg yolk. Dependent upon the initial temperature of the egg before the heat treatment and the size of the egg, this in-shell pasteurization method involves temperatures of approximately 58.8°C (138°F) for 40–48 min in a water bath. Heat transfer to the center of the egg occurs more rapidly when vibration, shaking, or ultrasound is employed. Special precautions must be taken to rapidly temper the eggs before heat treatment to avoid shell cracking. In addition, the eggs must be rapidly cooled in a way that prevents recontamination of the egg's interior by microbes in the cooling medium passing through the shell during cooling.

Although the in-shell pasteurization process was primarily developed to eliminate *Salmonella*, this process also destroys some spoilage bacteria and, hence, likely extends the shelf life of shell eggs. However, many potential egg spoilage bacteria are more heat resistant than *Salmonella*. One patent related to the in-shell egg pasteurization stated "It is to be understood, however, that pasteurization of eggs, similar to pasteurization of milk, does not extend the shelf life of the eggs nor does it lessen the necessity for proper handling and cooling of the eggs, in the same manner as pasteurized milk" (Davidson, 2001). To our knowledge, a thorough microbiological shelf life study for in-shell pasteurized eggs has not been published.

Liquid Egg Products

Liquid egg products are available as whole eggs, yolks, or whites and a variety of scrambled egg mixes and are typically pasteurized and held under refrigerated or frozen conditions until used. Liquid egg products are sold primarily to restaurants and institutions, but a few of these products are also available to consumers through retail outlets. Cholesterol-free egg (egg white-based) products are the predominant retail products. The shelf life of liquid egg products is coincidentally enhanced by pasteurization, which is mandated in most countries.

Liquid whole eggs are typically sold as blends of egg whites and yolks. The microbes responsible for the spoilage of unpasteurized liquid eggs were identified in a detailed evaluation of the spoilage microflora of unpasteurized liquid whole eggs (MacKenzie & Skerman, 1982). Liquid whole egg samples were incubated at 5, 10, 20, 25, and 30°C until signs of spoilage became apparent. From these samples, the following bacteria were isolated and identified: *Acinetobacter calcoaceticus, Aeromonas hydrophila, Bacillus cereus, Citrobacter freundii, Enterobacter aerogenes, E. cloacae, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Serratia marcescens, Pseudomonas putida, Salmonella typhimurium, Streptococcus faecalis, S. lactis*, and *Vibrio metschnikovii*. Gram-negative bacteria were dominant at all temperatures evaluated. Genera of the *Enterobacteriaceae* were predominant in the temperature range of 20–30°C, whereas the *Pseudomonadaceae* predominated at 5°C. Very few Gram-positive bacteria were isolated.

Pasteurization Processes

Pasteurization of all processed egg products is a regulatory requirement in the USA. Conventional pasteurization processes for liquid whole eggs involve heating to a minimum temperature of approximately 60°C for 3.5 min. This process is targeted to eliminate *Salmonella*; however, some more heat-resistant microbes can survive. A finished product receiving this process may have a shelf life of only 10–12 days when held at 2°C (York & Dawson, 1973). Hence, eggs pasteurized using these processing parameters are typically frozen to further extend the shelf life.

The need to extend the shelf life of pasteurized liquid eggs without freezing led to the development of a process referred to as "ultrapasteurization," although there is no legal definition of this process for egg products. The process involves heating the liquid whole eggs at temperatures between 63.7 and 72.2°C for times of 2.7–192.2 seconds (Ball, Hamid-Samimi, Foegeding, & Swartzel, 1987). Product heated at these temperatures and times and then carefully packaged to minimize recontamination will have a much longer shelf life than conventionally pasteurized liquid whole eggs. Unopened containers may last as long as 3–6 months when held at 4°C; however, spoilage can eventually occur. Both foodservice and retail products are marketed with a refrigerated shelf life of 4–12 weeks. The predominant microorganisms surviving the ultrapasteurization process are Gram-positive bacteria; however, product spoilage is most commonly caused by postpasteurization contamination with psychotrophic Gram-negative bacteria (Ray, 2004). Foegeding and Stanley (1987) preferentially selected 28 isolates from ultrapasteurized, aseptically packaged liquid whole egg product held at 4 and 10°C. Of the 28 isolates, 23 were Gram-positive bacteria and 11 were sporeformers. Isolates selected for further characterization were identified: *B. cereus, B. circulans, Pseudomonas* spp., and *Enterococcus faecalis.* Only the *B. cereus* and *Pseudomonas* spp. isolates could grow at 4°C. Generation times for these microorganisms were less than or equal to one day. All four isolates grew well at 10°C, with generation times less than 10 h. Prediction

of times to spoilage based on these experimentally determined generation times is much less than is actually observed for these products and therefore these predictions should be considered worst-case scenarios for such products when stored at these temperatures.

Egg yolks are also typically pasteurized to kill *Salmonella* and extend the product shelf life. Pasteurization requirements for yolks are somewhat higher than those for other egg products, as salmonellae and other microorganisms are more heat resistant in yolks due to the protective effects of higher solids and lipid levels. A typical process would involve heating at 60–65°C for 3.5–6.5 min.

Sometimes sugar or salt are added to frozen yolks to improve flowability. Yolks treated in this manner are used only in products that otherwise require the addition of sugar or salt, for example, ice cream mixes, mayonnaise, and bakery mixes. While this addition reduces the water activity of the yolks and can slow the growth of spoilage microorganisms prior to pasteurization, it also reduces the thermal transfer during heating and necessitates a higher heating temperature or longer heating time to affect an equivalent level of microbial inactivation.

Egg whites or albumen are pasteurized for the same reasons as whole eggs and egg yolks; however, the heat sensitivity of some components in the albumen requires that a more gentle heating process be used. A typical process would require heating to a temperature of 55–58°C for 2.5–9.5 min. The antimicrobial activity of the naturally occurring lysozyme present in the albumen is minimally impacted by this heating process and remains effective in eliminating or minimizing the growth of those microbes that either survive the pasteurization process or contaminate the product after heating.

Thermal processing is effective at eliminating pathogens of concern and reducing the overall numbers of spoilage microorganisms present, but heating can negatively affect the organoleptic properties and functionality of egg products. As a result, nonthermal preservation processes have been evaluated as preferred alternatives to processing with heat. Gongora-Nieto, Seignour, Riquet, Davidson, Barbosa-Canovas, and Swanson (2001) evaluated the use of pulsed electric fields (PEFs) and high hydrostatic pressure (HHP) in the presence of antimicrobials as treatments to inactivate *P. fluorescens* in liquid whole egg. They concluded that these nonthermal technologies can be effective in inactivating spoilage bacteria in liquid whole egg and negatively affect quality attributes. The presence of antimicrobials during these nonthermal treatments had a synergistic effect on the inactivation of *P. fluorescens*.

Processed Egg Products

Cooked Eggs

Numerous cooked egg products and cooked egg-containing products are manufactured today. Many of these products are used in foodservice applications, although some are sold at retail. Hard-cooked eggs, scrambled eggs, egg patties, eggs mixed

with other ingredients and wrapped in a tortilla, and French toast are some examples. These products are usually manufactured from previously pasteurized liquid eggs. They are cooked to target temperatures in excess of 71°C to coagulate proteins, changing the eggs from a liquid to a solid-gelled state. Conveniently, vegetative spoilage microorganisms and salmonellae are easily killed by cooking. Nonetheless, in order to provide sufficient shelf life during distribution and storage, virtually all of these products are sold in the frozen state. Freezing prevents microbial spoilage of these products provided that they are maintained frozen throughout distribution and then thawed and consumed promptly. Once thawed, these products should be maintained refrigerated and used within 3 days to ensure full flavor and quality and to avoid microbial growth. The USDA-recommended storage conditions for various egg products are shown in Table 3.

Table 3 Recommended egg and egg product handling and storage conditions (FSIS, 2007)

Product	Refrigerator	Freezer
Raw eggs in shell	3–5 weeks	Do not freeze
Raw egg whites	2–4 days	12 months
Raw egg yolks	2–4 days	Yolks do not freeze well
Raw egg accidentally frozen in shell	Use immediately after thawing	Keep frozen, then refrigerate to thaw
Hard-cooked eggs	1 week	Do not freeze
Liquid egg substitutes		
Unopened	10 days	Do not freeze
Opened	3 days	Do not freeze
Frozen egg substitutes		
Unopened	7 days after thawing*	12 months
Opened	3 days after thawing*	Do not freeze
Casseroles made with eggs	3–4 days	2–3 months after baking
Eggnog, commercial	3–5 days	6 months
Eggnog, homemade	2–4 days	Do not freeze
Pies, pumpkin, or pecan	3–4 days	1–2 months after baking
Pies, custard, and chiffon	3–4 days	Do not freeze
Quiche with any kind of filling	3–4 days	1–2 months after baking

*Or refer to "use-by" date on carton

Very few egg products are cooked distributed and sold under refrigerated conditions. Such products are cooked to 71°C or greater to destroy *Salmonella*, then cooled, and typically packaged in modified atmosphere packaging. The temperature during distribution and storage is maintained between 4 and 5°C. One of the greatest shelf life challenges for these products is not microbial spoilage, but rather a rapid loss of flavor that occurs during storage. This flavor loss is the basis for determining the end of product shelf life. Microbial spoilage, while a concern, generally will not occur until after the end of shelf life determined by flavor loss. The extension of shelf life of these refrigerated products relative to flavor loss is currently a very active area of research. Until this challenge is resolved, the number and variety of egg products sold under refrigerated conditions is likely to remain very small.

Dried Eggs

Whole eggs or their components may also be prepared and distributed in a dry form. Drying may be achieved in a number of different ways. Among the most common are spray drying, freeze drying, and pan drying (Cox, 2001). The products are typically dried to a moisture content of less than 10%. For whole eggs and yolks, a moisture level of 5% is common; for albumen, 8–10% is the targeted moisture range. At the resulting water activities of <0.70, growth of most spoilage microorganisms is not possible. However, some microorganisms can survive the drying process and may be able to grow if the products are not packaged, stored, and held in a manner that prevents water from reentering the product. Growth of the surviving microorganisms can also become a problem upon rehydration of the products if they are not properly handled and consumed promptly.

Glucose, present in the albumen, is often removed before drying in order to prevent Maillard browning. Browning of the albumen may be incorrectly interpreted as being caused by spoilage. Glucose is removed either by fermentation or by enzyme treatment.

Baked Eggs

Baked eggs are a relatively recently introduced form of processed eggs. These are whole shell eggs heated in an electric oven for 24 h. The eggs are baked under dry conditions with constant circulation of air within the oven. The product is sold in the shell and is reported to have a shelf life of 3 months when held at room temperature. The pH of the final product reportedly ranges from 7.2 to 7.4, and the water activity is <0.85. The shell is removed just prior to consumption revealing the solidified contents, which are brown in color. The finished product has slightly reduced moisture content as a result of the baking process. Based on the water activity of the finished product, bacterial spoilage would not be anticipated; however, the potential for mold growth is a possibility.

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Microbiological Spoilage of Fruits and Vegetables

Margaret Barth, Thomas R. Hankinson, Hong Zhuang, and Frederick Breidt

Introduction

Consumption of fruit and vegetable products has dramatically increased in the United States by more than 30% during the past few decades. It is also estimated that about 20% of all fruits and vegetables produced is lost each year due to spoilage. The focus of this chapter is to provide a general background on microbiological spoilage of fruit and vegetable products that are organized in three categories: fresh whole fruits and vegetables, fresh-cut fruits and vegetables, and fermented or acidified vegetable products. This chapter will address characteristics of spoilage microorganisms associated with each of these fruit and vegetable categories including spoilage mechanisms, spoilage defects, prevention and control of spoilage, and methods for detecting spoilage microorganisms.

Microbiological Spoilage of Fresh Whole Fruits and Vegetables

Introduction

During the period 1970–2004, US per capita consumption of fruits and vegetables increased by 19.9%, to 694.3 pounds per capita per year (ERS, 2007). Fresh fruit and vegetable consumption increased by 25.8 and 32.6%, respectively, and far exceeded the increases observed for processed fruit and vegetable products. If US consumption patterns continue in this direction, total per capita consumption of fresh fruits and vegetables would surpass consumption of processed fruits and vegetables within the next decade.

This shift toward overall increased produce consumption can be attributed, at least in part, to increased awareness in healthy eating habits as revealed by a broad field of research addressing food consumption and health and promoted by the

M. Barth (✉)
Responsible Source, 350 Berkshire Drive, Lake Forest, IL 60045, USA
e-mail: margaret.barth@sbcglobal.net

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National Cancer Institute with the 5-A-Day Challenge along with the USDA-revised Food Pyramid. Additional factors influencing greater fresh produce consumption are the increased availability of fresh produce throughout the country throughout the year, increased diversity of selection at the retail level (Kaufman, Handy, McLaughlin, Park & Green, 2001), and rapid growth in the fresh-cut, ready-to-eat produce sector (see "Microbiological Spoilage of Fresh-Cut Fruits and Vegetables").

According to a USDA-Economic Research Service study in 1995, 18.9 billion pounds of fresh fruits and vegetables were lost annually due to spoilage, which was 19.6% of all US losses of edible foods that year (Kantor, Lipton, Manchester, & Oliveira, 1997). The portion of loss specifically due to microbiological spoilage was not reported.

Most microorganisms that are initially observed on whole fruit or vegetable surfaces are soil inhabitants, members of a very large and diverse community of microbes that collectively are responsible for maintaining a dynamic ecological balance within most agricultural systems. Vectors for disseminating these microbes include soil particles, airborne spores, and irrigation water. Most bacteria and fungi that arrive on the developing crop plant either are completely benign to the crop's health or, in many instances, provide a natural biological barrier to infestation by the subset of microorganisms responsible for crop damage (Janisiewicz & Korsten, 2002, Andrews & Harris, 2000). The even smaller subset of bacteria and fungi responsible for causing spoilage to the edible portion of the crop plant is the subject of this section.

Spoilage microorganisms can be introduced to the crop on the seed itself, during crop growth in the field, during harvesting and postharvest handling, or during storage and distribution. Those same types of soil-borne spoilage microbes that occur on produce are the same spoilage microorganisms that are present on harvesting equipment, on handling equipment in the packinghouse, in the storage facility, and on food contact surfaces throughout the distribution chain. Therefore, early intervention measures during crop development and harvesting through the use of good agricultural practices (GAP) will provide dramatic reductions in yield loss due to spoilage at all subsequent steps in the food-to-fork continuum (Eckert & Ogawa, 1988). Examples of GAPs include foliar fungicide application in the field, cross-contamination prevention measures (stringent sanitation standard operating procedures) in the packinghouse and storage facility, and use of postharvest fungicides. These practices also will enhance substantially the food safety and shelf life of fresh-cut produce (see "Microbiological Spoilage of Fresh-Cut Fruits and Vegetables").

In 1998, FDA published the *Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables*, recommending GAPs that growers, packers, and shippers implement to address the common microbiological hazards that may be associated with their operations (FDA, 1998). These GAPs are organized in eight categories:

I. Water
II. Manure and municipal biosolids

III. Worker health and hygiene
IV. Sanitary facilities
V. Field sanitation
VI. Packing facilities sanitation
VII. Transportation
VIII. Traceback

In addition, FDA worked with the produce industry to develop commodity-specific food safety guidelines for sprouts, lettuce and leafy greens, melons, and tomatoes that provided metrics for soil and water amendments as well as adjacent land usage. In March 2007, FDA issued a draft final version of its "guide" (FDA, 2007). These should also improve substantially the food safety and shelf life of fresh-cut produce (see "Microbiological Spoilage of Fresh-Cut Fruits and Vegetables").

Unusual Characteristics of Spoilage Microorganisms

Many fruits and vegetables present nearly ideal conditions for the survival and growth of many types of microorganisms. The internal tissues are nutrient rich and many, especially vegetables, have a pH near neutrality. Their structure is comprised mainly of the polysaccharides cellulose, hemicellulose, and pectin. The principal storage polymer is starch. Spoilage microorganisms exploit the host using extracellular lytic enzymes that degrade these polymers to release water and the plant's other intracellular constituents for use as nutrients for their growth. Fungi in particular produce an abundance of extracellular pectinases and hemicellulases that are important factors for fungal spoilage (Miedes & Lorences, 2004). Some spoilage microbes are capable of colonizing and creating lesions on healthy, undamaged plant tissue (Tournas, 2005b). Spoilage microorganisms also can enter plant tissues during fruit development, either through the calyx (flower end) or along the stem, or through various specialized water and gas exchange structures of leafy matter. Successful establishment, however, requires the spoilage microbe to overcome multiple natural protective barriers. Fruits and vegetables possess an outer protective epidermis, typically covered by a natural waxy cuticle layer containing the polymer cutin (Lequeu, Faucconnier, Chamma, Bronner, & Blee, 2003). A diverse community of epiphytic microorganisms that present a further competitive barrier to the spoilage organism also typically colonizes the outermost fruit surface. Overcoming these barriers requires an exquisite set of biochemical tools that allow the spoilage microorganism to (1) identify and recognize the plant surface; (2) employ one or more strategies to achieve irreversible attachment to the plant surface; and (3) initiate steps leading to internalization of the tissue (Mandrell, Gorski & Brandl, 2006). On plant structures other than the fruit, internalization can be achieved through a number of specialized vessels and surface structures employed by the plant to absorb and release water and to provide CO_2 and O_2 exchange (Bartz, 2006).

Table 1 Bacterial fruit pathogens[a]

	2004 Annual US per capita consumption (lbs)[b]	*Pseudomonas*	*Erwinia*	*Xanthomonas*	*Acidovorax*
Apples	18.8		+		
Bananas	25.8				
Berries	6.1				
Citrus	22.7	+		+	
Grapes	7.9				
Melons	14.7				+
Peaches	5.1				
Pears	3.1		+		
Pineapple	4.4				

[a]Important postharvest diseases retrieved from Sholberg et al. (2004) and other sources.
[b]85.2% of all fresh fruits consumed per capita in the United States in 2004 (ERS, 2007).

However, the fruit of the plant lacks many of these structures, requiring the spoilage microbe to employ other methods to become internalized (Lindow & Brandl, 2003; Agrios, 1997). This may partially explain the rather limited success of bacteria to spoil fruits (Table 1) and an improved ability to spoil vegetables that are not the fruit of the plant (Table 3). The natural acidity of most fruits also serves as a barrier to many spoilage microbes, especially bacteria. By contrast, spoilage fungi that typically produce more diverse and greater amounts of extracellular depolymerases successfully attack and spoil both fruits and vegetables (Tables 2 and 4).

Colonization and lesion development more typically and more rapidly occurs within damaged or otherwise compromised plant tissue. External damage such as bruising, cracks, and punctures creates sites for establishment and outgrowth of the spoilage microbes. Lesion development can be relatively rapid, occurring within days or weeks. This presents the risk that rapidly reproducing spoilage microorganisms will arrive within open wound sites at the packing facility, and thereby, through shedding from the asymptomatic wound, present the potential for cross-contamination within the facility during handling, culling, washing, sorting, and packing before storage. Such cross-contamination to some degree is inevitable and, if not carefully managed with a robust facility sanitation program, could lead to the establishment of a population of spoilage microbes endemic to the facility that may be difficult to eradicate. A further and potentially more serious complication is the introduction into the cold storage facility of spoilage microorganisms already established in wound sites on product, whether the product is in bins or boxed and palletized. Depending upon storage conditions and storage time (greater than 12 months for certain robust crops), and if not carefully managed, these “primed” spoilage microorganisms can have a devastating impact on the stored product. Apples, for example, are stored in very large, controlled atmosphere storage rooms, either in wooden bins or boxed and ready for distribution (Watkins, Kupferman, & Rosenberger, 2004).

Table 2 Fungal fruit pathogens[a]

	2004 Annual U.S. per capita consumption (lbs)[b]	*Penicillium*	*Geotrichum*	*Fusarium*	*Botrytis*	*Colletotrichum*	*Mucor*	*Monilinia*	*Rhizopus*	*Phtyophthora*
Apples	18.8	+			+	+	+	+		
Bananas	25.8			+		+				
Berries	6.1	+			+	+	+	+		+
Citrus	22.7	+	+			+				+
Grapes	7.9	+			+				+	
Melons	14.7									
Peaches	5.1	+			+			+	+	
Pears	3.1	+			+		+			
Pineapple	4.4			+						

[a]Important postharvest diseases retrieved from Sholberg et al. (2004) and other sources.
[b]85.2% of all fresh fruits consumed per capita in the United States in 2004 (ERS, 2007).

Table 3 Bacterial vegetable pathogens[a]

	2004 Annual US per capita consumption (lbs)[b]	*Pseudomonas*	*Erwinia*	*Xanthomonas*	*Bacillus*	*Clostridium*	Lactic acid bacteria
Broccoli	5.9	+	+	+			
Cabbage	8.3	+	+	+			
Carrots	8.9	+	+		+		
Corn, sweet	9.6						
Cucumbers	6.3		+		+		
Lettuce, head	22.5	+	+	+			
Lettuce, leaf	12.0	+	+	+			
Mushrooms	2.6	+					
Onions	21.7		+		+		
Potatoes	46.5	+	+		+	+	
Spinach	2.1						
Tomatoes	19.3	+	+	+	+		+

[a]Important postharvest diseases retrieved from Sholberg et al. (2004) and other sources.
[b]81.0% of all fresh vegetables consumed per capita in the United States in 2004 (ERS, 2007).

Fig. 1 Extensive *blue* mold infestation on apples

Two wound pathogens, *Penicillium expansum* and *Botrytis cinerea*, if not scrupulously cleaned from fruits prior to storage or if fruits with infected wounds have not thoroughly been culled from the lot, can cause significant crop loss as these spoilage fungi eventually degrade the wound sites, create lesions, and cross-contaminate adjacent fruits. If fruits receive improper preharvest fungicide application, poor washing, and/or inadequate culling, an expanding infestation of spoilage microorganisms can destroy a substantial portion of a stored lot of fruits (Figs. 1 and 4). *P. expansum* (Miedes & Lorences, 2004) and *B. cinerea* (van Kan, 2006) are pathogens of apples, pears, and a number of other pectin-rich fruits. *B. cinerea* is an especially sophisticated and selective plant pathogen that possesses multiple cutinases and lipases that are capable of degrading plants rich in pectin (van Kan 2006).

The bacterium *Erwinia carotovora* subsp. *carotovora* is a highly effective spoilage microbe that causes soft rot across a broad host range of vegetables and some fruits (Lund, Baird-Parker, & Gould, 1983; Table 4). One of six known genera of soft-rot bacteria (including *Xanthomonas*, *Pseudomonas*, *Clostridium*, *Cytophaga,* and *Bacillus*), *E. carotovora* subsp. *carotovora* is one of several species of *Erwinia* that infect and destroy plant tissues both pre- and postharvest and is the species that causes the greatest damage to harvested vegetables. Soft rot is a form of decay characterized by a watery transparency in infected leafy plant parts and

Table 4 Fungal vegetable pathogens[a]

	2004 Annual US per capita consumption (lbs)[b]	*Geotrichum*	*Rhizopus*	*Phytophthora*	*Fusarium*	*Pythium*	*Alternaria*	*Colletotrichum*	*Botrytis*	*Sclerotinia*
Broccoli	5.9		+				+		+	+
Cabbage	8.3		+				+			+
Carrots	8.9	+							+	+
Corn, sweet	9.6				+					
Cucumbers	6.3		+		+	+		+		
Lettuce, head	22.5	+							+	+
Lettuce, leaf	12.0	+							+	+
Mushrooms	2.6									
Onions	21.7	+			+		+	+	+	
Potatoes	46.5		+	+	+	+				
Spinach	2.1									
Tomatoes	19.3	+	+	+	+		+	+	+	+

[a]Important postharvest diseases retrieved from Sholberg et al. (2004) and other sources.
[b]81.0% of all fresh vegetables consumed per capita in the United States in 2004 (ERS, 2007).

watery disintegration of nonleafy plant materials. "Soft-rot erwinia" tend to initiate infection and decay at wound sites and, once established, can quickly advance to total destruction of the product. Soft-rot erwinia express four pectin-degrading extracellular enzymes: pectin lyase, polygalacturonase, pectin methylesterase, and pectate lyase. Of these enzymes, pectate lyase is primarily responsible for extensive decay. *E. carotovora* has built-in redundancy for this apparently critical pathogenicity factor, expressing four distinct extracellular pectate lyase isozymes (Barras, van Gijsegem, & Chatterjee, 1994).

Soft-rot erwinia are active only at temperatures of 20°C and above, which reinforces the need to maintain a continuous cold chain from immediately postharvest to retail to successfully manage this ubiquitous spoilage bacterium. Another group of soft-rotting bacteria, the fluorescent pseudomonads (i.e., *Pseudomonas fluorescens* and *Pseudomonas viridiflava*), can decay plant tissue at temperatures at or below 4°C. This is one explanation for the high prevalence of these bacteria on decayed vegetables at wholesale and retail markets (Liao & Wells, 1987). Liao, Hung, & Chatterjee (1988) revealed through several *P. viridiflava* mutants defective in pectate lyase expression that these mutants completely lost the ability to induce soft rot on potato tuber slices. The soft-rotting fluorescent pseudomonads, when considered together with soft-rot erwinia, present a formidable challenge to commercial fresh product operations, and fresh vegetables in particular, from the farm to retail and wholesale outlets.

Pseudomonas tolaasii, another fluorescent pseudomonad and fresh produce spoilage bacterium, has a much narrower range of host-specificity than *P. fluorescens* and *P. viridiflava*. *P. tolaasii* causes spoilage of the white mushroom, *Agaricus bisporus*. Similar to *P. fluorescens* and *P. viridiflava*, *P. tolaasii* produces siderophores that fluoresce under ultraviolet light (Munsch, Geoffroy, Alatossans, & Meyer, 2000). However, unlike the soft-rot pseudomonads, *P. tolaasii* does not cause soft rot on plants (i.e., it does not produce pectin depolymerases) but instead creates unsightly blemishes on the caps and stems of the *Agaricus* fruiting body as a result of localized infection and decay of those parts of the mushroom. Wells, Sapers, Fett, Butterfield, Jones, Bouzar, & Miller (1996) identified three pathotypes of mushrooms based on pathology and fatty acid analysis: *P. tolaasii* and *P. gingeri* which cause severe and yellowed lesions and *P. reactans* which causes a mild discoloration of the infected area.

Prevention and Control Measures

Preharvest and Harvest Factors

Fresh fruits and vegetables are among the more challenging of food products to commercially produce and distribute. Fresh produce remains metabolically and developmentally active as it proceeds from the commercially appropriate time to harvest (horticultural maturity), to physiological maturity, to senescence and complete deterioration. During this period of development, several physiological and

compositional changes occur. This process can be summarized chronologically as growth, maturation, physiological maturity, ripening, and senescence (Watada, Herner, Kader, Romani, & Staby, 1984). Although infection and microbiological spoilage can proceed at any time during this developmental continuum, the period of greatest susceptibility to decay onset is during ripening and senescence. Prior to ripening, fruits and vegetables are equipped with defensive barriers to infection including active wound healing and the production of phytoalexins which are phenolic substances that are toxic to fungi (Kader, 1992; Sommer, Fortlagae, & Edwards, 1992).

Losses due to postharvest spoilage or pathological decay are a result either of latent infections in the field that become active following harvest or of cross-contamination during harvest, cleaning, storage, and distribution. Presence of the pathogen on a susceptible host fruit or vegetable, combined with suitable environmental conditions such as high temperature, provides the three components required for disease expression such as host, environment, and pathogen (Sommer et al., 1992). Therefore, spoilage management should begin in the field using an integrated strategy of GAPs. Balanced crop nutrition influences susceptibility to spoilage. For example, Sugar, Righetti, Sanchez, and Khemira (1992) determined that adjusting pear orchard nutrition, specifically for low nitrogen and high calcium, reduced fruit decay postharvest. High nitrogen in plant tissues generally increases susceptibility to decay, whereas high calcium content reduces postharvest decay on several crops (Conway, 1984, 1989; Conway, Janisiewicz, Klein, & Sams, 1999). Removing dead and decaying plant matter and other organic material from the crop plant and soil surface will eliminate a major harborage for spoilage microbes as well as other crop pests. To the extent possible, isolating the agricultural field from wild and domestic animals will not only reduce total microbial pressure on the crop, but also reduce food safety risks. Aerial fungicide applications preharvest also will reduce postharvest spoilage in storage. For example, a single application of the fungicide ziram to pome fruit reduced postharvest decay by 25–50% (Sugar & Spotts, 1995). Other preharvest fungicides are also available (e.g., iprodione and cypronidil) and several new fungicides are under development (Sholberg & Conway, 2004). In addition, insect pest management will reduce insect damage to crops and also will reduce microbial cross-contamination by the insect vector. This is especially important for chewing insects that create wounds on the fruit or vegetable and can simultaneously inoculate the wound site (Mahovic, Sargent, & Bartz, 2005).

At time of harvest and throughout handling before storage and distribution, it is important to minimize wounds and bruising and to cull all damaged and diseased product. A few spoilage microbes, primarily fungi, can infect healthy tissues by forming appressoria, external structures that enable the pathogen to penetrate the cuticle and epidermis (Sommer et al., 1992). The developing appressorium ramifies through these protective layers and into the pulp through a combination of mechanical pressure and tissue destruction by extracellular enzymes (Collmer & Keen, 1986). However, most spoilage microbes infect and initiate decay at punctures and splits in the epidermal layer or, in far fewer cases, through natural openings such as stomata and lenticels.

Postharvest Factors

Product integrity at time of harvest and stringent temperature management from harvest to consumption are two critically important factors contributing to acceptable storage and shelf life of all fresh fruits and vegetables. Upon harvest, fresh fruits and vegetables benefit from immediate surface sanitation and rapid cooling to slow product metabolism and growth of spoilage microbes. Reducing the rate of metabolism likewise reduces product respiration which, in turn, reduces the rate of deterioration, or perishability, of the crop (Kader, 1992). In many instances, product cooling and sanitation are accomplished simultaneously through one or more washings with chilled water amended with a sanitizing chemical. Chlorine, as sodium hypochlorite, calcium hypochlorite, or chlorine gas, is the most commonly used sanitizing chemical in the produce industry. Chlorine at a rate of between 50 and 200 ppm is added to prechilled water which is then applied to harvested fruit as a dip or as a spray or as some combination of these two methods. Concentrations below 50 ppm may not be particularly effective on some fruit, and concentrations above 200 ppm may damage the product and also create a potential worker safety issue due to off-gassing. To achieve and maintain maximum sanitizing efficacy, it is important to maintain water pH at or slightly below neutrality (pH 6.5–7.0). This can be achieved using any of a number of food-grade acids such as citric acid. It is also important to maintain as low an organic load as possible in the wash water because chlorine is unstable in the presence of organic matter and is rapidly inactivated. Other sanitizing chemicals such as ozone, chlorine dioxide, and peroxyacetic acid also are approved for use on fresh produce and are available commercially (Sapers, Miller, Pilizota, & Mattrazzo, 2001). Methods for monitoring sanitizer concentration are available for all commercially available sanitizers, and it is also strongly recommended to perform routine treatment efficacy assessments. This can be determined by collecting a minimum of three, and preferably five, individual samples immediately prior to washing and another three or five samples immediately following washing, and determining the total aerobic plate count on each sample. A properly functioning wash system should reduce the average total aerobic plate count by 10- to 100-fold. Sequential wash steps will further improve product sanitation by providing greater reductions in microbial load on the product.

Some additional commonly used methods for removing the field heat of harvested produce include forced air refrigeration, vacuum cooling, and immersion in ice. Mushrooms, for example, are not as amenable to water washing as many other products, and hence forced air and vacuum cooling are common in the mushroom industry (although mushroom wash systems are beginning to see increasing use). Selecting the optimum cooling method or combination of methods for a given product is beyond the scope of this chapter, but there are excellent resources available that provide specific technical guidance in this area (Kader, 1992).

Methods for Detection and Isolation of Spoilage Microorganisms

Methods to detect and isolate spoilage microbes from fresh fruits and vegetables depend largely on whether the sample of interest is currently infected with a visible

lesion or the sample has no visible lesions. If the sample has no visible signs of disease, it is reasonable to assume any spoilage microbes present will be residing at or near the outer surfaces of the sample. In this case the objective of sample preparation is to dislodge as many of the viable microorganisms as possible from the sample surface for subsequent isolation and detection. Several different strategies may be used to release microorganisms, and all typically begin by adding the sample to a volume of sterile diluent to obtain a 1:10 dilution in a sterile Whirlpak® or Stomacher® bag. Sterile, deionized water can be used for this purpose, but this is not recommended as osmotic shock may inactivate a portion of the total microbial population. Phosphate-buffered saline, Butterfield's buffer, and 1% buffered peptone water are all acceptable diluents for this purpose and can be prepared easily in the laboratory or purchased preformulated. Physically dislodging the microbes can be accomplished by palpating the sample in a Stomacher for up to 2 min, or by pulping the sample in a sterile, commercial food blender for up to 60 s, or by vigorous shaking on a wrist-action shaker for up to 30 min. Indeed, when sample preparation must be conducted outside the laboratory setting, dislodging surface microbes can be accomplished, albeit less efficiently and with lower yields than the aforementioned methods, by hand shaking the sample bag for up to 2 min. Each of these mechanical methods has advantages and disadvantages. The Stomacher method, probably the most widely applied in the food industry, is rapid, does not come into physical contact with the diluted sample (does not require re-sterilization between samples), and reportedly provides a high rate of recovery of viable microbes from the sample (Sharpe, Hearn, & Kovacs-Nolan, 2000; Wu, Jitareerat, & Fung, 2003). Blending the sample is rapid and efficient, but the blender jar and blades must be re-sterilized between samples (or multiple blender jars must be used). Shaking the diluted sample on a wrist-action shaker is efficient and, depending upon the length of the shaker arms, up to 16 samples can be prepared simultaneously, and the shaker reused immediately as the sample does not come into direct contact with the equipment. Another advantage of agitation by a wrist-action shaker is that the sample remains more or less intact. This is unlike either the Stomacher or blender that macerates the tissue and makes subsequent sample handling steps such as pipetting more difficult. A relatively new piece of equipment, the Pulsifier, offers the same advantage as the wrist-action shaker by preparing the sample with very little maceration of the sample (Fung, 2006). Wu et al. (2003) compared total viable bacterial cell and total coliform, recovered from samples of 30 different fresh vegetables, with the Stomacher and with the Pulsifier and found no difference in viable recovery between the two methods. Irrespective of the initial sample preparation step, the next step depends on whether the investigator is interested in attempting quantitative recovery of a specific pathogen (or pathogens) or simply desires to determine if the microbe of interest is present on the sample. Quantitative recovery can be difficult if a suitably selective medium for the pathogen of interest does not exist. In this instance, it is typically necessary to streak the plate directly onto several nutritionally different media and subsequently identify those colonies resembling the microbe of interest. However, if a suitable selective medium does exist, the next step after sample preparation is serial dilution, followed by spread-plating (0.1 ml)

and incubation. Incubation time and temperature depend very much on the temperature range of the pathogen of interest, compared with the typical temperature range of the background flora the investigator wishes to suppress.

Several texts are available that describe routine diagnosis of fungal and bacterial diseases of fresh fruits and vegetables. Many spoilage microbes develop very distinctive lesions depending upon the fruit or vegetable afflicted. For this reason, initial diagnosis often is conducted in the field or in the packing facility based on macroscopic appearance of the lesion. *A Color Atlas of Post-Harvest Diseases and Disorders of Fruits and Vegetables* (Snowdon, 1990) is a two-volume set of texts that provides a comprehensive review of the biology, epidemiology, and physical appearance of a large number of fruit and vegetable spoilage microorganisms. Included are several photomicrographs of the pathogens described, illustrating their appearance either as single cells, mycelia, and fruiting bodies.

Basic Plant Pathology Methods (Dhingra & Sinclair, 1985) is a very comprehensive reference text that includes not only methods for enrichment, isolation and identification of most plant pathogens, but also provides several chapters on methods for pathogen isolation from soils and other strata, manipulation and handling of pure cultures in the laboratory, microscopy methods (including several staining techniques), fungicide efficacy assays and biological control assays, and histological techniques.

Two other manuals that address specific methods for the diagnosis of plant bacterial diseases are *Methods for the Diagnosis of Bacterial Diseases of Plants* (Lelliott & Stead, 1987) and *Laboratory Guide for Identification of Plant Pathogenic Bacteria* (Schaad, 1988). The former text provides both macroscopic diagnosis on plant and fruit tissues and isolation and identification methods, whereas the latter focuses more on laboratory procedures. Supplemental information can be found in more general references (Downes & Ito, 2001; Jackson, 1998; Gerhardt, et al., 1981),

Microbiological Spoilage of Fresh-Cut Fruits and Vegetables

Introduction

Fresh-cut fruits and vegetables are “any fresh fruit or vegetable or any combination thereof that has been physically altered from its original form, but remains in a fresh state” (IFPA, 2001). Fresh-cut fruits and vegetables offer consumers ready-to-eat produce that is one of several convenient, nutritious and fresh-like tasting, and are a rapidly growing category of value-added produce products that are minimally or lightly processed. Processing of fresh-cut products involves sorting, cleaning, washing, heating/pasteurization (such as Biosteam®), trimming/peeling, coring, slicing, shredding, packaging and/or other related steps, depending on the product. The processing can be as simple as fresh-cut grape tomatoes for which raw tomato fruit is only sorted and washed with sanitized water or as complicated as cut cantaloupe, for which cantaloupe is sorted, cleaned with brush and spray water, heat treated

with hot water or steam, peeled, deseeded, chunked, and rinsed with sanitized water before packaging in rigid containers. The fresh-cut fruits include melon chunks and slices; cored and sliced pineapple; apple wedges treated with antibrowning preservatives; peeled citrus fruits and segments; de-capped strawberry; de-stemmed and washed grapes; sliced kiwifruits, and fruit salads. Examples of fresh-cut vegetables are shredded lettuce, shredded and diced cabbage, washed and trimmed spinach, peeled "baby" carrots, cauliflower and broccoli florets, sliced or diced tomatoes, peeled and sliced potatoes, snapped green beans, trimmed green onions, cleaned and diced onions, and mixed salads. Compared with whole fresh produce, fresh-cut produce is ready-to-use (ready-to-eat), contains 100% usable product, and always requires processing, refrigeration (including chilling-sensitive fruits and vegetables that can be injured after a period of exposure to chilling temperatures below 10–15°C (50–59°F) but above their freezing points), and packaging, specifically modified atmosphere packaging (MAP, a packaging technique that utilizes package atmosphere other than air, <0.1% CO_2, 20.9% O_2, 78% N_2, in a sealed package to extend shelf life of foods). Fresh-cut products are in a raw state or fresh-like, nutritious, and contain live tissues without freezing, canning (heat sterilization), dehydrating, fermentation, acidification, or treatments with additives or preservatives to prevent spoilage. Fresh-cut is also described as precut, minimally processed, minimally processed refrigerated, lightly processed, partially processed, fresh processed, and pre-prepared in literature and application communications. Fresh-cut fruit and vegetable sales are approximately $12 billion per year in the North American foodservice and retail market and account for nearly 15% of all produce sales (IFPA, 2001). Fresh-cut products offer produce growers/shippers an opportunity to increase sales by adding value to raw agricultural commodities. The largest portion of US fresh-cut vegetable sales at retail is fresh-cut salads, with sales of $2.4 billion. Retail fresh-cut fruit products are the fast growing fresh-cut produce category. In 2004, 3.5 million units of fresh-cut fruits were sold for $719 million in sales. Between January and February of 2005, those numbers were up 17% over 2004 (Warren, 2005).

Shelf Life of Fresh-Cut Fruits and Vegetables

The shelf life (i.e., the length of time that corresponds to a tolerable loss in quality of a processed food and other perishable items) of fresh-cut fruits and vegetables ranges from 1 to 35 days depending on types of shelf life (such as marketing shelf life, food safety shelf life, sensory shelf life, or microbiological shelf life), food safety concerns, marketing strategies of fresh-cut processors, produce commodities, raw materials, refrigerated storage temperatures, preparation methods, and packaging methods. Marketing shelf life of fresh-cut products, indicated by sell-by-date, best if use-by-date, use-by-date, or best use-by-day, is not necessarily the same as sensory shelf life or microbiological shelf life, depending on marketing concerns and competition. The average shelf lives of fresh-cut fruits and vegetables are typically 10–14 days (Cantwell & Suslow, 2002). However, the shelf life for kitchen- or

store-prepared fresh-cut fruits for the catering industry and restaurants is only 1–2 days (Ahvennainen, 1996). FDA (2000) has suggested a 7-day shelf life at 5°C for fresh-cut melons prepared at home for microbiological safety reasons. Sensory shelf life and microbiological shelf life are usually the same; however, they can differ significantly. For example, white surface formation on "baby" carrots can occur weeks before microbial spoilage is observed even though the peeled carrots are packaged in polyethylene pouches and stored at 2°C (Bolin & Huxsoll, 1991). Poubol and Izumi (2005) reported that fresh-cut mango made from cultivar "Nam Dorkmai" (50–60% yellow) had a shelf life as short as 2 days at 5°C due to browning discoloration and a water-soaked appearance. However, fresh-cut celery of 10-cm sticks or 1.9-cm crescents retained its fresh appearance and flavor up to 28 days when stored at 2°C (Robbs, Bartz, McFie, & Hodge, 1996a). O'Hare (1994) reported that cut pineapple had a shelf life of more than 5 weeks at 1°C. Fresh-cut melons (such as cantaloupe and watermelon) prepared by retail stores (store-cut) and displayed on retail shelves typically have a 2-day shelf life. The shelf life of cut cantaloupe that is prepared by fresh-cut processors could be as short as 5 days at 7°C. Cantaloupe washed with hydrogen peroxide, cut and packed at research laboratories could last for more than 18 days at 4°C (Sapers, Miller, Pilizota, et al., 2001). Sterile diced cantaloupe stored in controlled atmospheres at 4.5°C had a shelf life of up to 28 days (O'Connor-Shaw, Roberts, Ford, & Nottingham, 1996). Powrie, Wu, and Skura (1988) reported a shelf life of 12 weeks at 1°C for sliced melon, using modified atmosphere packaging (MAP) and gas-impermeable containers.

Impact of Microbiological Spoilage

As processing and packaging technologies have improved during the last decade, microbiological spoilage or microbiological shelf life has become a major reason for sensory quality shelf life failure for most packaged fresh-cut fruits and vegetables, followed by surface discoloration (e.g., pinking of cut lettuce, browning of cut potato, graying and browning with processed pineapple, and gray discoloration with cabbage), water-soaked appearance or translucency (e.g., cut watermelon, papaya, honeydew, and tomatoes), moisture loss (e.g., "baby" carrots and celery sticks), off-aroma (e.g., broccoli florets and diced cabbage in low % O_2 and high CO_2 packages), flavor changes (e.g., cut kiwifruit), and texture changes (e.g., processed strawberry, grated celery, kiwifruit, and papaya). Microbial spoilage including off-flavor (e.g., fermented aroma with cut lettuce, sour taste with cantaloupe and bell pepper) formation, slimy surface (e.g., "baby" carrots), wetness and soft rot (e.g., cut bell pepper), discoloration (e.g., apple wedges), and visual microbial growth/colonies (such as apple wedges, cantaloupe chunks, and cored pineapple) has been used as a main or exclusive objective criterion to determine shelf life of fresh-cut products (Sapers, Miller, Pilizota, et al., 2001, O'Connor-Shaw, Roberts, Ford, & Nottingham, 1994). Brackett (1994) concluded that microbial decay can be a major source of spoilage of fresh-cut produce. O'Connor-Shaw et al. (1996) reported that microbial spoilage is a limiting factor for shelf life of fruit pieces stored under con-

trolled atmosphere conditions. Shelf life, including microbial spoilage, results in 30–50% shrinkage of fresh-cut fruits (Warren, 2005). Microbial spoilage has been used by quality assurance departments in the fresh-cut industry as the objective indicator for quality failure for more than 50% of fresh-cut vegetable commodities and almost 100% of fresh-cut fruit products that have been treated with preservatives (such as antibrowning reagents) and/or packaged properly using MAP technologies. Under equilibrium modified atmosphere (MA) conditions, mixed fresh-cut bell pepper (including green, yellow, and red bell pepper) was unacceptable by day 6 of storage at 7°C due to acidic flavor, water loss, and texture change (Jacxsens, Devlieghere, Ragaert, Vanneste, & Debevere, 2003). Processed Lollo Rosso lettuce had a shelf life of shorter than 7 days at 5°C due to high microbial counts and off-odor formation under MAP (Allende, Aguayo, & Artes, 2004). Grated carrots became wet and slimy, lost firmness, and produced off-odors during storage at 10°C under MAP (Buick & Damoglou, 1987; Carlin, Nguyen-the, Cudennec, & Reich, 1989, 1990). The first indicator of changes in freshness for fresh-cut lettuce packed using active/passive MAP to prevent pinking or browning is fermented aroma formation (Table 5). Studies of cut cantaloupe revealed evidence of visual spoilage, including presence of microbial colonies, slime, and turbidity in juice, within 15 days of storage at 4°C (Sapers, Miller, Pilizota, et al., 2001).

Table 5 Changes in intensity of sensory attributes of fresh-cut lettuce during storage at refrigeration temperature (15-point universal scale was used in the test)

Shelf life (days)	Flavor	Hardness (texture)	Fermented aroma
0	6.3	5.3	3.8a
4	5.8	5.2	4.3a
5	5.6	5.8	6.1ab
6	4.6	7.3	5.9ab
7	6.0	6.7	4.6ab
10	4.3	7.6	6.3ab
11	6.2	6.3	6.8b
12	5.9	6.6	6.9b
P-value	0.671	0.131	0.047

Sources of Microbial Contamination

Contamination sources of fresh-cut fruits and vegetables include raw materials and contact with processing equipment. The microorganisms that exist on the surfaces of raw, whole produce appear to be the major source of microbial contamination and consequent spoilage of fresh-cut fruits and vegetables. Sapers, Miller, Jantschke, and Mattrazzo (2001) reported that, compared with good surface sanitization practices, no decontamination treatment or an ineffective antimicrobial treatment on whole cantaloupe resulted in premature microbiological spoilage of fresh-cut cantaloupe. Studies have also revealed over a 1-year period of sampling that there is a close relationship between the total mesophilic aerobic counts on lettuce

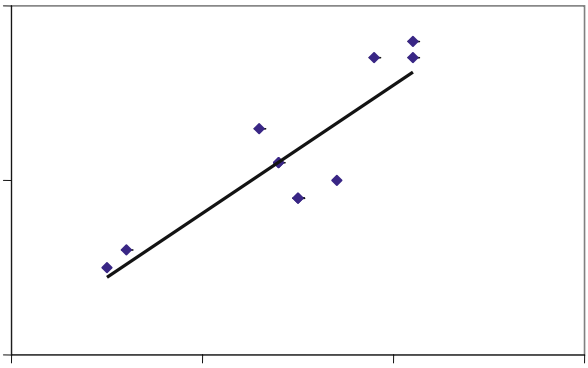

Fig. 2 Relationship between microbial population of raw lettuce and shredded finished product

raw material and those on finished shredded lettuce product (Fig. 2). Robbs et al. (1996a) determined that the most common bacteria on raw celery plants, including fluorescent *Pseudomonas* spp. and *Aeromonas* spp., were also the most common bacteria on cut celery products. Boyette, Ritchie, Carballo, Blankenship, and Sanders (1993) reported that the microbial decay of fresh-cut lettuce is largely due to the growth of microorganisms originating from preharvest environments. Delaquis, Stewart, Toivonen, and Moyles (1999) determined that the types of microorganisms found on shredded lettuce were highly associated with the microorganisms detected on lettuce before shedding. Several studies (Magnusson, King, & Torok, 1990; Geeson, Churey, & Splittstoesser, 1990; Torok & King, 1991) have revealed that yeast species identified on fresh-cut produce can also be isolated from raw materials prior to processing. Garg, Churey, and Splittstoesser (1990) concluded that peel is the major source of microbial contaminants on carrot sticks. Several outbreaks of salmonellosis that were associated with cut cantaloupe and watermelon have resulted from *Salmonella* present on the rind contaminated in the field or packinghouse (Harris et al., 2003). Inoculation of *Listeria monocytogenes* and *Salmonella* on the surface of entire cantaloupes resulted in the contamination to fresh-cut pieces during cutting (Ukuku & Sapers, 2001, Ukuku & Fett, 2002). These results indicate that the bacteria on the surface of whole produce are the same as those on fresh-cut produce and can contaminate finished product through processing. Fresh-cut products can also be contaminated by spoilage microorganisms through contact by people or equipment during processing possibly by air during processing and packaging steps, especially in facilities that have been used for produce processing over an extended period of time. Cantwell and Suslow (2002) found significantly higher bacterial counts during processing on automated cutters and package fillers of a lettuce processing line, indicating that clean product can become recontaminated after passing through operations where vegetable and fruit debris can accumulate, such as cutters and package-filling equipment. Shredding and slicing steps in fresh-cut processing resulted in increased microbial populations by 1–3 logs on cut cabbage, lettuce, and onions (Garg et al., 1990) and at least a 1-log increase for lettuce and chicory salads (Jockel & Otto, 1990). Legnani and Leoni (2004) found statistically significant differences for total plate counts and total and fecal coliforms in the last

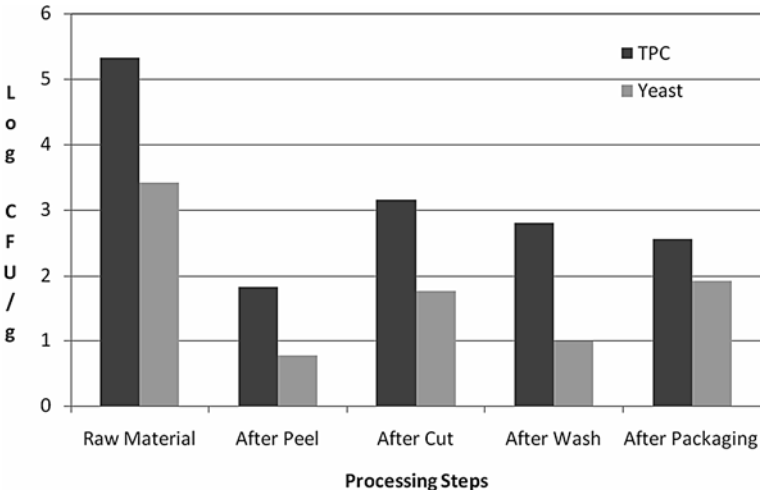

Fig. 3 Changes in mesophilic aerobic bacterial (TPC) and yeast populations of cantaloupe during processing

wash water step (rinsing water) compared to the water used during the previous disinfecting stage (3.52 $\log_{10}$ vs. 0.94 $\log_{10}$ for TPC, 1.86 $\log_{10}$ vs. 0.04 $\log_{10}$ for total coliforms). Zhuang, Barth, and Hankinson (2003) reported that the microbial population significantly increased on broccoli florets after the washing step. Allende et al. (2004) determined that shredding, rinsing, and centrifugation of red lettuce Lollo Rosso increased coliform, lactic acid bacteria, and psychrotrophic bacterial counts. Yeast populations on cut cantaloupes increased after packaging (Fig. 3). Several researchers have suggested that the large numbers of lactic acid bacteria and fungi present on fresh-cut products indicate likely contamination from processing, such as cutting machines (Brocklehurst, Zaman-Wong, & Lund, 1987; Dijk et al., 1999). For example, *Geotrichum candidum* has been termed "machinery mold" because it can accumulate on fruit-processing equipment.

Microbial Populations and Varieties on Fresh-Cut Fruits and Vegetables

Since raw materials can contribute to contamination of produce products during cultivation, harvesting, packaging, and shipping, and there are no definite decontamination steps during processing, it is not surprising that a variety of microbial populations can be present. Goepfert (1980) reported that mesophilic aerobic bacterial populations on vegetables sampled at processing plants ranged from 4.6 (carrots) to 7.5 (peas) $\log_{10}$ CFU/g fresh weight. The mesophilic aerobic bacterial counts ranged from 4 to 6 $\log_{10}$ CFU/g fresh weight on finished cut vegetables and from 2 to 5 $\log_{10}$ CFU/g fresh weight on finished cut fruits, depending on the commodities, seasons of the year, and growing regions (Zhuang et al., 2003). The mesophilic

aerobic bacterial counts on bagged salads from the retail market ranged from 4.0 to 9.0 $\log_{10}$ CFU/g (Heard, 2000). High numbers of yeast and mold populations were also present on many ready-to-eat packaged salads, including lettuce, coleslaw, celery chunks, and baby carrots, and salad bar items including broccoli, cauliflower, iceberg and romaine lettuce, spinach, sliced green peppers, cucumbers, and tomatoes, ranging from 1.6×10^3 cfu/g on iceberg lettuce to 9.2×10^6 cfu/g on sliced tomatoes (Tournas, 2005a).

Many types of microorganisms can be found on a cut fruit or vegetable, including Gram-negative bacteria, Gram-positive bacteria, and fungi (yeasts and molds). Some viruses have been identified as plant pathogens of whole produce and presumably result in quality loss of fresh-cut root or tuber vegetables. Parasites can be a food safety concern but do not affect the sensory qualities/spoilage of either whole or fresh-cut fruits and vegetables. Buick and Damoglou (1987) reported that the microflora isolated from chlorinated sliced carrots included 70% *Erwinia* spp., 20% *Pseudomonas* spp., and 10% *Bacillus* spp. Babic, Hilbert, Nguyen-the, and Giraud (1992) identified a variety of yeasts, including *Candida* spp., *Cryptococcus albidus*, *Rhodotorula* spp., *Trichosporon penicillatum*, and *Saccharomyces cerevisiae,* on packed grated carrots. Carlin et al. (1989) isolated lactic acid bacteria, specifically *Leuconostoc mesenteroides*, and yeasts from grated carrots stored in MAP. Liao and Fett (2001) isolated lactic acid bacteria, yeasts, and 48 strains of pectolytic bacteria of the genera *Pseudomonas, Erwinia, Bacillus*, *Xanthomonas,* and *Flavobacterium* from baby carrots. Poubol and Izumi (2005) reported that bacteria isolated from "Nam Dokmai" mango cubes were predominantly Gram-negative rods of which about 60% were Enterobacteriaceae*,* including the genera *Klebsiella* and *Pantoea*. Phytopathogenic bacteria that cause rot in vegetables such as *Pantoea agglomerans* (synonymous with *Erwinia herbicola* and *Enterobacter agglomerans*) and *Burkholderia cepacia* (synonymous with *Pseudomonas cepacia*) were also isolated frequently. The most common Gram-positive bacteria were of the genus *Curtobacterium*. Robbs et al. (1996a, 1996b) found Gram-negative bacteria fluorescent *Pseudomonas* spp., *Pantoea herbicola (E. herbicola)*, *P. agglomerans*, *Aeromonas, Arthrobacter*, *Aureobacterium,* and *E. carotovora*, Gram-positive cocci (*Leuconostoc*), and Gram-positive rods on decayed fresh-cut celery. Garg et al. (1990) detected large populations of Gram-negative psychrotrophic bacteria, particularly *Pseudomonas* spp., lactic acid bacteria, and fungi on freshly prepared carrot sticks. The filamentous fungi isolated from ready-to-eat salads included *Alternaria, Cladosporium and Penicillium, Alternaria, Cladosporium and Aspergillus, and Fusarium* (Tournas, 2005b; Acevedo, Mendoza, & Oyon, 2001).

Characteristics of Spoilage Microorganisms on Fresh-Cut Fruits and Vegetables

Fresh-cut produce differs substantially in physical properties (no protective epidermic tissues and high moisture), biochemical characteristics (wounding response), and handling environment (processed and stored under refrigerated temperatures

and packed under MAP) from whole produce. These differences likely influence the types of spoilage microflora present. Fresh-cut vegetables and melons, including cantaloupe, honeydew, and watermelon, have high water and nutrient contents (water activity, a_w>0.90), with pH values higher than 4.5. These characteristics make them suitable hosts for most microorganisms. Refrigerated conditions and high oxygen transmission rate film packages (in which equilibrium $\%O_2$ is >5% and $\%\ CO_2 < 5\%$) (Lund, 1982) enable bacteria to more successfully compete with the fungi in these foods. Hence, bacteria are more often responsible than fungi for postharvest spoilage of refrigerated fresh-cut vegetables and melons, with the majority being psychrotrophic and Gram-negative. The most common and important spoilage microorganisms of refrigerated fresh-cut vegetables are the fluorescent *Pseudomonas* species of which *P. marginalis* is an example. *Pseudomonas* spp. is a Gram-negative rod and strict aerobe. These species can be divided into four groups based on RNA homology and nine groups based on cellular fatty acid composition. Pseudomonads are widely distributed in nature and are found on both animal and plant products. They are able to utilize a wide variety of organic compounds and produce acids oxidatively from glucose and/or maltose. Some pseudomonads species produce pyoverdine or fluorescein that are water soluble, fluorescent pigments and can be observed in spoiled foods with an ultraviolet light. They are usually yellow-green but may appear blue or orange depending on the species and environmental factors. Pseudomonads produce catalase, oxidase (most), and enzymes that catalyze proteolytic and lipolytic reactions that contribute to spoilage of refrigerated fresh animal products, and pectolytic enzymes that can cause soft rot of fleshy vegetables. Pseudomonads are heat sensitive, are not found in heat-processed foods unless there is post-processing contamination, and are not very sensitive to drying or gamma irradiation. However, they are able to grow at refrigeration temperature (the minimal temperature for growth is ca. 4°C) and have been found in a variety of frozen and refrigerated foods, including fresh-cut produce. Pseudomonads can cause soft-rot decay of many types of vegetables including celery, potato, chicory, lettuce, Chinese chard, and cabbage (Brocklehurst & Lund, 1981). Many researchers (Brocklehurst et al., 1987; Carlin et al., 1989; Garg et al., 1990; Magnusson et al., 1990; Manvell & Ackland, 1986; Marchetti, Casadei, & Guerzoni, 1992; Nguyen-the & Prunier, 1989) have determined that 80–90% of mesophilic bacteria in the aerobic plate counts of vegetables are Gram-negative rods, *Pseudomonas* spp., *Enterobacter* spp., or *Erwinia* spp. with pseudomonads prevailing over other genera. Koek, De Witte, and De Maaker (1983) found that Pseudomonadaceae were 5–10 times more numerous than other families on prepared raw vegetables. King, Magnusson, Torok, and Goodman (1991) determined on cut lettuce the frequency of pseudomonads as 56.7% of total bacterial population; other species identified were *Flavobacterium* spp., *Xanthomonas* spp., *Chromobacterium* spp., *Chryseomonas* spp., *Rahnella aquatilis*, *Serratia* spp., *Alcaligenes* spp., and *Bacillus* spp. Robbs (1996a) found that the predominant bacteria in soft rot of fresh-cut celery were fluorescent *Pseudomonas* spp. *P. marginalis* and *P. chlororaphis* were isolated from 15 of the 16 samples, whereas *P. fluorescens* was found in 9 of the 16 samples. Large populations of *Pseudomonas cichorii*, *P. syringae*, and *P. viridiflava*

were also isolated from the celery. Brocklehurst and Lund (1981) found fluorescent *Pseudomonas* species to be the principal microbes on chopped salad 1 day after the listed "sell by" date. Marchetti et al. (1992) found that *P. fluorescens* represented 90% of the pseudomonads in ready-to-eat vegetables salads, whereas yeasts and molds and lactic acid bacterial populations generally remained low. Ukuku and Sapers (2005) reported that one of the most common types of spoilage microbes associated with cut melon were *Pseudomonas* spp. *Pseudomonas* spp., *Escherichia coli*, *Enterobacter* spp., and *Micrococci* were the predominant microflora on sliced watermelon (Abbey, Heaton, Golden, & Beuchat, 1988). Inoculation with either large cell numbers of *P. marginalis* or filtrated *P. marginalis* cultures isolated from spoiled shredded endives produced soft rot of endive leaves (Nguyen-the & Prunier, 1989).

Erwinia spp. are another common Gram-negative spoilage microbe associated with fresh-cut vegetables. *Erwinia*, a genus within the family Enterobacteriaceae, are small rods and facultative anaerobes. Their optimum growth temperature is 30°C, and they can ferment sugar anaerobically to form acids. *Erwinia* cause rapid necrosis, progressive tissue maceration called "soft-rot" occlusion of vessel elements called "vascular wilt," and hypertrophy leading to gall or tumor formation in plant tissues. The genus *Erwinia* spp. consists of three species or subspecies, including *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *E. chrysanthemi*. *Erwinia* spp. are the major single cause of microbial spoilage of whole vegetables (Liao, 2005; Lund, 1983). Brocklehurst et al. (1987) and Manvell and Ackland (1986) identified *E. carotovora* as a principal spoilage microbe of both fresh-cut and fresh vegetables. Buick and Damoglou (1987) found that *E. carotovora* was the dominant spoilage microorganism on sliced carrots packed in air, consisting of more than 80% of total detectable microflora. Robbs et al. (1996a) identified *Erwinia* in 5 of 16 soft-rot samples of fresh-cut celery.

Several Gram-positive bacteria, most notably the lactic acid bacteria, have been associated with spoilage of fresh-cut fruits and vegetables that are packaged under modified atmosphere with <2% O_2 and >10% CO_2 and stored at 7°C or above, regardless of the produce. Lactic acid bacteria are Gram-positive, usually nonmotile, nonspore forming rods and cocci. They lack the ability to synthesize cytochromes and porphyrins (components of respiratory chains) and therefore cannot generate ATP by creation of a proton gradient. The lactics can only obtain ATP by fermentation, usually of sugars. Since they do not use oxygen in their energy production, lactic acid bacteria grow well under anaerobic conditions, but they can also grow in the presence of oxygen. They are protected from oxygen byproducts (e.g., hydrogen peroxide) by peroxidases. These organisms are often termed aerotolerant anaerobes or microaerotrophic. They are differentiated from other microbes by their ability to ferment hexoses to lactic acid, hence their name. Because of the low energy yields, lactic acid bacteria often grow more slowly than microbes capable of respiration and produce smaller colonies. The genera of lactic acid bacteria include *Lactobacillus*, *Leuconostoc, Pediococcus, Lactococcus,* and *Enterococcus.* They are often associated with animal oral cavities and intestines (e.g., *Enterococcus faecalis*), plant leaves (*Lactobacillus, Leuconostoc*) as well as decaying plant or animal matter such

as rotting vegetables, fecal matter, and compost. Lactic acid bacterial fermentation lowers the pH due to lactic acid production and produces acetylmethylcarbinol and diacetyl that provide an off-flavor similar to buttermilk. Other fermentation products include acetic acid, ethanol, formic acid, and CO_2. Lactic acid bacteria were detected in almost every fresh-cut product, including honeydew, papaya, pineapple, cantaloupe, cabbage, carrots, chicory, celery, bell peppers, and various salad mixes (O'Connor-Shaw et al., 1994; Garg et al., 1990; Allende, Jacxsens, Devlieghere, Bebevere, & Artes, 2002; Carlin et al., 1989; Jacxsens et al., 2003). When packed under MAP and stored at 10°C, spoiled shredded carrots contained high numbers of lactic acid bacteria, identified as *L. mesenteroides,* and large concentrations of lactic acid, acetic acid, ethanol, and CO_2. Inoculation of shredded carrots with *L. mesenteroides* reproduced spoilage when the carrots were stored under conditions similar to those previously used (Carlin & Nguyen-the, 1989). Jacxsens et al. (2003) reported that the spoilage of mixed cut bell peppers and grated celery was related to the populations of lactic acid bacteria and yeasts, concluding that these microbes were responsible for spoilage during refrigerated storage.

The last group of major microorganisms that can cause spoilage of fresh-cut fruits and vegetables under refrigerated and MAP storage are fungi, including yeasts and molds. The optimum pH for growth of most microorganisms is near neutrality (pH 7.0). However, yeasts and molds are usually acid tolerant and are therefore associated with the spoilage of acidic foods. Yeasts can grow in a pH range of 3–10. Molds can grow from pH 2 to 11, but favor an acidic pH. In terms of water requirements for growth, yeasts are intermediate between bacteria and molds. Yeasts of the genera *Saccharomyces, Candida, Torulopsis,* and *Hansenula* have been associated with fermentation of fruits. In addition, other yeasts that can cause quality loss of produce include *Rhodotorula mucilaginosa*, *R. glutinis*, *Zygosaccharomyces bailii*, *Z. bisporus,* and *Z. rouxii* and have been isolated widely from fresh-cut fruits and vegetables, and salad mixes, even though the specific species were rarely identified (O'Connor-Shaw et al., 1994; Ukuku & Fett, 2002; Delaquis et al., 1999; Carlin et al., 1989; Jacxsens, Devlieghere, Ragaert, van der Steen, & Debevere, 2001). Yeasts have a slightly higher growth rate than molds, ferment sugars into alcohols, and are responsible for off-flavors and off-odors. Yeast growth has been responsible for shelf life failure of grated celery stored under MAP at 4°C and shredded chicory endives packed in high oxygen atmosphere with barrier film (Jacxsens et al., 2001).

Molds are fungi that cover surfaces as fluffy mycelia and usually produce masses of asexual, or sometimes sexual, spores. Mold is a growth of minute fungi forming on vegetable or animal matter, commonly as a downy or furry coating and associated with decay or dampness. Molds are overwhelmingly present in postharvest diseases of fruits and vegetables. These pathogens are commonly members of the class *Ascomycetes* and the associated Fungi *Imperfecti*. Mold spoilage of fresh produce, especially fresh fruit, is caused by species of *Penicillium, Phytophthora, Alternaria, Botrytis, Fusarium, Cladosporium, Phoma, Trichoderma, Aspergillus, Alternaria, Rhizopus, Aureobasidium, and Colletotrichum*. The symptoms include visible growth, rots and discoloration, such as blue mold rot, gray mold rot, botry-

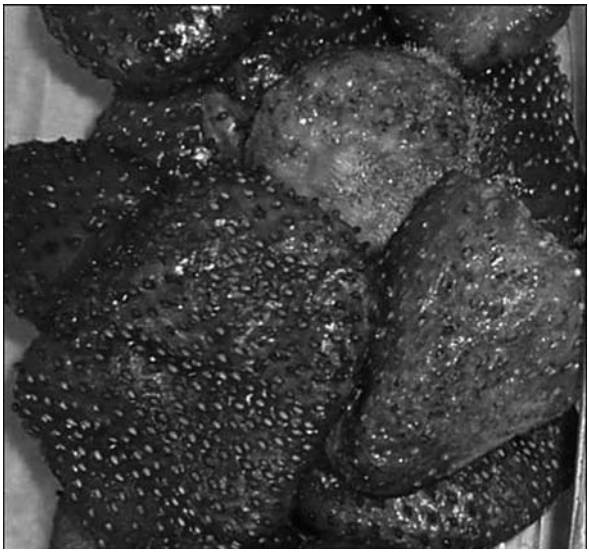

Fig. 4 Visible mold growth on cut strawberry

tis rot, and brown rot. Like yeasts, mold populations have been reported in various types of fresh-cut fruits and vegetables (Nguyen-the & Carlin, 1994; Tournas, 2005a; Hagenmaier & Baker, 1998) and visible molds have resulted in inedible fresh-cut fruits, such as strawberry, honeydew, pineapple, and cantaloupe (Fig. 4; O'Connor-Shaw et al., 1994; Ukuku & Fett, 2002). Since molds are usually detected and enumerated using the same plating media as yeasts and reported in the same category, their species most often are not identified and/or reported for contamination of fresh-cut produce.

Intrinsic and Extrinsic Factors of Fresh-Cut Fruit and Vegetable Products

Although microbes are largely responsible for the spoilage of fresh-cut produce, they can vary greatly for each type of fresh-cut product and accompanying storage conditions. The type of fresh-cut commodity and the pH of fresh-cut products are the two primary intrinsic factors that determine the microbiological spoilage profile of fresh-cut products. For example, under equilibrium-modified atmosphere spoilage of mixed lettuce (0.25 kg shredded lettuce mix with 20% each of endive, curled endive, radicchio lettuce, Lollo Rosso, and Lollo Bionda lettuces packed in 20 × 23.5 cm bags with 2,270 OTR, oxygen transmission rate) and chicory endive (0.15 kg of chicory endives packed in 19 × 15 cm bags with 3,704 OTR) leafy tissues was dominated by Gram-negative microorganisms at 7°C. However, spoilage of mixed bell peppers (0.15 kg of chopped green, red, and yellow bell peppers packed in 19 × 15 cm bags with 2,897 OTR) and grated celery (0.15 kg grated celery packed in 19 × 15 cm bags with 3,530 OTR) was dominated by lactic acid bacteria

and yeasts in the same study (Jacxsens et al., 2003). A relationship was observed between the number of pectinolytic *P. fluorescens* at the end of storage at 10°C and the extent of spoilage of shredded chicory; however, in similar experiments no relationship was noted between the presence of pectinolytic pseudomonads and spoilage with shredded carrots (Nguyen-the & Prunier 1989). Instead, spoilage of fresh-cut carrots in MAP at 10°C was highly associated with lactic acid bacteria and yeast populations (Carlin et al., 1989). In shredded cabbage stored at 4, 13, and 21°C, populations of aerobic bacteria were the dominant spoilage microbes. However, lactic acid bacterial populations increased to large cell numbers regardless of packaging treatment or storage temperature. The development of sourness and gassing of the packaging was related to extensive growth of these bacteria (Hao, Brackett, Beuchat, & Doyle, 1998). These illustrate that microbiological spoilage patterns are determined by the types of fresh-cut commodities. Intrinsic factors of plants affecting microbial spoilage can include the vegetable's nutrient content, biological structure, wounding response, self-defense system, and inherent antimicrobials. However, there are very few reports on the relationship between microbiological spoilage and these commodity-specific intrinsic variables. In addition to commodity differences, the pH of fresh-cut produce also significantly influences the microflora and the patterns of microbiological spoilage. Fresh-cut vegetables, melons, and some fruits, such as papaya, have a pH above 4.5 and the predominant spoilage microorganisms are mesophilic bacteria, specifically pseudomonads and *Erwinia.* Many reports indicate that fresh-cut lettuce packaged under oxygen-reduced atmospheres contain large populations of mesophilic and psychrotrophic bacteria, but small cell numbers of yeasts, molds, and lactic acid bacteria. The spoilage microflora of fresh-cut lettuce is largely dominated by *Pseudomonas* spp. (Garg et al., 1990; King et al., 1991; Delaquis et al., 1999). *Pseudomonas* spp., *E. coli, Enterobacter* spp., and *Micrococcus* spp. were predominant on watermelon slices during storage at 5°C (Abbey, et al., 1988). Mesophilic bacteria dominated the microflora of fresh-cut sweet potato slices, with lesser cell numbers of psychrotrophic bacteria and fungi, both initially and during storage (Erturk & Picha 2006). Mesophilic bacteria were also consistently predominant on finished cut honeydew and cantaloupe, including at the end of the shelf life (O'Connor-Shaw et al., 1994; Ukuku & Fett 2002). In contrast, the normal spoilage microflora of refrigerated, fresh-cut fruits differs markedly from that of vegetables. For most fresh-cut fruits such as apples, pineapples, strawberries, grapes, and a few fresh-cut vegetables, such as tomatoes, there is sufficient acid to limit spoilage primarily due to fungi (Splittstoesser, 1987) and aciduric bacteria (lactic acid bacteria, *Acetobacter, Gluconobacter*), *Leuconostoc* spp., and *Enterococci* spp. Martinez-Ferrer, Harper, Perez-Muroz, and Chaparro (2002) identified a relationship between increased shelf life and reduced populations of yeasts and molds on both cut mangoes and pineapples during storage. O'Connor-Shaw et al. (1994) reported that evidence of mold growth on cut pineapples stored in closed containers was the major quality defect at both 4 and 20°C. Under MAP and refrigerated storage (7°C), yeast and mold populations dominated the microflora of fresh-cut pineapples (Fig. 5) and strawberries

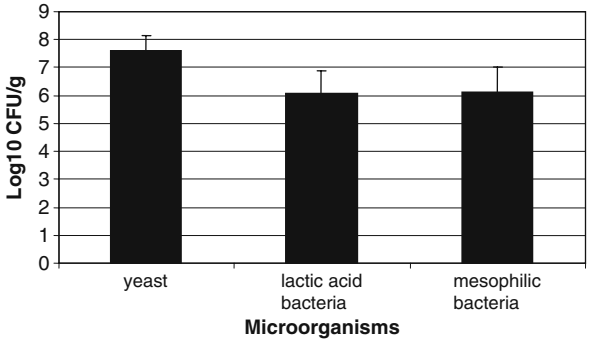

Fig. 5 Populations of microorganisms on cut pineapple held at 7°C for 9 days

Fig. 6 Microbial-related discoloration of fresh-cut apple

(Fig. 4). The most frequent reason for shelf life failure of antioxidant-treated apple wedges in MAP was fungi-related browning discoloration and decay (Fig. 6).

The major extrinsic factors that most significantly influence the microbial spoilage profile of fresh-cut produce are storage temperature and the modified atmosphere within the packages. Refrigerated storage temperature selects for psychrotrophic microbes over mesophilic microorganisms with high CO_2 (>10%) and low O_2 (<2%) content in fresh-cut packages favoring facultative and strict anaerobes instead of aerobic microbes. For example, cabbage (in coleslaw) deteriorated at the same rate at 7 and 14°C; however, at 7°C, the total mesophilic bacterial load was significantly reduced (King, Michener, Bayne, & Mihara, 1976). Similar phenomena have been reported for shredded chicory salads (Nguyen-the & Prunier, 1989) and shredded carrots (Carlin et al., 1989), in which the total mesophilic bacterial counts decreased with reduced storage temperatures. Characteristics of *P. marginalis* soft-rot spoilage are similar to those of *E. carotovora*. However, the more rapid growth of pseudomonads at refrigeration temperatures makes them more

likely to contribute to spoilage of refrigerated produce than would *E. carotovora* when the packaging atmosphere is not a limiting factor (Lund, 1982). *Erwinia* spp. (such as *E. carotovora* subsp. *carotovora* and *E. chrysanthemi*) grow poorly and fail to induce soft rot of fresh produce at 10°C or below, and the doubling time for *E. carotovora* was 15.4 h at 5.7°C (Lund, 1983). Growth of Gram-positive bacteria was more consistently affected by temperature. The optimal growth temperature range of these bacteria is 20–30°C, and they grow slowly, if at all, at refrigeration temperature. For example, the minimum growth temperature for a spoilage strain of *Clostridium puniceum* was ca. 7°C; however, most strains of *C. puniceum* are unable to grow at 10°C (Lund, Brockelhurst, & Wyatt, 1981). On shredded carrots, growth of lactic acid bacteria and subsequent spoilage was greatly delayed at 2°C compared to 10°C, but not significantly reduced at 6°C compared to 10°C (Carlin et al., 1989). Lactic acid bacteria predominated in vegetable salads held at 30°C and large amounts of lactic acid are produced. In salads held at 7°C even for prolonged periods, the lactic acid level remained low and Gram-negative bacteria predominated. The ratio of Gram-positive to Gram-negative bacteria in vegetable salads was consistently larger than 0 when held at 20 and 30°C; however, it was approximately 0 at 7°C. A Gram-positive to Gram-negative bacteria ratio of 0.5 or greater has been recommended as an indicator of temperature abuse for fresh-cut produce (Manvell & Ackland, 1986). Although many different types of fungi can be associated with spoilage of vegetables and fruits, only a few are able to spoil vegetables at refrigeration temperatures. Pitt and Hocking (1985) concluded that species of *Fusarium*, *Cladosporium*, *Penicillium*, and *Thamnidium* can grow and spoil foods at refrigeration temperatures. However, since psychrotrophic bacteria are more likely to cause spoilage in products with a neutral pH, fungal spoilage of vegetables and fruits is more likely to occur when adequate refrigeration is not maintained. Gimenez, Olarte, Sanz, Lomas, Echavarri, and Ayala (2003) found in their study of minimally processed artichokes packaged using different films that the populations of spore-forming bacteria remained virtually constant during storage at 4°C regardless of packaging films (ca. 2.5 and 2 log cfu g^{-1} of spore-forming aerobic and anaerobic bacteria, respectively). They concluded that the low storage temperature prevented germination of these spore-forming bacteria from occurring.

Pectolytic fluorescent pseudomonads that are involved in spoilage of cut produce are strictly aerobic bacteria. Gill and Tan (1979) and Wells (1974) reported that decreased O_2 content or increased CO_2 in the atmosphere reduced the growth of pectolytic fluorescent pseudomonads as well as reduced their capability of inducing soft rot on fresh produce. However, production of soft rot in potatoes by *Erwinia* and *Clostridium* is greatly enhanced by the depletion of oxygen (Perombelon, Cullings-Hander, & Kelman, 1978). The increased CO_2 and decreased O_2 concentrations formed in MAP generally favor the growth of lactic acid bacteria. Lactic acid bacterial populations were small on ready-to-use butterhead lettuce packaged with air as the initial headspace; however, higher counts were observed (with loss in quality) on samples packaged in a modified atmosphere containing 0% O_2 and 10% CO_2 as the initial headspace (Mazollier, Bardet, & Bonnafous, 1990). Growth of lactic acid bacteria on cut chicory leaves stored in modified atmosphere containing 20% CO_2 as the

initial headspace was more rapid than in air at 2, 6, and 10°C (Carlin & Nguyen-the, 1989). Generally, lactic acid bacteria grow slowly, if at all, at refrigeration temperature, and only become predominant under low O_2 and/or high CO_2 atmospheric storage. Studies have revealed that modified atmosphere storage of graded carrots at 10°C resulted in shelf life failure by *Leuconostoc*, a lactic acid bacterium (Babic et al., 1992; Carlin et al., 1989, 1990). Buick and Damoglou (1987) identified four different bacterial genera, i.e., *Erwinia*, *Pseudomonas*, *Bacillus,* and *Leuconostoc*, on sliced carrots and further study on the effect of vacuum packaging and nonvacuum packaging on microbial spoilage of sliced carrots revealed that in nonvacuum pouches, 100% of the bacteria were Gram-negative (90% was *Erwinia* and 10% was *Pseudomonas*) at 8 days of storage at 10 and 15°C, and 90% of bacteria was Gram-negative and 10% was *Bacillus* at 5°C storage. No *Leuconostoc* were detected as spoilage bacteria. Reducing the temperature had no effect on the percentage of *Pseudomonas* present, but reduced the percentage of *Erwinia* and increased the percentage of *Bacillus*. In the vacuum packages held at 15°C, 100% of the bacteria were *Leuconostoc*. Reducing the storage temperature to 5°C reduced the *Leuconostoc* population from 100 to 40% and increased the *Erwinia* population from 0 to 50%. No *Pseudomonas* spp. were detected.

Yeast populations on fresh-cut fruits and vegetables that have a neutral pH are generally not affected by modified atmospheres. Yeast growth on cut lettuce was unaffected by controlled atmosphere containing 3% O_2 with or without 10% CO_2 (Barriga et al., 1991). No differences in yeast counts were observed on grated carrots stored at 10°C under modified atmospheres containing 20–50% CO_2 and from 10 to 2% O_2 (Babic et al., 1992). However, yeasts can be facultatively anaerobic; hence in the absence of oxygen, fermentative yeasts can convert sugars into carbon dioxide and ethanol (alcohol) and result in spoilage of cut fruits and vegetables with pH values <4.5. In contrast, molds are strictly aerobic microorganisms and their spoilage depends on the availability of oxygen in the environment. MAP with high CO_2 (>10%) inhibits mold growth (Molin, 2000), although the effect is not fungicidal.

Based upon above research observations, the relationships between predominating spoilage microorganisms and the factors that influence the spoilage of fresh-cut produce are summarized in Table 6.

Table 6 Factors influencing spoilage of fresh-cut produce (Barriga, et al., 1991; Babic, et al., 1992, & Molin, et al., 2000).

pH of fresh-cut products	Storage atmosphere	Storage temperature	Predominating microorganisms
>4.5	Air	Refrigerated (<3°C)	Pseudomonads
<4.5	Air	Refrigerated and abuse temperature (>3°C)	Fungi and lactic Acid bacteria
>4.5	MAP (%O_2 < 1 and % CO_2 > 10)	Abuse temperature (>3°C)	*Erwinia*
<4.5	MAP (%O_2 < 1 and % CO_2 > 10)	Abuse temperature (>3°C)	Yeasts and lactic acid bacteria

Microbiological Spoilage Defects of Fresh-Cut Fruits and Vegetables

Microbiological spoilage defects of fresh-cut fruits and vegetables include microbial colony formation or visible microbial growth mainly due to microorganism proliferation, off-aroma and off-flavor formation mainly due to fermentation of sugar, soft-rot/water soak and sliminess due to enzymatic pectolyzation, and discoloration. For example, O'Connor-Shaw et al. (1994) observed mold growth at 14 days on cut pineapples held at 4°C and at 4 days when held at 20°C. White mold colony formation was observed at 11 days on cut cantaloupe held at 4°C and at 7 days on cut honeydew held at 8.5°C. Visual evidence of bacterial spoilage of cut cantaloupes results from the presence of bacterial colonies (Fig. 7), slime, juice turbidity, and off-odor (Sapers, Miller, Pilizota, et al., 2001). Mixed lettuce stored in bags with an oxygen transmission rate (OTR) of 15 mL $O_2/(M^2$ 24 h 1 atm) became inedible within 4 days at 7°C due to the off-odor and unacceptable taste that were described as alcoholic and fermented (Jacxsens et al., 2003). Mixed bell peppers held at 7°C were rejected by a trained sensory panel within 6 days due to an acid odor and taste. Fresh-cut cantaloupe held at 4°C developed an off-odor within 11 days (O'Connor-Shaw et al., 1994), and mixed vegetable salads held at 4°C were spoiled by off-odors within 7 days (Allende et al., 2002). Shredded carrots packaged in a modified atmosphere and held at 10°C developed off-flavors and became slimy (Carlin et al., 1989).

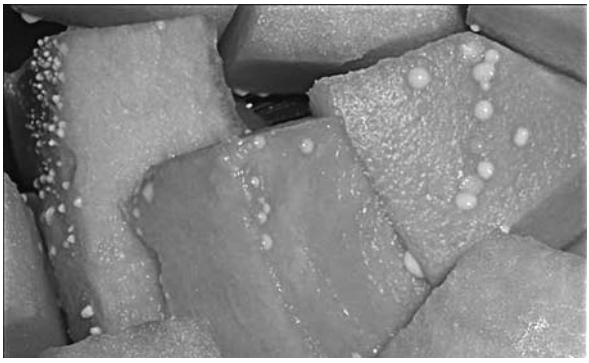

Fig. 7 Bacterial colony formation on fresh-cut cantaloupe

Another very common defect of fresh-cut vegetables attributed to microbiological spoilage is water soak/soft-rot. The decay of fresh-cut celery segments stored at <5°C in sealed film bags begins with water soaking at the cut surface and slimy moisture accumulation inside the bags (Robbs et al., 1996a). In a salad mix, endive and Lollo bionda developed soft rotting more rapidly than the other components (Artes & Martinez, 1996). Water soaking has been most commonly associated with spoilage of cut cantaloupe, honeydew, and watermelon (Ukuku & Fett, 2002; O'Connor-Shaw et al., 1994), especially under abusive storage temperature (>4°C), although there is no direct evidence that this results from the activity of pectolytic

bacteria. Studies have revealed that spoilage microbes such as *Gluconobacter and Acetobacter* can cause discoloration of whole produce, and fungal spoilage has discolored cut apples treated with antioxidants and packed in a modified atmosphere.

Microbiological Spoilage Mechanisms in Fresh-Cut Fruits and Vegetables

Growth of microorganisms subsequently forming visible colonies is a common cause of spoilage of fresh-cut fruits and vegetables. There is in general a linear relationship between the microbial cell numbers and spoilage of cut produce during refrigerated storage (Zhuang et al., 2003; Barriga, Richie, Willemot, & Simard, 1991). This relationship is stronger for fresh-cut melons and fruits compared with fresh-cut vegetables. In Europe, microbial specifications have been established for quality of fresh-cut produce. For example, in France and Germany, microbiological specifications for mesophilic aerobic bacterial populations or aerobic plate counts (APC) of salad vegetables at production (fresh) are 5×10^6 cfu/g, for separating good quality from marginally acceptable quality, and at use by date are 5×10^7 cfu/g (Francis, Thomas, & O'Beirne, 1999; Lund, 1993). Debevere (1996) proposed 10^8 cfu/g of aerobic psychrotrophic bacteria, 10^5 cfu/g of yeast, and 10^7 cfu/g for lactic acid bacteria as the limiting criteria for ready-to-eat vegetables. The Spanish legal limit (RD 3484/2000, 2001) for microbial populations on minimally fresh-processed fruit for safe consumption are 7, 5, and 3 $\log_{10}$ cfu/g for aerobic bacteria, yeasts, and molds, respectively.

Carlin, Nguyen-the, Abreu Da Silva, and Cochet (1996) and Nguyen-the and Carlin (2000) reported that the limits of microbial populations associated with spoilage of minimally processed endive packaged in sealed polypropylene film was associated with storage temperature. At storage temperature = 6°C, the bacterial counts when spoilage was first noticeable were $>10^8$ cfu/g; at 10°C, the bacterial counts were between 10^7 and 10^8 cfu/g; at 20°C, the average count was $10^{6.5}$ cfu/g. In cut cantaloupe, white colony formation was associated with high APC ($>10^8$ cfu/g) and yeast counts ($>10^5$ cfu/g) (O'Connor-Shaw et al., 1994). However, the exceptions to this overall positive linear relationship have been reported in many studies (Zhuang et al., 2003; Nguyen-the & Carlin, 1994). Sapers, Miller, Jantschke et al. (2001) did not observe a consistent difference in total APC between unspoiled and spoiled cut cantaloupe during refrigerated storage. However, in the same report, a lower APC was consistently associated with unspoiled products. These phenomena suggest the following:

1. Most, if not all, of the analyses that are currently conducted to determine microorganism populations of cut produce during storage are not specific enough to be associated with the shelf life or spoilage.
2. Low microbial counts are necessary to avoid or reduce spoilage of cut produce; however, high total microbial populations do not always correlate with spoilage.

3. It is not practical to use a microbiological specification based only on nonspecific microbial test results to reject fresh-cut products on a commercial level. APC and yeast, or lactic acid bacterial counts cannot be used solely to judge or predict shelf life or spoilage of lot of products, although there is an overall linear relationship between microbial load and quality of fresh-cut produce.

Formation of organic acids such as lactic acid and acetic acid associated with decreased pH values and generation of volatile compounds such as ethanol from fermentation of sugar by yeasts are additional mechanisms that result in aroma and flavor defects of fresh-cut products packaged under MAP. Carlin et al. (1989, 1990) determined that storage of shredded carrots under MAP at 10°C resulted in a pH decrease caused by the growth of lactic acid bacteria. Jacxsens et al. (2003) concluded that the production of detectable organic acids and reduction of pH caused by the growth of lactic acid bacteria and yeasts in mixed cut bell peppers and grated celery stored under MAP at 7°C resulted in unacceptable organoleptic properties, including off-flavor, odor, and taste. Lopez-Galvez, Peiser, and Nie (1997) reported the off-odors produced in salad mixes sensorially correlated with ethanol and acetaldehyde concentrations. Babic et al. (1992) and Fleet (1992) reported that large cell numbers of yeasts ($>10^5$ cfu/g) produce off-flavors in fresh-cut produce from the production of ethanol, organic acids, and volatile esters.

Bacterial soft rot, which is characterized by water-soaking and formation of a slimy surface on plant tissues, has been identified as the leading cause of storage disorders in many types of whole produce (Lund, 1983), and is frequently observed in fresh-cut fruits (Ukuku & Fett, 2002; O'Connor-Shaw et al., 1994) and vegetables (Artes & Martinez, 1996; Carlin et al., 1989; Robbs et al., 1996a, 1996b, Hakim, Austin, Batal, Gullo, & Khatoon, 2004) during storage. Bacterial soft rot results from degradation of plant cell walls by pectolytic enzymes produced by a variety of microorganisms, including *E. carotovora*, *P. marginalis* (Lund, 1983), *Botrytis, Clostridium, Alternaria*, *Geotrichum,* and *Fusarium* (Bulgarelli & Brackett, 1991). Many microbes use pectolytic enzymes to overcome plant defense mechanisms and access plant nutrients. The pectolytic enzymes, including pectin methyl esterase (PME), polygalacturonase (PG), pectin lyase (PNL), and pectate lyase (PL), can degrade pectins in the middle lamella of the cell, thereby resulting in liquification of the plant tissue leading to conditions such as soft rots. Other enzymes such as hemicellulase, cellulases, and proteases are also involved in the spoilage process but are usually secondary to pectinases (Liao, 2005). In the last two decades, molecular genetic research has revealed that multiple isozymic forms of the pectolytic enzymes exist. These are inducible and are not equally involved in the degradation of plant tissues. Their importance in soft-rot formation is dependent on bacterial genus. For example, there are three to five isozymes of PL in *E. chrysanthemi*, and at least one is inducible by plant constituents. The biological function and pathological effect of each PL isozyme and the specificity of its genetic expression are not yet clear. No single pectolytic enzyme in *E. chrysanthemi* has been shown to be essential to initiate a spoilage defect (Py, Barras, Harris, Robson, & Salmond, 1998). In vitro, the alkaline isozyme usually displays the highest degree of tissue-degrading

ability (Payne, Schoedel, Keen, & Collmer, 1987; Py, et al., 1998). In contrast, in *P. viridiflava*, PL is the principal or sole pathogenicity factor causing soft rot (Liao, et al., 1988; Liao, Sullivan, Gardy, & Wong, 1997). Mutants that were defective in the production or secretion of PL were unable to induce soft rot on potato tuber slices. When cloned PL gene was mobilized into the mutants, the PL-producing and soft-rotting ability of monopectolytic mutants was restored. In vitro, purified PL from *Pseudomonas* can induce soft rot of potato tuber slices. Production of *Pseudomonas* PL in vivois mediated by the product of the genes *gac*S/*gac*A, which encodes a sensory and a regulator protein in a two-component regulatory protein family (Liao, Mc Callus, Wells, Tzean, & Kang, 1996). Some *P. fluorescens* strains can be induced by calcium ions, although most are induced by pectic substrates (Liao, Mc Callus, & Wells, 1993). In vitro, purified PG was able to cause soft rot of potato tuber slices, whereas purified PME and PNL were not.

In summary, microbial spoilage of fresh-cut produce can be caused by microbial growth and/or by microbial metabolic processes. There is a strong relationship between microbial populations and shelf life if the cause of quality failure is visible growth of microflora on the surface. However, there is a relationship between microbial cell numbers and shelf life if the cause of quality failure requires specific metabolic activity, particularly under conditions of temperature abuse.

Prevention and Control of Microbiological Spoilage

Many thermal and nonthermal technologies have been developed to control microorganisms on fresh-cut produce. These have been summarized by Farber et al. (2003) and Sapers, Gourney, and Yousef (2005). Types of thermal processing used to treat fresh-cut produce include hot water, hot steam, and hot sanitizing solution. Thermal processing is a relatively new technology to the fresh-cut produce industry. Biosteam® Technologies trademarked ThermoSafe system is an example of thermal technology developed for fresh-cut processing. Biosteam's equipment is the size of a commercial dishwasher and uses hot steam to surface sanitize raw fruits and vegetables before peeling. Laboratory studies with inoculated whole melons revealed that the ThermoSafe process can effectively reduce microbial cell numbers on the surface of produce by 5 log units. The process time (from seconds to minutes) and temperature (from 60 to 100°C) are dependent upon the commodities being treated. There are a number of difficulties associated with the application of thermal processes to fresh-cut fruits and vegetables. For example, thermal processing cannot be used for fresh-cut commodities such as leafy vegetables and berries because of deterioration of quality characteristics. Also, can other processed products be called fresh-cut after a thermal process treatment is applied? Another limitation is that thermal processes are generally an inefficient use of energy and create a challenge for cold chain management which is needed by fresh-cut processors for product distribution. Further research and development is needed to validate thermal processes that can achieve a 5-log microbial reduction without affecting the quality of processed produce.

Nonthermal processing technologies can be classified as either physical or chemical. Physical technologies include high pressure, irradiation, pulsed electric fields, pulsed white light, ultrasound, and ultraviolet radiation. Some of these methods are generally not applicable commercially because they are too expensive (high pressure and pulsed electric fields), do not have consumer acceptance (irradiation), or require process validation of efficacy (UV and pulsed white light). The mechanisms and application of these methods have been well reviewed by Lund et al. (2000) and Ohlsson and Bengtsson (2002).

Chemical technologies can be divided into gas-phase sanitation and liquid-phase sanitation based on the physical state of the chemical used. Examples of gas-phase sanitation include ozone and chlorine dioxide. One of the difficulties in the application of gas-phase technologies is that a special in-line closed system is needed for the treatment of produce. These applications could also pose an employee safety issue. The most widely used chemical treatment in the fresh-cut produce industry is chlorinated water.

In addition to these active control measures, other factors important in the prevention of microbial spoilage include raw material quality, processing technologies, good manufacturing practices (GMP), packaging, and temperature management. High-quality raw materials can both reduce the potential for surface contamination and maximize the plant self-defense system. Diseased or damaged products are difficult to decontaminate using current methods of prevention and treatment and can contaminate products with low levels of microbes (Sapers, Miller, Jantschke, et al., 2001; Sapers, Miller, Pilizota, et al., 2001; Wiley, 1994). Zhuang et al. (2003) found that during storage yeast populations were significantly higher on cut honeydew melons having soft tissue than from melons that were firm. It is commonly known that in climacteric fruits, the increase in respiration just prior to full ripening generally coincides with a major reduction in fruit resistance to pathogens. Damaged cells have greater rates of respiration subsequently leading to cellular senescence or death and increased susceptibility to fungal colonization.

Processing techniques, including peeling, cutting, washing, and dewatering, also influence the vulnerability of fresh-cut fruits and vegetables to microbiological spoilage. Fresh-cut processing by peeling or abrasion or exposing inner nutritious fruit cells by cutting removes natural epidermal barriers to microbial attack. These processing steps result in accumulation of surface moisture and exposure of tissue to microbial contaminants. Barry-Ryan and O'Beirne (2000) determined with carrot slices that an abrasive peeling method resulted in significantly more lactic acid bacteria compared to hand peeling. Coarse abrasion resulted in significantly more total mesophilic aerobic bacteria during storage compared to hand peeling and fine abrasion peeling of product. Wash water with sanitizers can affect microbial contamination of fresh-cut produce (Zhuang et al., 2003), especially when the microbial load is low on the cut surface. Francis and O'Beirne (1997) found that dipping cut lettuce into 100 ppm chlorinated water significantly increased *Listeria innocua* levels compared with undipped samples. Bolin, Stafford, King, and Huxsoll (1977) determined that dewatering with centrifugation extended shelf life of cut lettuce by at least 5 days and concluded that the presence of any free moisture or cellular fluids

on the lettuce surface reduced shelf life. Although Bolin et al. (1977) did not determine microbial populations fresh-cut processors have observed for a long time that wet fresh-cut products decay considerably more rapidly compared to those that are well dewatered such as peeled carrots. Fresh fruits and vegetables have a high water activity (>0.90), hence reducing water activity is not an option for the prevention of microbial spoilage in fresh-cut products. Therefore, it is important to minimize the amount of residual surface moisture after processing.

GMPs also influence the microbial spoilage and subsequent shelf life of fresh-cut produce. It is important to train all employees in GMPs before they can work in processing operations. Fresh-cut fruits and vegetables are minimally processed and usually consumed raw. To date, intervention strategies that have been developed cannot completely eliminate microbial growth due to the fragility of raw plant tissues, particularly leafy vegetables. Therefore, preventing contamination, especially through effective implementation of GMPs, is the most effective strategy to minimize microbial contamination during processing. It is well known in the fresh-cut industry that fresh-cut samples prepared in the laboratory often have substantially longer shelf lives than samples produced by the commercial process. This is largely due to special precautions taken in the laboratory in handling the produce which reduces both the damage and the opportunity for contamination. Hence, this reinforces the importance of GMPs. Studies have revealed that during commercial processing both microbial populations and the composition of the microbial flora change dramatically on fresh-cut product (Garg et al., 1990; Zhuang et al., 2003, Fig. 2). The contaminating microorganisms can be transferred from gloves and utensils (Cantwell & Suslow, 2002). Beginning with proper training and employing good hygienic practices will have a positive influence on the microbiological shelf life of fresh-cut produce at the commercial level.

Packaging affords several means to minimize microbial spoilage. As mentioned previously, MAP is one of the most important extrinsic factors that affect microbial spoilage of fresh-cut produce. In recent years, several new modified atmosphere packaging treatments have emerged aimed at preventing microbial growth by actively introducing antimicrobial agents into the packages (Han, 2003; Farber et al., 2003). One method is the use of large concentrations of oxygen or super-atmospheric oxygen packaging (>70% O_2). The key to successful implementation of MAP of fresh-cut produce in commercial practice is establishing equilibrium-modified atmospheres (usually 2–5% O_2 and 5–15% CO_2) that can slow down the metabolism of processed produce and inhibit microbial growth by the selection of correct packaging material permeability for O_2 and CO_2 and the correct ratio of product weight and surface area of packages. However, due to the high-level respiratory activity of fresh-cut produce and the potential variation in storage temperature due to placement of packaged products on display shelves in retail stores, O_2 levels in MAP products can decrease to create anaerobic conditions (<2% of O_2 and >20% of CO_2). Anaerobic conditions are favorable for growth of anaerobic bacteria and can result in undesirable fermentation reactions and spoilage. Using high O_2 concentrations can overcome the disadvantage of anaerobic atmosphere packaging. Several studies have revealed that high O_2 concentrations have been

effective in reducing microbial growth, preventing anaerobic fermentation, and also inhibiting enzymatic discoloration (Day, 1996, 2000; Jacxsens et al., 2001; Allende et al., 2002). Kader and Ben-Yehoshua (2000) suggested that high O_2 levels (> 40%) could generate reactive oxygen species (O_2^-, H_2O_2, $OH^\bullet$) in plant cells, damage vital cell components, and thereby reduce cell viability. A concern with this technology is potential worker safety implications during packaging in the production environment. Oxygen concentrations higher than 25% are explosive (BCGA, 1998). Another concern associated with antimicrobial packaging is how these treatments will change or modify the normal microbial flora on fresh-cut produce during storage, and whether this change is advantageous remains to be determined. Jacxsens et al. (2001) observed that growth of the plant pathogen *E. carotovora* at 4°C was stimulated by increased O_2 levels, although yeast growth was reduced. Other new packaging developments include the use of microperforated films and Intelimer packaging (Landac film).

Another important technology to minimize microbial spoilage is adequate cold chain temperature management. Effective cold chain management begins with raw product cooling in the field through processing and retailing and ends at the restaurant or consumer's table. Unfortunately, there are often breaks in the cold chain, which have an additive effect on reducing the optimum shelf life of a product. Sales representatives in the fresh-cut industry have correlated poor performance of fresh-cut produce in retail stores with breaks in cold chain temperature management. Regardless of raw material quality, GMPs, processing conditions, antimicrobial treatments, types of antibacterial packaging, and abusive temperatures shorten the shelf life of fresh-cut produce.

Methods for Detection and Isolation of Spoilage Microorganisms

The methods used to detect and isolate spoilage microorganisms are mainly based on cultural procedures. For example, for fresh-cut fruit O'Connor-Shaw et al. (1994) extracted microorganisms from the fruits (1:5 dilution) using sterile 0.1% peptone water and 0.5% sodium chloride, macerated this preparation by stomaching for 1 min and using the following methods for enumerating different microorganisms: standard methods agar (SMA) with incubation at 25°C for 3 days for aerobic plate counts; dichloran rose bengal chloramphenicol agar with incubation at 25°C for 5 days for yeasts and molds; Man, Rogosa and Sharpe (MRS) agar with anaerobic incubation at 30°C for 6 days for lactic acid bacteria. Ukuku and Fett (2002) enumerated microbes on cut melons (20 g), using plate count agar (PCA) and incubation at 30°C for 3 days for mesophilic aerobic bacteria; PCA + crystal violet at 30°C for 3 days for Gram-negative bacteria; *Pseudomonas* isolation agar and incubation at 27°C for 3 days for pseudomonads; MRS agar with 0.08% sorbic acid and incubation at 30°C for 3 days for lactic acid bacteria; and Czapek malt agar (CMA) for yeasts and molds. Allende et al. (2002) enumerated microbes on mixed vegetable salads (30 g) by plating homogenates in peptone saline on:

PCA and incubating at 22°C for 3 days for total psychrotrophic bacteria; MRS agar and incubating at 30°C for 3 days for lactic acid bacteria; yeast glucose chloramphenicol agar and incubating at 30°C for 3 days for yeasts; violet red bile glucose agar and incubating at 37°C for 2 days for Enterobacteriaceae. Kakiomenow, Tassou, and Nychas (1996) enumerated pseudomonads on shredded carrots (25 g) by plating homogenates in sterile ¼ strength Ringer's solution on cetrimide–fucidin–cephaloridine agar and incubating at 25°C for 48 h. Garg et al. (1990) homogenized various fresh-cut vegetables (20 g) with sterile water and enumerated mesophilic bacteria on PCA held at 30°C for 2 days, psychrotrophic bacteria held at 3.3°C for 10 days, and molds and yeasts on potato dextrose agar adjusted to pH 3.5 with tartaric acid held at 20°C for 5 days. Gimenez et al. (2003) macerated artichokes with 0.1% peptone plus 0.5% sodium chloride and enumerated mesophilic aerobic bacteria on PCA held at 30°C for 3 days and mesophilic anaerobic bacteria using the same methods but under anaerobic conditions, and psychrotrophic bacteria on PCA held at 7°C for 10 days. Liao and Wells (1987) enumerated pectolytic bacterial populations on crystal violet pectate (CVP) agar. In the fresh-cut produce industry, 3 M Petrifilm methods are widely used to enumerate total plate counts, coliforms, lactic acid bacteria, and yeasts and molds because of convenience and minimal need for incubator and operating space. Specificities and detailed operating procedures of many of these methods are described by Downes and Ito (2001) and Sapers et al. (2005).

Microbiological Spoilage of Fermented and Acidified Vegetable Products

Introduction

Spoilage of fermented and acidified vegetable products is prevented by low pH (typically between pH 3 and 4) and the presence of organic acids, including lactic and acetic acids. Several excellent reviews of microbial metabolic end products of lactic acid bacteria (LAB) from vegetable fermentations have been published (Adams & Nicolaides, 1997; DeVuyst & Vandamme, 1994; Holzapfel, Geisen, & Shilling, 1995). Fermented vegetables are considered in the United States to be acid foods, having a pH below 4.6. Examples include cucumber pickles and sauerkraut. Acidified vegetables are foods to which acids or acidic food ingredients have been added to lower the pH below 4.6 (CFR, 1979). The acidified foods regulation was promulgated to prevent botulism in improperly acidified foods. Research had shown that *Clostridium botulinum* cannot grow and produce toxin at or below pH 4.6 (Ito et al., 1968). The market for acid and acidified vegetable products is currently dominated by acidified, not fermented, cucumber pickles (Fleming, Kyung, & Breidt, 1995). The largest sales of fermented products in the US market are hamburger dill chip pickles. Other fermented and acidified vegetable products include olives, sauerkraut, pickled peppers, and assorted pickled vegetables. Acidified cucumber

pickles are typically produced with a pH value of ca. 3.7 and have acetic acid as the primary acidulent. These pickles are typically heated (pasteurized) or refrigerated to prevent spoilage. Other acidified products, including pickled peppers, can be preserved primarily by acetic acid if the pH values are below 3.3 and acid concentrations are above 2.5%. These products may be shelf stable without pasteurization.

Organic acids have widespread application for preventing food spoilage in fermented and acidified foods (Brul & Coote, 1999; Shelef, 1994; Sofos, 1993). The bactericidal activity of weak organic acids is presumably due to acidification of the cell cytoplasm, facilitated by the diffusion of uncharged protonated acid across bacterial membranes, as well as the accumulation of acid anions in the interior of the cell. The acid anion, which does not readily cross bacterial membranes, may accumulate to molar concentrations in bacterial cells, depending on the difference between intracellular and extracellular pH (Diez-Gonzalez & Russell, 1997a, 1997b). The type, concentration, and pH of organic acid solutions help to determine the effectiveness of acids in preventing spoilage. Types of organic acids added to foods include salts of benzoic acid and sorbic acid, as well as acetic, lactic, and propionic acids. A given acid concentration and pH may independently affect the growth and death of bacterial cells, or these factors may interact (Passos, Ollis, Fleming, Hassan, & Felder, 1993). Reported pH values for the inhibition of growth of *E. coli* O157:H7 varied by 1.0 pH unit, depending on whether acetic acid (minimum inhibitory concentration [MIC] = pH 5.5) or HCl (MIC = pH 4.5) was used to lower pH (McKellar & Knight, 1999). In addition, the effectiveness of weak organic acids in inhibiting the growth of bacteria may be modulated by factors other than pH. Temperature is a primary factor influencing antimicrobial activity, with increasing temperature resulting in increasing effectiveness (Breidt, Hayes, & McFeeters, 2004; Brudzinski & Harrison, 1998; Hsin-Yi & Chou, 2001; Presser, Ross, & Ratkowshy, 1998; Uljas & Ingham, 1998).

An important quality factor for cucumber pickles is a solid and crisp texture for the cucumbers. Gas pockets that can form within cucumbers during fermentation result in bloating of cucumbers, a form of spoilage making them undesirable for commercial sale. Yeasts and heterofermentative lactic acid bacteria are largely the source of this gas (Bell & Etchells, 1956; Etchells, Borg, & Bell, 1968). In addition, homofermentative lactics have been implicated in this problem (McFeeters, Fleming, & Daeschel, 1984). *Lactobacillus plantarum* and other lactic acid bacteria decarboxylate malic acid (present in cucumbers), thereby producing lactic acid and carbon dioxide via a malolactic enzyme. This one-step reaction results in the net loss of one proton from solution, so it raises the pH, possibly conferring a selective advantage for microorganisms in acidic environments. To prevent bloater damage to cucumbers from internal gas pockets of carbon dioxide, a gas purging technology was developed in the 1970s (Costilow, Bedford, Mingus, & Black, 1977; Fleming, Etchells, Thompson, & Bell, 1975; Fleming, Thompson, Etchells, Kelling, & Bell, 1973). Prior to this development, the formation of hollow centers or bloated cucumbers was a significant economic problem for the cucumber fermentation industry.

Microbiological and Nonmicrobial Spoilage

Spoilage of fermented or acidified vegetables due to softening can result from enzymatic or nonenzymatic activities. The softening of cucumber pickles has been extensively studied. Cucumbers naturally contain polygalacturonases which are involved in the natural maturation process of the fruit (Saltveit & McFeeters, 1980). Another source of polygalacturonase enzyme is fungi associated with the cucumber fruit flowers (particularly on small fruit), which can cause softening problems (Bell, Etchells, & Costilow, 1958; Etchells, Bell, Monroe, Masley, & Demain, 1958). However, this problem can be minimized by simply removing the flowers prior to brining. Storing fermented cucumbers in high concentrations of NaCl (greater than 10%) can reduce polygalacturonidase activity to prevent softening (Etchells, Borg, & Bell, 1961; McFeeters & Fleming, 1989), but this can lead to problems with salt waste disposal. Nonenzymatic softening can also occur with cucumber pickles (McFeeters & Fleming, 1989, 1990). The use of low concentrations (less than 0.5%) of calcium chloride can help retain firmness of brined vegetables (Doesburg, 1965; Fleming, McFeeters, & Thompson, 1987; Van Buren, 1986). Calcium is widely used in acidified and fermented vegetable products to prevent softening, although the mechanism of how calcium works to retain firmness remains unclear (McFeeters & Fleming, 1990).

The growth of film yeasts on the surface of cucumber brine fermentation tanks can also be a source of enzymatic softening. Commercial cucumber fermentation tanks (fiberglass tanks, ca. 40,000 L or 10,000 gallons) are usually situated in outdoor "brine yards," so that the brine surface is exposed to the UV rays of the sun, hence preventing yeast growth. The cucumbers are held below the brine surface by wooden headboards. While some yeasts may produce polygalacturonidase (Bell & Etchells, 1956), softening may also result from yeasts consuming lactic acid, resulting in an increase of pH. This can result in the growth of other microorganisms, including propionibacteria and clostridial species (Fleming, Daeschel, McFeeters, & Pierson, 1989) which produce degradative enzymes. In this secondary fermentation, lactic acid is converted anaerobically to propionic and butyric acids with a concomitant increase in pH, although the details of the microbial ecology and biochemical changes occurring remain to be elucidated. This type of secondary fermentation can also occur if the salt concentration in the fermentation brine is too low. Similar spoilage fermentations are responsible for malodorous butyric acid production in olive fermentation. Known as "zapatera" spoilage, this can occur when the pH of the fermentation remains about ca. 4.5, possibly due to residual sodium hydroxide from the initial lye treatment used for olive fermentation (Delmouzos, Stadtman, & Vaughn, 1953; Fernandez, Garcia, & Balbuena, 1995; Plastourgos & Vaughn, 1957).

Fermented cabbage, including sauerkraut and Korean kimchi, is also subject to spoilage due to softening. Yeasts, propionibacteria, and clostridial species may be involved. Sauerkraut is typically fermented in the United States in indoor cement fermentation tanks holding up to 90 tons of cabbage. The cabbage is spread to form a concave surface which is covered by a plastic film that is flooded with water to

maintain anaerobic conditions under the film. If the temperature (ca. 18°C) and salt concentration (equilibrated around ca. 2%) are correct, and anaerobic conditions are maintained, high-quality sauerkraut is produced by a natural lactic acid bacteria fermentation (Pederson & Albury, 1969). Softening problems can occur with commercial sauerkraut when the dry salting process used for preparing the fermentation tanks is at too low a salt concentration or the sale is unevenly distributed. Exposure of the fermenting cabbage to air can allow the growth of a variety of spoilage microorganisms, including molds. Formation of pink color in the sauerkraut is also a frequent spoilage problem. The pink color has been studied since the 1920s and is attributed to yeasts, including *Rhodotorula* species (Fred & Peterson, 1922; Pederson & Kelly, 1938), and possibly lactic acid bacteria (Stamer, Hrazdina, & Stoyla, 1973).

A spoilage problem for both sauerkraut and kimchi is an excess accumulation of lactic acid. Cabbage fermentations, including both sauerkraut and kimchi, typically occur in two stages: an initial heterolactic fermentation, dominated by *L. mesenteroides* and related species, as well as *Weissella* species (Plengvidhya, 2003), followed by a homolactic phase dominated by *Lactobacillus* species, typically *L. plantarum* (Fleming et al., 1995; Pederson & Albury, 1969). A homolactic phase is needed to produce good-quality sauerkraut; however, kimchi is commonly preferred as a lightly fermented, somewhat carbonated, product resulting from the heterolactic phase of fermentation. Accumulation of excess lactic acid is considered a spoilage problem with both sauerkraut and kimchi fermentations, although some people prefer a more acidic product. Unlike cucumbers which typically contain ca. 2% sugar, commercial cabbage used for sauerkraut may have 5% or more sugar (Fleming et al., 1995), primarily glucose and fructose, and the homolactic stage of fermentation may continue for an extended period, resulting in acid concentrations of 3% or greater.

Prevention and Control of Spoilage

The spoilage microflora for acidified and fermented vegetable products may consist of yeasts, molds, and lactic acid bacteria. Yeasts and molds that can tolerate low water activity are the primary spoilage agents of fermented products, including sweet pickles (Bell & Etchells, 1952; Etchells, 1950). A preservation prediction chart having the combined concentrations of acetic acid (1–4%) and sugar (10–50%) needed to preserve cucumber pickles is used to prevent spoilage of sweet pickles (Bell & Etchells, 1952). In addition, sorbic acid can be added to inhibit yeasts (Etchells et al., 1961). Most acidified vegetable products are pasteurized to prevent growth of acid-tolerant microorganisms. Acidified cucumber products by definition are not fermented, and acid-tolerant lactic acid bacteria may grow by fermenting the sugars naturally present. Recommended pasteurization treatments to prevent spoilage and ensure safety have been determined (Breidt, Hayes, Osborne, & McFeeters, 2005; Etchells & Jones, 1942). The temperatures and times typically used in commercial pasteurization processes today (5–15 min at 70–80°C) kill the

lactic acid bacteria and yeasts. However, if the necessary time–temperature conditions are not obtained, growth of lactic acid bacteria may occur resulting in turbidity, gas production, pressure formation, and leakage of brine from jars. One particularly hazardous type of spoilage that can occur is due to improper sealing of jars or containers, thereby allowing entry of oxygen. This can result in the growth of oxidative molds that can aerobically metabolize lactic acid or acetic acid. As a result, the pH can increase above 4.6, and if aerobic conditions are maintained in the bottom of the container, clostridial spores may germinate, potentially leading to the production of botulinum toxin.

Future Needs

There are several needs for further investigation to improve our overall understanding of microbiological spoilage of produce. Some examples are given here:

1. Identification of the specific spoilage microorganisms for different types of pre- and fresh-cut products stored under unique packaging conditions. Currently, mesophilic aerobic bacteria are widely used as indicators of both general quality and microbiological quality of fresh-cut produce products. This is insufficient for knowing if pathogen contamination occurs or for predicting sensory quality changes. Additionally, storage temperature and packaging methods have a significant impact on microbiological spoilage patterns. There have been many studies on microbial spoilage patterns on specific types of fresh-cut produce packaged in specific types of packaging films and stored at a specific refrigerated temperature, such as fresh-cut carrots (Nguyen-the and Carlin, 1994) held at 10°C and fresh-cut celery (Robbs et al., 1996a, 1996b) held at 5°C. However, there are few research reports on spoilage patterns of fresh-cut produce products stored under the MAP conditions and at different temperatures such as 2 vs. 10°C. More information on the relationship of microbial spoilage to product preservation will be valuable, especially considering the direction being taken to improve cold chain management in retail and foodservice environments and distribution that can lead to extending the shelf life of products.
2. There is a pressing need to develop simple and rapid assays to measure specific spoilage microorganisms and to better establish relationships between spoilage microbial populations and spoilage of whole and fresh-cut produce products, especially fresh-cut fruits. For example, current methods to enumerate psychrotrophic bacteria require 3–10 days to complete which encompasses the entire shelf life of most whole and fresh-cut products. The shelf life of most whole and fresh-cut products is approximately 8–14 days. Hence, it is difficult to use existing methods as a basis for making corrective actions in the processing facility not only at the time of production but also to remove contaminated material at retail. In addition, quality limit specifications need to be better defined for fresh processed and end-of-shelf life products relative to spoilage microorganisms.

3. There is a need for investigation of spoilage patterns and microflora of fresh-cut products packaged with new, emerging MAP technologies, including antimicrobial packaging, microperforated packaging, Intelimer® packaging, and high O_2 backflush, in commercial practice. With the continuing development of packaging technologies and changes in marketing fresh and fresh-cut produce, the spoilage microflora of packaged produce in the future may be completely different from today. For example, the headspace profile (ratio of CO_2/O_2 at equilibrium) of fresh-cut produce in microperforated packages is significantly different from that packaged with conventional films. There is very limited information regarding how changes in atmospheric composition affect spoilage microflora profile during refrigerated storage.
4. There is a need for a better understanding of the plant defense systems of fresh-cut produce and their role in controlling the microflora and spoilage patterns under refrigerated conditions. Plant defense responses appear to influence the spoilage pattern of fresh-cut produce. For example, the mesophilic aerobic bacterial population of cut pineapple treated with methyl jasmonate (both by vapor and dipping) decreased by 3 log CFU/g after 12 days of storage (Martinez-Ferrer and Harper, 2005). A purified ethanolic extract of peeled and shredded carrots had an antimicrobial effect against a wide range of microorganisms, including *L. mesenteroides, P. fluorescens, Candida lambica,* and *E. coli*. C_6-aldehyde, a common volatile product produced by plant tissue through enzymatic lipid peroxidation, inhibited spoilage microbes on fresh-cut produce and has been hypothesized as a factor in the plant defense mechanism (Zhuang, Barth, & Hildebrand, 2002). There are few studies addressing the understanding of plant defense systems after wounding fruit or vegetable tissue under refrigerated conditions and their impact on microbial spoilage of different commodities and during a variety of seasons.

Temperature is one of the most impactful factors affecting the quality and microbiological characteristics of produce, hence there is need to further investigate the effect of breaks in the cold chain on microbial flora and shelf life of both intact and fresh-cut produce, especially fresh-cut fruits.

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Microbiological Spoilage of Canned Foods

George M. Evancho, Suzanne Tortorelli, and Virginia N. Scott

Introduction

Nicolas Appert (1749–1841) developed the first commercial process that kept foods from spoiling in response to an offer from the French government for a method of preserving food for use by the army and navy. Appert, a confectioner and chef, began to experiment in his workshop in Massy, near Paris, but since little was known about bacteriology and the causes of spoilage (Louis Pasteur had yet to formulate the germ theory), much of his work involved trial and error. In 1810, after years of experimenting, he was awarded the prize of 12,000 francs for his method of preservation, which involved cooking foods in sealed jars at high temperatures. He described his method of preserving food in a book published in 1811, "*L'Art De Conserver, Pendant Plusiers Annes, Toutes les Substances Animales et Végétales*," which translated means "*The Art of Preserving All Kinds of Animal and Vegetable Substances for Several Years*." He later built a bottling factory and began to produce preserved foods for the people of France and is credited with being the "Father of Canning."

Appert's developments in preservation led to the creation of the canning industry. The science of food preservation using thermal processing has advanced considerably since Appert first applied heat to food in a sealed jar, and much is known today about heating times and temperatures required to destroy microorganisms of public health significance and other microorganisms capable of growing in canned food at normal non-refrigerated temperatures.

Any discussion of the microbiological spoilage of canned foods requires a definition of "canning." For the purposes of this chapter, canning will be defined as a method of food preservation wherein a food is rendered commercially sterile by the application of heat, alone or in combination with pH and/or water activity or other chemicals. In traditional canned food processing, the heat process is

G.M. Evancho (✉)
19693 Marimar Court, Lewes, DE 19958-3500, USA
e-mail: george_evancho@verizon.net

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applied to the product in a hermetically sealed container. Commercially sterile, aseptically processed and packaged foods are also considered canned foods even though the methods used to produce these foods differ considerably from conventional canning (product and package are sterilized separately, then combined in a sterile environment). Commercial sterility is defined in the US *Code of Federal Regulations* Title 21 Part 113 as the condition achieved by (1) the application of heat which renders the food free of (a) microorganisms capable of reproducing in the food under normal non-refrigerated conditions of storage and distribution; and (b) viable microorganisms (including spores) of public health significance; or (2) the control of water activity and the application of heat, which renders the food free of microorganisms capable of reproducing in the food under normal non-refrigerated conditions of storage and distribution. The sterility of the food is maintained by the hermetic seal. Canned foods, then, are defined as thermally processed shelf-stable foods in hermetically sealed containers, regardless of the type of container (metal, glass, plastic, paper, etc.) or how the thermal process was delivered.

Federal regulations govern the production of commercially canned foods in the United States. United States Department of Agriculture – Food Safety and Inspection Service (USDA-FSIS) regulations that govern thermally processed meat and poultry products packaged in hermetically sealed containers are set out in Title 9 of the *Code of Federal Regulations* Parts 318.300 and 381.300. Food and Drug Administration (FDA) regulations that govern thermally processed foods other than meat and poultry packaged in hermetically sealed containers are contained in Title 21 of the *Code of Federal Regulations* Parts 108, 113, and 114.

The principles by which thermal processes are established are well documented elsewhere (see Appendix 1) and will not be discussed in detail in this chapter; however, the principal steps involved in establishing a thermal process are outlined in Table 1. In establishing a thermal process, the organism of greatest public health significance targeted for destruction is *Clostridium botulinum*. Studies have shown that the critical pH that determines whether or not this organism will grow in a food is pH 4.8 (Hauschild, Aris, & Hilshimer, 1975); below this pH growth of the organism is inhibited in foods (studies in laboratory media where conditions are critically controlled have demonstrated growth at lower pH values). A maximum pH of 4.6 has been established in regulations to incorporate a safety factor for acid and acidified foods (Codex, 1993). Therefore, thermal processes for products with a pH of 4.6 or below are not required to destroy the spores of *C. botulinum*.

Food products can be classified according to their acidity into low-acid, acid, high-acid, and acidified foods. Low-acid foods are any foods (other than alcoholic beverages) with a finished equilibrium pH greater than 4.6 and a water activity (a_w) greater than 0.85. Low-acid foods are foods that will support the growth and toxin production by *C. botulinum*, and therefore require a thermal process to inactivate spores of this organism. Foods with natural pH values of 4.6–3.7 are classified as acid foods. High-acid foods have pH values below 3.7. US federal regulations define acidified foods as those low-acid foods that have had their pH reduced to 4.6 or lower by the addition of acids or acid foods. Acid foods, high-acid foods, and acidified

Table 1 Principal steps involved in establishing a thermal process for a commercially sterile canned food

Step	Accomplished by
1. Determine heat resistance of target microorganism (*D* and *z* values)	Conducting thermal death time studies in the product or using *D* and *z* values reported in the literature
2. Obtain product heating data (heat penetration) for the specific formula and container (where applicable) and establish critical factors which affect heat penetration and delivery of the calculated process	Placing thermocouples at various points in product-filled containers and measuring how the product formulation heats (e.g., locate slowest heating point) in a processing simulator. Conduct studies to establish impact of factors such as initial temperature, fill weight, viscosity, container headspace. Knowledge of factory processing capabilities is essential
3. Calculate the thermal process	Performing mathematical calculations (e.g., using commercially available software) using the thermal resistance of the target microorganism and the data obtained from heat penetration tests
4. Inoculated pack – biological validation of the calculated process (optional: not always conducted since it involves use of viable microorganisms)	Inoculating product with a specific number of spores or cells of the target organism and processing containers for several time/temperature combinations (the calculated process as well as conditions of lower lethality where spoilage would be expected) followed by incubation of all containers and examination for spoilage
5. Incubation pack (optional)	Producing a predetermined number of containers on a commercial production line, usually over several days, to demonstrate that the product can be routinely produced without issue

foods do not require thermal processes to inactivate spores of *C. botulinum*, because these products will not support the growth and toxin production by this organism.

The severity of a thermal process designed to deliver commercial sterility (the scheduled process, defined as the process selected by the processor as adequate under the conditions of manufacture for a given product to achieve commercial sterility) is largely influenced by product pH. Because *C. botulinum* can grow in low-acid foods, thermal processes for this class of products must be designed to destroy a given number of *C. botulinum* spores (established at 10^{12} and called a 12*D* process, where the *D* value is the time in minutes to inactivate 90% or 1–log of cells of a microorganism). Based on the thermal resistance of *C. botulinum* spores, processes for these products typically have a minimum lethality (F_0) of 3 min or the equivalent. F_0 is the time in minutes at a reference temperature of 121.1°C, or its equivalent calculated with a *z*-value (the number of degrees for a microbial thermal death curve to change by one log) of 10°C to destroy spores of a particular microorganism. The process to achieve commercial sterility of low-acid foods is generally an $F_0 \geq 5$ to inactivate sporeformers that could spoil the product. Since pH values of 4.6 or lower inhibit the growth of *C. botulinum*, thermal processes for products with a finished equilibrium pH of 4.6 or lower are not designed to destroy spores of

C. botulinum. Consequently, the lethality of thermal processes for products with pH values below 4.6 are often expressed at a reference temperature of 93.3°C, rather than 121.1°C as with low-acid foods. Products with a pH below 4.6 therefore may contain viable spores of *C. botulinum*, but these are of no public health significance, because the pH of the product will inhibit the growth and toxin production by this organism.

If the water activity (a_w) of a food is adjusted to 0.85 or less, a thermal process is not required by US regulations, although one may be applied to some products to inactivate yeasts and molds capable of growing in the product. Studies have shown that most strains of *C. botulinum* will not grow below an a_w of 0.93 (ICMSF, 1996). For products with an a_w between 0.93 and 0.85, a pasteurization process is necessary to inactivate vegetative cells of pathogens such as *Salmonella* and data must be available to show the conditions result in commercial sterility. Pasteurization has been defined by the National Advisory Committee on Microbiological Criteria for Foods as "any process, treatment, or combination thereof, that is applied to food to reduce the most resistant microorganism(s) of public health significance to a level that is not likely to present a public health risk under normal conditions of distribution and storage" (NACMCF, 2006). Pasteurization processes must usually be combined with other factors such as reduced pH or a_w in order to produce a commercially sterile product. It is important to recognize that there are commercially sterile products in distribution that may contain viable spores of *C. botulinum*, but growth and toxin production are inhibited either by the pH or a_w of the product.

Once a thermal process is established for a food product, the delivery of that process must be carefully controlled to ensure the safety of the food. An improperly delivered thermal process can result in spoilage, either of economic or public health significance. Likewise, loss of the hermetic seal can result in spoilage of commercially sterile product. Table 2 lists factors that could contribute to the cause of spoilage.

Types of Spoilage

Most microorganisms produce gas when they grow. If they grow in a canned food, the gas produced generally cannot escape and causes the container to swell, providing visible evidence that the product inside might be spoiled. The appearance of the container can range from nearly flat (a loss of vacuum where a container end no longer is concave) to highly swollen (where one or both ends are severely distended). Metal cans may bulge to a point where the container may be buckled, or, if sufficient gas is produced, they may explode from excessive internal pressure. Flexible or non-rigid containers will appear "ballooned." However, not all microorganisms produce gas. If a non-gas-producing microorganism spoils a canned food, there is no visible means of identifying the spoiled container. The only means of identifying spoilage in normal-appearing containers is by destructive

Table 2 Factors that may contribute to the cause of spoilage of commercially sterile canned foods

Manufacturing-related factors	Product was excessively held prior to delivery of the thermal process
	The scheduled process was not delivered because of mechanical or personnel failure
	The formula and/or procedure were not followed or there was an inappropriate ingredient substitution which negatively affected the delivery of the process
	Processed product not properly cooled or product held at elevated temperature
Process-related factors	Excessive heat resistance of contaminating spores
	Excessive number of bacterial spores contributed by product ingredients
	Inadequate thermal process due to process being improperly established
	One or more critical factors not met, e.g., product pH, viscosity, solids/liquid ratio, size/shape of solids, clumping of particles, initial temperature, and container rotation
Container-related factors	Improperly or inadequately formed seals/seams
	Defective containers or closures
	Rough handling (excessive container abuse)
	High microbial counts in cooling water
	Inadequate disinfection of cooling water
	Dirty can lines and handling equipment
	Container corrosion
	Food debris in the container seam/seal

analysis, where the container is opened and the product examined in a number of ways to determine if microbial growth has occurred. Spoilage may be detected by appearance or odor, by a shift in pH, by the appearance of large numbers of microorganisms in a product smear, or by isolating viable microorganisms in subculture. Some products might appear to be broken down and mushy, or normally clear broth or syrup may appear cloudy. In jars or transparent plastic containers, a white deposit may sometimes be seen.

All spoilage has an economic impact, but not all spoilage is of public health significance. Because sophisticated procedures are often required to distinguish nonpathogenic from pathogenic spoilage, all spoiled containers should be handled with care and treated as if they contained a pathogen. Every effort should be exercised to prevent spoiled containers from reaching the consumer, and consumers should be advised not to purchase or consume product from a swollen container or consume visibly spoiled product from a container that appears normal.

Spoilage of thermally processed products may be caused by

(1) Growth of microorganisms to high numbers before thermal processing (incipient spoilage)
(2) Growth of microorganisms that gain entrance into a product through faulty or inadequate seals or seams (post-process or leakage spoilage)

(3) Growth of microorganisms that survive the thermal process (inadequate thermal processing)
(4) Growth of thermophilic microorganisms due to storage at high temperatures
(5) Growth of acid-tolerant sporeforming microorganisms in products with a pH equal to or less than 4.6
(6) Non-microbial causes

Incipient Spoilage (Spoilage Before Processing)

If food is held too long during blending, or between filling and closing the containers and the delivery of the thermal process, the microorganisms normally present in the ingredients may have sufficient time to replicate and spoil the product. This type of spoilage is referred to as "incipient spoilage." The microorganisms that grow will be subsequently killed by the thermal process; however, if sufficient growth occurred to alter the product characteristics, the product would be considered spoiled. Typically, if product was held too long between container closing and processing, spoilage may manifest itself as low or no vacuum in the container and/or a change in product characteristics (e.g., a reduction in pH, loss of viscosity, or development of an off-odor or flavor in the product). Generally, no viable microorganisms are recovered in subculture media, although large numbers of cells may be visible in a product smear. Although the product typically presents no risk to public health, if there is sufficient growth (e.g., if the product characteristics have changed), the product may be considered adulterated.

The degree of incipient spoilage depends on the specific product characteristics, the type and number of microorganisms present, and the time and temperature conditions during the delay. For example, if the product has been heat treated (e.g., cooked) prior to excessive holding, there may be low levels of bacteria present such that holding product for several hours may result in bacterial increases that are not significant. Products that contain inhibitors, those that are held at low temperatures (e.g., below 21°C), or those that are held hot and filled hot (above growth temperatures for most microorganisms) may also demonstrate only limited bacterial growth over several hours.

If sealed containers are held too long prior to processing, there may be a loss of vacuum or increase in internal pressure in the container. The increase in internal pressure may disrupt the container seams or seals during processing and increase the potential for post-processing or leakage spoilage. Occasionally, if pressures are high enough, some containers may actually buckle or rupture during processing. There have been instances with batch retort processes where containers removed from the retort were buckled because product sat too long between closing and retorting. Delays between blending, filling, and retorting should be avoided or minimized in order to prevent incipient spoilage.

Any delay encountered during production that results in product being held longer than normal prior to processing should be viewed with caution, and samples should be randomly taken from affected product and tested for pH and sensory

properties, looking for perceptible changes. Any reduction in pH (generally 0.2 pH units or greater) or change in sensory properties should be considered unacceptable and the involved product should be removed from production and appropriately destroyed. Product smears may also be examined microscopically, but these are often difficult to interpret. Unless these smears can be compared to a product smear taken before the product was excessively held, it may be difficult to distinguish between the numbers of microorganisms normally present from the ingredients and those present as a result of multiplication during excessive holding.

During blending, it is advisable to limit the number and size of batches or units that are prepared so that any interruption in the operation will not put a large volume of product at risk of spoilage. In aseptic systems, the volume of product in the product sterilizer supply tank should be limited. Production procedures should limit the length of time (typically less than 4 hours) product can be held at bacterial growth temperatures, and if this time is exceeded, product samples should be evaluated prior to processing the product.

The spoilage pattern is influenced by the manufacturing process and the particular event(s) that led to incipient spoilage. Spoilage can involve an entire production batch or be limited to a specific time code within the batch. If incipient spoilage is suspected in a production lot, the entire lot should be placed on hold until the spoilage pattern and the cause can be determined. Production records should be examined and product samples should be taken throughout the lot and examined for signs of spoilage. Sometimes it is possible to segregate normal (good) product from spoiled product based upon the production records and spoilage pattern, thereby salvaging some of the lot.

Extreme caution should be exercised, however, in releasing production lots in which incipient spoilage has been identified. If incipient spoilage occurred as a result of cans of product sitting for an excessive length of time prior to processing, it may be possible to remove those containers from the lot and release the remainder of the lot for sale. However, if product was held for an excessive time in a blending kettle and then mixed with normal product in a filler supply tank, it might be more difficult to distinguish spoiled from non-spoiled product. In an aseptic system, where product is often held in large sterilizer supply tanks, sterilized, and then held in an aseptic surge tank prior to the filler, it may be difficult to isolate incipiently spoiled product from normal product because of the large amount of admixing. Unless production practices, production records, and results from finished product analysis can clearly segregate normal from spoiled product, the entire production lot should be destroyed.

As noted before, most incipient spoilage will be of non-public health significance because the microorganisms that grew and spoiled the product will be destroyed during thermal processing. Some microorganisms also produce toxins, and again most of these would be destroyed during the thermal processing. Staphylococcal entertoxin, however, is extremely heat resistant, and may not be inactivated during thermal processing. If large numbers of cocci are observed in a product smear, or the conditions under which the product was held prior to processing would allow for the growth of *Staphylococcus aureus*, great care should be exercised

in determining disposition of the affected production lot. Several spoilage incidents have occurred where *S. aureus* grew in a product before the thermal process was applied, and although the products were rendered commercially sterile, people who consumed the products became ill from staphylococcal enterotoxin food poisoning.

Post-process Contamination (Leakage Spoilage)

Spoilage of shelf-stable thermally processed foods can occur whenever there is an opportunity for microorganisms to enter the container. Thus, maintaining the hermetic seal is essential to preventing spoilage due to post-process contamination (leakage spoilage). Spoilage due to recontamination after thermal processing is probably the most common type of spoilage of canned foods.

Causes of Post-process Contamination

Leakage is generally due to inadequately formed seams, defective containers or closures, cooling water contaminated with large numbers of microorganisms, container damage, rough handling of processed containers, dirty can-handling equipment, or a combination of these. Post-process contamination most commonly results from improperly formed seams, and it can be expected that there may be an occasional spoiled container in a lot simply due to a defective can that is undetected prior to filling and processing. Can manufacturers check the integrity of the "manufacturer's end seam" on three-piece cans, as well as the integrity of two-piece cans, and reject defective containers from lots sent to producers. More extensive spoilage often results when seams or seals are improperly formed during food manufacturing. These defects may be large enough that product leaks out of the container, but in most instances they are small enough and infrequent enough that they may not be detected at the time of production, even with seam teardowns or other seal evaluations that are routinely conducted. In canned foods, the seam (known as a double seam) is a five-layer construction formed from the body of the can and the end (cover or lid) – three thicknesses of the can end and two thicknesses of the can body, with sealing compound distributed between the layers of the folded metal. Many things can go wrong in forming the seams, such as defects in the flange on the body of the can or on the end curl; improperly adjusted seaming operations that result in loose or tight seams, fractured seams, or false seams; improperly distributed seaming compound; and other structural defects or seam abnormalities (Fig. 1). Glass containers may have defective seals due to chips in the sealing surface, gasket defects, or improper application of the cap. Heat seals on semi-rigid and flexible containers may be inadequately fused. Many of these defects, which are described more completely in *Canned foods –principles of thermal process*

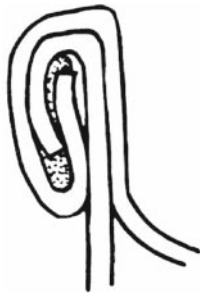
	Diagram	Photograph
Normal Can Seam		
Loose Can Seam		
False Can Seam		

Fig. 1 Double seam construction showing the appearance of normal and defective seams

control, acidification and container closure evaluation (Weddig, 2007) can result in a lack of a hermetic seal, allowing for recontamination with microorganisms.

Whether recontamination occurs depends on the severity of the defect and, in part, on the environment to which the container with the defect is exposed. Recontamination is less likely when the environment is dry. However, almost all canned foods where the thermal process is applied to the hermetically sealed container, are cooled after processing using water. The number of microorganisms in the cooling water will be important in determining the amount of contamination that occurs as a result of container defects. Leakage may also occur in containers with normal seams if the cooling water contains high levels of microorganisms, because as containers cool, they draw a vacuum. Minute amounts of water may be drawn into the container before the seaming compound sets. These minute amounts

Table 3 Botulism from post-process contamination of commercially canned foods[a]

Year	No. of deaths/no. of ill	Country (outbreak/product origin)	Product	Cause of contamination
1982	1/2	Belgium/USA	Salmon	Malfunctioning can reformer
1978	2/4	England/USA	Salmon	Damage to can
1963	2/3	USA/USA	Tuna	Defective seam
1941	1/3	USA/USA	Mushroom sauce	Suspected leakage
1934	1/3	USA/Germany	Sprats	Suspected leakage

[a] Based on Stersky et al. (1980) and NFPA/CMI Container Integrity Task Force (1984).

of water are unlikely to contain a microorganism that can result in spoilage if cooling water is properly maintained. It is generally accepted that cooling water should ideally contain <100 organisms/ml (Graves, Lesniewski, & Lake, 1977; Put, Van Doren, Warner and Kruiswijk, 1972).

For economic reasons, cooling water is generally recirculated or reused. Any containers damaged during the retorting process can leak and contaminate the cooling water with food debris. This debris can reduce the effectiveness of the chemical disinfectant (e.g., chlorine) and can provide sufficient nutrients to support bacterial growth. Such cooling water can lead to increased rates of spoilage in subsequent production lots.

Containers may be damaged either prior or subsequent to processing. Depending on the severity of the damage, the container may or may not be detected and removed from the line before casing. For example, damage to can flanges may not be noticed unless the lot of cans has been extensively damaged. Two incidents of botulism illustrate the importance of preventing can damage. An outbreak of botulism in Birmingham, England in 1978 (Table 3) was due to a single can that developed a corrosion pinhole on the rim of the double seam after the container was gouged by some undetermined means. In examining over a million cans from the 1977 pack, 8,068 cans with defects (0.05%) including false seams, droops, cable burns, cutovers, dents, and swells were detected. Of 296 cans that were subcultured, only four contained viable microorganisms, none of which were *C. botulinum* (NFPA/CMI Container Integrity Task Force, 1984). An outbreak of botulism in Belgium in 1982 (Table 3) was the result of recontamination through a triangular-shaped opening in the can body at the double seam caused by a malfunctioning can reforming machine (NFPA/CMI Container Integrity Task Force, 1984). (For economic reasons can bodies were shipped to Alaska as flattened cylinders, then reformed, flanged, and the end attached.) Although other containers with the defect were found, none contained toxic product (NFPA/CMI Container Integrity Task Force, 1984).

Rough handling of containers after processing and unsanitary container handling equipment can result in recontamination. Containers contacting wet, dirty roll tracks or conveyors, or those receiving rough treatment (such as bumping each other) may become contaminated through dirty equipment. Again, the seaming

compound may not have had a chance to set and form the hermetic seal at this point. Rough handling sufficient to result in post-process contamination may not be evidenced by visible dents; conversely, visible dents do not necessarily result in leakage (Denny & Parkinson, 2001). Studies by the National Food Processors Association (now the Grocery Manufacturers Association [GMA]) and others have shown that bacterial contamination is transferred in varying degrees to the can double seam areas as the cans pass through the can handling system. In general, counts increase as cans move down the processing line and the increase is greater if water is unchlorinated. Put, Van Doren, Warner, and Kruiswijk (1972) found in a plant using chlorinated, non-circulating well water with acceptable microbial counts ($<10^2$/ml) that low counts in the double seam area were only maintained when the surface of all wet can handling equipment that contacted the can seams was cleaned and disinfected every 2–3 hours. Probably every processor, at one time or another, has experienced leakage type spoilage due to improperly cleaned and sanitized lines coupled with rough handling of containers while seams were wet.

One additional source of post-process contamination is the presence of food in the container seam or seal that results in a path through the seam/seal that acts as a "wick" to draw contaminants in. Microbial growth can occur in the food trapped in the seam and eventually contaminate the product in the container. This type of spoilage can be particularly troubling with aseptic paper brick packaging where the package seal is applied through the product. This is also a common problem when processing leafy greens such as spinach, kale, and turnip greens. Any fibrous material from the product that is trapped in the seal area can provide a mechanism whereby bacteria can enter the package and spoil the product.

Characteristics of Leakage Spoilage

Leakage spoilage usually manifests itself as swollen containers. Swelling occurs when the hermetic seal is breached, allowing a variety of microorganisms from the environment, many of which are gas producers, to enter the container. The defect that allows entry of the microorganisms may be small enough to prevent the gas from escaping, or the defect may become plugged by food particles, preventing the gas from escaping and resulting in the container swelling (Denny & Parkinson, 2001). The product pH may be depressed and there may be obvious sensory changes, including off-odors, decomposed solids, and gassy liquids. Mixed cultures of microorganisms are usually recovered from spoiled product – short and long rods, cocci, cocco-bacilli, spores, and sometimes yeast and mold. In most instances the majority of the organisms are heat sensitive, and they would not have survived the thermal process delivered to the product. Occasionally, spoilage resulting from severe underprocessing or no process (e.g., a retort by-pass) may be confused with leakage spoilage because of the mixed flora observed in product smears. The color change in heat sensitive ink and the appearance of the product can sometimes be used to distinguish this type of spoilage from leakage spoilage.

It must be noted that the type of spoilage will reflect the type of microorganisms in the environment, and in rare instances leakage spoilage may be due to a single

type of microorganism, including sporeformers, which are generally associated with underprocessing. We have experience with several leakage spoilage incidents involving pure cultures of sporeformers. In one incident, makeup cooling water was drawn from concrete settling tanks. The water contained large numbers of spores as a result of growth and sporulation in the sediment at the bottom of the tanks. Normal chlorination levels were inadequate to inactivate the spores, and less-than-perfect seams allowed the spores to enter the containers and spoil the product. In another incident, inadequate chlorine treatment time for the cooling water contributed to spoilage. Chlorine was being injected into the cooling water stream immediately prior to the cooling water entering the cooling shell of the cooker, and seaming compound problems allowed the surviving spores to enter the containers and spoil the product. In both incidents, however, identification of container integrity issues allowed us to rule out underprocessing and establish leakage as the cause of spoilage.

Once post-process spoilage is detected in a production lot, it may take several weeks until all spoilage in the lot has ceased. If there are many swells in the lot, some small percentage of non-swollen spoiled containers (flat sours) may be expected, and consequently, destructive analysis of a percentage of the containers in the lot would be prudent.

Consideration should be given to the type of container defect identified in a production lot and the potential for additional spoilage to occur due to stresses placed on the seams/seals during shipping and distribution. In some circumstances, it may be prudent to conduct a shipping test where pallets of product are placed on a truck and shipped a reasonable distance, then returned to the plant for additional incubation and examination. If additional spoilage is detected in the lot after shipping, it would be wise to withhold the product from distribution.

Public Health Significance

While, theoretically, leakage could result in post-processing contamination with pathogens, it is unlikely in plants operating under good manufacturing practices. Historically, post-process contamination in commercially canned foods has caused almost no significant public health risk to consumers in the United States. Although incidents of foodborne illness associated with post-process contamination have occurred in other countries in the past, it appears to be less common today. A review of incidents of foodborne illness associated with post-process leakage in canned foods between 1921 and 1979 was published by Stersky, Todd, and Pivnick (1980). These included 100 outbreaks due to staphylococcal enterotoxin, 6 from *Salmonella typhi*, 9 from other *Salmonella,* and 3 from *C. botulinum*. The incidents of staphylococcal poisoning resulted from faulty seams (inadequate overlap, crack in double seam at a dent, badly soldered seam, etc.); damaged cans (e.g., can puncture); or contamination through handling by an employee with staphylococcal skin infections. The incidents of typhoid fever resulted from consumption of meat products imported into the UK from Argentina in the 1950s and 1960s that were

contaminated when cans were cooled using river water contaminated with sewage (Stersky et al., 1980).

Table 3 provides information on five outbreaks of botulism, all due to type E toxin, from commercially canned foods due to recontamination. In addition, there was one incident in which *C. botulinum* type C was detected in a single can of tuna fish opened at home by a consumer, in which the can was malformed (turned or cocked body) (CDC, 1974). Based on existing reports of foodborne disease for which package defects are alleged or proven to have contributed to the problem, the probability of post-process contamination with organisms of public health significance is extremely low. In fact, it was estimated in 1984 that the probability of botulism from container leakage is about one chance in every 260 billion cans of food consumed (or one potential botulism incident about every 9 years) (NFPA/CMI Container Integrity Task Force, 1984). The report concluded that this probability compares well to the risk associated with the minimum acceptable thermal process for low-acid canned foods.

A concern that has been raised with post-process spoilage deals with metabiosis, or the ability of one microorganism to change conditions which are unfavorable to another microorganism, thereby allowing the second microorganism to grow. This is a mechanism whereby *C. botulinum* could grow in an acid food, for example (Huhtanen, Naghski, Custer, & Russell, 1976; Odlaug & Pflug, 1978; Odlaug & Pflug, 1979; Montville, 1982). Molds have been shown to raise the pH of foods such as tomato juice and allow *C. botulinum* to grow and produce toxin (Huhtanen et al., 1976; Odlaug & Pflug, 1979). *Bacillus licheniformis* has also been shown to raise the pH of a model system and allow toxin production by *C. botulinum*, although botulinum toxin was not detected in pH-elevated jars of tomatoes containing *C. botulinum* spores, possibly due to this being a low-probability event difficult to detect in small trials (Montville, 1982). The factors that determine whether or not *C. botulinum* will grow and produce toxin will depend on the composition and characteristics of the food (pH, a_w, nutrients, antimicrobial constituents, redox potential), the conditions of storage (temperature, exposure to oxygen), and microbial interaction (Odlaug & Pflug, 1978). Incidents have been reported in which acid foods have caused botulism in the absence of an elevated pH and microorganisms such as *Lactobacillus*, yeast, and others have been isolated from the product; however, it is possible that microenvironments of elevated pH allowing toxin production were present but not detected (Odlaug & Pflug, 1978). Odlaug and Pflug (1978) listed the following factors as necessary for a botulism hazard to result from an acid food: (a) contamination of the food with large numbers of *C. botulinum* spores, (b) contamination with other microorganisms due to process failure or post-process contamination, (c) composition of the food and storage conditions favorable to growth of *C. botulinum*, and (d) metabiosis (the reliance of one organism on another to produce a favorable environment for growth). The probability of all these factors occurring in commercially canned acid foods is extremely low, and this low probability is supported by the excellent history of safety of these products. Toxicity due to metabiosis

is a possibility in home canning of acid foods, where conditions are much less controlled.

Control and Prevention

Although there have been many technological changes in the canning and can-manufacturing industries in the last 70 years, some of these have offset the effects that others have had in reducing spoilage. For example, in the late 1930s, chlorination of cooling water came into practice and this greatly reduced the potential for product recontamination and spoilage. However, economic factors have led to lighter base plate and thinner tin coatings in cans. With the lighter base plate, greater care is needed to ensure proper seams and to minimize container damage. Economic pressures dictating faster production lines and more mechanical handling increased container damage, but the reduction in manual handling of containers has decreased recontamination with *S. aureus*. Since the 1970s there has been a considerable reduction in the incidence of post-process contamination involving defective containers. Spoilage rate reductions are directly related to improved container closure control, improved post-processing handling practices, and improved container integrity as a result of newer can making technology (e.g., drawn cans, which only have a single double seam and no side seam) (NFPA/CMI Container Integrity Task Force, 1984). To minimize the potential for spoilage, and a potential health hazard from recontamination of a sealed container or package, manufacturers must take steps to avoid container defects and container abuse; control levels of microorganisms in cooling water by use of chlorine or other sanitizers; and reduce microbiological loads on container handling equipment by frequent cleaning and sanitizing. Bee and Hontz (1980) described a program to detect and prevent post-processing container handling damage. Since it is not possible to achieve "zero defects" in container integrity, manufacturers should implement procedures to detect defective packages before they reach the consumer. Destructive testing of seams and seals is common practice in the industry. Non-destructive testing of food packages (e.g., dud detectors) maximizes the detection of defects while minimizing product loss.

Inadequate Thermal Processing

Heat processes for thermally processed shelf-stable foods are designed to destroy all microorganisms of public health significance as well as microorganisms of non-public health significance that could grow in the product under normal non-refrigerated storage conditions. "Inadequate thermal processing" indicates that the thermal process that was applied to the product was insufficient to destroy these organisms. In some instances, the product may receive sufficient heat to inactivate pathogenic sporeformers such as *C. botulinum*, but more heat-resistant spoilage organisms might survive and spoil the product. However, if the heat process is inadequate to destroy *C. botulinum* spores, the situation can be very hazardous,

since botulinum toxin could be produced and, if the product is consumed without adequate heating, cause botulism in the consumer.

An inadequate thermal process may occur for a number of reasons, including but not limited to the following:

(1) The scheduled thermal process is not properly established.
(2) The time and/or temperature (or equivalent) specified in the scheduled thermal process for the product and specified container size is not used.
(3) The scheduled thermal process is not properly delivered because of some mechanical or personnel failure.
(4) One or more of the scheduled process critical factors is not met.
(5) The formulation is changed such that the delivery of the scheduled process is negatively impacted.

When spoilage is detected in a production lot (usually appearing as swollen containers), and there is no evidence of leakage (seams or seals cannot be made to leak and structurally they meet the criteria of acceptability), it is generally safe to conclude that the cause of spoilage is probably under sterilization or inadequate processing. The degree of underprocessing will influence the type and extent of spoilage that occurs as well as the time it takes for swells or spoilage to appear in the pack. In some cases, underprocessing spoilage may occur without containers being visibly swollen, such as "flat sour spoilage" or, if insufficient fermentable carbohydrate is present in the product, thus restricting gas production. We are aware of spoilage incidents in chicken broth caused by *Clostridium sporogenes*, where containers appeared normal because gas was not produced. If bacterial spores are seen in a product smear, or aerobic and/or anaerobic sporeforming organisms are isolated in subculture from a low-acid product, and no container integrity issues can be identified, inadequate thermal processing is almost confirmed.

Spoilage that occurs as a result of underprocessing of low-acid canned foods is usually caused by a single sporeforming type of organism, whereas spoilage that results from leakage or post-processing contamination is usually the result of mixed cultures of non-sporeforming and possibly sporeforming bacteria. On occasion, where underprocessing is severe, multiple types of sporeforming and non-sporeforming organisms may be seen in a product smear or recovered in subculture. There has been spoilage in beets in which a mixed microflora of heat-resistant and non-heat-resistant microorganisms were recovered, suggesting post-processing contamination, but no container defects or other causes of leakage could be detected. Occasional firm beet slices could be found, suggesting underprocessing. Ultimately it was determined that the cause of spoilage was underprocessing resulting from stacking of beet slices because the slices were too large in relation to the can diameter. We have also seen underprocessing spoilage in acid products when processors have switched from using refrigerated components to frozen components without accounting for the impact of the change in initial temperature on the thermal process.

All spoilage incidents of low-acid canned foods caused by an inadequate thermal process should be handled with caution due to the potential for toxic spoilage to occur.

Thermophilic Spoilage

The spores of thermophilic bacteria usually have a greater heat resistance than the spores of mesophilic bacteria. Therefore, heat processes designed to kill mesophilic bacterial spores are not adequate to destroy thermophilic bacterial spores. Since the production of shelf-stable, "commercially sterile" foods involves the destruction of microorganisms that would grow under non-refrigerated conditions of distribution and storage (i.e., mesophilic microorganisms), most shelf-stable products probably contain some viable thermophilic spores. Given the high heat resistance of thermophilic spores, it would be impractical to establish thermal processes to destroy these organisms because the quality of the food products would suffer greatly. Therefore, products must be properly cooled (preferably below 40–43°C) after thermal processing, and held below 35°C during subsequent storage, in order to prevent thermophilic spoilage. During production, thermophiles may grow in products or on equipment in contact with food if the temperature is within their growth range. Consequently, product should always be held at 76.7°C or above or at room temperature or below to prevent the growth of thermophiles. Thermophilic spoilage has economic significance, but not public health significance, since there are no known thermophilic foodborne pathogens.

The growth of heat-resistant, thermophilic, facultative anaerobic sporeforming bacteria in a food product results in non-swollen containers because these bacteria do not produce gas but do produce acid (hence the term "flat sour" spoilage). On the other hand, thermophilic, obligate anaerobic sporeforming bacteria usually swell the containers. Prompt and adequate cooling is important in preventing thermophilic spoilage.

For food products containing ingredients known to be a source of thermophiles, such as sugar, starch, and/or spices, where thermophilic spoilage may be a problem, processors should exercise great care in preventing product contamination by thermophilic bacteria. Processors should use ingredients – such as sugar, starch, and spices – that the supplier certifies are free of thermophilic spores or that the supplier certifies meet specifications for thermophiles for canning processes (Olson & Sorrells, 2001). This is particularly important if the product is to be hot-vended.

Growth of Acid-Tolerant SporeForming Microorganisms

The growth of acid-tolerant sporeforming microorganisms can occur in product with a pH less than or equal to 4.6. Butyric acid anaerobes can be a problem in products with a pH between 4.2 and 4.6. The butyric acid anaerobes are discussed more fully in the section entitled Types of Spoilage Microorganisms. Heat-resistant molds have caused spoilage problems in acid and acidified foods and

beverages, but beverages will be dealt with in a separate chapter. More information on heat-resistant molds can be found later in this chapter.

Non-microbial Spoilage

Spoilage in canned foods can sometimes occur as the result of container deterioration; this may at times result from chemical interactions between the food and the container. Container deterioration may result in swollen containers resulting from the production of hydrogen gas (hydrogen swells) or in leaking containers due to pinholes or cracks.

There are four main types of corrosion inside plain tinplate containers: normal corrosion, rapid detinning, pitting corrosion, and cosmetic corrosion. The normal corrosion process is slow, and the canned product may have a shelf life of about 2 years. Rapid detinning involves rapid tin dissolution of the tinned surface, with complete detinning occurring in as little as 3 months. If hydrogen is produced into the can headspace, the process is called rapid electrolytic detinning. Products traditionally involved have been fruits (e.g., pineapples and pineapple juice, tomatoes, peaches), but canned vegetables such as mushrooms, green beans, spinach, and carrots have also demonstrated this problem. A number of factors have been shown to cause rapid detinning (Charbonneau, 2002): incorrect tinplate, high levels of oxygen, sulfites or nitrates, incorrect organic acid acidulant, and storage temperature. Proper headspace and a good vacuum are necessary to minimize the level of oxygen in the container and avoid detinning. Sulfur dioxide, a clarifying agent used in the manufacture of corn syrup, can promote detinning if residual levels in the finished product are above 2 ppm. High levels of nitrates from fertilizer used to grow vegetables are no longer a factor in rapid detinning of food products because of the use of enameled cans (Charbonneau, 2002). EPA has established a maximum contaminant level of 10 ppm for nitrate, but some water sources have been shown to exceed 50 ppm, which can result in complete detinning within a year. Nitrate levels of water must be <5 ppm to avoid detinning.

Pitting corrosion involves rapid dissolution of iron, with or without tin dissolving. This can lead to early failure by either hydrogen swells or perforations in the metal. Pitting corrosion can arise from some of the same factors as rapid detinning (e.g., sulfites, organic acid), and the influence of these and other factors has been described by Charbonneau (2002).

Many products are packed in enameled cans rather than plain tinplate. Corrosion inside enameled cans is localized at fractures in the coating where the plate is exposed to the product. At one time, can codes were embossed on the container end, which sometimes led to fractures in the enamel, leading to premature corrosion. This problem has almost completely disappeared with the use of video jet coding of containers. There are five main manifestations of corrosion in coated cans – normal corrosion, pitting corrosion, under-enamel corrosion and enamel flaking, stress corrosion cracking, and sulfide black corrosion. The normal corrosion process involves iron dissolution from small pores, and the corrosion shelf life will

exceed 18–24 months. Pitting corrosion involves rapid iron dissolution from the container walls at coating defects. Under-enamel corrosion is detinning or staining through the coating at areas where the coating has lost adhesion. Stress corrosion cracking involves a reaction between "worked" areas in the container with stress-inducing components in the product. Cracks through the container have been observed in as little as 4 months. These three types of corrosion may lead to early failure. Sulfide black corrosion involves rapid iron dissolution through the coating, with black deposits forming about 24 hours after processing. Sulfide black discoloration is a type of cosmetic corrosion that is objectionable to the consumer.

Laboratory analysis is necessary to determine whether spoilage is the result of microbial action or container failure. It is important to record the condition of the container interior when conducting the analyses and to test for hydrogen gas when opening swollen containers, as these will provide useful information, particularly if no microorganisms are recovered. Metallic or rotten egg (hydrogen sulfide) odors may also be a clue to spoilage due to container–product interaction.

Non-microbial spoilage rarely results in a health hazard. However, in high-acid products where detinning of containers has occurred, there have been instances of illness due to high levels of tin (>200 ppm), which can cause acute toxicity (nausea, vomiting, cramps, and diarrhea). In 1997 there was an outbreak of illness associated with pineapple juice. Groups of children in Texas and in Florida became ill with nausea and vomiting 0.5–5 hours after consuming the juice. The cause of the outbreak was originally ascribed to *Bacillus cereus*, which was reportedly isolated from the product by a private laboratory. However, since *B. cereus* would not be able to grow and produce emetic toxin in pineapple juice due to its low pH, another explanation was sought. It was eventually discovered that tin levels between 200 and 300 ppm (sufficient to cause illness) were present in the juice and high nitrate levels were found in the pineapple concentrate used to manufacture the product.

Unknown or Unidentified Causes

Occasionally, spoilage may be suspected or identified in a production lot for which no explanation can be found. Signs of bacterial spoilage may be evident (gas production resulting in swollen containers, reduced product pH, off-odor, or bacterial cells visible in product smear), yet no viable microorganisms can be isolated in subculture. This condition is often referred to as autosterilization, since the characteristics of bacterial spoilage are evident, but the microorganisms that caused the spoilage are no longer viable. Often this type of spoilage is difficult to distinguish from incipient spoilage.

Determining the Cause of Spoilage

Determining the cause of spoilage of commercially sterile canned food products requires knowledge and experience to conduct the analysis and to correctly interpret the results. There are several reference publications that outline the proper

procedures and media to use when investigating a spoilage incident in a thermally processed food. Chapters in the *Compendium of methods for the microbiological examination of foods* (Denny & Parkinson, 2001); the US Food and Drug Administration's *Bacteriological analytical manual* (Landry, Schwab, & Lancette, 1998); the *Recommended international code of hygienic practice for low and acidified low acid canned foods* (Codex Alimentarius Commission, 1993); and the *Procedures for diagnosis of spoilage* (Moir, Eylles, & Richardson, 2001) are good source documents that outline specific procedures and the necessary materials and equipment that are needed for a thorough examination. In addition, these references provide keys to interpretation of the results as an aid in determining the cause of spoilage based on the subculture results. Along with adherence to proper investigative procedures, it is important that the analyst performing the testing has experience in laboratory diagnostic procedures. There are many nuances to spoilage investigations that must be taken into consideration.

One of the most important aspects in the analysis of spoiled product is to ensure that the media being used for subculture are at the proper pH with respect to the product. The pH of any subculture media should be in the same range as that of the product prior to spoilage. For example, spoiled canned corn with an initial pH of 6.5 should be subcultured in media with neutral pH regardless of the pH of the spoiled product, because the organism responsible for spoilage initiated growth at the normal pH of the product. Likewise, acid products (less than pH 4.6) should be subcultured in media with a pH close to the initial pH of the product prior to spoilage. If acid products are subcultured in neutral laboratory media, viable microorganisms that are present in the product but are inhibited by the product pH may grow in the neutral media, confusing the spoilage diagnosis.

To the inexperienced analyst, growth in the bottom of a subculture tube of medium incubated aerobically may be interpreted as aerobic growth when in fact, it may be anaerobic. Conditions at the bottom of the tube may be anaerobic enough to allow for the growth of anaerobes, especially if the tube had been exhausted (oxygen driven off) through a mild heating step prior to inoculation. Any growth in media tubes should be streaked onto pour plates containing an appropriate medium, and duplicate plates incubated both aerobically and anaerobically.

Heat shocking as a means of identifying sporeformers should, in general, be performed on growth obtained from subculture rather than on the spoiled product (Denny & Parkinson, 2001). However, it is not inappropriate to heat shock spoiled product in culture media if caution is exercised in interpreting results. Growth obtained in subculture after heat shocking obviously indicates the presence of spores. However, no growth signifies that spores were absent, but not that the organism is not a sporeformer. Conditions in the product may not have been adequate for spore formation to occur. Subcultures must be allowed to incubate for the full recommended time in order for sporulation to occur. With *Bacillus* species, sporulation is generally quick. However, *Clostridium* species often take an extended period of time and often require specific nutrients or conditions. Most of the subculturing methods may miss the very fastidious sporeformers.

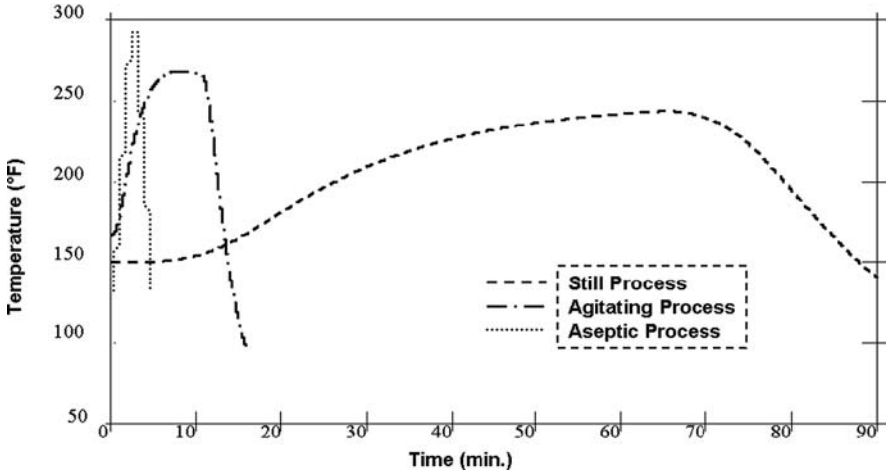


Fig. 2 Comparison of heating curves for various types of thermal processes

Perhaps the most frequent mistake made during a spoilage investigation involves interpretation of results. The keys to determining the cause of spoilage that are provided in selected references are general and meant to serve as a guide. The analyst must take into consideration much more than just the subculture results when determining a probable cause of spoilage. Spoilage analysis should include a review of all appropriate records. Processing records should be reviewed to determine if the thermal process applied was the correct one for the product, and that it was appropriately delivered (all critical factors adhered to). Any deviations should be noted to determine if there is a relationship to the spoiled container.

In some cases, it may even be appropriate to review the process establishment procedures to insure that the thermal process was appropriately established and all critical factors identified. In the design of a thermal process, sufficient lethality to destroy sporeforming microorganisms is delivered through a combination of time and temperature. Still processes (no agitation) are usually delivered at ~121°C and typically are quite lengthy because heat penetration is slow. Agitating processes (either axial or end-over-end) usually involve a slightly higher temperature (~132°C) and are shorter because heat penetration occurs faster due to the agitation. Aseptic processes (continuous flow through a heated pipe) are usually the shortest and often involve much higher temperatures (~140°C). Figure 2 provides a visual comparison of still, agitating, and aseptic processes. In general, factors that impede heat penetration have a greater effect on aseptic and agitating processes because these processes typically are shorter than still processes. What might have a slight negative effect on the lethality of a still process may have a significant effect on an agitating process, and consequently greatly influence the type and extent of spoilage that occurs. It is prudent to consider the type of thermal process when determining the cause of spoilage.

Blending records should be reviewed to determine that the correct formula was used and that there were no blending errors or excessive preprocess downtime. Product ingredients and packaging should be as specified in the purchase specifications and product formula. Seaming records or in-process container evaluation records should be reviewed. If leakage is suspected, disinfection of cooling water and container handling systems should be examined to insure procedures were being followed when the spoiled container was produced.

If the container is unopened when received in the laboratory, it should be examined externally for any signs of leakage (e.g., stained label, product residue near a seam). If the container is severely swollen, seals or seams may have been compromised, causing product to leak from the container, so this may be an effect rather than a cause of spoilage. Swollen rigid containers may be difficult if not impossible to analyze using most leak detection methods. Prior to opening the container, product gross weight should be measured, especially if fill weight or container head space is a critical factor in the process. Net weight should then be determined by subtracting the weight of the empty container from the gross weight. In some cases, drained weight may need to be determined to assess whether there was an overfill of solids. If the container is not severely distended or appears relatively normal, an appropriate leak detection test (e.g., pressure hold test, dye test, helium leak test) should be conducted prior to destructive analysis of the container. Results of destructive analysis should be compared to seaming/sealing specifications as a means of assessing the potential for seam abnormalities to result in spoilage.

Once the container is opened, the product should be examined for odor, general appearance, and pH (Denny & Parkinson, 2001; Landry et al., 1998; Moir et al., 2001). Control (non-spoiled or normal) samples, if available, should be analyzed at the same time and the results compared to those obtained from spoiled containers. A library or reference sample of spoiled product should be prepared and held refrigerated in the event that additional testing from the source product is required. It is important that the analyst test the product as soon as swells develop or are found. Over time, organisms can die off due to autosterilization.

The results obtained during the subculturing investigation provide the analyst with general information about the type of microorganism(s) present in the spoiled product. If the recommended procedures are followed, the identity of the organism's genus can be determined in most cases. Knowing the genus or growth characteristics of the spoilage organism(s) may be enough information to identify the root cause of spoilage and determine corrective actions. In other cases, the use of microbial characterization tools might be useful in assisting the analyst in pinpointing the source(s) of spoilage organism(s).

There are many types of rapid automated microbial characterization tools on the market today, including the Vitek®, RiboPrinter®, BAX®, and Micro-ID®. Some units provide the analyst with the genus and species of the organism, while others, like the DuPont RiboPrinter®, provide a DNA profile that can be compared to other profiles in the database. A current shortcoming of this instrument and others like it is the limited number of profiles available for most microorganisms encountered in the spoilage of foods (the majority of organisms in the database are of

clinical significance). However, it may not always be necessary to have an exact identification of the spoilage organism. In many cases, knowing the source of the organism will provide insight into determining the cause of spoilage, and this is where the genetics-based instruments are useful. We have had success in identifying the source of a spoilage microorganism in a number of spoilage incidents using the RiboPrinter®. In one incident involving a mushroom-containing product, the source of the spoilage organism was traced to the mushrooms. Further examination of the mushroom growing practices traced the source of the organism to the casing material (pasteurized peat moss, often containing various supplements), and finally to one of the ingredients in the casing material that was a by-product of another production process. By changing the source of the casing material ingredient, further occurrences of this type of spoilage were eliminated. The problem was solved without ever knowing the genus and species of the spoilage organism, but simply by matching genetic profiles of microorganisms from ingredients with the genetic profile of the organism recovered from spoiled containers. In another incident, contaminated equipment surfaces were identified as the source of the spoilage organism, and by modifying sanitation practices further occurrences of the spoilage problem were prevented. Any tool that provides information useful in solving a spoilage problem should be embraced.

Types of Spoilage Microorganisms

It is not possible to fully describe all the microorganisms that are capable of spoiling canned foods. The most significant types of microorganisms are described below. Information on their temperature and pH limits for growth and heat resistance is presented in Table 4.

Mesophilic Aerobic Sporeformers

Strains of *Bacillus* are the most common mesophilic aerobic sporeformers that cause spoilage of canned foods (Stevenson & Segner, 2001). These organisms are generally widespread in the environment. In most instances, containers remain flat, although a few strains of *Bacillus* (e.g., *B. macerans*, *B. polymyxa*) produce gas that can distend the containers. The presence of *Bacillus* species is generally the result of improper process application rather than a faulty process design, since most *Bacillus* species have only moderate heat resistance (Stevenson & Segner, 2001). *Bacillus* species have caused spoilage in a number of products where dry ingredients (pasta, tapioca, cocoa) have not been properly hydrated prior to or during processing. These organisms have also been responsible for spoilage in aseptic processing operations where contamination occurs on the warm side of the system (e.g., growth across a valve seat that has a malfunctioning steam block).

Table 4 Thermal resistance, pH, and temperature requirements of typical canned food spoilage organisms

Spoilage type	Organism	Growth temperature (°C)		Min. pH for growth	*D* value	*z* value (°C)
		Min	Max			
Mesophilic aerobic sporeformer	*Bacillus sporothermodurans*	20[a]	52[a]		$D_{140} = 3.4–7.9$ s	13.1–14.2
	Bacillus spp.				$D_{121 =} \sim 0.1 –0.5$ min[b]	6.7–8.9[b]
Mesophilic anaerobic sporeformer	*Clostridium botulinum* proteolytic	10	48	4.77[c]	$D_{121} = 0.1–0.2$ min	7.8–10[b]
	C. botulinum non-proteolytic	3.3	45	5	$D_{100} = <0.1$ min	~8–9
	C. sporogenes	10	50		$D_{121} = 0.1–1.5$ min	10
	C. perfringens	12	50	<5.5[d] (3.7[e])	$D_{100} = 0.5–124$ min	
Butyric acid anaerobe	*C. butyricum* non-toxigenic			4.2[f]	$D_{100} = 4.7$ min[f]	~8.5[f]
	C. butyricum toxigenic			5.2[f]	$D_{76.6} = 2.3–2.5$ min[f]	~4–6[f]
	C. pasteurianum			4.2[g]	$D_{100} = 0.1–0.5$ min[b]	6.7–8.9[b]
Aciduric flat sour sporeformers	*Bacillus coagulans*	18[h]	60	3.8	$D_{120} = 0.01–0.07$ min[b]	7.8–10[b]
Thermophilic flat sour sporeformer	*B. stearothermophilus*	30	75	5.3	$D_{121} = 4–5$ min[b]	7.8–12.2[b]
H_2S-producing thermophilic anaerobic sporeformer	*Desulfotomaculum nigrificans*	30	70	5.6	$D_{120} = 2–3$ min[b]	8.9–12.2[b]

(Continued)

Table 4 (Continued)

Spoilage type	Organism	Growth temperature (°C) Min	Max	Min. pH for growth	*D* value	*z* value (°C)
Non-H_2S-producing thermophilic anaerobic sporeformer	*Thermoanaerobacterium thermosaccharolyticum*	32	60	4.1[i]	$D_{121} = 3–50$ min[i]	6–7
Heat-resistant mold	*Byssochlamys fulva*	~7	~50	2.0	$D_{90} = 1–12$ min	6–7

[a] Hammer et al. (1995).
[b] Stumbo (1973).
[c] Hauschild et al. (1975).
[d] ICMSF (1996).
[e] De Jong (1989).
[f] Morton et al. (1990).
[g] Morton (1998).
[h] Gordon and Smith (1949).
[i] Ashton (1981).

An organism known as *Bacillus sporothermodurans*, a Gram-positive, mesophilic, strictly aerobic organism that produces extremely heat-resistant spores, has been isolated from UHT milk, cream, chocolate milk, and milk powder; although it has been found at levels as high as 10^5/ml, only rarely has it had a negative impact on product (Herman, Vaerewijck, Moermans, & Waes, 1997). The organism grows at temperatures between 20 and 52°C, and the spores are activated by boiling for 30 min (Hammer, Lembke, Suhren, & Heeschen, 1995; Herman et al., 1997). The source appears to be raw milk. However, there is no evidence that these organisms have been involved in spoilage of food products other than UHT dairy products.

Mesophilic Anaerobic Sporeformers

The mesophilic anaerobic sporeformers of significance in foods primarily belong to the genus *Clostridium*, and those of greatest interest in foods fall into two main groups (Scott, Anderson, & Wang, 2001). One group consists of *C. sporogenes*, the proteolytic strains of *C. botulinum*, and other relatively heat-resistant "putrefactive anaerobes." The other group consists of *Clostridium perfringens*, the non-proteolytic strains of *C. botulinum*, and a variety of other similar clostridia, such as the butyric acid anaerobes (e.g., *Clostridium butyricum*, *Clostridium pasteurianum*) and some recently recognized psychrotrophic clostridia, such as *Clostridium laramie*, that have caused spoilage in refrigerated meat and seafood (Kalinowski & Tompkin, 1999; Segner, 1992). It is the first group that contains the strains of primary importance in the spoilage of low-acid foods packed in hermetically sealed containers, including the pathogenic sporeformer with the greatest heat resistance – *C. botulinum*. Because of the high heat resistance of spores of mesophilic anaerobes, and because the canning process provides the anaerobic environment ideal for germination and outgrowth of the spores, these organisms are the basis for the thermal processes established to provide safe, shelf-stable canned foods. It is the butyric acid anaerobes, however, that pose a risk of spoilage to acid foods with a pH above 4.0.

Putrefactive Anaerobes

Proteolytic mesophilic anaerobes may cause spoilage of canned foods having a pH of 4.8 or above if the spores are not destroyed by the thermal process to which the canned foods have been subjected, or if they gain access through container leakage after processing (see section "Post-process Contamination") (Scott et al., 2001). The lowest pH at which *C. botulinum* spores have been observed to germinate and grow in commercial food products is pH 4.77 in marinated mushrooms (Hauschild et al., 1975). A pH of 4.6 is used to delineate low-acid from acid foods to provide a margin of safety. Putrefactive sporeformers are widely distributed in nature, with soil (and soil contamination of foods and ingredients) being a primary source of the foodborne strains.

Spoilage by these anaerobes is generally characterized by gas production that swells the container, a reduced product pH, and a foul, putrid odor, hence the generic name "putrefactive anaerobes." There are, of course, exceptions to this spoilage pattern. Cameron and Esty (1940) noted that putrefactive anaerobes can cause spoilage without gas in low-acid products in the range of pH 4.6–5.0, and Montville (1983) has shown that cultural conditions influence gas and protease production by *C. botulinum*. Also, if there is a hole in the container that allows gas to escape, the can will not appear swollen. A microscopic examination of product will usually reveal a mixture of rods, spore-containing rods, and free spores. Depending on the strain(s) present, spores may be subterminal, central, or terminal, and may or may not swell the sporangium. The appearance of rods containing subterminal spores in a product smear is cause for concern, as this is the typical appearance of *C. botulinum*. It may be necessary in such cases to send samples to a laboratory that can conduct analyses for botulinum toxin, since most laboratories of food manufacturers are not adequately equipped to handle toxic samples or conduct the appropriate analysis to identify the toxin.

Since some strains of mesophilic anaerobic sporeformers are able to grow in the presence of relatively high levels of oxygen, they may appear to grow in media designed to recover aerobic spoilage organisms. Signs of growth in aerobic media may also occur when there is a heavy load of microorganisms in the volume of product subcultured. The organisms seen in the subculture smear may simply be due to carryover. Generally if the culture is heat shocked, the remaining spores will only germinate and grow out anaerobically. It is important to determine if only mesophilic anaerobic sporeformers are present.

The recovery of pure cultures of anaerobic sporeformers is usually an indication of underprocessing; however, in a low percentage of spoilage incidents spoilage by mesophilic anaerobic sporeformers can be attributed to post-process contamination. Lake, Graves, Lesniewski, and Anderson (1985) found that 27 of 770 canned food spoilage incidents over a 4-year period could be attributed to growth of clostridia that had entered the can due to post-processing leakage. This is not entirely unexpected, as spores are more likely to remain viable in chlorinated can cooling water than vegetative cells. However, Lake et al. (1985) attributed such spoilage to abusive handling of wet cans and cannery insanitation, since mesophilic anaerobic sporeformers are usually present in very low numbers in can cooling waters (Thompson & Griffith, 1983).

It is important to remember that there is a fairly wide range of heat resistance among strains of mesophilic anaerobic sporeformers (Table 4). A process with a sterilizing value (F_0 value) of 3.0 is generally employed as a "minimum public health" process to inactivate spores of *C. botulinum* (this assumes a $D_{121^\circ C}$ value of 0.25 min). However, since there are mesophilic anaerobic sporeformers with much greater heat resistance than that of *C. botulinum*, greater F_0 values are generally employed in order to prevent economic spoilage. In general, thermal processes for commercially sterile products have an F_0 value of 5.0–6.0 (and sometimes greater). This process is designed to eliminate a reasonably likely number of spores with a $D_{121^\circ C}$ value of 1–1.25 min (more typical of spores of *C. sporogenes*, which are

roughly 4–5 times more heat resistant than *C. botulinum*). Consequently, spoilage due to marginal underprocessing typically is economic in nature rather than of public health significance.

Butyric Acid Anaerobes

The butyric acid-producing anaerobes are mesophilic sporeformers that produce butyric acid as well as carbon dioxide and hydrogen. The spores are capable of germination and growth at pH values as low as 4.2 and consequently are of spoilage significance in acid and acidified foods with pH values above 4.2. Growth of these organisms in foods is characterized by a butyric odor and the production of large quantities of gas.

The most common butyric acid anaerobes (also referred to as butyric anaerobes) are *C. butyricum* and *C. pasteurianum*. These organisms are capable of germination and growth at a pH as low as 4.2–4.4, although vegetative cells can grow at somewhat lower pH (Morton, 1998). As a result, they can be a problem in non-pressure-processed acid foods such as tomatoes, tomato products, and certain fruits (e.g., pears) with pH values above 4.2. Since the pH of these products normally is 4.2 or below and the processes are designed to inactivate low levels of spores of butyric acid anaerobes, spoilage by these organisms is not a common problem. However, because of factors such as fruit variety and growing conditions, where the pH of the product may exceed 4.2, there is an increased likelihood of spoilage by butyric acid anaerobes. In those cases where the acidity of these products is too low (e.g., the pH exceeds 4.2), spoilage by butyric acid anaerobes may be controlled by increasing the acidification of the product or by increasing the thermal process (Scott et al., 2001). Butyric acid anaerobes are also of spoilage significance in underprocessing of acid and acidified foods, and occasionally in low-acid canned foods as well. Occasionally, strains will be encountered that can grow at a pH lower than 4.2; if these strains are present in high numbers, the heat process will be inadequate and spoilage can occur (Scott et al., 2001).

As with other clostridia, soil is the primary source of the microorganisms. Growth of butyric acid anaerobes in foods is characterized by a butyric odor (described by many as a "vomit" odor), increased acidity, and the production of carbon dioxide and hydrogen, usually in copious amounts. Although strains of *C. butyricum* producing botulinum neurotoxin have been isolated from cases of infant botulism in Italy (Aureli et al., 1986), the toxigenic strains were shown to possess little heat resistance and they could not grow below pH 5.2 (Morton, Scott, Bernard, & Wiley, 1990). Thus, there is no indication that strains of *C. butyricum* are of public health concern in commercially produced shelf-stable products.

De Jong (1989) reported an unusual spoilage incident in canned, acidified mung bean sprouts in which acid-tolerant strains of *C. perfringens* and *Clostridium barati*, as well as *C. butyricum*, were isolated. The sprouts were acidified with citric acid to a pH 4.0–4.5 and pasteurized to a minimum core temperature of 85°C. Many of the cans swelled within 2 weeks, and a number of cans burst. The strains of *C. perfringens* and *C. barati* isolated from the product were able to grow at pH

values as low as pH 3.7, whereas the lowest pH at which the *C. butyricum* isolate grew was 4.0. Multiplication of clostridia to high numbers and sporulation occurred during the mung bean sprouting process, apparently as a result of reduced oxygen concentration due to growth of Enterobacteriaceae and lactobacilli, along with sprout respiration.

Aciduric Flat Sour Sporeformers

Aciduric "flat sours" are facultative anaerobic sporeformers that seldom produce gas in spoiled products. The ends of spoiled cans remain flat; hence, the term "flat sour." Spoiled products have an off-flavor that has been described as "medicinal" or "phenolic." These organisms (e.g., *Bacillus coagulans*) have caused spoilage in acid foods such as tomato products and could cause problems in other products with tomato sauces if the sauces are prepared from fresh tomatoes (the problem is unlikely if the tomato ingredients are previously processed to inactivate these organisms, as with commercially sterile products). It is advisable to ensure that the thermal process is adequate to inactivate an expected number of spores, which can be determined through bacteriological surveys if necessary. Pinpointing the ingredient that is contributing the most to the total spore load may prove beneficial in process control. For example, proper handling of vegetables prior to use, such as washing and culling, may help to reduce spore loads.

Most food processing operations do not provide anaerobic conditions; therefore, heavy buildup of acid-tolerant anaerobic sporeformers seldom occurs. However, "dead ends" (dead legs) in processing lines must be avoided because these can provide conditions appropriate for growth and sporulation of *B. coagulans*. If this situation should be encountered, spoilage will almost certainly result because the process lacks adequate lethality to address the high spore load in the product.

If flat sour spoilage is encountered in a lot, the spoilage pattern is often spotty and scattered, more typical of post-processing spoilage that is due to container leakage than of the pattern expected from sporeformers that survive a thermal process. Thermal processing records and other processing parameters usually give no indication of any irregularities. In most cases, the problem can be identified only by investigation at the factory, which would include a bacteriological survey, plus the absence of demonstrable leakage and package defects in the spoiled containers.

Alicyclobacillus spp. such as *A. acidoterrestris* are acidophilic sporeformers that can grow at a pH as low as 2.0 and have been associated with spoilage of canned juices (Walls & Chuyate, 1998). Although the source of the spores appears to be soil that contaminates the raw fruit, there has been no evidence to date of spoilage in any product other than fruit-containing beverages. Further information on this type of spoilage is presented in the chapter "Microbiological Spoilage of Beverages."

Thermophilic Flat Sour Sporeformers

The organisms in this spoilage family generally grow between 40 and 90°C, with optimum growth seen between 55 and 65°C (Ray, 2001). The most common species of concern are *B. coagulans*, which has been discussed in relation to aciduric flat sour spoilage, and *Bacillus stearothermophilus.* It is interesting to note that some thermophiles will grow at mesophilic temperatures (~37°C) once their spores have germinated at more elevated temperatures (Ray, 2001; Ashton, 1981; Ashton & Bernard, 2001; Bergey's 1986). This may cause issues with regulatory bodies who typically analyze spoiled products for mesophilic growth only. Growth in subculture that "appears" to be caused by a mesophilic sporeformer may force a product recall because of the potential for toxic spoilage and its implications on public health. It is very important that analysts determining the cause of spoilage are aware of this unique characteristic of some thermophiles and conduct additional analyses, such as thermophilic incubation and microbial identification to determine whether or not the spoilage was due to thermophilic or mesophilic microorganisms.

The pH range for growth varies with the different types of organisms. In general, most of the thermophilic sporeformers can spoil a wide range of thermally processed products, including acid products such as tomato sauce. The spores of these organisms have a very high heat resistance, often higher than the current processes designed to kill mesophilic sporeformers. However, since thermophilic sporeformers do not produce toxins or infections in humans, they are not of public health significance. They are more of a nuisance organism that can cause great economic loss if the proper controls are not in place. The key to preventing spoilage by thermophilic sporeformers is to quickly cool thermally processed products to below 40–43°C and store them below 35°C (Ray, 2001; Ashton, 1981; Ashton & Bernard, 2001).

A special problem arises for products intended to be sold in hot vending machines or destined for shipment and sale to areas with warmer climates. Since the product may be stored at elevated temperatures (greater than 43°C) for extended periods of time, the ingredients that are used to produce the product should be screened to insure very low levels of thermophilic spores to minimize potential spoilage. Many manufacturers will screen ingredients such as sugar, starch, chicken stock, and mushrooms for thermophilic spores because these ingredients traditionally have high loads of thermophilic spores due to either the nature of their processing or growing environments (i.e., soil, compost). Other manufacturers will go beyond screening and will treat higher risk ingredients with heat or chemicals to eliminate or greatly reduce the spore load. Chicken stock is processed using elevated temperatures for an extended period of time in order to drive water out of the ingredient, thus concentrating the stock. This high-temperature holding process is conducive to growth and sporulation of many thermophilic organisms. Therefore, some manufacturers will presterilize their chicken stock prior to its use in a thermally processed product. Presterilization involves very high temperatures for a specific period of time designed to eliminate several logs of thermophilic spores. There are some chemicals that can also be added to products that will inhibit germination of

spores. Nisin is one such chemical that prevents the outgrowth of spores in certain applications. In acid and acidified canned foods such as beans in tomato sauce, canned tomato products, and fruits, nisin has been used to prevent spoilage from *B. coagulans* and *Thermoanaerobacterium thermosaccharolyticum* (Hoover, 2000). In rare cases, these products may be given a severe heat process (F_0 of 30) to minimize the potential for thermophilic spoilage (i.e., hot-vended products).

In the 1920s the National Canner's Association (currently known as GMA) investigated the occurrence of spores in US canning factories. Their investigation led to bacterial spore advisory levels for many different ingredients, including starches and sugars. A total thermophilic spore count advisory standard was developed for sugars and starches that recommended a maximum of no more than 150 spores and an average of no more than 125 spores per 10 g out of five samples. Specific types of spores such as flat sour, thermophilic anaerobic, and sulfide spoilage spores have individual standards as well, which are outlined in the *Compendium of methods for the microbiological examination of foods* (Ashton & Bernand, 2001).

Thermophilic Anaerobic Sporeformers

Sulfide "Stinkers"

Desulfotomaculum nigrificans (formerly known as *Clostridium nigrificans*) is a thermophilic anaerobe that produces hydrogen sulfide and discoloration upon spoilage, known as sulfide stinker spoilage. *D. nigrificans* is the only sulfur-reducing organism that has been associated with spoilage of thermally processed foods (Speck, 1981; Chapman, 2001). The first incident of spoilage caused by *D. nigrificans* was reported in 1919 by an Iowa sweet corn cannery (Speck, 1981). Over the years, *D. nigrificans* has been found to be the cause of spoilage in canned vegetables (predominantly in canned corn and peas), mushroom products, infant formulas, canned cream, condensed milk, and non-fat dry milk solids (Speck, 1981; Donnelly & Hannah, 2001). It has also been seen as a problem in the sugar industry, spoiling molasses on occasion (Chapman, 2001). *D. nigrificans* can be isolated from sugar, starch, soy, soil, fresh water, geothermal regions, low-acid spoiled food, and from insect's intestines and rumen contents (Bergey's 1986; Donnelly & Hannah, 2001).

This slightly curved, Gram-negative, sporeforming rod grows at thermophilic temperatures (55°C optimum with a range of 45–70°C). *D. nigrificans* is sensitive to acid and will only grow in low-acid foods at a pH range of 6.6–7.4, with pH 6.2 generally being the lower limit for growth (Chapman, 2001; Donnelly & Hannah, 2001). *D. nigrificans* has the unique ability to reduce sulfates, sulfites, and other reducible sulfur compounds producing hydrogen sulfide with its characteristic rotten egg odor (Chapman, 2001, Bergey's 1986). *D. nigrificans* utilizes sulfate ions as oxidizing agents in much the same way other organisms use oxygen during aerobic respiration (Chapman, 2001). Growth of *D. nigrificans* in low-acid foods results in an off-odor and product discoloration with no gas production (i.e., flat

can). The interior of the can and the product blackens from the interaction of the dissolved H_2S with the iron in the container (Speck, 1981, Ray, 2001).

The spores of *D. nigrificans* are highly heat resistant and can survive typical commercial sterilization processes. Various studies have shown that the spores of *D. nigrificans* have $D_{120°C}$ values in the range of 2–3 min (Donnelly & Hannah, 2001). In general, the spores of *D. nigrificans* are more heat resistant than *T. thermosaccharolyticum*, but less resistant than *B. stearothermophilus* (Speck, 1981). In order to cause spoilage of thermally processed foods by *D. nigrificans*, a significantly high spore level along with elevated post-processing temperatures is needed (Donnelly & Hannah, 2001). It is very unlikely that this organism will establish itself in a cannery and become a reoccurring source of contamination due to its anaerobic, temperature, and pH requirements along with its limited source (Speck, 1981).

Currently, the *Compendium of methods for the microbiological examination of foods* provides the best direction on sampling and detection of *D. nigrificans* spores from food products (Donnelly & Hannah, 2001). In general, a recovery medium with a source of sulfur is necessary for growth of this organism. An Iron Sulfite Agar is commercially available. Other researchers add 6-d nails or iron strips to sulfite agar in order to observe the characteristic blackening that results from the interaction between the hydrogen sulfide and the iron (Speck, 1981; Donnelly & Hannah, 2001).

Non-H_2S Gas Producers

T. thermosaccharolyticum (formerly *Clostridium thermosaccharolyticum*) is a thermophilic anaerobic, non-H_2S gas-producing sporeformer important to the spoilage of thermally processed foods (Dotzauer, Ehrmann, & Vogel, 2002; Ray, 2001). These obligate anaerobic, saccharolytic, rod-shaped organisms have been responsible for spoilage of many different thermally processed products, including spaghetti with tomato sauce, tomatoes, sweet potatoes, pumpkin, green beans, mushrooms, asparagus, vegetable soup, dog food, and even high-acid products such as canned fruit (Ashton, 1981; Ashton & Bernard, 2001). These sporeformers, which produce copious amounts of gas, occur naturally in the soil (Ashton, 1981; Ababouch, 1999; Ashton & Bernard, 2001; Bergey's, 1986). Ingredients that are known sources of *T. thermosaccharolyticum* include vegetables, sugar, starch, flour, mushrooms, dehydrated milk, cereals, alimentary pastes, rendered meat, and spices (Ashton, 1981; Ababouch, 1999; Ashton & Bernard, 2001). Some ingredients such as chicken stock, beef extract, and yeast hydrolysate can contain high levels of this sporeformer due to the heat processing applied during concentration or hydrolysis (Ashton, 1981; Ashton & Bernard, 2001).

T. thermosaccharolyticum produces an abundant amount of gas, mainly carbon dioxide and hydrogen, along with acetic acid, butyric acid, lactic acid, succinic acid, and ethanol (Vicini, Previdi, & Pirone, 1992; Ashton, 1981; Pirone, LaPierta, Impembo, Longo, & Squitieri, 2005; Ashton & Bernard, 2001). The optimum temperature for growth of *T. thermosaccharolyticum* is 55–60°C. Some species have

shown the ability to grow at mesophilic temperatures (<37°C) once they have germinated at higher temperatures (Ashton, 1981; Pirone et al., 2005; Bergey's, 1986; Ashton & Bernard, 2001). The optimum pH for growth is 6.2–7.2 but some strains can grow as low as 4.1 (Ashton, 1981; Ashton & Bernard, 2001). The pH range for sporulation is 5.0–5.5 (Ashton, 1981). Spores of *T. thermosaccharolyticum* are extremely heat resistant. $D_{121°C}$ values have been reported from as low as 3–4 min all the way up to 50 min (Ashton, 1981). As noted before, most commercial processes are not designed to eliminate thermophilic spores. Therefore, it is imperative that thermally processed products are quickly cooled down to below 40–43°C immediately after processing and then stored at temperatures below 35°C (Ray, 2001; Ashton, 1981; Ashton & Bernard, 2001).

T. thermosaccharolyticum does not readily grow in the production facility unless an anaerobic environment containing nutrients and moisture at elevated temperatures is provided (Ashton, 1981; Ashton & Bernard, 2001). Some ingredient processing practices such as concentrating meat stocks and hydrolysis of yeast can promote the development of thermophilic spores due to the extended times at elevated temperatures. In order to prevent the buildup of thermophilic spores in processing equipment, thorough sanitation of tanks, blanchers, and washers is recommended on a daily basis. The organism has also been known to reach high loads in the cooling leg of hydrostatic cookers. There is evidence that this high spore load can lead to increased leakage type spoilage (Ashton & Bernard, 2001).

Heat-Resistant Molds

Most fungi have very limited heat resistance, except for a group of fungi referred to as heat-resistant molds. Only a few species of heat-resistant molds have been isolated from heat processed shelf-stable foods, but, in many cases, these fungi, though viable, did not cause spoilage (Pitt & Hocking, 1997). The most frequently isolated heat-resistant molds that have been associated with the spoilage of heat-processed food are from the genera *Byssochlamys*, *Neosartorya*, *Talaromyces*, and *Eupenicillium* (Pitt & Hocking, 1997; Tournas, 1994; Beuchat & Pitt, 2001). *Byssochlamys fulva* was first recognized as a food spoilage organism in the 1930s in canned strawberries packed in England (Pitt & Hocking, 1997; Tournas, 1994). *Neosartorya fischeri* has also been repeatedly isolated from canned strawberries, although it usually has not caused spoilage. *Eupenicillium* was responsible for the spoilage of canned blueberries (Williams, Cameron, & Williams, 1941). Heat-resistant molds have been isolated mainly from fruit and fruit products such as fruit juices, juice concentrates, and canned fruits and purees. Heat-resistant molds have primarily caused spoilage in juice products.

Heat-resistant molds have several unique characteristics that enable them to grow in heat-processed foods. They produce ascospores that can withstand normal thermal processing times and temperatures given acid foods (Pitt & Hocking, 1997; Tournas, 1994; Beuchat & Pitt, 2001). *B. fulva* and *B. nivea* in particular have the ability to grow at very low oxygen tensions, enabling them to grow in packages

with very limited oxygen. Pitt and Hocking (1997) suggest that these species appear to grow anaerobically and produce CO_2. Even packages with relatively minimal headspace or very slow oxygen transfer, such as plastic and paperboard cartons, have a sufficient amount of oxygen for growth of these types of organisms (Tournas, 1994). Although there are reports of production of mycotoxins (e.g., patulin) and other toxic metabolites such as byssochlamic acid and fumitremorgins (Tournas, 1994; Beuchat & Pitt, 2001) by heat-resistant molds, there is little evidence that mycotoxins of significance are produced in commercially processed products (Pitt & Hocking, 1997).

Heat-resistant molds are mainly found in soil and dust. Fruits that are in contact with the soil, such as strawberries, are more likely to be contaminated with these organisms (Pitt & Hocking, 1997; Tournas, 1994). The most effective approaches to minimizing spoilage by heat-resistant molds are (1) selecting sound fruit (avoiding windfall fruits which have higher contamination rates due to their contact with the soil), (2) using good handling practices such as washing with chlorinated water, (3) preventing injury to fruit during handling, (4) removing of damaged fruit or peeling to remove skin, (5) blanching, and (6) employing good sanitation practices to prevent buildup in the processing facility (Beuchat & Rice, 1979; Pitt & Hocking, 1997). Other factors that affect the rate of growth in fruit include the initial level of contamination, types of organisms, product composition (i.e., stage of growth: green versus ripe), pH, water activity, oxygen tension, and mechanical injury to the fruit (Tournas, 1994). In some cases it may be possible to filter fruit juices through diatomaceous earth to reduce or eliminate the ascospores. Antimycotic agents such as sulfur dioxide, benzoate, and sorbate are sometimes used either during harvest or in the final product to minimize growth (Tournas, 1994; King, 1986). Destruction of ascospores in fruits by thermal processing alone is usually impractical due to the adverse effects of the required heat treatment on sensory and nutritional quality (Beuchat & Rice, 1979), although processes of 104.4–110°F for 6 s have occasionally been used to destroy ascospores.

There are several methods for identifying and enumerating heat-resistant molds from a food product. The *Compendium of Methods for the Microbiological Examination of Foods* offers the latest review in acceptable methods (Beuchat & Pitt, 2001). Most methods begin with a heat treatment at 75–80°C for 5 min or more to germinate the ascospores. There are direct plating methods as well as flat bottle techniques that are used to grow the mold cultures. These molds are not fastidious and can utilize many types of media. Potato Dextrose and Malt Extract agars are most frequently used (Tournas, 1994). Incubation times, however, are substantial, requiring 30 days at 30°C (Pitt & Hocking, 1997).

Non-sporeformers

Non-sporeforming microorganisms are frequently isolated in the examination of spoiled canned foods. When these organisms are present, they indicate the product was not processed, was significantly underprocessed, or that post-process contami-

nation occurred. The presence of non-sporeformers due to inadequate processing is not common in a low-acid canned food, as most non-sporeformers are killed by even a mild heat treatment. However, severe underprocessing (and "retort bypasses") can result in a mixed flora of more resistant non-sporeformers (e.g., *Enterococcus*) and sporeformers (e.g., *Bacillus*). Non-sporeformers are more common in underprocessed acid and acidified foods where the thermal process is much less severe than for low-acid foods. Mixed flora is generally considered to be indicative of post-process contamination (Denny & Parkinson, 2001). The types of non-sporeforming microorganisms found in spoiled canned foods can vary widely, depending on the types of microorganisms present in the raw product (for underprocessing) or in the environment (post-processing contamination). They include yeast, mold, Gram-positive bacteria such as lactic acid bacteria, and Gram-negative bacteria such as *Enterobacter* and *Pseudomonas*. In most cases the spoilage organisms are never speciated, as this is not necessary to assess the cause of spoilage.

Conclusion

Bacterial spoilage, primarily of economic significance, occurs as a normal event in the production and marketing of shelf-stable canned foods. On rare occasion, and under specific circumstances, there is the potential that the spoilage may also have public health significance. Knowledge of thermal processing, the manufacturing process, and spoilage bacteria are necessary for rapidly establishing the correct cause of spoilage and determining product disposition. The importance of good laboratory and analytical skills cannot be overemphasized for quickly distinguishing between economic spoilage and that of public health significance in order to minimize loss of life and negative impact on the business and the food industry.

Appendix 1: References for Establishing Thermal Processes

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Microbiological Spoilage of Cereal Products

Frederick K. Cook and Billie L. Johnson

Introduction

A wide range of cereal products, including bakery items, refrigerated dough, fresh pasta products, dried cereal products, snack foods, and bakery mixes, are manufactured for food consumption. These products are subject to physical, chemical, and microbiological spoilage that affects the taste, aroma, leavening, appearance, and overall quality of the end consumer product. Microorganisms are ubiquitous in nature and have the potential for causing food spoilage and foodborne disease. However, compared to other categories of food products, bakery products rarely cause food poisoning. The heat that is applied during baking or frying usually eliminates pathogenic and spoilage microorganisms, and low moisture contributes to product stability. Nevertheless, microbiological spoilage of these products occurs, resulting in substantial economic losses.

Economic Impact of Spoilage

The quality and the safety of bakery products are of major concern to bakery producers, quality control authorities, and end product consumers. In addition to the economic losses incurred because of bakery spoilage, possible foodborne illnesses could cost billions of dollars to the industry because of costly adverse health effects, the loss of productivity, medical expenses, and most importantly, adverse publicity for the bakery industry. Additional costs in international trade include the costs of rejections, detention of products, recalls, and the resulting adverse publicity for the industry and even for the country. Food spoilage and the resulting waste of nutritious food is a problem worldwide. Approximately 5–10% of the world food supply

F.K. Cook (✉)
Malt-O-Meal Company, 701 West 5th St, Northfield MN 55057
e-mail: fred_cook@malt-o-meal.com

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is lost annually because of the presence of fungi and mycotoxins alone. The total yearly cost in the USA is estimated to be in the hundreds of millions to billions of dollars. Waste due to spoilage of perishable products accounts for substantial losses in grocery store markets. Bakery products alone account for 4–5% of these losses. In order to curb this loss, many stores must offer this perishable good at a discount which will result in overall loss of economic revenue (Tsiros & Heilman, 2004).

For many years, "stales" and spoilage returns in the baking industry have amounted to 10% of the total production volume. Staling of bread is a physico-chemical deterioration resulting in hard and crumbly texture and loss of fresh baked flavor. Although staling is not the result of microbial spoilage, it has contributed to huge economic loss for the bakery industry (Bechtel, Pomeranz, & de Francisco, 1978). Recently, the level of these losses has been reduced. Bakers report that stales and spoilage returns currently vary between 1 and 10%; 5% is a realistic average. Although this level of spoilage is an improvement, it is still a staggering financial burden for the industry. No other industry must deliberately produce an additional five units for every 100 units manufactured to cover the losses incurred through spoilage. A cost estimation considered for one loaf of bread suggests that the ingredient cost correlates to $0.23, transportation cost at $0.15, container or packaging costs at $0.01, spoilage costs at $0.05, and labor costs estimated at $0.50, making the total cost $0.94 a loaf. This suggests that spoilage contributes to about 5% of the total cost of bread (Kulp, 1979). This 5% estimation of bread returned to bakeries as "unsellable" corresponds to approximately 600 million pounds of bread annually.

As the bread industry has seen diminished sales in recent years as a result of "low-carb" diets, numerous large baking companies have been looking for ways to cut costs and increase margins to compensate for lower sales. Suppliers to the industry have been helping by developing antistaling enzymes that promise a longer life for bread. As bread usually lasts only a few days before spoilage begins to occur, the bakery industry has been forced to maintain more bakeries to minimize the distances trucks must be driven for delivery. This allows drivers to check and restock stores every few days. A longer shelf life would mean bakeries could close extra production facilities with fewer deliveries necessary, a colossal savings to the industry as a whole. Typical shelf life lengths for various cereal products are shown in Table 1.

A rather small group of microorganisms is responsible for most of the spoilage of cereal products. Molds are the most common, especially with baked goods, because of their ability to grow at the relatively reduced water activity levels. Several genera of bacteria, yeasts, and molds can be involved in the spoilage of various products (Table 2). The types and numbers of microorganisms present in cereal products are dependent on the microbiological quality of raw materials, processing steps during manufacture of products, hygienic conditions during production, properties of products that affect microbial growth rates, and storage and distribution conditions.

Table 1 Shelf life of bread, cereal, and pastry foods

Food type	Room temperature	Refrigerator	Freezer
Bread	5–7 days	1–2 weeks	3 months
Doughnuts	4–5 days	NA	3 months
Pasta	2 years	NA	NA
Pies and pastries	NA	3 days	4–6 months
Pies and pastries (baked)	NA	NA	1–2 months
Pies and pastries (cream)	NA	2–3 days	3 months
Pizza	NA	3–4 days	1–2 months
Rice (white)	1 year	5–7 days (cooked)	6 months (cooked)
Rice (brown)	6 months	NA	NA
Waffles	NA	4–5 days	1 month

NA – not applicable.

Table 2 Microbes involved in spoilage of cereal products

Organism	Types of food spoiled	Type of spoilage
Fungi		
Aspergillus	Bread	Black mold
	Grains	Black mold rot (aflatoxin)
Candida	Breads	Yeasty
Cladosporium	Bread	Brown/black mold rot
Claviceps purpura	Corn, grain	Ear rot (ergotism)
	Breads	Black rot
Fusarium	Corn	Pink mold rot (fumonisins)
Penicillium	Breads	Blue-green mold
Rhizopus	Breads	Black mold
Saccharomyces	Breads and Pastas	Yeasty
Zygosaccharomyces	Breads and Pastas	Yeasty
Bacteria		
Bacillus	Bread	Slime
Clostridium	Bread	Ropy
Lactobacillus	Bread	Ropy
Leuconostoc	Bread	Ropy

Bakery Products

Types of Foods and General Properties

A wide variety of bakery products exist, including leavened and unleavened breads, rolls, buns, croissants, English muffins, crumpets, cakes (including snack cakes), doughnuts, pastries, pancakes, waffles, biscuits, scones, tortillas, muffins, cupcakes, and sweet rolls. These products are typically baked at temperatures that provide sufficient lethality to destroy all vegetative bacteria, yeasts, and molds in the product. Most baked goods have a dry outer crust that prevents the growth of bacteria that

may recontaminate the products after baking. Because they are rarely involved in food poisoning incidents, bread and most baked goods are not considered high-risk foods. Nevertheless, bakery products are highly perishable. Because of typical pH values near neutrality, relatively high moisture content, and ambient storage temperatures, these products provide a favorable environment for the growth of spoilage microorganisms (Jay, 1996).

Ambient temperatures, product pH levels between 5.4 and 7.5, and water activity in the range of 0.75–0.98 promote spoilage of baked cereal foods with mold, yeast, and rope bacteria. Water activity is a particularly important factor influencing spoilage of cereal products, and many bakery products such as breads and cakes have levels above 0.94. Although relatively harmless, their visible presence deters customers and can result in substantial economic losses to wholesale bakeries.

Mold and Yeast Spoilage

Mold growth contributes far more to spoilage of bakery products than any other spoilage organism encountered. Yeast is a much less prominent cause, but growth of either can cause surface spoilage. Although shelf life of bakery products like bread is limited by the physicochemical deterioration called "staling" mentioned previously, shelf life is often further limited by rapid mold growth. Mold spoilage is first evident as white, filamentous, or "fuzzy" colonies which gradually turn various colors from blue-green to black as spores are produced. Typically, untreated bread will become moldy within 5–6 days (Vora & Sidhu, 1987). Surface yeast growth can create white or pink areas.

Mycotoxins are secondary metabolites produced during the growth of many species of molds. Although not directly involved in spoilage issues, the potential presence of mycotoxins in grains and cereal products is a lingering concern because of the high incidence of mold spoilage of these materials. Specific spoilage fungi of the genera *Aspergillus* and *Fusarium* produce mycotoxins when growing on food commodities or animal feedstuffs (Diekman & Green, 1992). Since the eleventh century AD, when the French discovered that *Claviceps purpurea*-contaminated rye baguettes caused people to hallucinate (ergotism), hundreds of additional mycotoxins have been identified. Aflatoxin, produced by *Aspergillus flavus, A. parasiticus*, and other closely related fungi; fumonisins, produced by *Fusarium verticillioides*; and deoxynivalenol (DON, or vomitoxin), produced by *F. graminearum* are the principal mycotoxins produced in grains and cereal products. Other fungal toxins, including cyclopiazonic acid in peanuts, zearalenone from corn, and ochratoxin from coffee and grains, can be of concern in particular ingredients (Robens & Richard, 1992). The public health effects of mycotoxins are complex. Some mycotoxins are carcinogenic, some are vasoactive, and some cause central nervous system damage. Often, a single mycotoxin can cause more than one type of deleterious effect.

Despite the fact that bakery products are commonly spoiled by visible molding, the potential presence of mycotoxins in spoiled bakery products is not considered to be, and has not been demonstrated to be, a public health problem for this simple reason: for centuries consumers have been educated to not eat moldy, or otherwise spoiled food. The occurrence of mold spoilage usually means that the spoiled food has been stored too long and, in addition to visible mold, the food may have also developed other quality defects. People at risk for mycotoxin-induced health problems are those who are economically or environmentally forced to consume moldy grains and cereal products in an effort to avoid starvation.

Typical genera of mold involved in spoilage are *Penicillium, Aspergillus, Cladosporium, Fusarium, Monilia, Endomyces, Rhizopus*, and *Mucor* (Legan & Voysey, 1991). Yeasts that can cause surface spoilage of bakery products include *Saccharomyces, Debaryomyces, Kluyveromyces, Pichia, Candida*, and *Zygosaccharomyces* (van der Zee & Huis In't Veld 1997). A number of strains of *Saccharomyces* spp. are used to produce millions of tons of bakery products each year (Demain, 1998), and some yeasts such as *Saccharomyces, Pichia, Candida, and Torulopsis* (Spicher, 1983) are used in the manufacture of sourdough breads.

Microbial Sources and Effects

Typically, post-process contamination is responsible for bakery product spoilage. Although heat applied during baking destroys all yeasts and molds, bakery products are readily subjected to recontamination from the air, equipment surfaces, and handling during cooling, slicing, and packaging operations. The types and numbers of yeast and mold initially present on bakery products are dependent on hygienic conditions of the manufacturing environment and equipment during production. Ambient air usually has sufficient mold spores to cause spoilage, since a single spore can produce a visible mold colony when it has the opportunity to grow. Therefore, virtually all bakery products are vulnerable to mold contamination and spoilage.

Sources of mold or yeast contamination within the bakery environment may originate from their natural presence in the ingredients. If these contaminants are able to find a niche and grow within the plant environment, they could become dispersed into the atmosphere of the bakery operation and contaminate food contact surfaces such as shelves, racks, conveyor belts, and slicing or packaging machines. Contamination of bakery products can also result when workers preparing dough subsequently handle the baked products or from surfaces or utensils that contact both the raw dough and the finished product (Seiler, 2000). Unsanitary equipment including utensils can be a source of contamination by osmotolerant yeast.

Factors Influencing Spoilage

In addition to contamination, spoilage of bakery products is influenced by several other factors: the type of product (bread or sweet baked goods), ingredients

(type of flour and other dry ingredients), leavening sources (chemical, baker's yeast, or sourdough), size and architecture of the bakery, and conditioning and packaging of the products (cooling, slicing, wrapping, and materials used for packaging) (Lavermicocca et al., 2000).

Control of moisture and water activity is critical for microbial stability of bakery products. In general, molds and yeasts are more tolerant to lower water activity levels than are bacteria, with minimum levels for growth in foods generally well below the 0.85–0.92 lower levels that typically limit many types of bacteria. This is why bread spoils more predominantly because of mold growth rather than bacterial growth (Worobo & Padilla-Zakour, 1999). Most molds will not grow significantly below a_w 0.80, but some are very resistant to dry conditions and grow very slowly at a_w of 0.60 or below. *Zygosaccharomyces rouxii* is an osmotolerant spoilage yeast. Wrapping of warm bakery products after baking can lead to condensation on the inner package and on the product's surface, promoting mold and yeast growth.

Prevention and Control Measures

Excellent post-baking hygiene, aseptic conditions (including sterile air, packaging, and equipment contact surfaces), destruction of contamination by a post-baking treatment, and design of product to prevent growth (low a_w, preservatives, low pH) can contribute to the reduction of mold and yeast spoilage.

Use of good manufacturing practices and excellent sanitation programs are important to control spoilage of bakery products, particularly for high volume production. These programs minimize microbial contamination and growth for ingredients, components, and finished product. Raw materials should also be carefully assessed as these can be prime sources of contamination. Yeasts tend to be more resistant to the effects of disinfectants and preservatives than are molds, thereby causing greater difficulty in controlling bakery contamination. For disinfection of surfaces, quaternary ammonium compounds are preferred over hypochlorite because of greater effectiveness for suppressing multiplication of yeast cells.

Dry nonperishable ingredients such as flours, sugar, cocoa, coconut, and spices can contribute to microbial contamination, therefore must be stored in areas of the bakery separated from those where perishable products are prepared. This is particularly important with powdered materials like flours where the dust raised during unloading can enter the bakery atmosphere and cause contamination (Seiler, 2000). Hygienic measures which aim at reducing the number of mold spores that contaminate the product either from the atmosphere or through contact with surfaces during the slicing, finishing, and wrapping operations can be achieved by cleaning floors, walls, and ceilings at intervals to prevent accumulations of flour dust, pieces of dough, crumbs, and so on.

Some manufacturing procedures aim to prevent contamination of baked products entirely. Some bakery industries have used special, heat-resistant laminated films to package the baked product before baking. The products are baked at a relatively low temperature, and final sealing occurs shortly after leaving the oven which prevents

post-baking contamination. Packaging products as soon as possible after baking, frying or steaming, and sealing at temperatures high enough to destroy mold spores that may come from the environment can also prevent mold contamination. For example, this method of preservation is used for canned fruitcakes. The cake is baked in the can and the lid is sealed as soon as possible after leaving the oven. The lids are sterilized by heating them in an oven at the same time when the cakes are being baked. Canned fruitcake has a shelf life of several years (Seiler, 2000). Nylon or polypropylene containers may be used for steamed products because of the lower processing temperatures.

Packaging in sterile atmosphere can also be used to prevent post-baking contamination. Absolute filters are used to blow sterile air over the area, thereby eliminating air as a source of contamination. Absolute filters and sterile atmospheres, however, are extremely expensive and are best suited in situations that are highly mechanized (Anonymous, 2005).

Post-baking treatments can also be effective for reducing contamination of bakery products such as the use of heat treatments or ultraviolet (UV) irradiation. When poorly conducting material such as bread or cake is placed in the field of high frequency energy produced by microwave or dielectric ovens, heat is generated throughout the product by molecular friction. Most wrapping materials are good conductors; however, the product will be largely unaffected by this high frequency energy. The surface temperature of the wrapped goods can be raised to 70°C, which is sufficient to destroy most bacterial and mold contaminates. Infrared heat treatment may also be used without affecting the appearance of most conventional cellulose and plastic wrapping materials. The advantages that infrared treatment has over microwave or dielectric heat treatment are that it may be cheaper overall to install and operate and does not require a second cooling stage. The main difficulty is that not all surfaces of the baked product can be treated at the same time, thereby requiring two infrared projectors mounted above and to the side of a wire mesh conveyor system. UV irradiation of the surface of the bakery product can reduce or even eliminate mold spores from baked products (Snyder & Poland, 1995). UV light penetrates transparent packaging films and has an advantage over other methods for destroying mold contaminates in that no heat is generated that can char wrappers or give rise to internal condensation. As with infrared techniques, two separate installations are necessary to ensure all surfaces are treated. Using a battery of 2537 A, germicidal lamps are set 1 inch from the surface of the baked good for a 30-s exposure. This procedure is successful for products with a smooth, even surface, but much less effective in products that have uneven surfaces or cracks where the UV light cannot penetrate (Seiler, 2000).

Another treatment to prevent the contamination of bakery products is ionizing radiation or "cold pasteurization." Small doses of radiation (2–7 kGy) can eliminate nonspore-forming bacteria such as *Salmonella* species, *Staphylococcus aureus, Campylobacter jejuni, Listeria monocytogenes*, or *Escherichia coli O157:H7* (Grolichova, Dvorak, & Musilova, 2004). Applications for food irradiation have focused mainly on poultry and red meat, egg products, fruits and vegetables, and fishery products so its use in cereal products has been limited.

Ionizing radiation has been used for dry spices, seasoning blends, and flours with a claim that the irradiated flour allows for enhanced "elasticity" and faster rising of the dough (Thayer, 1990).

Methods used to control or prevent microbial growth in bakery products involve reducing water activity of the baked good, using chemical antimicrobial agents, gas packaging, and refrigeration. Reformulating products to reduce a_w has been fairly successful in reducing overall mold growth of products; however, eating quality, texture, and appearance of the product may decline as the water activity is reduced (Troller & Christian, 1978). Humectants such as sugars or glycerine can be used to reduce water activity.

Although water activity is the most important factor affecting the type and rate of spoilage in bakery products (Membre, Kubaczka, & Chene, 1999), alteration of the water activity levels can only be used to a limited extent without affecting palatability of bakery products. Other ingredients that are used to enhance safety and to suppress the growth of molds and yeast as well as bacteria include antimicrobial preservatives (calcium propionate, potassium sorbate, sorbic acid), acids to reduce pH (vinegar, citric acid, phosphoric acid, malic acid, fumaric acid), spices with antimicrobial properties (cinnamon, nutmeg, garlic), and water-binding agents to control free water (gums, starches) (Jay, 1996). Several kinds of preservatives permitted for use in bakery products are listed in Table 3. Of the preservatives allowed for use in bread, propionic acid, sodium propionate, and calcium propionate are the most widely used; however, at maximum levels of usage, large loss of product volume may occur because of the inhibition of yeast activity, coupled with loss in flavor and odor. Guynot, Ramos, Sala, Sanchis, and Marin (2002) reported potassium sorbate as the most effective weak acid preservative to suppress the growth of spoilage fungi on bakery products. Ethanol is also permitted for use in some bakery products including pizza crust. In general, these mold inhibitors may be added to product for

Table 3 Preservatives permitted for use in bakery products. Adapted from Seiler (2000)

Products	Preservatives	Maximum permitted levels
		By flour weight (%)
Baked goods (breads, rolls, buns, etc.)	Propionic acid	0.300
	Calcium propionate	0.375
	Sodium propionate	0.375
	Potassium propionate	0.387
		By weight of product (%)
Flour confectionaries, crumpets, and muffins	Sorbic acid	0.100
	Potassium sorbate	0.134
	Sodium sorbate	0.119
	Calcium sorbate	0.117
	Propionic acid	0.100
	Calcium propionate	0.127
	Sodium propionate	0.129
	Potassium propionate	0.152

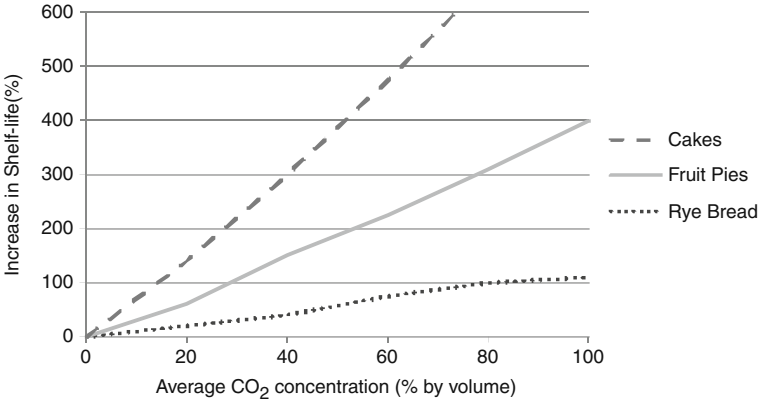

Fig. 1 Increase in mold-free shelf life of various bakery products packaged in differing concentrations of carbon dioxide. Adapted from Seiler (2000)

increasing shelf life; however, this often results in only 1 or 2 day's extension. For this reason, the manufacturer must depend more on the baking process, temperature control, and overall standards of hygiene employed within the bakery.

Another technique employed by bakery manufacturers to prevent microbial growth in some situations is modification of headspace composition. Oxygen can be excluded; however, it is not always removed completely and O_2 can penetrate through many types of packaging films (Vermeiren, Devlieghere, van Beest, de Kruijf, & Debevere, 1999). Increase in shelf life has been obtained in baked products packaged in an atmosphere rich in carbon dioxide gas. CO_2 has an inhibitory effect on the growth of aerobic microorganisms, including mold growth. Figure 1 illustrates the increase in mold-free shelf life obtained in various bakery products packaged in different concentrations of carbon dioxide (Seiler, 2000). As a means of increasing mold-free shelf life, gas packaging in carbon dioxide has the advantage that all parts of all surfaces of the product are protected. Carbon dioxide does not affect the odor, flavor, or appearance of the product, as well.

Research has demonstrated that shelf life of sliced bread held at room temperature conditions can be extended up to 3 weeks or more by incorporating 1–2% ethanol into the package or by packing under a CO_2 environment (Vora & Sidhu, 1987). Figure 2 illustrates the shelf life extension in sliced bread by using ethanol and a CO_2 environment. Ethanol acts by reducing the water activity of the bakery item. CO_2 acts by displacing oxygen in the package, thereby interfering with respiratory metabolism of molds that may contribute to bakery spoilage. Vacuum packing or the use of modified atmosphere packaging (MAP) utilizing nitrogen and carbon dioxide minimizes oxidation of bakery products and limits microbial spoilage by ensuring that oxygen is not available for reaction or respiration. However, the equipment necessary to package products in a carbon dioxide atmosphere, such as use of a CO_2 chamber or gas packing machinery, is expensive. Another major disadvantage

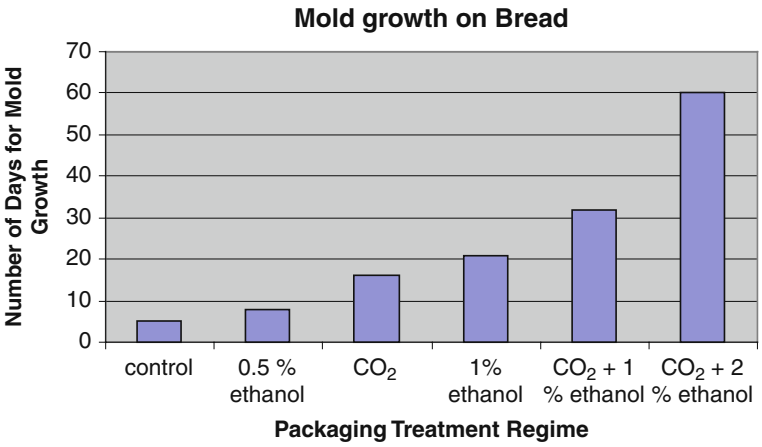

Fig. 2 Packaging treatments to prevent mold growth using varying concentrations of ethyl alcohol and carbon dioxide in sliced bread. Adapted from Vora and Sidhu (1987)

is the possibility that growth of anaerobic bacteria may occur in the gas packaged baked goods.

Storage techniques such as deep freezing, refrigeration, and even hot storage may also contribute to increased control of microbial spoilage for baked goods. Typical refrigeration temperatures (2–10°C) will retard microbial growth; however, chemical reactions will still occur at a very slow rate. Although chemical reactions such as oxidation may occur at a much reduced rate during typical freezer temperatures (+2°C to –20°C), growth of nearly all microorganisms will be completely suppressed. Unfortunately, less than optimum temperature control during storage by retail stores or by consumers can lead to higher temperatures, which can accelerate microbial spoilage.

Deep freezing has been used very effectively to control microbiological spoilage in bakery products. Under these conditions, neither molds nor yeasts can grow; however, once product is removed from frozen condition, growth may resume. Generally, deep freezing tends to be expensive and can only be justified for use in certain types of baked goods such as cream-filled products, cheesecake, or pizza. Refrigeration is widely used in the baking industry as a means of preservation as it is very useful for preventing bacteriological spoilage problems, but it may not be possible if the product is subject to a high rate of chemical staling. Cakes, which stale less rapidly, are readily preserved using refrigerated storage techniques. Hot storage techniques may also be used to retard mold growth if heated storage occurs at 120°F. This is only effective if the storage procedure is for a short period of time (no longer than 56 hrs) because of the fact that breads stored in this manner may demonstrate discoloration of the crumb after 48 hrs. The bread should also be allowed to cool slowly from this short-term storage. Any method of storage, whether deep freezing, refrigeration, or hot storage, can result in loss of product quality if storage of the bakery product is prolonged.

Rope Spoilage

Development of rope is the second leading cause of bakery product spoilage. Rope is characterized by a distinctive, unpleasant, fruity odor, such as overripe melons or pineapple followed by enzymatic degradation of the crumb which becomes soft and sticky and slightly discolored (Pepe, Blaiotta, Moschetti, & Villani, 2003). When broken in two and pulled apart, ropy bread loaves will demonstrate long, slimy threads, or sticky strings. Most types of bakery products can develop rope including bread, doughnuts, crumpets, and cakes; however, whole-meal and rye breads seem to have a higher propensity for rope spoilage.

Ropiness is bacterial spoilage caused primarily by *B. subtilis*, sometimes by *B. licheniformis*, *B. pumilus*, and *B. cereus* (Pepe et al., 2003), and also by *B. clausii* and *B. firmus* (Andersson, Rönner, & Granum, 1995). These bacteria are common in soil where they are active in the decomposition of organic matter. Their heat-resistant spores can survive the baking process, especially in the center of loaves where temperatures sometimes do not exceed 36°C. The water activity, pH, and temperature during storage of the baked product affect spore germination and growth of the *Bacillus* spp. vegetative cells. Ropiness occurs typically during storage of moist bakery products with a water activity value of 0.95 or above, at ambient temperatures or higher (Pepe et al., 2003). These conditions enable the rope bacilli to grow faster and to produce higher levels of amylase and protease activity (Quintavalla & Parolari, 1993; Rocken & Voysey, 1995), resulting in enzymatic degradation of the crumb and stickiness because of the production of extracellular mucilaginous polysaccharides by the bacilli (Thompson, Waites, & Dodd, 1998).

The types and numbers of microorganisms initially present in bakery products are dependent on the microbiological quality of raw materials and hygienic conditions during production. Microbial contamination of dried flours and meals often originates from raw materials during the milling process (Richter, Dorneanu, Eskiridge, & Rao, 1993). Poor milling procedures can contribute to increased concentrations of rope bacteria in flours used in the manufacture of bakery goods. While some of the ingredients of doughs or batters may be contaminated with rope spores, this contamination occurs at low levels and is not typically the cause of rope spoilage. Rather, rope spoilage is the result of inadequately cleaned and sanitized dough mixing and handling equipment that introduces high numbers of rope spores into the dough. Rope spores may contaminate mixers, dough bowls, pipelines, filters, water tanks during dough production, and cooling racks, conveyor belts, slicing blades, and wrapping materials after baking.

Bakeries can provide the perfect environment for rope bacteria to multiply and spread; however, the widespread implementation of good cleaning and sanitation procedures has greatly diminished the incidence of rope spoilage (Pepe et al., 2003; Thompson et al., 1998). Other effective measures that can contribute to the control of rope spoilage include use of certificates of analysis (COA) for incoming raw ingredients to assure low rope spore count, ensuring adequate baking, rapidly cooling, storage of bread products at low temperatures, and in some cases, the use of acid preservatives such as propionic acid and calcium propionate.

Sour Spoilage

Souring of bakery products is most commonly associated with the presence of a sour odor or flavor. This is caused by bacterial metabolism of carbohydrates with the resulting production of organic acids such as lactic acid. In some cases, this sour effect is beneficial and intentionally produced in products such as sourdough breads. For example, *Lactobacillus sanfranciscensis* is used to lower the pH during production of sour rye bread, thereby enabling sourdough yeast to grow and leaven the dough (Salovaara & Savolainen, 1984). Conversely, the same type of bacterial growth in other bakery products is highly undesirable and constitutes product spoilage.

Lactobacillus spp. found responsible for bakery product spoilage include *L. plantarum*, *L. curvatus, L. casei, L. farciminis, L. alimentarius, L. sanfranciscensis, L. fermentum, L. brevis lindneri, L. fructivorans, L. buchneri*, and *L. acidophilus.* Additional bacterial genera that can spoil bakery products include *Pediococcus, Carnobacterium, Enterococcus, Oenococcus, Streptococcus, Tetragenococcus, Vagococcus*, and *Weissella* (Stiles & Holzapfel, 1997). In addition to souring bakery products by production of lactic acid, these bacteria can also produce off-flavors, slime and CO_2, which can expand or burst packaged product. Growth of *Serratia marcescens* on the surface of bread stored at high humidity can produce red colonies that appear to be drops of blood. Once considered to be a divine signal, especially when the red colonies appeared on sacramental wafers, *S. marcescens* has been responsible for religious conflicts (Doyle, Beuchat, & Montville, 1997).

Control of these types of bacterial spoilage depends on sufficient baking to destroy vegetative microorganisms present in the dough, as well as moisture control at product surfaces to prevent post-baking contaminants from growing.

Refrigerated Dough and Pasta Products

Types of Foods and General Properties

Refrigerated cereal products such as dough and fresh pasta are marketed as convenient products requiring less preparation than alternative products traditionally available to bakers, food service operators, and consumers. Refrigerated, unbaked biscuits first appeared in the retail market in 1930, the idea derived from a Louisville, Kentucky, baker, who recognized the potential in reducing the work load required to make fresh made biscuits on a daily basis (Hesseltine, Graves, Rogers, & Burmeister, 1969). Fresh pasta products are especially desirable for present day consumers who desire a more convenient alternative to dry pastas which generally take 10–20 min to cook. Fresh pasta has significantly shorter cooking time because it requires less hydration during preparation.

At the time refrigerated biscuit dough was first available, the concept of supplying a product that was completely mixed and ready for baking was revolutionary,

as most bakery products were baked fresh shortly before consumption. The baking industry has expanded its production capabilities to include a wide variety of fresh-baked, partially baked, refrigerated, and frozen products. This includes proliferation of packaged refrigerated dough products such as biscuits, dinner rolls, breakfast rolls, breads, pie crust dough, and cookie dough. Chemically leavened refrigerated doughs for biscuits, rolls, and breads are typically packaged in foil-lined, cylindrical composite containers with metal lids crimped onto each end. As the packaged dough expands by leavening action, it seals pores present in the crimped ends, thereby creating a hermetically sealed package with an internal pressure of approximately 20 psi. It is not possible to pack unbaked yeast-leavened doughs in this manner as the continued metabolic activity of the yeast will quickly generate excessive pressure and burst the container (Sperber, 2006).

The development of efficient refrigerated distribution methods has led to the marketing of many types of fresh pasta products. Freshly made pasta and noodles can be purchased either uncooked (raw) or cooked. These products are usually prepared from wheat flour, with or without egg or other ingredients (Deibel & Swanson, 2001). They are highly perishable and must be consumed within a few days after manufacture. Under efficient refrigeration, the shelf life can be extended by a week or two, or up to about a month. Even with an efficient refrigerated distribution system, fresh pasta is still considered to be quite perishable because of the growth of mold, bacteria, or yeast.

Spoilage and Influential Factors

Spoilage of refrigerated dough and fresh pasta products can occur because of the growth of several microbial types. The types and numbers of microorganisms present are dependent on the microbiological quality of raw materials used during production. Ingredients used to manufacture dough such as flour, dry milk, sugar, spices, and eggs contain microorganisms and, since lethal heat steps are not used, microbial content of the finished products will reflect that of the ingredients. Hygienic conditions of manufacturing equipment can also impact the numbers and types of spoilage microorganisms present.

One of the more common microbiological spoilage problems in refrigerated dough products with high sugar contents, such as cookie doughs or fruit-filled products, is fermentation by osmophilic yeasts which are capable of growing at water activity values as low as 0.62 and pH values as low as 2.0. The enzymes produced by these yeasts metabolize sugars to carbon dioxide gas and alcohol (Seiler, 2000). Growth of osmophilic yeasts in cookie dough produces an undesirable odor and flavor upon baking. The aroma is often described as “alcoholic,” “fruity,” or “acetone.” The most common yeast to cause an “acetone” or “fruity” aroma is the yeast, *Hansenula anomala*. This yeast spoilage is characterized by the production of CO_2 gas, which causes bubbling or expansion of flexible packaging (Membre et al., 1999).

Spoilage of refrigerated doughs can also be caused by lactobacilli and leuconostocs especially under temperature abuse conditions (Hesseltine et al., 1969). Lactic acid produced by these spoilage bacteria shifts the equilibrium of the chemically leavened system so that excess carbon dioxide is produced, eventually bursting the container. This spoilage defect will occur whether or not the lactic acid bacterium is homofermentative (nongas producing), or heterofermentative (gas producing) (Sperber, 2006).

Elliot (1980) found that the microflora of spoiled doughs subjected to higher temperature consisted of such bacteria as *Leuconostoc dextranicum, Leuconostoc mesenteroides, Lactobacillus, Streptococcus, Micrococcus,* and *Bacillus* species. Research conducted by Hesseltine et al. (1969) suggest that 92% of bacterial spoilage isolates from refrigerated dough products belonged to the family *Lactobacillaceae*. Other heterofermentative lactic acid bacteria may also contribute to the spoilage of refrigerated doughs because of gas production and expansion or bursting of packaging containers (Deibel & Swanson, 2001).

If improper storage or refrigeration conditions occur whereby the refrigerated dough is subject to higher temperatures, yeasts, molds, and bacterial growth may contribute even more to spoilage of the products.

Spoilage Prevention and Control

The microbiological stability of refrigerated doughs is provided by controlled refrigeration temperature and by the reduction of water activity caused by leavening salts and other solutes. The water activity of canned refrigerated doughs is typically about 0.95 or lower, similar to that of the interior of baked, yeast-leavened products. If refrigerated doughs are stored at 5°C or lower, they will not spoil during the expected shelf life of about 3 months.

Usually, lactic spoilage of refrigerated doughs will not occur unless an excessive contamination with spoilage lactics occurs before the dough is packaged. As in the case of rope spoilage of bakery products, lactic spoilage of canned, refrigerated doughs is rarely the result of contaminated ingredients. It is usually caused by inadequately cleaned and sanitized equipment containing large numbers of lactic bacteria. Poor sanitary design of dough handling equipment preventing effective cleaning and sanitation can contribute to this problem. Modern equipment with good sanitary design and the use of adequate cleaning and sanitation procedures have greatly reduced the spoilage incidence of canned, refrigerated doughs (Sperber, 2006).

Refrigerated cookie dough is usually chub-packed in impermeable films. The low water activity of the cookie dough, about 0.80, prevents bacterial growth. Gas production by osmophilic yeasts can spoil cookie doughs that are poorly refrigerated (>10°C) for several weeks or longer. Under these conditions, osmophilic molds will not grow because of the carbon dioxide in the product.

The use of fungistats such as sorbic acid and sorbates, propionates, and such compounds have been used in fresh pasta products, and their use can extend the storage life (Deibel & Swanson, 2001). The shelf life of fresh pasta may also be extended by heat treatment with steam, microwave heating, or by other treatments before packaging. Pasteurization, however, substantially adds to the cost of processing and packaging and can cause undesirable partial cooking or gelatinization of the pasta (Anonymous, 2005). Very careful control over microbiological recontamination is also necessary during packaging. Fresh pasta should be packaged in an oxygen-free atmosphere to suppress growth and spoilage by aerobic microorganisms and to reduce oxidative changes to color and flavor. While oxygen-free packaging allows increase in shelf life unfortunately these conditions may select for growth of pathogenic anaerobic bacteria.

Fresh pasta packaged in gas-impermeable containers under modified atmosphere conditions and then stored under temperature abusive conditions were found to support the growth of *Clostridium botulinum,* thereby exacerbating the process of eliminating spoilage microorganisms (Schebor & Chirife, 2000).

Dried Cereal Products

Types of Foods and General Properties

Dried cereal products include such items as ready-to-eat grain-based foods (including breakfast cereals, crackers, cookies, wafers, granola bars, and many dried snack foods), dry bakery mixes of all kinds (including cake, brownie, muffin, and bread mixes), grain ingredients (including flours, rice, oats, corn meal, and corn grits), and dried pasta. Grain-based snack foods include such products as corn chips, tortilla chips, extruded snacks (such as cheese puffs), popcorn, trail mix bars, cereal bars, crackers (such as peanut butter-filled crackers), cereal snack mixes, rice cakes, and pretzels. These are all low moisture products, with a_w below 0.60.

Ready-to-eat grain-based foods are very popular, and demand for these products is increasing worldwide. The world snack food market alone reached an estimated $66 billion in 2003. The USA continues to be the largest market, accounting for about a third of the world's total. Since 1986, US snack food exports alone have quadrupled (Huthoefer, 1992; Kuehm, 2005). Since 1997, US exports of snack foods have hovered around $1.1 to $1.3 billion.

Many types of ready-to-eat grain-based products are considered convenience foods designed for quick "on-the-go" consumption between or instead of traditional meals, readily available treats requiring no refrigeration, or easily prepared foods for breakfast or other times. The spread of Western eating habits to other parts of the world continues as lifestyles in those parts of the world become busier, with traditional family meal times becoming a thing of the past (Hodgen, 2003). As a result, the demand for ready-to-eat grain products will continue to increase.

Prepared dry mixes are readily available for home use and for small- and medium-sized commercial bakeries. The manufacture of dry mixes is a dry blending of such ingredients as flour, dried eggs, flavorings, sugar, and dried dairy products. The complexity of these dry mixes has risen over the years. Historically, pancake mixes were available to the US public in the early 1930s. By the 1950s, cake mixes were "revolutionary" to the overall consumer base. They were touted as having greater "accuracy" for baking a "perfect" cake. Today, cake mix use predominates over the cake from "scratch" method.

The wide popularity of pasta as a food and the necessity for shelf stable economical distribution have resulted in the widespread production of dried pasta products having a moisture content of about 10–12%. Shelf life of dried pastas is estimated as 2 years with longer periods possible if the pasta is stored in airtight containers. Spoilage possibilities in dried pastas are very rare, an indication why shelf life can be so long as compared to other food products.

Spoilage and Influential Factors

Manufacturing of breakfast cereals generally occur through three differing procedures called "flaking," "puffing," or "extrusion" (Deibel & Swanson, 2001). For each of these procedures, moisture is introduced into the cereal formulation, thus providing a higher water activity by which microbial growth can be sustained. During this time in the manufacture of the breakfast cereal product, microbial loads may increase, especially if this time is prolonged. A subsequent "baking" or cooking process then reduces microbial levels substantially. Hygienic conditions may cause post-heat contamination. Low water activity of these finished products prevents any microbial growth.

The snack food industry relies on supplier's certificates of analysis to assure incoming supplies, and ingredients have been tested and found to be acceptable regarding presence or numbers of microbial contaminates. Because of the rare incidences of salmonellosis, the snack food industry randomly tests finished products for microbial pathogens coupled with sanitation verification programs. Sensitive ingredient programs have been readily implemented including separation of allergen-related ingredients as well as known ingredients for microbial contamination. Correct frying or baking procedures should ensure reduction in microbial contamination; it is the packaging process that must be most closely monitored. Excess moisture during the packaging step may result in mold growth. Use of high salt content, low water activity, and mold inhibitors greatly reduces the possibility of snack food spoilage.

Microbial contents of dry bakery mixes depend on microorganisms present in the ingredients used to make these dry blends, since no antimicrobial process is usually used. Hygienic conditions of the manufacturing plant, including equipment and storage, are also important. Maintenance of dry conditions will prevent spoilage of bakery mixes. Most spoilage microorganisms present in bakery mixes will be

destroyed during baking that is required for the preparation of finished products made from the mixes.

Raw cereal grains contain numerous types of microorganisms that originate from soil, plants, animals, air, and equipment surfaces used to handle grain. Numbers of these microorganisms are influenced by weather (temperature and rainfall), exposure to insects, birds and other animal, and conditions used to store, handle, and mill grain. Raw cereal grain products are typically dried and protected from moisture to prevent microbial spoilage. Measures to protect grains from moisture include ventilation of storage bins to remove moisture, equilibrate temperature, and prevent condensation. Moisture levels of 12% or below will not support any microbial growth (Hesseltine & Graves, 1966). Although presence and numbers of microorganisms may not be of spoilage consequence in the cereal grains, they are important considerations for microbial stability of products in which grains are used as ingredients.

Spoilage is rarely seen in dry cake mixes. This is due to the low water activity present within the mix itself, just as with dried pastas. Shelf life storage for the consumer is suggested at 6–9 months if stored in the original packaging. If spoilage does occur, it is usually attributed to mold growth, such as *Rhizopus* species, *Penicillium* species, *Absidia* species, *Mucor* species, and *Eurotium* species, because of "clumping" of the mix, whereby a pocket of moisture may become trapped in the packaged product. Osmophilic yeasts may also contribute to spoilage in dry bakery mixes. Sorbic acid has been used for many years as a fungistatic agent in protecting cake mixes against mold spoilage (Melnick, Vahlteich, & Hackett, 1956). If dried egg has been incorporated into the dry mix, there may be potential hazard for *Salmonella* contamination; however, no cases of foodborne outbreaks have been recorded.

Manufacture of both egg-based pasta and macaroni-type pasta involves mixing of ingredients (generally flour, water, enrichment nutrients, and in some cases eggs), extrusion, shaping, cutting, and drying. During manufacture, the unheated moist dough can support microbial growth (*Staphylococcus aureus* must be controlled) and spoilage can occur if time and temperature permit. Activity of microorganisms is prevented in the finished product by the drying process. This results in water activity so low even the most osmotolerant yeast and molds cannot grow.

Spoilage Prevention and Control

Microbial spoilage rarely occurs in dried cereal products primarily because of their low water activity (below 0.60), which prevents the growth of all bacteria, molds, and yeasts. Reduced water activity is also effective for decreasing enzyme activity which also serves to extend shelf life. Proper packaging and storage conditions are essential for maintaining low moisture levels in dried cereal products, to prevent microbial spoilage.

Compound Cereal Products

Types of Foods and General Properties

Compound cereal products contain a nongrain-based component. These products include tarts filled with fruit, pies filled with fruit or meat, sausage rolls, cream-filled pastries, cream cakes, cheesecake, quiche, pizza, calzones, and dough-enrobed sandwiches. Nongrain-based components may be added to grain components before baking as in the case of dough-enrobed sandwiches, or added after the cereal component is baked as in the case of filled pastries or pizza. Spoilage of these products is often a result of microorganisms contributed by the nongrain-based components that grow more readily in the nongrain components' higher moisture level environment.

Spoilage and Influential Factors

Because of their general inability to grow at reduced water activity levels, many types of microorganisms, especially bacteria, are not frequently involved in the spoilage of cereal products, but can be involved in the rapid spoilage of high-moisture fillings or toppings such as those containing meats, vegetables, fish, or dairy-based ingredients. Therefore, careful consideration must be given to combination products to accurately assess the need for time, temperature, and/or other controls. For example, egg and dairy ingredients baked inside a pastry, such as cream–cheese croissant, may receive sufficient heat treatments to destroy vegetative pathogens and are therefore stable at room temperature with water activities above 0.86. However, if these ingredients are added after the baking process as in the case of a cream-filled éclair, microorganisms may be present that could grow in the product; therefore, refrigeration and shelf life control may be required for microbial quality and safety.

Many fillings, toppings, and other high-moisture components can support the growth of spoilage organisms including sporeformers that survive the cooking process. Meat and vegetable-filled cereal products with high water activities (>0.94) and neutral pH generally require time and temperature control because the baking process can activate and permit growth of sporeformers such as *Clostridium botulinum* that are present in these ingredients (USFDA, 2001).

Microbial growth can spoil foods in numerous ways, through its metabolism and resulting by-products that can alter flavor, odor, appearance, and texture of products. Products such as cakes with cream fillings or pies that contain meat, fish, or vegetables can also become potential safety hazards to consumers if not packaged, stored, and handled correctly (Fellows, Axtell, & Dillon, 1995). For example, cream or custard-filled baked products have been implicated in foodborne illness because of the growth of *Bacillus cereus* and *Staphylococcus aureus*.

Spoilage Prevention and Control

Microbial content of nongrain-based components is important for reducing spoilage rates in many types of compound products. Any high-moisture components are of high risk for spoilage if not of suitable quality upon receipt and if not properly stored and processed. Appropriate care and attention to microbial content of ingredients, time/temperature control, cleaning and sanitation, and good manufacturing practices are essential with these products (Fellows et al., 1995).

Mold growth can contribute to lipolytic rancidity in products having lipid-based components such as cream fillings, butter, margarine, desiccated coconut, or nuts. In this case, specific control of mold growth and its generation of lipase in ingredients or finished product may be important for increasing the overall stability and shelf life.

Techniques to suppress microbial growth can be used to extend shelf life of compound products. With flour confectionary products, e.g., humectants, such as glycerol, sorbitol, salt, or sugar, can be used to reduce water activity while maintaining "moist" texture. Antimicrobial additives such as potassium sorbate and various organic acids can also be used to extend shelf life of compound products.

Methods for Determination of Spoilage Microorganisms Associated with Cereal Products

Aerobic plate counts, coliform counts, and *E. coli* counts can be useful for indicating sanitary quality of equipment and the manufacturing environment. These counts can also be helpful for assessing overall safety and quality of cereal products. As a note of caution, the finding of these bacteria in cereal products, especially those made from raw grains not subjected to a lethality step, does not necessarily indicate health concern. Coliform and *E. coli* bacteria are known to be present in grains and other products not necessarily associated with illness hazards (Sperber & NAMA, 2007).

Determination of mold and yeast counts can also be helpful for assessing sanitary quality of equipment and manufacturing environments. Quantitative counts of mold can also be useful for assessing quality of air and for locating and eliminating sources of mold contamination.

Since some cereal products may contain moisture levels that prevent all but the most tolerant yeast and mold, tests selective for osmotolerant yeast and mold can be useful.

Quantitative determination of rope spores on equipment, in dough, or in finished product can be used to assure lack of buildup from these microorganisms or to evaluate a cause of spoilage.

Lactic acid bacteria counts can be determined as a measure of risk for causing spoilage of certain cereal products.

Methodologies for the above purposes are described in detail in *Compendium of Methods for the Microbiological Examination of Foods* (Downes & Ito, 2001).

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Microbiological Spoilage of Beverages

Kathleen A. Lawlor, James D. Schuman, Peter G. Simpson, and Peter J. Taormina

Introduction

Commercially packaged, non-alcoholic, ready-to-drink (RTD) beverages comprise a diverse group of products, both carbonated (sparkling) and non-carbonated (still), that appeal to consumers of all ages and provide refreshment, hydration, energy, and nutrition at home and "on-the-go." Examples of such products include purified, mineral, and spring waters, flavored or enhanced waters, colas, fruit-flavored sodas, sports and energy drinks, fruit or vegetable juices, teas, coffees, smoothies, dairy and yogurt drinks, and fusion beverages (hybrid products that bridge multiple beverage categories).

While carbonated beverages dominate the global RTD beverage market, outnumbering non-carbonated beverages by a factor of 6 to 1 (Anonymous, 2006a), non-carbonated beverages continue to gain ground in both popularity and market share. Bottled water and energy/health drinks constitute the fastest growing segments of the global beverage market. Worldwide consumption of bottled water grew by 34% between 2000 and 2005, to 163.9 billion liters (Rodwan, 2006), and is projected to grow another 25% by 2009 (Anonymous 2006b). Sales of fortified and functional and "better-for-you" beverages totaled nearly $40 billion in 2005, and are expected to exceed $53.9 billion in 2010 (Fuhrman, 2007).

Ensuring the safety and stability of today's increasingly complex RTD beverages is a challenge for manufacturers and microbiologists alike. Conventional and state-of-the-art processing/packaging technologies, together with established microbiological hurdle strategies, effectively control pathogens and most spoilage microbes in these beverages, but some heat-tolerant and/or preservative-resistant spoilage microorganisms can survive and compromise beverage quality, stability, and shelf life. This chapter will address the spoilage microorganisms of concern in

K.A. Lawlor (✉)
PepsiCo, Inc., 100 Stevens Avenue, Valhalla, NY 10595, USA
e-mail: kathy.lawlor@pepsi.com

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packaged RTD beverages, as well as the sources of contamination, factors influencing spoilage, control strategies, and recommended detection methodologies.

Bottled Water

Bottled water is generally defined as water intended for human consumption that is sealed in bottles or other containers and has no added ingredients, except for optional safe and suitable antimicrobial agents and fluoride within established regulatory limits (CFR, 2008; Posnick & Kim, 2002). Bottled water (also called drinking water) is regulated as a foodstuff in many countries around the world, and its composition, sourcing, treatment, and packaging are governed by the hygienic and quality standards/guidelines established by such bodies as the US Food and Drug Administration (USFDA), Health Canada, European Union (EU), Codex Alimentarius Commission (Codex), and the World Health Organization (WHO) (Dege, 2005). The recognized categories of bottled water vary by country and region and generally include:

- Spring water, which is derived from an underground formation from which water flows naturally to the surface of the earth at an identified location;
- Well water, which is derived from a hole bored, drilled, or otherwise constructed in the ground which taps the water of an aquifer;
- Mineral water, which originates from a geologically and physically protected underground water source, and contains not less than 250 ppm total dissolved solids;
- Purified water, which is produced by distillation, deionization, reverse osmosis, or other suitable processes, and meets the definition of "purified water" in the US Pharmacopeia, 23rd Revision, January 1, 1995; and
- Sparkling water, which after treatment and possible replacement of carbon dioxide, contains the same amount of carbon dioxide that it had at emergence from its source (Posnick & Kim, 2002).

Not included in the bottled water definition/standard of identity are seltzer, soda water, tonic water, and certain sparkling waters, which are considered to be soft drinks.

Regardless of source or type, all bottled waters must be safe to drink – i.e., free from pathogenic bacteria, viruses, and protozoa, as well as certain chemical contaminants identified by WHO as having "health significance" (e.g., arsenic, volatile organic compounds, and pesticides). Water that originates as ground water from protected sources such as naturally flowing springs, aquifers, and deep wells or boreholes may require little, if any, treatment to ensure fitness for consumption. Surface water originating from unprotected sources such as reservoirs, lakes, streams, and rivers, on the other hand, may require extensive treatment for wholesomeness to be assured. The nature and extent of treatment depends upon a number of factors,

including the quality of the raw water supply, the available treatment technologies, and the degree of purification required. Typical treatments may include:

- filtration to remove particulate matter such as suspended solids, colloidal compounds, metal complexes, and biological species;
- microfiltration and/or ultrafiltration to remove turbidity, microbes, and particulates;
- nanofiltration to soften and to remove disinfection by-product precursors;
- reverse osmosis to remove salts and metal ions;
- adsorption onto activated carbon or alumina to remove biological contaminants (including protozoa, mold, algae, bacteria, and viruses), natural organic matter, volatile organic compounds, disinfection by-products, and chlorine or ozone;
- ion exchange to remove hardness and to demineralize;
- chemical/biological oxidation to remove iron, manganese, ammonium, color, and taste- or odor-causing compounds; and
- chemical (ozone, chlorine, or chlorine dioxide) or photochemical (UV irradiation) disinfection to inactivate microorganisms.

These and other treatment technologies used in the production of bottled water are discussed in further detail in the review of Croville and Cantet (2005).

Types and Sources of Spoilage Microorganisms

The principal sources of spoilage microorganisms that can be introduced into bottled water include raw water, extraction and collection equipment, bulk transportation and delivery equipment, treatment system components, finished water storage vessels and piping systems, bottling plant and filling environment, compressed air, carbon dioxide (for sparkling waters), and packaging materials and associated conveying equipment (IBWA, 2003; Senior, 2005a). Depending upon the source, raw water may harbor a wide variety of heterotrophic microorganisms (those requiring organic carbon for growth), including bacteria, yeasts, and molds. Aerobic, Gram-negative bacteria belonging to the genera *Pseudomonas, Flavobacterium, Acinetobacter, Moraxella*, and *Chromobacterium* are widely distributed in shallow and deep groundwater habitats, and are often found at levels of 10^6–10^8 CFU/g (ICMSF, 1998c; Leclerc & da Costa, 2005). Actinomycetes (filamentous, branching bacteria) and fungi are widespread in subsurface habitats, but their populations are typically low, on the order of 10 CFU/g. Several metabolic groups of bacteria can be found in anaerobic aquifer habitats, at levels of 10^5–10^6 CFU/g, including nitrate reducers (*Pseudomonas* spp., *Paracoccus* spp., *Thiobacillus* spp., *Bacillus* spp.), iron (Fe(III)) reducers (*Shewanella putrefaciens*), sulfate reducers (*Desulfovibrio* spp., *Desulfotomaculum* spp., *Desulfobacter* spp., *Desulfonema* spp.), and methanogens (Leclerc & da Costa, 2005).

During the extraction and collection processes, source water can become contaminated by *Bacillus* spp., *Enterobacter* spp., *Klebsiella* spp., *Actinomyces* spp.,

Streptomyces spp., and other microbes present in soil, decaying vegetation, and on drilling/pumping equipment. Pipelines or tankers that convey raw water to bottling plants can serve as additional sources of contamination, if their design or operation allows for pooling/stagnation of water. At the bottling plant, various components of the water treatment system (such as sand/carbon filtration beds, reverse osmosis (RO) membranes, and treated water storage tanks/transfer lines) can be colonized by pseudomonads and other heterotrophic species, which form persistent biofilms that can shed over long periods of time into downstream equipment and finished water. Unfiltered carbon dioxide (used for sparkling waters) and compressed air (used for bottle blow-molding and cleaning) can serve as sources of microbe-laden particulates (such as oil or water droplets), as can bottles, closures, and conveying equipment contaminated with condensate or corrugate dust.

Factors Affecting Spoilage

The natural pH of bottled water varies by source, and generally ranges from 4.5 to 6.5 for carbonated water, 5.0–7.0 for purified water, and 6.5–8.5 for non-carbonated water (Dege, 2005). Spoilage of bottled water is usually the result of regrowth or recontamination. Regrowth is the growth of injured or naturally occurring microorganisms after treatment (or, in the case of non-treated natural mineral waters, after bottling), and is often the result of pseudomonads and other heterotrophs being released from stagnant parts of piped distribution systems or from biofilms on inadequately cleaned/sanitized surfaces of treatment system components (e.g., RO membranes and carbon beds). Recontamination is the entry of contaminants (via leaks, cross-connections, back-siphoning, biofilm shedding) after treatment, and is often the result of a breakdown or loss of integrity in the distribution or treatment system. Regrowth and recontamination are influenced by storage temperature, nutrient availability, and greatly reduced levels (or absence) of residual disinfectants (WHO, 2002; ICMSF, 1998c; Hutchinson & Ridgway, 1977).

Spoilage of bottled water can be characterized by cloudiness, sliminess, stagnant or musty off-odors or off-flavors, and visible mold growth resulting from, the presence of metabolizable organic compounds in the water. Trace minerals, corrugate dust on filling equipment and packaging materials, non-water product residue left in bottling lines, and biofilm constituents can all serve as substrates for the growth of spoilage microorganisms in bottled water.

Prevention and Control Measures

The most important measures for preventing and controlling contamination and spoilage of bottled water are proper maintenance of treatment, processing, and filling systems, adequate cleaning and sanitation, and on-going microbiological monitoring of target spoilage and indicator organisms. Routine audits of treatment and bottling plants should be performed, in order to identify potential microbiological harborage or biofilm formation points, process inadequacies, and testing capabilities or deficiencies. RO membranes in treated water systems should be cleaned

and heat-sanitized per manufacturer's protocols after every use, and replaced as necessary to prevent buildup of contaminating pseudomonads and heterotrophs that can be shed into finished product. Sediment or surface scum removal and backwashing of granular-activated carbon beds, sand filters, etc., are important preventive measures for controlling buildup as well.

In plants that bottle both water and beverage products, adequate flooding and flushing of processing equipment during cleaning and sanitation procedures is required to eliminate yeast and mold contamination potential. Additional sanitation steps on non-dedicated equipment may be necessary, to ensure that specified microbiological and sanitation parameter limits are continuously met. The frequency of any needed additional cleaning/sanitation steps will be determined by data collection and trends. Periodic acid-washing may be required to completely remove residual substances that provide nutrient sources for organisms. Typical parameter measurements may include yeasts, molds, aerobic bacteria counts, coliforms, *Escherichia coli*, *Pseudomonas aeruginosa*, fecal streptococci, and sulfite-reducing anaerobes such as *Clostridium perfringens*.

Visible mold spoilage is often indicative of ineffective sanitation procedures and/or GMPs. Air, wet cardboard or corrugate dust, standing water, clothing, and equipment can all be sources of mold spores that can contaminate conveyors, line covers, crown/closure hoppers, filler/equipment surfaces, ceilings, overhead pipes, air vents, and cracks/crevices/joints of walls and floors/drains in the bottling plant, and eventually find their way into bottled water. Supplemental causes of mold spoilage may include misapplied closures that allow for mold growth on/around the bottle neck/closure, and plasticizers that may leach from PVC bottles into product and serve as a nutrient source for mold growth.

Methods for Detection and Isolation of Spoilage Microorganisms

The standard methods used for detection and enumeration of spoilage and indicator microorganisms are detailed in Downes and Ito (2001) and SMEWW (1998). The microorganisms and methods typically of interest in bottled water include:

- Heterotrophic (aerobic) plate count
- Yeast and mold counts
- Coliforms and *E. coli* counts
- *P. aeruginosa*
- Fecal streptococci
- Sulfite-reducing anaerobes

Carbonated Beverages

This category of products – often referred to as carbonated soft drinks or CSDs – includes shelf-stable, acidic (pH typically 2.5–4.0) beverages that have been carbonated in a range of 1.5–5.0 volumes of carbon dioxide; the typical carbonation level

is about 3.0 volumes. CSDs contain carbonated water and most, if not all, of the following ingredients: nutritive or non-nutritive sweeteners, natural or artificial flavors, acidification agents, colors, emulsifiers, stabilizers or viscosity-producing agents, foaming agents, fruit juice, and chemical preservatives. Nutritionally fortified CSDs may also contain added vitamins, minerals, antioxidants, protein, fiber, and a wide variety of functional or bioactive compounds, such as omega-3 fatty acids, ginseng, caffeine, taurine, and plant sterols (Goel-Lal, 2007). The vast majority of CSDs are chemically preserved and cold filled, but some CSDs are nonchemically preserved and require thermal processing in order to ensure microbiological stability.

Cold-Filled Preserved CSDs

Examples of cold-filled preserved CSDs include colas, fruit- or juice-flavored sodas, ginger ale, root beer, and flavored/enhanced sparkling waters. These beverages are typically formulated with chemical preservatives, and are cold-filled into PET, glass bottles, or aluminum cans.

Types and Sources of Spoilage Microorganisms

To spoil a CSD, a microorganism must be capable of surviving and growing in an acidic (generally pH< 4.0) environment that is depleted in oxygen and rich in carbon dioxide. Molds generally do not grow under depleted oxygen or in the presence of significant amounts of carbon dioxide; hence. they are not considered major spoilers of CSDs and will not be discussed further. Likewise, most bacteria (including pathogens) are not considered significant causes of CSD spoilage because of their intolerance to acidic environments. Yeasts and aciduric bacteria can survive and grow in the physical and chemical conditions of a CSD and are, therefore, the main spoilage agents of this type of beverage.

Yeasts: Yeast cells can enter the plant environment from many different sources. Ingredients, equipment, packaging, and personnel are the major vehicles for potential introduction. Elevated yeast populations in bottled soft drinks may be linked to infrequent or inadequate use of sanitizer or heat in CIP systems, flaws in equipment design, inadequate control of yeasts in incoming ingredients, or lack of control of general plant hygiene leading to cross-contamination of processing equipment. Their osmophilic nature enables yeasts to survive and grow in sugars, syrups, and product residues. Generally, high sugar concentrations can increase the heat resistance of yeast ascospores (McKelvey, 1926; Put, De Jong, Sand, & van Grinsven, 1976), but both acid and carbonation decreases thermal resistance (Aref & Cruess, 1933). If incidental contamination is not addressed with rigorous sanitation and good manufacturing practices (GMP), yeasts may become indigenous in bottling plants and eventually contaminate products.

Ingredients such as sweeteners, flavorings, stabilizers, cloudifiers, and fruit juice concentrates may contain elevated populations of yeasts when entering the plant. These ingredients and their contaminants will be diluted upon proportioning with

water and other ingredients, but the distribution of yeast cells and associated spoilage risks are increased compared to those associated with ingredients having lower incoming yeast loads. Since yeasts are susceptible to heat and to most sanitizers used in CIP treatment of processing lines, they can be eliminated by adequate treatment of linear portions of processing lines prior to production if water quality control factors are in place. However, areas such as filler valves, dead legs in piping, and tanks/lines that are not self-draining represent key harborage points in bottling plant equipment that require extra attention, as CIP processes may not adequately remove product residues from these areas.

Yeasts are not likely to be introduced by air into product at the filling stage. However, packaging and closures can be sources of yeasts if exposed to moisture during storage prior to use. Therefore, proper protected storage and handling of preformed packaging as well as adequate package rinsing prior to filling is essential to limit introduction of yeasts into the product during filling.

Lactic Acid Bacteria: Lactic acid bacteria are facultative, anaerobic, Gram-positive bacilli or cocci. They are the most common spoilage bacteria found in CSDs. Although many species of lactic acid-producing bacteria can grow in CSDs, *Leuconostoc mesenteroides* and *Lactobacillus paracasei* are the most common causes of bacterial spoilage of these products (Stratford, Hofman, & Cole, 2000). Lactobacilli typically enter a plant from juice ingredients, raw materials (including sweeteners, dairy-derived proteins), and packaging materials, and can quickly colonize poorly sanitized equipment surfaces.

Acetic Acid Bacteria: The acetic acid bacteria are Gram-negative short or coccoidal rods that exhibit strictly aerobic metabolism. Acetic acid bacteria therefore only grow and spoil CSDs that have lost their anaerobicity via loss of carbonation or oxygen permeation through PET. Two genera of acetic acid bacteria are responsible for CSD spoilage: *Acetobacter* spp. and *Gluconobacter* spp.

Factors Affecting Spoilage

The pH of CSDs is normally below 4.0 and, as such, is below the pH range of growth of most bacteria. Thus, bacterial spoilage of CSDs is only of limited concern, and restricted to acidophilic genera such as *Lactobacillus* spp. and acetic acid bacteria (*Gluconobacter* spp. and *Acetobacter* spp.). In contrast, fungi typically have pH optima for growth between pH 3.0 and 6.5, and are therefore well adapted to growth in the acidic environment of a CSD.

CSDs generally contain between 1.5 and 5.0 volumes of carbonation. It is widely accepted that carbonation levels greater than 1.5 volumes are inhibitory to beverage spoilage molds and most bacteria, with the exception of acidophilic facultative anaerobic bacteria (e.g., *Lactobacillus* spp.). Yeasts are significantly resistant to carbonation, and have been recovered from products that contain over 3.0 volumes of carbonation.

Spoilage by Yeasts: Yeast spoilage of CSDs is usually characterized by abundant carbon dioxide production which can lead to package bloating and rupture or excessive effervescence on opening, turbidity or sediment, and fermentation-related

(fruity, ester-like, alcoholic) off-flavors and off-odors. Examples of fermentative yeasts causing spoilage of CSDs are shown in Table 1. Osmotolerance and acid tolerance, traits common to most fungi and some bacteria, enable yeasts to survive and grow in CSDs. Osmotolerance is a selective advantage only in sugar-sweetened soft drinks because of the quantity of sugar used and its effects on ºBrix (i.e., percent soluble solids expressed as sucrose). Acid tolerance is required for growth in all CSDs. Carbonation tolerance provides certain yeasts a selective advantage in these beverages. Several *Dekkera* spp. (formerly *Brettanomyces* spp.), including *Dekkera anomala*, can grow in the presence of up to 4.45 volumes CO_2 (Ison & Gutteridge, 1987). *Zygosaccharomyces bailii* and *Pichia* (*Hansenula*) *anomala* are tolerant to up to 3.34 volumes CO_2, whereas *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Zygosaccharomyces microellipsoides* are moderately tolerant to up to 2.23 volumes CO_2. Fructophilly (preference for fructose), as manifested in the *Zygosaccharomyces*/*Torulaspora* superfamily (Stratford et al., 2000), has become a key attribute for yeasts spoiling CSDs in North America, where high fructose corn syrup is the sweetener of choice for caloric drinks.

Nitrogen generation under nitrogen starvation conditions enables growth of some yeasts such as *Saccharomyces* and *Zygosaccharomyces*. Under conditions of nitrogen starvation, eukaryotic cells may resort to disassembly of cell structures, such as mitochondria, for scavenging of their nitrogenous compounds. This phenomenon, called macroautophagy, provides significant advantage to yeasts in CSDs compared to other spoilage microbes. There is evidence that benzoic acid helps inhibit macroautophagy, thereby successfully preserving beverages against certain yeasts (Hazan, Levine, & Abeliovich, 2004).

S. cerevisiae and *Z. bailii* are known for resistance to the primary preservative agents in soft drinks, i.e., benzoic and sorbic acids. Both yeasts can limit intracellular accumulation of weak acids compared to other yeasts, but they rely on different mechanisms to do so. *S. cerevisiae* undergoes ATP-dependent extrusion of dissociated acid molecules and hydrogen ions from the cell using transporter plasma membrane proteins (Piper, Calderon, Hatzixanthis, & Mollapour, 2001). Although adequate for cell survival, this high-energy resistance strategy also results in glycolysis inhibition and limits cell proliferation. By contrast, *Z. bailii* is much more efficient in its preservative resistance mechanisms. It uses modifications to its cell envelope to restrict acid from entering by diffusion, thereby reducing the need to spend energy to export acid or hydrogen ions. The oxidative degradation of sorbate and benzoate and use of these compounds as carbon sources by *Z. bailii* is thought to be catalyzed by a mitochondrial monooxygenase (Mollapour & Piper, 2001). Preservative degradation by *Z. bailii* can occur even in high sugar concentrations and causes significant enhancement of resistance to weak acid preservatives. Rather than inhibit glycolysis, weak acid preservatives can stimulate glycolysis in *Z. bailii*. *Candida krusei* is also believed to be capable of resistance to weak acid preservatives.

Although very little research has been published regarding the metabolism of yeasts in protein-deficient, acidified beverages, general *S. cerevisiae* metabolism is well studied and has been summarized by Moat, Foster, and Spector (2002).

Table 1 Examples of fermentative yeasts causing spoilage of CSDs

Species	Types of CSDs	Defects reported[a]	References
Saccharomyces cerevisiae	Cola, citrus, fruit juice flavored, ginger beer	Sourness, foreign matter, strong fermentation	Back (2005); Mohamed and Mustafa (1978); Oranusi, Ezeogu, and Okolo (1994); Put et al. (1976)
Saccharomyces bayanus	Cola	Flocculation	Mohamed and Mustafa (1978)
Saccharomyces spp.	Orangeade	Destabilization of cloud	Röcken, Finken, Schulte, and Emeis (1981)
Saccharomyces cerevisiae var. *uvarum*	Various, cola	Strong fermentation	Back (2005); Put et al. (1976)
Saccharomyces kluyveri	Various	Fermentation	Back (2005)
Zygosaccharomyces bailii	Various	Strong fermentation	Back (2005)
Zygosaccharomyces florentinus	Cola	Off flavor	Back (2005)
Zygosaccharomyces fermentati	Orange soda		Sand and van Grinsven (1976)
Zygosaccharomyces microellipsoides	Various	Fermentation	Back (2005)
Dekkera bruxellensis	Cola		Jong, Lee, and Bengston (1985)
Dekkera naardenensis	Carbonated water and soft drinks	Acetic acid	Back (2005); Jong and Lee (1986)
Dekkera anomala	Fruit-based soft drinks, ginger ale		Smith and van Grinsven (1984); Put et al. (1976)
Torulaspora delbrueckii			Sand, Kolfschoten, and van Grinsven (1976); Back (2005)
Candida krusei	Cola	Sedimentation	Mohamed and Mustafa (1978)
Candida boidinii			Put et al. (1976)
Candida (*Torulopsis*) *stellata*	Orange soda		Sand and van Grinsven (1976)
Pichia (*Hansenula*) *anomola*	Cola	Slight haze or sediment, surface ring or pellicle, off-flavor	Back (2005)

[a] Yeast growth in CSDs is accompanied by fermentation with CO_2, alcohol formation, and usually turbidity and/or sedimentation. Other specific defects are species- and product-dependent.

S. cerevisiae uses the Embden Meyerhof-Parnas (EMP) pathway for glucose metabolism under neutral to slightly acidic pH and anaerobic environments. The major products formed under these conditions are carbon dioxide and ethanol. In high glucose concentrations, as would be encountered in high fructose corn syrup or sucrose-sweetened beverages, *S. cerevisiae* cells undergo glucose repression. The tricarboxylic (citric) acid cycle is only utilized by growing yeasts in chemostatic conditions, which could not be maintained in beverages supporting growth in closed containers. Some of the glucose which is consumed may enter the oxidative pentose-phosphate pathway under certain conditions, although it has been difficult to pinpoint the relative importance of this pathway in relation to the EMP pathway. Interestingly, modifications of the pentose-phosphate pathway may be used for carbohydrate utilization by *Acetobacter* spp. and *Gluconobacter* spp., which are also spoilage agents of soft drinks. The products and by-products of these processes lead to alteration of the sensory characteristics of beverages. All yeasts isolated during a survey of spoiled carbonated beverages could ferment glucose to acid and gas when cultured (Turner, 1925). Growing yeast cells may respire only 3–20% of catabolized sugar and the remainder is fermented with production of CO_2 and ethanol.

Spoilage by Lactic Acid Bacteria. L. mesenteroides can ferment glucose and fructose to produce carbon dioxide, ethanol, lactic acid, and diacetyl. In addition, *L. mesenteroides* can produce dextran from sucrose. *L. paracasei* ferments glucose and fructose. Spoilage by lactic acid bacteria can cause a variety of different appearance changes, including cheesy notes (from diacetyl production), loss or decrease in astringency, as well as a loss of carbonation. Spoilage by *L. mesenteroides* can also lead to the product becoming filamentous or ropy, due to the production of dextran.

Spoilage by Acetic Acid Bacteria: *Acetobacter* spp. and *Gluconobacter* spp. produce acids from glucose and have an optimum temperature growth range of 25–30°C, but typically do not grow above 37°C. Beverages spoiled by this group of bacteria typically have a vinegary or acidic off-taste and may be slightly hazy. Acetic acid bacteria are commonly found in bottling plants, particularly in pools of spilled product or on neglected equipment surfaces.

Prevention and Control Measures

As with all RTD beverages, the physical and chemical properties of a CSD and its ingredients, combined with processing and manufacturing conditions, influence the type and magnitude of beverage spoilage. If correctly managed, these physicochemical attributes combine to inhibit microbial growth and produce a safe and stable product, susceptible only to rare spoilage events. Figure 1 illustrates locations at which microorganisms can enter and contaminate product in a typical CSD production process. Awareness of common CSD contamination vectors (as described below) is necessary for assessing the risk of microbiological spoilage and applying appropriate control measures.

Ingredient and Beverage Properties: Controlling microbial contamination levels in CSD ingredients is important to minimize the potential for finished beverage spoilage. Water is used both as an ingredient (beverage water) and a utility (process

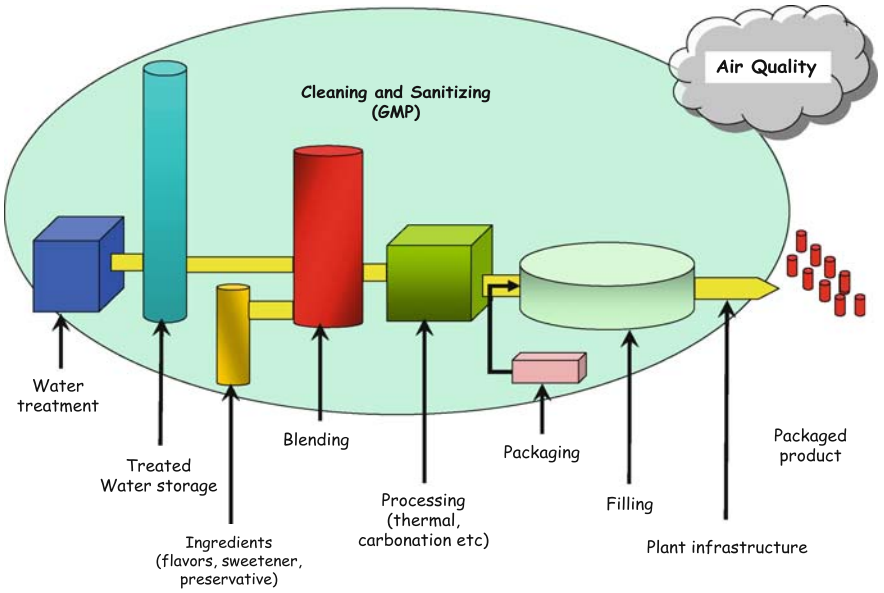

Fig. 1 Overview of carbonated soft drink production

water) in the manufacture of CSDs and other beverages. Its quality and chemistry are of critical importance in ensuring finished beverage safety, quality, and stability, as well as optimal equipment condition and operational performance. Impurities such as turbidity, microorganisms, dissolved/suspended solids, carbonates/bicarbonates (which contribute to alkalinity), calcium/magnesium ions (which contribute to hardness), chlorines/chloramines, and heavy metals, can result in equipment degradation/failure, product defects (e.g., off-flavors/-odors, cloudiness or haze, sedimentation, flocculation, color fading, changes in pH or titratable acidity, microbiological instability), and potential consumer health concerns. Beverage and process water are therefore treated by a variety of means in order to remove these impurities. Refer to the "Bottled Water" section of this chapter, and the review of Croville and Cantet (2005) for details of water treatment technologies.

Incoming municipal or well water is typically subjected to a disinfection step (e.g., chlorination or UV irradiation) that significantly reduces the incoming microbial load. Together with frequent, typically daily, back-flushing of components of the water treatment system and scheduled hot sanitation of carbon towers, this prevents most bacterial colonization and biofilm buildup that could detach from equipment and piping surfaces during beverage manufacture and result in high bacterial cell densities in packaged product.

With the exception of artificially sweetened beverages, CSDs normally contain 7–12% (w/w) sweetener. Depending upon a variety of factors, including product type and the geographic location of the production facility, sweetener is added as a syrup (e.g., high fructose corn syrup) or as a granulated sugar that is

subsequently dissolved during manufacturing. Sugar in the final beverage is a significant source of fermentable carbohydrate for microorganisms that can grow in carbonated acidic environments. Moreover, in many cases, the type of fermentable carbohydrate present will dictate the species of microorganism present in the spoiled beverage. If handled incorrectly, ingredient sugar can be a significant vector for introducing spoilage yeasts into a CSD.

Most sugar syrups used in a CSD production facility are approximately 67°Brix and have low water activity (approximately 0.865). Sugar syrups are therefore susceptible to spoilage by osmophilic yeasts. Populations of slow-growing yeasts do not normally reach a sufficiently high cell density to have spoilage implications for finished beverages. However, if the water activity of the syrup increases during storage, then growth of yeasts may accelerate and sufficient cells can carry over into the finished beverage and cause spoilage. To prevent more rapid growth of osmophilic yeasts, it is important to prevent condensate formation inside syrup-holding tanks. Microenvironments of higher water activity caused by drops of condensate allow more robust growth of yeasts already present in the syrup.

Granulated sugar has a very low water activity and therefore does not support the growth of microorganisms. Maintenance of the low water activity of granulated sugar is critical to its microbiological stability. GMPs related to dry storage of the ingredient ensure that the ingredient does not absorb moisture.

Other ingredients, such as acids, flavorings, minerals, preservatives, typically do not contribute significant microbial loads to the manufacturing process; correct storage and handling will ensure their stability. Note, however, that nitrogen, phosphorous, and mineral contents of these other ingredients can have an influence on the microbiological stability of CSDs. Organic flavorings used in CSDs contribute low levels of nitrogen to the beverage matrix; this is usually the only source of nitrogen in CSDs and, therefore, most CSDs contain very low nitrogen levels. An important exception are beverages artificially sweetened with Aspartame. In this case, nitrogen may be available to microorganisms via decomposition of aspartame to phenylalanine. Cola-type beverages contain significant amounts of phosphorous in the form of phosphoric acid but, in general, CSDs contain relatively low levels of phosphate. CSDs also historically have had low mineral and vitamin contents. In recent years, however, the trend toward health and wellness beverages has resulted in many new CSD formulations having added calcium, zinc, vitamin B complexes, and vitamin C, for example. Addition of these minerals and vitamins may increase the sensitivity of such beverages to microbial spoilage.

Weak acid preservatives are the most common preservatives used in CSDs. Both benzoic acid and sorbic acid inhibit microbial growth by causing an imbalance in the intracellular pH. Importantly, some yeasts are resistant to benzoic and sorbic acids and, hence excellent sanitation practices must be followed in order to prevent spoilage by these microorganisms.

Processing and Manufacturing Conditions: Plant production equipment, process environment, and packaging materials have the potential to be significant sources of contamination in CSD materials. Ineffective cleaning and sanitation practices can

result in product residues on plant equipment that, in turn, provide growth substrates for acidophilic microorganisms. Effective sanitation is crucial to a CSD operation. Two types of sanitations are employed in CSD bottling plants: hot sanitation using hot water at about 85–90°C, and chemical sanitation using one of a variety of chemicals (e.g., peracetic acid, chlorine). Hot sanitation is generally recognized as more effective than chemical sanitation because the penetrative effect of heat kills microorganisms that colonize surfaces (e.g., reverse side of gaskets, valves, filler head components) which would otherwise not be exposed to chemical sanitizers. Each sanitation method has limitations. Certain microbial structures (e.g., heat-resistant mold ascospores and acidophilic bacterial spores) are resistant to heat and may not be inactivated by hot sanitation. Also, some microorganisms are thought to develop resistance to chemical sanitizers over the course of time. To avoid this possibility, chemical suppliers usually recommend alternating sanitizers on a routine basis. Regardless of the sanitation method, effective sanitation of CIP systems is dependent upon appropriate process monitoring of temperature, chemical concentration, flow rate, and exposure time.

For CSDs, mold contamination via air that has a high microbiological load is not significant. There is some evidence that insects may be an airborne vector of yeast contamination. To prevent airborne contamination, filling room air filters should be changed on a regular basis and, ideally, the filling room should have a positive air pressure to prevent air flow into the room.

Microbiological assessments conducted by these authors demonstrate that nonreturnable glass and PET bottles have very low microbial counts, usually less than 10 CFU per package. Due to the low microbial load, most plant operations do not sanitize nonreturnable containers for CSD production. Although plants may use chlorinated water to rinse particulates (e.g., cardboard fibers) from empty bottles, this should not be considered as a sanitation step for packaging materials. Instead, the chlorine is present in the water to keep the feed line clear of biofilms that could slough-off and contaminate bottles during the rinsing process.

Returnable glass bottles (RGB), on the other hand, are a significant reservoir of yeasts in a CSD plant. Moreover, residual preserved product in RGB provides a natural process for selection and enrichment of preservative-resistant yeasts. In addition, the process of washing RGB produces large amounts of condensation and standing water in a CSD plant – conditions that support growth of spoilage microorganisms. CSD plants can minimize contamination risk from returnable bottles by storing unwashed bottles away from blending and filling areas. GMPs that limit the amount of water and condensation associated with bottle washing also help reduce microorganism load associated with the washing process.

Pasteurization is regarded as the most effective way to control spoilage in high-acid beverages. In geographies where the microbial content of certain ingredients (e.g., liquid sugar) is high, or where there are strict limits on preservative concentration, CSD manufactures may pasteurize sugar syrup prior to proportioning. Only in rare cases will a manufacturer pasteurize beverage-strength, cold-filled, preserved CSD products.

Methods for Detection and Isolation of Spoilage Microorganisms

Membrane filtration is the standard industry method used for detection of yeasts, molds, and aciduric bacteria in filterable ingredients, CSDs, and environmental samples (swabs and sanitation rinse waters), because of the method's simplicity and ability to detect low levels of the target microbes. M-green yeast and mold medium is used for detection of yeast, mold, and aciduric bacteria; preservative-resistant yeast (PRY) medium is used for detection of highly preservative-resistant *Zygosaccharomyces* spp.; and *Brettanomyces* selective medium (BSM) is used for detection of preservative-sensitive, but highly carbonation-resistant, *Brettanomyces* spp. For nonfilterable beverage and ingredient samples, pour plating onto orange serum agar (pH 5.5) for detection of aciduric bacteria, acidified potato dextrose agar (pH 3.5) for detection of yeasts and molds, non-acidified potato dextrose agar for detection of acetic acid bacteria, and MRS agar for the detection of lactic acid bacteria, may be used (Downes & Ito, 2001).

Thermally Processed Non-Preserved CSDs

Examples of thermally processed non-preserved CSDs include energy drinks, organic or "all natural" sodas, and sparkling juices, that are formulated to pH 2.5–4.0 and do not contain preservatives. These beverages are carbonated with 1.5–2.5 volumes of CO_2, filled cold into glass or heat-stable plastic bottles and/or aluminum cans, and tunnel-pasteurized (65°–75°C for 10–20 min) to ensure microbiological stability. Products in this category may contain 5–10% (or more) fruit juice or juice blends.

Types and Sources of Spoilage Microorganisms

The principal spoilage microbes of concern for thermally processed non-preserved CSDs are yeasts – such as *Saccharomyces* spp., *Pichia* spp., and *Kloeckera* spp. – that form heat-resistant ascospores capable of surviving pasteurization, and germinating and growing in the finished beverages (Pitt & Hocking, 1999). $D_{60°C}$-values of 5.1–17.5 min have been reported for *S. cerevisiae* ascospores (Put & deJong, 1982b). These and other ascospore-forming yeasts are often soilborne and may originate from ingredients, environmental growth niches in processing plants, and packaging materials. Certain beverage ingredients, including sweeteners, fruit juices/concentrates, herbals/botanicals, and minimally processed or "all natural" flavors/colors/extracts, are particularly susceptible to contamination by yeast ascospores. Bottles and closures, and cans and lids may also be sources of yeast ascospores that can contaminate beverages, particularly if these packaging materials are stored unprotected from dirt, dust, and other sources of ascospores (e.g., fruit-washing operations, RGB operations) in the processing and filling environments.

Other heat-resistant spore-forming microorganisms, such as heat-resistant molds and thermoacidophilic bacilli, may be present in thermally processed, non-preserved

CSDs, but typically do not pose spoilage concerns, because of the inhibitory effect of beverage carbonation.

Factors Affecting Spoilage

Because of their acidity, carbonation level, and substantial quantities of readily fermentable carbohydrates, energy drinks, natural sodas, and sparkling juices provide favorable environments for yeast growth. Spoilage manifestations typically include abundant CO_2 production (with the potential to result in package rupture/explosion), off-flavor/off-odor (ethanol, ester, organic acid, acetaldehyde) generation, cloudiness/turbidity, and sediment or pellicle formation (ICMSF, 1998c). Poor harvest or postharvest practices, insufficient pretreatment or pasteurization, and improper handling of many natural/organic ingredients (including juices/juice concentrates, sugar syrups, and herbal extracts) can significantly increase incoming levels of sporogenous yeasts that can potentially overwhelm the finished beverage thermal process. Inadequately designed or delivered tunnel pasteurization processes can fail to inactivate ascospores, resulting in increased spoilage risk. Packaging materials that are not thermally rated for tunnel pasteurization applications can undergo permanent deformation, leading to loss of seal integrity and carbonation, and the potential for post-pasteurization contamination.

Prevention and Control Measures

Ingredient monitoring and control and proper design and delivery of the thermal process, including uniform temperature control throughout the pasteurizer heating zones, are the principal means used for preventing yeast spoilage in thermally processed CSDs. Container/closure composition and thermal stability must be appropriate for the intended thermal process, if package and seal integrity is to be achieved and maintained. Because of the need to accommodate product headspace expansion, flood filling is not an option for tunnel-pasteurized CSD products.

Post-process contaminants such as yeasts, molds, or aciduric bacteria in cooling water sprays are generally not a threat to beverage stability, because product carbonation provides sufficient over-pressure to prevent ingress of these microbes into the finished beverage container during cooling. Failure to promptly and adequately remove cooling water from package exteriors before casing, palletizing, and shrink-wrapping, however, can lead to aesthetic problems such as mold growth or slime development, especially on bottle closure threads. Rough handling or stacking of cooled product before the package seal is set should be avoided, as this increases the potential for rupture or other breach of seal integrity that can result in a loss of carbonation and subsequent beverage spoilage.

Methods for Detection and Isolation of Spoilage Microorganisms

The standard industry procedure for assessing the microbiological stability of tunnel-pasteurized products is evaluation of commercial sterility. This evaluation

involves incubating the product under controlled-temperature conditions (commonly 28–30°C for 14 days), followed by visual examination, pour-out, and screening for evidence of spoilage (e.g., cloudiness, off-odor, excess effervescence). Should spoilage be evident, microscopic examination and/or plating of product can be performed to determine the responsible microorganism(s). Malt extract agar, MY50G agar, or MY10-12 agar (Pitt & Hocking, 1999) can be used for pour plating of samples, followed by incubation at 25°C for 3–5 days.

Non-carbonated Beverages (NCBs)

Chilled Fruit and Vegetable Juices

The commercial chilled fruit and vegetable juice category is diverse and includes products with juice contents ranging from approximately 20 to 100%. Exceptions to this trend include certain lemonade and "limeade" products (10–12% juice content) and a variety of reduced-calorie or "light" juice products containing only 4–10% juice. Juice products containing less than 100% juice are commonly referred to as juice drinks, juice beverages, or punches. Citrus juices (orange, grapefruit) are the most widely consumed variety, accounting for over 50% of the global fruit juice trade. Other high-volume items include apple juice, pineapple juice, grape, and berry fruit juices and a variety of juice blends (Varnum & Sutherland, 1999). The most commonly consumed vegetable juices are tomato and carrot juice, followed by beet juice and several minor components used to enhance the flavor, color, and vitamin content of vegetable juice blends.

In recent years, consumer health and wellness trends have prompted the development of a variety of juice products fortified with specific nutrients such as B vitamins, extra vitamin C and other antioxidants, minerals (especially calcium and potassium), and components added to help manage serum cholesterol levels (e.g., plant sterols). Other additives commonly used in the formulation of commercial juice drinks include sweeteners (nutritive and non-nutritive), acidulants (e.g., citric acid, malic acid), natural flavors and colors, and emulsifiers. Of these ingredients, only calcium fortification has a substantial effect on the microbial spoilage potential of juice products, due to increased product pH and buffering capacity compared to nonfortified juices. The pH ranges and major acid constituents of various juice commodities are listed in Table 2. Lemon and lime juices are among the most acidic commodities used in juice processing (mean pH approximately 2.3). In commercial practice, the titratable acidity of juice concentrates with pH values above approximately 4.3 is typically reduced via blending practices or the controlled addition of citric or malic acids, with appropriate labeling as required under local regulations.

Chilled fruit juices and juice drinks are commonly packaged in single- or multiserve bottles made of food-grade plastic such as PET or HDPE. Paperboard "gable-top" cartons represent another major packaging category. In the early years after gable-top cartons were developed, chilled shelf lives of 4–5 weeks were common in the US marketplace. The widespread use of cartons incorporating oxygen barriers

Table 2 Typical pH values and the naturally occurring organic acids of fruit juices

Fruit juice	pH range	Major acid types
Apple	2.9–4.2	Malic, citric
Cherry	3.2–4.4	Malic, citric
Grape	2.9–4.5	Tartaric, malic
Grapefruit	2.9–3.6	Citric
Kiwi	2.8–4.0	Citric, malic
Lemon	2.0–2.6	Citric
Lime	1.6–3.2	Citric
Mango	3.7–4.4	Citric, tartaric
Orange	3.0–4.3	Citric, malic
Pear	3.0–4.6	Malic, citric
Pineapple	3.1–4.0	Citric, malic
Raspberry	2.5–3.1	Citric
Strawberry	3.0–3.9	Citric
Tomato	3.9–4.5	Malic, citric

Sources: Data from Stratford et al. (2000); Landry, Schwab, and Lancette (1998)

has allowed the shelf life of pasteurized, chilled citrus juices to be extended to 9–10 weeks at $\leq$4°C, without significant deterioration of key citrus flavor components or vitamin C content (Narciso & Parish, 1997). Shelf life extensions of this kind place greater pressure on juice processors to produce microbiologically "clean" products and to maintain consistent refrigeration temperatures during warehouse storage and distribution. This is because psychrotrophic spoilage microorganisms – if present – will have ample time to multiply to levels triggering sensory spoilage and consumer complaints.

In the United States, microbial spoilage of chilled or ambient high-acid beverages may be the basis for a class III recall action by the US Food and Drug Administration. The economic costs of such spoilage events are difficult to quantify; however, the following examples help to illustrate the potential scope of the problem when it does occur. In five randomly selected recent class III recalls of juice products removed from store shelves in the United States recently due to "mold contamination" or "fermentation", the estimated lost retail value of the affected production lots ranged from $57,000 to $7,136,000 (2006 US dollars), with an average value of $2,262,000 (FDA, 2005). Another way to consider the economic impact of chilled juice spoilage on juice manufacturers is as follows. If a single standard half-gallon (1.9 l) carton filler operates out of specification for just 4 h because of post-pasteurization contamination with yeasts, molds, or aciduric bacteria, the retail value of the cartons produced – and now at risk of spoilage and financial losses – ranges from $116,000 to $134,000.

Types and Sources of Spoilage Microorganisms

The nature of chilled single-strength juice products – i.e., low pH/high acidity, reduced oxygen content, and low protein and amino nitrogen content – renders them susceptible to spoilage by fermentative yeasts, molds, and a small number

of aciduric bacteria. The microorganisms of greatest concern during chilled warehousing and distribution of juices are those capable of multiplying at temperatures at or below 5–7°C. Fruits and vegetables commonly used in juice processing are exposed to a variety of potential spoilage microorganisms during agricultural production, and harvesting, and transportation to fruit sorting and juice extraction facilities. The primary source of these microorganisms is the soil, both in the immediate vicinity of the field or grove and in the surrounding environment. Environmental vectors of soilborne microorganisms include strong wind currents, rain/moisture droplets, and insects, which also may harbor significant yeast populations (Stratford, Hofman, Cole, 2000). During the growing and postharvesting stages, fruits and vegetables are susceptible to contamination with a wide range of soilborne yeasts, including ascospore-forming genera such as *Saccharomyces*, *Debaryomyces*, *Hansenula*, *Kluyveromyces,* and *Pichia*. Major fungal groups of concern include ascospore-producing, heat-resistant molds (e.g., *Byssochlamys* spp., *Talaromyces* spp., *Neosartorya* spp.) and molds that frequently contribute to fresh fruit spoilage (Splittstoesser, 1987; Parish & Higgins, 1989). Major mold genera responsible for the spoilage of industrially important fruit crops include:

- *Alternaria* – Tomatoes, apples, stone fruits*, citrus
- *Botrytis* – Citrus, apples pears, berry fruits (especially strawberries)
- *Cladosporium* – Stone fruits, raspberries, grapes, citrus
- *Fusarium* – Citrus fruit
- *Geotrichum* – Tomatoes, citrus, peaches
- *Penicillium* – Citrus, apples, grapes, pears, stone fruits
- *Rhizopus* – Apples, pears, stone fruits, berry fruits

Within the category of pasteurized and chilled single-strength citrus juices, the predominant fungal species isolated are the molds, *Penicillium* spp., *Cladosporium* spp., *Aspergillus niger*, *Aspergillus fumigatus*, *Botrytis* spp., and the *black yeast*, *Aureobasidium pullulans* (Parish & Higgins, 1989). These fungi are readily destroyed by typical industry pasteurization procedures; hence, their presence in packaged products represents post-thermal process contamination introduced by contaminated packaging materials, environmental air and dust (i.e., in the package filling zone), or poorly sanitized and maintained packaging equipment (e.g., carton mandrels, filling nozzles, unsanitary cap/fitment tracks, sealing jaws).

With the exception of certain spore formers (e.g., *Bacillus* spp., *Alicyclobacillus* spp., *Clostridium butyricum,* and *Clostridium pasteurianum*), bacteria are usually present in relatively low numbers on fresh fruit and vegetables after harvesting (ICMSF, 1998a). During extraction, blending, thermal processing, and packaging, other important nonspore forming bacterial spoilage flora may establish a niche

*Peaches, nectarines, plums, apricots, and cherries are all members of the *Prunus* genus, and therefore are closely related. They are referred to as "stone fruits" because the internal seed is large and very hard.

in the juice-processing environment. These microbes include heterofermentative lactic acid bacteria (e.g., *Lactobacillus* spp., *Leuconostoc* spp.), acetic acid bacteria (*Acetobacter* spp., *Gluconobacter* spp., *Gluconoacetobacter* spp., *Asaia* spp.), and certain Gram-negative enterobacteria (Parish, 1991; Yamada et al., 2000; Bott, 1997). The most frequent sources of microorganisms introduced during processing are the fruit extraction equipment, process waters (fruit wash water and flume water, especially if recycled evaporator condensate water is used), and the internal surfaces of major juice-processing equipment (e.g., piping, pumps, valves, evaporator systems, intermediate juice storage tanks). During the filling process, establishing and maintaining the sanitary integrity of the packaging equipment and its local environment is of paramount importance. This is because a variety of common airborne molds are capable of multiplying within both single-strength and concentrated juice products. Efforts must also be made to minimize cross-contamination of juice and concentrate packaging areas, with microorganisms originating from the incoming fruit handling, sorting, and washing areas of the production facility.

Juice-packaging materials such as plastic drum liners and PET or HDPE bottles may also harbor substantial levels of fungal and bacterial spores, if not protected from excessive airborne environmental exposure during manufacture and storage. A significant but underappreciated source of juice spoilage molds is the material used to manufacture paperboard cartons. Narciso and Parish (1997) analyzed internal paperboard fibers from four different carton manufacturers and isolated 40 different viable mold species – primarily from the genera *Penicillium* and *Aspergillus*. Two *Penicillium* species (*P. biforme* and *P. spinulosum*) were isolated from both paperboard fibers and from juice-filled cartons from the same supplier, which were stored at 5°C before analysis.

Another hazard that must be monitored is the microbiological quality of the air within the juice processing and packaging facility. Mold spores tend to predominate in unfiltered air supplies, especially during the spring and summer seasons, when substantial fruit and vegetable harvesting activity takes place. Surveys have shown that molds comprise approximately 95% of the aerial microflora over fruit orchards (Adams, 1964). Juice processors must design their air handling, filtration, and pressure balancing systems to accommodate various geographical and site-specific risks. For example, location of a beverage-processing plant in close proximity to fruit orchards/harvesting areas or corrugate manufacturing facilities will heighten airborne fungal risks substantially. Insect vectors such as *Drosophila* spp. (fruit flies) represent a significant source of yeast contamination in beverage-processing plants (Stratford et al., 2000).

The primary categories of microbial spoilage defects affecting chilled fruit and vegetable juices are summarized in Table 3.

Because of their versatility – i.e., psychrotrophic nature, low pH tolerance, osmotic tolerance, and ability to metabolize carbohydrates in the presence or absence of oxygen – fermentative yeasts represent the greatest spoilage threat to the producers of chilled juice (citrus, apple, grape) and tomato products. Yeasts also have minimal nutritional requirements for growth, including the ability to multiply in the presence of very low levels of nitrogen (0.2–0.5 mg/L) (ICMSF, 1998b). While their primary metabolic by-products of ethanol and carbon diox-

Table 3 Microorganisms responsible for major spoilage defects in chilled juices

Spoilage group (Examples)	Juice spoilage defects	Key metabolic by-products	References
Fermentative yeasts (*Saccharomyces* spp., *Candida* spp., *Hanseniaspora* spp.)	- Off-odors/off-flavors: "yeasty", "fermented", "alcohol" - Turbidity, flocculation sediment, sliminess - Effervescence, bloated/swollen packages	- Ethanol, CO_2, and trace fermentation products (occasionally including diacetyl) - Isoamyl alcohol (3-methyl-1-butanol) - Conversion of ferulic acid (from fruit) to 4-vinylguaiacol	Parish (1991); Parish and Higgins (1989); Kimball (1999); González et al. (2000)
Molds (*Cladosporium* spp., *Penicillium* spp., *Aspergillus* spp., *Botrytis* spp.)	- Visible mold "mats" or mycelial fibers in juice or adhering to the package interior, carton seams, closure or external bottle threads - Subtle off-flavors: "musty" or "stale" - Juice cloud loss due to activity of pectinesterase enzymes	- Slight increases in juice pH due to metabolism of acids - Degraded pectin molecules	ICMSF (1998b); Parish (1991)
Heterofermentative lactic acid bacteria (*Lactobacillus* spp. *Leuconostoc* spp.)	- Off-odors/off-flavors: "sour", "green apple"; sometimes "buttery" or "buttermilk-like" - Turbidity, severe sliminess (ropiness) - Effervescence, bloated/swollen packages	- Production of lactic acid, ethanol, CO_2, and trace amounts of acetic and gluconic acids. - Production of diacetyl (2,3-butanedione) from citrate causing "buttermilk" off-flavors - Extracellular dextran- or levan-based polymers	ICMSF (1998b); Jay (2000a); Drinan, Tobin, and Cogan (1976)

Table 3 (Continued)

Spoilage group (Examples)	Juice spoilage defects	Key metabolic by-products	References
Acetic acid bacteria (*Acetobacter* spp., *Gluconobacter* spp., *Gluconoacetobacter* spp.)	- Off-odors/off-flavors: "sour", "vinegar-like" - Effervescence, bloated/swollen packages	- Oxidation of ethanol to acetic acid (all three genera listed) - Oxidation of acetic acid to CO_2 (*Gluconobacter* and *Gluconoacetobacter*)	Yamada et al. (2000)
Enterobacteria (*Klebsiella* spp., *Citrobacter* spp., *Serratia* spp.)	- Off-odors/off-flavors: "sour", "unclean", or occasionally "sulfur-like" - Effervescence, bloated/swollen packages - Turbidity, sliminess	- Mixed acids (acetic acid, formic acid) - CO_2 - Other trace fermentation products - extracellular capsule material	Bott (1997); Fuentes et al. (1985)

ide are familiar to many juice consumers, yeasts may also produce trace amounts of 4-vinylguaiacol in spoiled citrus juices. Sensory descriptors associated with 4-vinylguaiacol include phenolic, medicinal, "clove-like" or "ham-like" off-flavors and aromas. Kimball (1999) reported that the taste threshold of isoamyl alcohol (3-methyl-1-butanol), a yeast spoilage metabolite in concentrated orange juice, is only 1 ppm. Industry experience has shown that certain yeast species and strains (e.g., *Candida* spp., *Torulopsis* spp., *Hanseniaspora*, and *Saccharomyces* spp.) are true psychrophiles, capable of spoiling single-strength grape and citrus juices stored at constant temperatures as low as 0–1.6°C (Lawrence, Wilson, & Pederson, 1959; Murdock, 1979).

Overt mold spoilage of chilled juices is typically detected in several key areas of commercial packaging: the oxygen-rich headspace area, the inner seams/folds of paperboard cartons, or clinging to the hydrophobic surfaces of plastic closures or the inner surface of plastic pull-rings and fitments used in "easy-pour" cartons of chilled juice. Mycelial strands may also be found floating or suspended in juices subject to mold spoilage. Molds capable of producing pectinesterase enzymes can reduce the stability of the "cloud" (suspended solids, pectic substances) that is characteristic of high-quality pasteurized citrus juices. For example, strains of the common environmental mold *Aspergillus niger* are known to produce polygalacturonase, polymethylgalacturonase, and pectin lyase enzymes (Naidu & Panda, 1998).

Among bacterial genera, the heterofermentative lactic acid bacteria represent the greatest challenge in single-strength chilled juices and juice beverages. Murdock (1979) observed relatively rapid growth of *Leuconostoc* spp. in single-strength orange juice stored at 4.4°C (40°F), 7.2°C (45°F), and 10°C (50°F). For example, off-flavors from fermentation by-products developed in the juice after only 12 days at 45°F in one set of inoculated challenge studies. In addition to typical lactic acid bacterial spoilage defects (i.e., production of lactic acid along with lesser amounts of acetic and gluconic acids, ethanol, and carbon dioxide), certain species are notorious for their ability to produce diacetyl and acetoin as metabolites in spoiling fruit juices. These compounds contribute a highly undesirable buttery or buttermilk-like off-flavor to citrus juices. The sensory detection threshold of diacetyl flavor in orange juice is only 50 ppb (unpublished data). The most important diacetyl- and acetoin-producing lactic acid bacteria include *L. mesenteroides* ssp. *cremoris, Leuconostoc paramesenteroides, Leuconostoc dextranicum*, and *Lactococcus lactis* ssp. *diacetylactis* (Drinan, Tobin, & Cogan, 1976). These species may also cause incipient spoilage in juice concentrates when juice evaporation equipment becomes heavily contaminated with diacetyl-producing strains. A final defect caused by strains such as *L. mesenteroides* ssp. *dextranicum* is the production of copious amounts of extracellular polysaccharides resulting in undesirable changes in the viscosity and "mouthfeel" of spoiled juices. This strain was among the lactic acid bacteria commonly isolated from unpasteurized orange juice by Parish and Higgins (1988).

The final two categories of Gram-negative bacteria listed in Table 3 are of less significance, but occasionally cause spoilage incidents in chilled juices and juice ingredients. The obligately aerobic acetic acid bacterial group includes strains of

importance in the vinegar production industry. Their ability to spoil fruit juices and other acidic beverages is limited to single-strength juices that have a high oxidation–reduction potential and contain at least low levels of ethanol (e.g., due to the presence of other spoilage flora in co-culture, such as fermentative yeasts). Certain psychrotrophic bacteria belonging to the family Enterobacteriaceae, including *Klebsiella* spp., *Serratia* spp., *Citrobacter* spp., and *Cedecea* spp., are capable of multiplying in citrus juices with pH values below 4.3. These strains carry out a mixed-acid fermentation resulting in citrate, acetate, and CO_2 production, along with "unclean" flavor and aroma defects (Bott, 1997). In certain cases, enteric bacteria may produce a sulfur-like off-aroma in spoiled citrus juices (unpublished data). Fuentes, Hazen, López-Torres, and Rechani (1985) isolated *Klebsiella pneumoniae* from orange juice concentrate and determined that it could multiply rapidly in single strength from-concentrate juice at 4°C. This bacterium is a capsule producer, making it both easy to identify on standard microbiological plating media and difficult to remove if biofilm formation is allowed to occur within processing lines, gaskets, and batching systems.

Factors Affecting Spoilage

The critical factors affecting the spoilage potential of chilled juices may be divided into those that are intrinsic or extrinsic to the product. Intrinsic characteristics of beverages include beverage pH, oxidation–reduction potential, water activity, availability of nutrients, the presence of antimicrobial compounds, and competing microflora. Common extrinsic factors include storage temperatures and times, relative humidity conditions during storage, packaging material characteristics, and headspace oxygen levels in both packages and juice storage tanks. Table 4 presents the minimum critical growth requirements of the fungal and bacterial species with greatest potential impact on the microbiological shelf life of chilled juices and juice drinks. Of the intrinsic factors, the pH (including acid type and content) and water activity of the juice or concentrate are the most influential factors affecting spoilage rates. Yeasts and molds present a formidable challenge since they are capable of growth at pH values as low as 1.5–3.5 and at water activity values below 0.89 (most species) and as low as 0.61 (osmophilic yeasts and xerophilic molds).

The latter characteristic is of particular relevance during the transport and storage of juice concentrates and bases with ºBrix levels (i.e., percent soluble solids expressed as sucrose) of 60 or higher. For reference purposes, 70°Brix apple juice concentrate has a water activity (a_w) of approximately 0.76 at ambient, and 65°Brix orange juice concentrate has an a_w of 0.81. Pineapple juice concentrated to 61°Brix has a water activity value of 0.84. If exposed to marginal refrigeration conditions or elevated temperature stress, such products may undergo overt spoilage by molds or incipient spoilage due to growth of psychrotrophic yeast species. For example, Kitchell and Miller (1960) observed rapid growth of the yeast *Candida magnoliae* in 70°Brix orange juice concentrate at temperatures of 16–27°C. In 60°Brix orange juice concentrate the yeast population increased significantly at a constant refrigeration temperature of 4.4°C. Beuchat (1983) published a concise review of

Table 4 Minimum growth requirements of the primary microorganisms responsible for spoilage defects of chilled juices

Spoilage group (Examples)	pH	Water activity (at 25°C)	Temperature	Oxygen Requirement
Fermentative yeasts (most spoilage species)	1.5–3.5	0.88	−5–0°C	Facultative anaerobes
Osmophilic yeasts (e.g., *Zygosaccharomyces bailii, Z. rouxii, Candida glucosophila*)		0.61–0.80		Facultative anaerobes
Molds (most spoilage species)	1.5–3.5	0.80	−10–0°C	Aerobes (with exceptions)
Xerophilic molds (e.g., *Eurotium* spp., *Wallemia*, spp., *Xeromyces* spp.)		0.61		Aerobes
Heterofermentative lactic acid bacteria	2.9–3.5	0.90–0.94	3–10°C	Microaerophiles
Acetic acid bacteria	3.0–4.5	0.91	5–10°C	Obligate aerobes
Enterobacteria (selected acid-adapted, cold-adapted species)	3.6–4.5	0.93–0.95	4–10°C	Facultative anaerobes

Sources: Data from Banwart (1981); Jay (2000b); and industry experience.

the sporulation, germination, and survival behavior of important food and beverage spoilage fungi as influenced by the water activity of the surrounding medium.

Juice spoilage molds and yeasts are also capable of growth at very low temperatures, especially in highly concentrated juices and purees (Table 4). Strategies to minimize fungal spoilage risks include use of high-quality raw materials (with particular attention to fruit selection, sorting, and washing systems), filtration processes where applicable (e.g., apple juice concentrate), pasteurization processes, and hygienically designed drum or tote filling systems. Certain industrial juice concentrates are packaged using carefully selected thermal pasteurization processes coupled with aseptic filling equipment. In certain cases, these products still require either chilled or frozen storage to extend shelf life by minimizing potential for oxidative changes in the color/flavor of the concentrates (e.g., banana puree, mango concentrate, pineapple concentrate). When present, molds generally thrive in aerated juices or in the oxygen-rich headspace of storage tanks and packaging containers (drums, totes, bottles, or cartons). In certain instances, the use of oxygen-impermeable packaging can significantly extend the shelf life of chilled orange juice (Wyatt, Parish, Widmer, & Kimbrough, 1995). Notable exceptions to this trend include molds capable of growth at very low dissolved oxygen concentrations including certain strains of *Fusarium*, *Rhizopus*, *Mucor rouxii,* and *Geotrichum candidum* (Scholte, Samson, & Dijksterhus, 2004).

In comparison to fungi, aciduric bacteria have more exacting growth requirements and have slower growth rates under refrigeration temperatures (Table 4). The

minimum pH values permitting growth of lactic acid bacteria (pH 2.9–3.5), acetic acid bacteria (pH 3.0–4.5), and enteric bacteria (pH 3.6–4.5) are substantially higher than those for growth of yeasts and molds. The osmotic tolerance of aciduric bacteria is such that there is little chance of growth in juices concentrated to greater than approximately 45°Brix (Kimball, 1999). Since most single-strength fruit juices have a_w values of greater than 0.97, all three categories of bacteria listed in Table 3 represent a spoilage threat if any contaminate the freshly extracted juice or diluted juice concentrates. The minimum growth temperature requirements of lactic acid bacteria vary considerably by species and strain. For example, Murdock (1979) determined relatively rapid growth of a *Leuconostoc* strain in single-strength orange juice stored at 4.4–10°C. Industrial experience with single-strength citrus juices has revealed that strains of *Leuconostoc citreum* and *Leuconostoc oenos* can multiply at temperatures as low as 2–4°C in bulk pasteurized juice storage tanks.

Prevention and Control Measures

Production and distribution of high-quality chilled juices and juice beverages requires careful attention to several manufacturing design and quality assurance principles. This is especially true in situations in which high-volume juice filling operations in only a few manufacturing locations are required to supply product to a wide geographical sales area. The six key factors that determine the microbiological shelf life of pasteurized, chilled juices (pH $\leq$ 4.3) may be summarized as follows.

Selection and Use of High-Quality Juice Ingredients: A truism of food and beverage manufacturing is that the quality of the end-product is in large measure dictated by the quality of the ingredients of which it is comprised. Among the critical considerations in selecting raw material suppliers is the ability to provide a juice, concentrate, or puree which consistently meets all relevant physicochemical specifications, sensory requirements, and microbiological specifications. Juice concentrate quality is directly influenced by the fruit selection and sorting practices, fruit-washing or sanitizing procedures, extraction procedures, and any further processing before packaging or bulk transport of the juice concentrate. For example, certain juices can withstand both heat treatment during the evaporation process and a terminal pasteurization of the concentrate as a means of extending nonfrozen shelf-life and/or inactivating specific heat-tolerant microorganisms (e.g., ascospore-forming molds). Examples of industrial concentrate pasteurization parameters are temperatures of >92°C for 30–60 s. In the case of not-from-concentrate juice, the pasteurization process must be adequate to stabilize the juice both enzymatically and microbiologically. To prevent cloud loss or gelation of freshly extracted citrus juices it is necessary to inactivate pectin methyl esterase (PME). PME is generally more heat-stable than the predominant juice spoilage microorganisms in chilled juices. A thermal treatment of at least 95–98°C for 10–30 s has been cited as an effective means to deactivate PME in single-strength, freshly extracted orange juice (Tetra Pak Processing Systems, 1998).

Bulk juices and juice concentrates may be transported to customers or end-users in containers ranging from small packages (pails, drums, totes) to very large storage

tanks or tanker trucks. To assure microbiological control and to avoid incipient spoilage incidents, the sanitary integrity of the juice, base, or concentrate filling operation must be maintained. A robust and well-designed sanitation program for washing and sanitizing tanker trucks, delivery hoses, and hose junctions is also vitally important. Lastly, it is important to consider the effects of juice concentrate pH, a_w, and distribution conditions on the microbiological risk profile of a given juice ingredient. For example, concentrates and bases with water activity values of 0.88 and above will support growth of a wide range of spoilage yeasts, some of which may multiply at very low temperatures (Table 3). Where opportunities exist to remove water from a given base formulation, this may not only improve shipping efficiencies, but also result in a more microbiologically robust raw material. Transportation, storage, and thawing conditions that allow condensate to form on the inside headspace area of tanks or totes can result in localized increases in moisture content and a_w. The net effect is that nonosmophilic yeasts, molds, and even certain aciduric bacteria may multiply in these microenvironments, often resulting in an inferior, organoleptically spoiled juice concentrate or base.

Application of Appropriate Pasteurization Processes: As described previously, the pasteurization of juices is designed to stabilize the product from both an enzymatic and microbiological standpoint, and to "lock in" the fresh flavor that consumers demand. While a number of alternatives to thermal treatment are being explored among the academic and industrial research communities (e.g., ultraviolet light, high-pressure processing, pulsed electric field processing), the use of lethal heat to inactivate microorganisms remains the approach used by the vast majority of commercial juice companies (Sizer & Balasubramaniam, 1999). The time–temperature combinations used by industrial juice processors remain closely guarded secrets and depend heavily on juice composition, the presence and levels of added pulp, calcium and other additives, and whether freshly extracted juice and pectin esterase enzymes are present.

In the United States, juice processors that employ heat pasteurization of juices are required by the Food and Drug Administration to assure a minimum 5-log cycle reduction of the pathogens *E. coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* using a process applied at the facility in which final packaging of the juice takes place (FDA, 2001). Mazzotta (2001) determined *D*- and *z*-values for these pathogens in single-strength apple, orange, and white grape juices adjusted to pH 3.9. The resultant thermal inactivation model indicated that a treatment of 3 s at 71.1°C (*z*-value of 5.3°C) would provide the required 5-D pathogen reduction.

To produce commercially successful chilled juice products, a thermal treatment in excess of the 5-D pathogen performance standard is required, particularly to address the challenge posed by yeast ascospores. Put and DeJong (1982a) reported *D*-values for the yeasts *S. cerevisiae*, *Z. bailii,* and *Kluyveromyces bulgaricus* at 60°C that were 30–350 times greater than those for vegetative cells of the same species. Shearer, Mazzotta, Chuyate, and Gombas (2002) evaluated the heat resistance of a variety of yeasts, nonascospore-forming molds, and lactic acid bacteria in citrate buffer adjusted to pH 3.0, 3.5, and 4.0. *S. cerevisiae* was the most

thermotolerant, and was further evaluated by determining *D*-values at 57–63°C in apple juice, calcium-fortified apple juice, grapefruit juice, tomato juice, and a juice blend containing high fructose corn syrup. In nonfortified apple juice, the holding time required to reduce the viable yeast population by 5 log cycles was 13 s at 71.1°C, more than four times that required for bacterial pathogen inactivation (Mazzotta, 2001). It was noted that calcium fortification of the apple juice (final pH 3.9) increased the heat resistance of *S. cerevisiae* even more. The minimum pasteurization parameters recommended by Parish (1994) included 65–77°C for 30 s for lemon–lime juice, 85–99°C for 1.75–42.6 s for grapefruit juice, and 86–99°C for 1.4 s for orange juice. It is not uncommon for tomato-based juices (pH $\leq$ 4.2) to be pasteurized at temperatures in excess of those listed above, such as 90.6–93.3°C for 2.5–3.0 min (Clavero, 2001).

Use of Properly Designed and Maintained Pasteurized Juice Delivery Systems: This aspect of the process involves the engineering, maintenance, and internal sanitation procedures that together prevent the juice from becoming recontaminated between the exit of the pasteurization hold tube and the filler bowl of the packaging system. Fundamental requirements include the use of appropriate-grade stainless steel and other food-grade construction materials. Sanitary welds must be used to create junctions, and all valves, pipe connection fittings and pumps must be of sanitary (and preferably aseptic) design. Filler supply tanks should be designed so as to be easily cleaned and sanitized, and they must be secured to prevent environmental exposure during production. Tanks that require the introduction of pressurized air require an air-handling system equipped with in-line, redundant microbiological-grade filters. Other common pitfalls to avoid include the presence of "dead legs" within juice or beverage water delivery lines, deterioration of critical wear and tear parts such as gaskets, O-rings, seals, and "pinch" valves, and the failure to properly weld and seal individual tubes within tubular heat exchangers. Foundational to the security of these systems is a cleaning and sanitation program that is effective, well documented, and applied consistently. For example, for internal clean-in-place (CIP) systems to be effective, the chemical concentration, temperature, contact time, and mechanical action (i.e., flow velocity in meters/s) must be tailored to the needs of the specific product(s) and packaging system. Juices fortified with calcium present a unique cleaning challenge. Effective removal of calcium deposits using an appropriate acid-based solution (e.g., phosphoric and/or nitric acid) is essential to prevent calcium from "plating" onto the interior walls of the juice-processing system. If not removed efficiently, such mineral deposits provide a means for microbial/organic biofilms to develop. After CIP and just before beginning the production cycle, sterilization of the system using chemicals or steam-heated water must be carried out using validated parameters.

Use of Sanitary Packaging Materials: Distributors and processors of chilled juices must be aware of the positive or negative contribution that packaging materials can make to the microbiological, chemical, and sensory shelf-life of their finished products. For example, Castberg, Osmundsen, and Solberg (1995) list the following eight criteria that must be met to produce high-quality gable-top cartons for chilled juices:

(i) high standard of hygiene (low microbial bioload);
(ii) good mechanical strength, including internal fibers;
(iii) liquid tightness;
(iv) barrier to light;
(v) low migration of components into the packaged juice;
(vi) barrier to gases and flavors;
(vii) good sealability; and
(viii) acceptable mechanical performance and efficiency in carton assembly and filling equipment.

Managing the microbial bioload on the juice contact surfaces of paperboard and plastic packaging (cartons, preforms, and fully-blown or thermoformed bottles) is a shared responsibility of the packaging supplier and the juice processor.

For juices marketed with chilled shelf lives longer than about 4 weeks, cartons are typically coated with an interior layer of a barrier material such as aluminum foil, nylon, or extra layers of polyethylene. These linings are selected to limit oxygen permeation into the juice and so-called "flavor scalping" (transfer of desirable flavor components from the juice into the paperboard). If the carton is to be used within packaging equipment that sanitizes or sterilizes the carton interior, the carton material and internal layers must be compatible with the chemical(s), heat treatments, and/or ultraviolet light exposures that are applied during processing. Inventories must be managed so that incoming lots of packaging material are used in the same sequence in which they are received ("first in, first out" approach). Quality assurance (QA) procedures should be established to protect packaging materials from excessive environmental exposure to dust, insects, or open air currents that may occur near exit doors and receiving docks. Procedures should also address situations in which a portion of a previously unwrapped pallet of bottles or case of cartons remains at the end of a production shift. As described previously, Narciso and Parish (1997) demonstrated that paperboard material may be a significant source of viable mold spores which can survive the extraction, thermal processing, and bleaching processes used to manufacture food-grade cartons. One simple but effective means to manage this potential source of fungal spoilage is to require the use of internally folded flaps called "skived" panels or "J-flaps" on the base of the cartons to prevent direct contact between raw edges of the paperboard and the beverage (Castberg et al., 1995). Establishing and maintaining seal integrity of the package is a final critical component in preventing post-filling contamination and microbial spoilage of juice beverages.

Use of Well Designed and Maintained Hygienic Package Filling Systems: This section focuses on the design and features of the equipment used for filling chilled juices into cartons, bottles, or jugs. It is helpful to conceptualize the shelf-life of a given chilled juice product in terms of a *population* of bottles packed using the same equipment and at the same time, rather than the length of time that any *individual* bottle will remain free of microbial spoilage defects or other undesirable changes in flavor, color, or consistency. In this respect the "defect rate" may be defined as the percentage of bottles or cartons, produced on the same line under standard

commercial operating conditions, showing the presence of microorganisms capable of spoiling the product under normal chilled storage conditions. One of the factors with greatest influence on the microbial defect rate of chilled juices is the choice and maintenance of the juice filling and packaging equipment within a given factory or production line. Within the chilled juice category, juice filling/packaging equipment falls within three basic categories: conventional, "ultraclean," and aseptic filler technology.

While these terms have various definitions within the industry and among specific equipment vendors, the following descriptions help to clarify the key differences. Conventional juice packaging equipment involves the filling of chilled juice into previously manufactured cartons or bottles that do *not* receive an in-line rinsing or sterilization treatment. This equipment is designed to index the bottles or carton blanks through the system; form the cartons and establish the bottom seals; fill each container with chilled, previously pasteurized juice; seal each container (e.g., folding and sealing of carton top seals, rotary application of a plastic closure to a PET bottle); and discharge the filled/sealed container to a conveyor belt moving toward the secondary packaging area. The filling chamber is *not* securely isolated from the surrounding plant environment, nor are the doors or panels that allow access to the filling chamber monitored using an electronic alarm system. The filling chamber may range in size from small (e.g., linear carton fillers) to quite large (e.g., rotary fillers used for multiserve bottles) and it may or may not be supplied with a pressurized and highly filtered (HEPA or ULPA) air supply. While conventional filling technology is among the least expensive, it has serious drawbacks in terms of operational consistency (day-to-day and seasonal microbial defect rates), shorter production run cycles due to microbial buildup and accompanying sanitation issues, and vulnerability to occasional major post-pasteurization contamination events driven by heavily contaminated packaging materials, unsanitary filling valves or capping systems, and/or contamination of the filler enclosure by the surrounding environment (dust, aerosols), or due to unsanitary filler intrusion practices by employees.

Ultraclean or ESL (extended shelf-life) equipment takes the basic design features listed above and adds to them several important technical capabilities, including a means to consistently and automatically disinfect and sterilize the critical surfaces, air, and packaging material that come into direct contact with the juice or juice product. In the case of cartons, sterilization is typically achieved via the use of hydrogen peroxide sprays, heated sterile air, and/or controlled exposure to ultraviolet light of specific wavelengths and intensities. Equipment vendors have varying standards, but these in-line package treatment systems reduce the microbial bioload within the containers by 3–5 log cycles using microorganisms of known and relatively high resistance to heat and/or hydrogen peroxide. ESL bottle treatment systems involve the spray sanitizing of the bottle interior surfaces using solutions of chemicals such as hydrogen peroxide, peroxyacetic acid, or other aqueous chemical solutions. Sanitizer solutions and spray parameters used for in-line bottle rinsing must be optimized for efficacy and operated within applicable regulatory limits within the country of use. If a final water rinse is required, the water used must be sterile or of excellent microbiological quality. Use of a HEPA- or ULPA-filtered

air supply is standard, and the filling zone is maintained under higher pressure than the surrounding packaging room and any upstream areas within the package filling equipment. While manual or clean-out-of-place (COP) cleaning of sensitive components, such as valves and capper assemblies, is always required, ESL fillers are equipped with special auto-sparging systems to initially sterilize the filling enclosure and to periodically sanitize these surfaces during the production cycle. Gruetzmacher and Bradley (1999) identified several of the common sources of microbial contamination in chilled dairy carton fillers and the means to reduce such risks using laminar-flow filtered air and selected sanitizer treatments for product contact surfaces. Closures and fitments used in ESL systems are typically sanitized in-line to assure a low rate of dust and mold contamination from this source. In successful ESL installations, the net result is a product with a significantly lower microbial defect rate (as compared to conventional fillers), fewer QA product "on-hold" incidents, and a lower rate of consumer complaints. Statistically based microbial defect rates in the range of 0.1–1.0% are possible with properly designed and operated ESL juice filling systems. In certain cases, Ultraclean or ESL technology is an enabler of longer and more consistent filler production runs and the opportunity to extend the commercial shelf-life, thereby increasing the timeframe available for distribution to regions or countries far from the juice-processing facility.

Of the three technologies described in this section, aseptic processing and packaging (APP) technology has received the greatest attention and research over the past three decades. This technology involves application of the capabilities previously described for ESL fillers, but with significantly higher performance requirements and quality assurance expectations (Graumlich, Marcy, & Adams, 1986). APP has been defined as follows: "The filling of a commercially sterilized (and) cooled product into presterilized containers, followed by aseptic hermetic sealing with a presterilized closure in an atmosphere free of microorganisms" (Chambers & Nelson, 1993). In the beverage industry, aseptic packaging technology is most often applied to shelf-stable beverages with very long shelf life and/or microbial safety requirements. However, in certain regions and markets, APP systems are used for high-acid beverages distributed exclusively or primarily under refrigerated conditions. In these instances, the business case for APP must be made based on factors such as requirements for very low microbial defect rates (typically not more than 1 nonsterile container in every 5,000–30,000 packages produced), high levels of automation and internal process control, the efficiency of the process, potential for extended production run times, and opportunities for the product to experience periods during distribution in which the beverage is not refrigerated.

Maintenance of Proper Refrigeration of the Packaged Juice During Storage and Distribution: A final and obvious factor influencing the microbiological shelf-life of pasteurized, chilled juices is the use of adequate refrigeration systems to maintain the packaged juice within the temperature range required to maintain juice quality throughout distribution and storage. This factor is important because several of the microorganisms capable of spoiling high-acid chilled juice products can multiply at temperatures well below 5°C (Table 4). Elevated temperature storage at any point in the cold chain (i.e., warehousing at the factory, distribution to off-site

warehouses, shipping to retail outlets, retail display, and home storage) will increase the growth/spoilage rate of any microorganisms present. Curiale (1998) stated that, for general estimation and within reasonable temperature limits, a two-to four-fold increase in the microbial growth rate is predicted for a 10–11°C increase in the storage temperature. In the United States, a 1999 survey revealed that a large percentage of refrigerators in homes (27%) and in retail dairy coolers (40%) operate at temperatures above 5°C (41°F). In the same survey, 8% (homes) and 15% (retail) of the refrigeration units audited were found to operate at temperatures above 7°C (Audits International, 1999). In certain industrialized countries, maximum retail refrigeration temperatures of 8–10°C are permitted under prevailing regulations.

Recommended Methods for Detection and Isolation of Spoilage Microorganisms

Unless otherwise referenced, the following microbiological methods are described in Downes and Ito (2001).
Analysis of juice-based ingredients (freshly extracted juice, concentrates, and bases):

- Preparation of serial dilutions, pour plating, and colony counting procedures
- Use of tempered orange serum agar (pH 5.5 ± 0.1) for enumeration of "total aciduric" microbial populations
- Use of potato dextrose agar (acidified to pH 3.5 ± 0.1), potato dextrose agar (with antibiotics, pH 5.6 ± 0.1) or malt extract agar (pH 5.5± 0.1) supplemented with chloramphenicol (100 mg/L, added before autoclaving) for enumeration of yeasts and molds.

Analysis of pasteurized, packaged juice products:

- Pour plating of 1-L subsamples of chilled juice (undiluted) using the pour plate technique for enumerating "total acidurics" and yeasts and molds, as described above.
- Note: In-package stress incubation of the juice at 25–30°C for 48–72 h before physical examination (i.e., the absence of package bloating, visible mold, or sensory off-aromas) and plating of 1-mL subsamples is sometimes used as a routine QA technique (Murdock, 1979). To determine post-pasteurization microbial defect rates, the 25–30°C stress incubation time should be extended to 10–14 days.

Determination of the fungal bioload within empty/formed cartons or plastic bottles:

- Use potato dextrose agar (acidified), potato dextrose agar (with antibiotics), or malt extract agar supplemented with chloramphenicol.

Identification of microorganisms isolated from spoiled juice products

- Identify spoilage molds and yeasts using the methods and media described in Samson, Hoekstra, and Frisvad (2004).
- Identify spoilage bacteria using cultural methods described in *Bergey's Manual of Systematic Bacteriology* (Berlin: Springer-Verlag) or using advanced DNA-sequencing techniques.

Ambient Fruit Juices

Products in this category are pasteurized at temperatures of about 80–90°C and hot-filled or aseptically filled into glass, metal, plastic, or composite paper/foil containers for storage and distribution at ambient temperatures. Often packaged in individual serving-sized containers, the aseptically filled products are susceptible to spoilage by fermentative yeasts and environmental molds that may enter during packaging or juice transfer, if there is a loss of aseptic system integrity. The control measures needed to prevent this type of yeast spoilage are similar to those described above for chilled juices, with emphasis on equipment cleaning and sanitation and HEPA/ULPA air management, particularly within the aseptic filling zone. The larger and more complex the aseptic filling system, the more challenging this task may become.

The hot-fill-hold process for high-acid juices must be properly designed, qualified, operated, and managed to assure commercial sterility. Consumer preferences for plastic bottles (PET, polypropylene) over glass have driven significant changes in the hot-fill processes used in the juice industry. In the past, glass bottles were typically filled with juice at temperatures in excess of 88°C, and then sealed with steam-treated metal lug closures. In contrast, PET bottles may not be filled above approximately 84°C without compromising bottle integrity and appearance. The plastic closures applied must readily form a hermetic seal, while withstanding the hot-fill process regimen (filling, sealing, bottle inversion or "layover," and hot-holding/conveyance through the cooling tunnel system). This narrow operating window means the margin for error or process deviation is much smaller than when filling glass bottles. When spoilage occurs in plastic bottles due to growth of common environmental molds, a typical root cause is insufficient bottle headspace/closure sterilization due to excessive juice foaming, under-filling, or insufficient bottle inversion times. When yeast or aciduric bacterial spoilage occurs, the ingress of cooling tunnel water or unsanitary air should be investigated, as there may have been a loss of seal integrity during or after the cooling process. Maintaining a clean and properly sanitized cooling tunnel is a key part of establishing a commercially stable hot-fill-hold process.

Two additional types of spoilage microorganisms, heat-resistant molds, and alicyclobacilli, also present spoilage threats to ambient fruit juices, but not to chilled juices, as they are unable to grow at refrigeration temperature. Being aerobic microorganisms, neither can spoil carbonated beverages. Spoilage by these microorganisms is not immediately obvious as neither produces carbon dioxide that would distort or burst the container. Rather, their more subtle spoilage is usually noticed as visual, odor, and/or flavor defects.

Heat-Resistant Molds

The principal species of heat-resistant molds are *Byssochlamys fulva, Neosartorya fischeri, Eupenicillium brefeldianum*, and *Talaromyces macrosporus.* These molds are sometimes able to survive the juice pasteurization process because of their formation of heat-resistant ascospores, which possess typical $D_{90}{}^{\circ}{}_{C}$ values of about 2 min and *z*-values of about 7°C. Potential spoilage incidents can be minimized by cleaning and sanitation of the processing equipment, the use of chemical preservatives in the product, and sometimes by the testing of raw fruit materials for the presence of unusually high levels of the molds (Beuchat & Pitt, 2000; Pitt & Hocking, 1999). Procedures for the detection and enumeration of heat-resistant molds are described by Beuchat and Pitt (2000). Because of the typically low number of heat-resistant mold ascospores in fruits and fruit juices, rather elaborate procedures are required for their detection and enumeration. These include: analysis of a large amount of sample, often 100 mL or g; heat activation of ascospores by heating the sample at about 75°C for at least 30 min; plating on multiple agar plates of commonly used mold isolation media; and incubation at 30°C for up to 30 days.

Alicyclobacilli

An extremophilic microorganism capable of growth at very low pH values and very high temperatures was originally isolated from acid hot springs about 40 years ago and later classified as *Alicyclobacillus* spp. At this writing, more than 20 species of *Alicyclobacillus* have been identified (Goto, Tanaka, Yamamoto, & Tokuda, 2007). *Alicyclobacillus acidoterrestris* is the species most likely to be involved in the spoilage of fruit juices (Darland & Brock, 1971; Wisotzkey, Jurtshu, Fox, Deinhard, & Poralla, 1992; Bevilacqua, Sinigaglia, & Corbo, 2008). The major characteristics of *A. acidoterrestris*, a Gram-positive, spore-forming bacillus, are described in Table 5. It is the most heat-resistant microorganism that can spoil high-acid, shelf-stable fruit products (Silva & Gibbs, 2004). In addition to being recovered from acid hot springs, it has also been recovered from acidic soils commonly found in fruit orchards.

Table 5 Characteristics of *Alicyclobacillus acidoterrestris*[a]

	Parameter	Value
Growth temperature range (°C)		30–60
Growth pH range		2.0–6.0
Water activity minimum for growth		0.97
$D_{95}{}^{\circ}{}_{C}$ (min)	Range	1.0–5.3
	Mean	2.8
z-Value (°C)	Range	7.2–12.9
	Mean	9.7

[a]Bevilacqua et al. (2008); Silva and Gibbs (2004).

Spoilage of fruit juices by *A. acidoterrestris* in commercial distribution usually occurs during summer months because of its moderate thermophilic nature (Parish, 2006). The principal mode of spoilage is the production of a "phenolic," "medicinal," or "tar-like" odor caused by guaiacol, which is formed in juices by the decarboxylation of vanillic acid, which can be produced by metabolism of several precursor compounds in juices. All strains of *A. acidoterrestris* are reported to produce guaiacol. The lower limit of guaiacol detection by a trained sensory panel is 2.26 ppm; spoiled product samples have levels greatly in excess of this concentration (Chang & Kang, 2004; Walker & Phillips, 2008).

Although the first commercial outbreak of fruit juice spoilage by *A. acidoterrestris* was detected in 1982, it is likely that spoilage incidents have been occurring since the inception of this product category. It is also possible that the incidence of spoilage has been accelerated by the increased use of packaging materials such as polyethylene terephthalate (PET) that permit a slow but steady ingress of atmospheric oxygen. In some products it is possible to absorb oxygen by the use of antioxidants, such as ascorbic acid, in order to prevent this type of spoilage (Tokuda, 2007; Shebuski, 2009). Fruits are readily contaminated with *A. acidoterrestris* spores by contact with orchard soil and carried into the processing plant where parts of the production process resemble an acid hot spring. Most fruit juices and drinks are manufactured using frozen concentrated juices. In the interest of water conservation, during evaporation of the primary juice to produce concentrates, condensate water is collected and used in numerous processing operations, including fruit washing, equipment cleaning, and product blending. Stored in large central collection tanks, the condensate water is usually about 50°C, particularly in tropical and semitropical regions. Under such conditions, recycled condensate water has been found to contain high levels of alicyclobacilli. Some juice producers prevent the buildup of alicyclobacilli in condensate systems by chlorination of the condensate water at residual levels not to exceed 0.3 ppm (Parish, 2006; A.I.J.N., 2008).

Earlier occurrences of *A. acidoterrestris* spoilage were likely not accurately reported because this microbe is often isolated using the same microbiological media that are used to isolate yeasts and molds. Therefore, normal microbiological evaluation of a spoiled juice sample that did not include microscopic examination would have resulted in a report of yeast contamination, because both yeast and alicyclobacillus colonies appear similar on plating media. This understandable mistake occurs occasionally today.

Beginning with acidified potato dextrose agar, which is commonly used for yeast and mold detection, numerous media have been developed for the detection of alicyclobacilli. The most commonly used media for this purpose are indicated in Table 6, with YSG currently considered the most productive. Typical incubation conditions are 43°C for 2–3 days, although incubation temperatures vary from 40 to 55°C (Murray, Gurtler, Ryu, Harrison, & Beuchat, 2007). A word of caution may be in order for the use of media that are acidified to a target pH of 4.0 (some not tabulated here are acidified to target pH values greater than 4.0). *Bacillus coagulans* and several other bacilli are capable of growth at or near pH 4.0; therefore, they could

Table 6 Acidification of plating media for the detection of *A. acidoterrestris*[a]

Medium	Target pH	Acidulant
Yeast extract starch glucose agar (YSG)	3.7	1 N sulfuric acid
K agar, commercial (KAC)	3.7	25% malic acid
Potato dextrose agar (PDA)	3.5	10% tartaric acid
Bacillus acidoterrestris thermophilic agar (BAT)	4.0	1 N sulfuric acid
Alicyclobacillus medium (ALI)	4.0	1 N sulfuric acid
A. acidoterrestris medium (AAM)	4.0	25% malic acid

[a]Murray et al. (2007).

potentially be detected when media adjusted to pH 4.0 or higher are used. Therefore, additional tests are advisable to confirm the suspected presence of spoilage-causing alicyclobacilli. These include (Parish 2006; Walker & Phillips, 2008):

- microscopic examination to rule out the presence of fermentative yeasts or other types of bacilli;
- streaking isolated colonies onto a neutral pH medium, because alicyclobacilli cannot grow above pH 6.0;
- conducting a test to demonstrate the microorganism's ability to produce guaiacol,
- definitive identification of *A. acidoterrestris* by using the randomly amplified polymorphic DNA test (RAPD), a 6-h test.

The principal control measures to prevent spoilage by *A. acidoterrestris* include (Bevilacqua et al., 2008; Parish, 2006; A.I.J.N., 2008):

- cleaning the fruit before processing;
- maintenance of regular equipment cleaning and sanitation procedures;
- prevention of growth in condensate collection systems; and
- when permitted, addition of a chemical preservative to the product, e.g., 0.01–0.05% sodium benzoate.

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Microbiological Spoilage of Acidified Specialty Products

William H. Sperber

Introduction

Acidified specialty products or condiments are among the most microbiologically stable and safe food products. Often formulated, packaged, and distributed without heat treatments, they are microbiologically stable indefinitely at ambient temperatures in unopened containers. The packaged, acidified products are often intended for multiple uses, exposing them at the points of consumption to numerous opportunities for contamination with microorganisms. Nonetheless, they remain resistant to microbiological spoilage for many months, often under refrigerated conditions that are used to retard chemical reactions, flavor changes, and yeast growth.

The exceptional resistance to microbiological spoilage of the acidified specialty products is attributable to their pH values, usually below 4.0, to typically high concentrations of acetic acid, introduced as vinegar, and to limited headspace and oxygen availability. These conditions prevent the growth of most bacteria and all bacterial pathogens. Food safety incidents are very rarely attributed to these products. Those few that have been suspected were related to cross-contamination from other foods. Overt food safety hazards are sometimes created by the explosion of glass jars following fermentation. This hazard has been greatly reduced by the replacement of glass with plastic containers and by manufacturing improvements to reduce the incidence of contamination and potential spoilage. The types of spoilage microorganisms are limited to various lactic acid bacteria, yeasts, and molds that can tolerate acetic acid at low product pH values.

The above considerations of microbiological stability and safety apply to commercially produced products that are manufactured in accordance with standards of identity for mayonnaise and some salad dressings and closely related procedures for nonstandard of identity products. In the interest of cost and personal preference, similar products produced in homes or restaurants may be made with more water,

W.H. Sperber (✉)
Cargill, Inc., Corp. Food Safety & Reg. Affairs, 5814 Oakview Circle, Minnetonka MN 55345, USA
e-mail: bill_sperber@cargill.com

W.H. Sperber, M.P. Doyle (eds.), *Compendium of the Microbiological Spoilage of Foods and Beverages*, Food Microbiology and Food Safety,
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less oil, and less vinegar, thereby diminishing this type of product's stability and safety.

Economic loss associated with the microbiological spoilage of acidified specialty products are very sparse; in large part because of the lack of spoilage, and in small part because most spoilage incidents would not require a report to public health authorities. They would be readily handled by the manufacturer and/or retailer without publicity or the need for a product recall. However, in the absence or neglect of formulation and production controls, major spoilage incidents can occur even with such robust products. In an incident to be described later in this chapter, a major recall of salad dressing that cost about $1 million 40 years ago was required because of yeast spoilage (Lawrence, 1970).

The principal acidified specialty products addressed in this chapter are high-fat products such as mayonnaise, intermediate-fat salad dressings, and low-fat or no-fat salad dressings, ketchup, mustard, and vinegar. Other high-acid foods or condiments are addressed in chapter "Microbiological Spoilage of Fruits and Vegetables", including high-acid-fermented vegetables such as pickles and sauerkraut, and in chapter "Microbiological Spoilage of Canned Foods" including acidified canned foods such as tomato and pasta sauces.

General Microbiological Spoilage

General features and knowledge of the microbiological spoilage of acidified specialty products are described in this section.

Spoilage Microorganisms

The low pH and high acetic acid concentration in acidified specialty products prevent the growth of bacterial sporeformers and almost all vegetative bacteria, except for several species of lactic acid bacteria. About one fourth of the spoilage of these products is caused by lactic acid bacteria, particularly *Lactobacillus fructivorans*, *L. brevis*, *L. buchneri*, and *L. plantarum* (Sharpe & Pettipher, 1983).

Yeasts are responsible for about three fourths of the acidified specialty products spoilage (Sharpe & Pettipher, 1983). Sixty-two species of yeasts have been found to be capable of spoiling these low-pH products. Seven species have been most frequently isolated from spoiled products (Table 1). The most commonly isolated yeast is *Saccharomyces cerevisiae*, well known as a prolific fermentative microorganism for the production of yeast-leavened bakery products and alcoholic beverages. Its prolific production of carbon dioxide makes it an important spoilage microorganism in acidified specialty products that support its growth.

Yeasts are generally aerobic microorganisms, but most can readily grow under anaerobic conditions in the presence of a fermentable carbohydrate. About 40% of

Table 1 Most frequently isolated yeasts from low-pH products ($n = 62$ species)[a]

Yeast	Frequency (%)
Saccharomyces cerevisiae	7.37
Pichia anomola	5.52
Zygosaccharomyces bailii	5.52
Candida parapsilosis	5.26
Debaryomyces etchellsii	4.73
Issatchenkia orientalis	3.95
Pichia membranaefaciens	3.95
The remaining 55 species	Average: 1.16 each

[a]From Deak and Beuchat (1996, pp. 124–125).

all yeast species are nonfermentative, as determined by the lack of collection of carbon dioxide gas in a Durham tube. Some yeasts, principally *Cryptococcus* spp. and *Rhodotorula* spp., are strict aerobes (Deak & Beuchat, 1996). Commonly called "film yeasts," they can only grow on the surface of acidified specialty products as thin films or pellicles. As with molds, the growth of film yeasts in these products is often inhibited by the limited availability of oxygen.

The remainder of high-acid product spoilage is caused by molds. Molds are rarely involved in the spoilage of these products because they poorly tolerate the presence of acetic acid. Furthermore, molds are obligate aerobes while most high acid products are packaged with minimal headspace and have minimal oxygen available for mold growth.

Mold spoilage of these products can occur when adequate oxygen is available, e.g., on the inside of partially emptied containers and on the outside of containers that become contaminated with product and are not cleaned. The latter situation is more likely to occur in institutional settings where large product containers are used. The principal molds that can grow under these circumstances are *Moniliella acetobutans*, *Monascus ruber*, and *Penicillium glaucum* (Hocking, 1994; Pitt & Hocking, 1997).

Factors Affecting Microbiological Spoilage

Acidified specialty products are very resistant to microbiological spoilage because of many preservative factors and control measures that are often used in concert.

pH and Type of Acidulant

The products in this category are typically in the pH range of 3.2–3.9, with acetic acid being the most commonly used acidulant. While this pH range effectively prevents the growth of microorganisms, acetic acid provides a major additional barrier to those microorganisms that can grow below pH 4.0. For example, yeasts commonly found in low-pH foods can grow at a minimum pH value of about 2.0 in the presence of most commonly used inorganic and organic food acidulants. The

Table 2 Influence of acidulant on the average minimum pH value at which foodborne yeasts will not grow[a]

Acidulant	Average minimum pH value
Hydrochloric acid	1.8
Phosphoric acid	2.0
Citric acid	2.1
Malic acid	2.1
Tartaric acid	2.1
Acetic acid	2.9

[a]Adapted from Deak and Beuchat (1996, p. 28).

same yeasts, however, cannot grow below pH 2.9 when acetic acid is the acidulant (Table 2). The characteristic strong odor and flavor of acetic acid, added to these products as vinegar, has led some companies to attempt the development of acidified specialty products with a milder odor and flavor by using acidulants other than vinegar. Such attempts should be undertaken with due diligence to validate that the replacement of acetic acid with a "weaker" acidulant does not compromise the microbiological stability and safety of the new products (Vermeulen et al., 2008).

Water Activity

Independent of their pH value, the water activity of most products in this category is sufficiently high to permit the growth of most microorganisms. The water activity of high-fat products such as mayonnaise, however, is sufficiently low – about 0.90 – to inhibit the growth of most bacteria. The water phase of mayonnaise can be as low as 15–20% of the product weight. In it all of the fat-insoluble solutes are concentrated, primarily salt and sugars, that are necessary to flavor the entire product mass. A more extensive explanation of the antimicrobial effects of water activity is presented in chapter "Introduction to the Microbiological Spoilage of Foods and Beverages".

Pasteurization

As will be discussed later in this chapter, some products such as mayonnaise are chemically pasteurized by their low pH and high acetic acid contents. Acidified specialty products with low acetic acid contents are sometimes heat pasteurized to assure microbiological stability during shelf life. Ketchup, for example, is heated to 85–90°C to pasteurize the product and set the starch. It is hot-filled to assure the microbiological stability of the packaged product (Michels & Konig, 2000).

Chemical Preservatives and Partition Coefficient

Because of the combined robust inhibitory effects of pH, acetic acid, and water activity, the need for chemical preservatives is not very great in this product

category. Multiple use products with a high water activity, e.g., pourable salad dressings, are sometimes further protected by the addition of chemical preservatives to prevent yeast and mold spoilage. The frequent reuse of these products makes them more vulnerable to contamination and spoilage. The most commonly used preservatives are sorbic and benzoic acids at concentrations of 0.05–0.2% (Michels & Koning, 2000). Both preservatives have a moderately high partition coefficient, therefore, their effectiveness will be diminished as the product's fat or oil content is increased. An explanation of chemical preservatives and the influence of partition coefficient is presented in chapter "Introduction to the Microbiological Spoilage of Foods and Beverages".

Headspace Oxygen Content

Mold and oxidative yeast spoilage of acidified specialty products is greatly minimized by the use of acetic acid and by packaging products with a minimal headspace. Being obligate aerobes, molds and oxidative yeasts can grow only in the presence of molecular oxygen. In addition to the limited headspace atmosphere, the oxygen available for growth is quickly removed in properly sealed packages by oxidation of the product's oil. In those products that are repeatedly used for weeks or months, the repeated introduction of atmospheric oxygen may increase spoilage by aerobic fungi.

Refrigeration

While the commercially produced products in this category are shelf stable at ambient temperatures, many are labeled, "Refrigerate after opening." Refrigeration provides some protection for multiple-use packaged products against the threat of spoilage caused by contamination with high numbers of potential spoilage microorganisms, as well as retarding organoleptic degradation.

Sources of Spoilage Microorganisms

The major ingredients used in acidified specialty products – oil, water, salt, and vinegar – are relatively free of microorganisms and unlikely to cause product spoilage (Michels & Koning, 2000). Minor ingredients, including dry or liquid sugar, corn syrups, eggs or egg yolks, starches, herbs, and spices, are more likely to contribute microorganisms that could contribute to product spoilage. The various sugar ingredients can be the source of osmophilic yeasts and *Zygosaccharomyces bailii*. The remaining minor ingredients are known sources of lactic acid bacteria, yeasts, and molds. Lactobacilli counts in various types of mustard seeds ranged from 4,000 to 400,000/g (Kneifel & Slama, 1992).

Quite likely, the immediate source of spoilage microorganisms is the production environment, equipment, and in-process materials. Cross-contamination from these sources to finished products often occurs when the processing and packaging

equipment is not adequately cleaned and sanitized. It is possible that an in-process component may assist a potential spoilage microorganism to adapt to the harsher environment. In one major product recall, salad dressing was spoiled by yeast fermentation after the yeast had been acclimatized in starch paste used to produce the salad dressing (Lawrence, 1970).

Spoilage Defects

Relatively few types of microbiological spoilage defects are observed in acidified specialty products. Off-flavors can be produced by growth and fermentation by yeasts and heterofermentative lactic acid bacteria. In the presence of adequate oxygen, oxidative yeasts can grow as a film or pellicle on the surface of semi-solid or liquid salad dressings. Several species of molds, as noted above, can cause surface discoloration when adequate oxygen is available.

Spoilage of Acidified Specialty Products

The microbiological spoilage of specific acidified specialty products is described in this section.

Mayonnaise

Mayonnaise is an oil-in-water emulsion that is generally composed of 65–80% vegetable oil (usually soy or canola [rapeseed]), vinegar, at least 6% egg yolk, and optional ingredients (Michels & Koning, 2000). Commercial mayonnaise in the USA is produced with 80% oil, has a pH range of 3.6–4.0, and contains about 0.29–0.5% acetic acid. The aqueous phase contains 9–12% salt and 7–10% sugar (Smittle, 1977). Production of mayonnaise in the USA is regulated by a standard of identity that has the following principal requirements (FDA 2008a):

- Vegetable oil, not less than 65% by weight
- Vinegar, with an acidity not less than 2.5% by weight, calculated as acetic acid and/or lemon or lime juice, with an acidity not less than 2.5% by weight, calculated as citric acid
- Egg yolk-containing ingredients, such as liquid, frozen, or dried whole eggs or egg yolks
- Optional ingredients, including salt, nutritive carbohydrate sweeteners, monosodium glutamate, sequestrants, e.g., calcium disodium EDTA, citric acid, or malic acid not comprising more than 25% of the acidulants added with the vinegar and citrus juices, and crystallization inhibitors, e.g., oxystearin or lecithin

When produced according to the above standard, mayonnaise is remarkably resistant to microbiological spoilage. Mold and oxidative yeast spoilage can occur on the product surface if the jar or package is not hermetically sealed to prevent the ingress of oxygen. More important from the food safety perspective, the composition of mayonnaise provides a "chemical pasteurization," assuring that vegetative bacterial pathogens such as *Salmonella* and *Staphylococcus aureus* are quickly killed, even though the mayonnaise never receives a heat treatment. The chemical pasteurization is so effective that the standard of identity in effect for mayonnaise in the 1960s and 1970s allowed the cracking of shell eggs directly into the mayonnaise production. Experiments to validate this procedure showed a consistent 6-log reduction of *Salmonella typhimurium* and *S. aureus* within minutes after inoculation into mayonnaise (Sperber & Okada, 1972).

Substandard mayonnaises, usually not prepared for commercial distribution, contain as little as 25% vegetable oil, are much more susceptible to microbiological spoilage, and have a limited shelf life (Smittle, 1977). *Z. bailii* is the predominant spoilage microorganism in such products (Deak & Beuchat, 1996).

Outbreaks of foodborne illness caused by mayonnaise-containing foods such as salads and sandwiches often cast suspicion upon commercially produced mayonnaise as the cause of the outbreak. The early work of Sperber and Okada (1972) and Smittle (1977) indicated that mayonnaise was a self-pasteurizing product that could not be responsible for foodborne illnesses. Brocklehurst and Lund (1984) determined that the acetic acid content of mayonnaise can be reduced by absorption into vegetables, yet the mayonnaise provided a protective effect to maintain the product shelf life. They tested a variety of vegetable salads that included components such as shredded cabbage, potatoes, carrots, peas, and green beans. The mayonnaise used in the salads had an initial pH of 3.0 and an acetic acid content of 1.3%. After equilibration with the vegetable components for 6 hours, the mayonnaise pH increased to 4.0 and its acetic acid content was reduced to 0.4%. Vegetable, potato, coleslaw, and Florida salads had an average shelf life of 13 days at 10°C and 28 days at 5°C before signs of incipient spoilage occurred. The predominant spoilage microorganism was *Pichia membranaefaciens*. Fialová, Chumchalová, Mikoivá, and Hrůšová (2008) determined that the shelf life of mayonnaise-based foods could be extended by the use of one of several combinations of chemical preservatives: acetic acid and sodium acetate; acetic acid, lactic acid, and sodium lactate; or lactic acid, sodium lactate, and sodium diacetate.

Spoonable Salad Dressings

The products in this category of salad dressings are oil-in-water emulsions that have less oil and more water and vinegar than does mayonnaise. The emulsion is stabilized by starch paste which gives the salad dressings a semi-solid or "spoonable" texture, in contrast to the more fluid "pourable" dressings described in the next

section. The ingredients of salad dressings produced in the USA are regulated by a standard of identity that has the following requirements (FDA, 2008b):

- Vegetable oils, not less than 30% by weight
- Vinegar and/or lemon or lime juice (acid concentration not indicated)
- Liquid, frozen, or dried egg yolks or whole egg, not less than 4% egg yolk by weight
- Starchy paste prepared from water, food starch, modified food starch, tapioca flour, wheat flour, rye flour, or combinations of two or more of these
- Optional ingredients including one or more of the following: salt, nutritive carbohydrate sweeteners, spices (that do not simulate the color of egg yolk), monosodium glutamate, stabilizers and thickeners, citric or malic acid, sequestrants, and crystallization inhibitors
- The salad dressing may be mixed and packed in an atmosphere in which air is replaced in whole or in part by carbon dioxide or nitrogen

An example of a salad dressing formulation is presented in Table 3. The chemical characteristics of spoonable salad dressings include a pH range of 3.2–3.9, an acetic acid range of 0.9–1.2%, and water activity of 0.93, with an aqueous phase containing 3–4% salt and 20–30% sugar (Smittle, 1977). Salad dressings are more susceptible than mayonnaise to microbiological spoilage because of the greater variety of ingredients and the potential mishandling of starch paste, which was the reason for the major salad dressing spoilage incident cited in the introduction of this chapter. In that incident, the mixer operator noticed that a drum of starch paste "smelled funny." Rather than dumping the entire drum into a batch of salad dressing, or dis-

Table 3 Salad dressing formulation used to test the effects of chemical preservatives[a]

Ingredient	Percent (w/w)
Soybean oil	32.5
Egg yolk	5.2
Water	3.0
Vinegar	0.7
Flavor	0.01
(Experimental additions)	2.72
Starch paste	
Water	28.1
Corn syrup	10.1
Vinegar	7.6
Corn starch	4.1
Sucrose	2.2
Isomerose	2.2
Salt	1.5
Flavor	0.07
Total:	100.00

[a] Yang et al. (2003).

posing it as spoiled, he portioned small quantities into each of many batches over several weeks. Within several months, a major spoilage outbreak was underway in this product at the retail level. Microbiological investigations revealed that the yeast responsible for the spoilage could not grow when inoculated directly into the salad dressing. It could, however, readily grow in the starch paste. When pilot plant batches of salad dressing were produced with the contaminated starch paste, they were readily spoiled by the yeast. In addition to the economic losses incurred by the manufacturer, consumers and product handlers were put in jeopardy because of exploding glass jars (Lawrence, 1970). This incident illustrates how a well-intentioned employee, not wanting to "waste" a drum of ingredient, can cause a far greater problem.

The predominant spoilage microorganisms of this product category are *Z. bailii* and *L. fructivorans*. Both are rapid fructose fermenters and carbon dioxide producers. Fructose occurs in the product from the acid hydrolysis of sucrose. Occasional spoilage incidents are caused by *L. plantarum*, which does not produce carbon dioxide (Kurtzman, Rogers, & Hesseltine, 1971; Smittle & Flowers, 1982). The generation of carbon dioxide can cause the explosion of glass containers or the distortion of plastic containers. The minimum pH values at which *Z. bailii* and *L. fructivorans* can grow are 3.60 and 3.55, respectively; and the minimum water activity values that support their growth are 0.88 and 0.91, respectively (Meyer, Grant, Luedecke, & Leung, 1989). The growth in salad dressings of both microorganisms can be inhibited by 1% sucrose monoesters of lauric (C_{12}), myristic (C_{14}), or palmitic (C_{16}) acids. At these levels the preservatives are as effective, or more effective, than the use of 0.1% sodium benzoate (Yang, Luedecke, Swanson, & Davidson, 2003). Commercial salad dressing better protected the product quality and prolonged the shelf life at 4°C of a shredded carrot and cabbage salad (one part salad dressing to four parts vegetables) than did pretreatment of the vegetables for 5 or 30 min in 0.2 or 1.0% citric acid dips (Eytan, Weinert, & McGill, 1992).

Pourable Salad Dressings

Pourable salad dressings can be manufactured as stable oil-in-water emulsions, as colloidal suspensions stabilized by gums, or as products with separate oil and water phases that require shaking before serving. Representative products in this category are dressings such as French, Italian, Ranch, and vinaigrettes, as well as barbecue sauces and marinades for raw meat and poultry. In the United States, French dressing is the only pourable dressing that is the subject of a standard of identity, with the following requirements (FDA, 2008c):

- Vegetable oil, not less than 35% by weight
- Vinegar, with or without lemon or lime juice. Both may be diluted with water
- Optional ingredients:

- salt,
- nutritive carbohydrate sweeteners,
- spices and/or natural flavorings,
- monosodium glutamate,
- tomato paste or puree, catsup, sherry wine,
- eggs and ingredients derived from eggs,
- color additives that will impart the color traditionally expected,
- stabilizers and thickeners,
- citric or malic acid,
- sequestrants, and
- crystallization inhibitors.

French dressing may be mixed and packed in an atmosphere in which air is replaced in whole or in part by carbon dioxide or nitrogen.

The microbiological stability of pourable dressing products may be provided by pasteurization at 85–90°C followed by hot filling, or the addition of chemical preservatives. As with mayonnaise, some products in this category are chemically stabilized. They will be microbiologically stable without heating or chemical preservatives as long as the retail package remains unopened.

The pH range of this category is 3.3–4.1, with a modern preference for reduced-oil, reduced-calorie products (Table 4). A 30-g serving of salad dressing containing 40% oil typically has about 160 calories, whereas a dressing containing 23% oil has about 80 calories. Commercially produced buttermilk ranch dressing can be spoiled by *L. brevis* subsp. *lindneri*. Experimental formulations adjusted to pH 3.8

Table 4 Examples of French and Ranch dressing compositions (% w/w)[a]

Ingredient	French dressing	Ranch dressing
	(% w/w)	
Soy oil	37.5	60.0
Vinegar (5% acetic acid)	21.0	12.0
Water	9.55	1.9
Salt	3.0	0.75
Buttermilk	0	20.0
Sugar	20.0	0.75
Egg yolk (10% salt)	0	3.5
Tomato paste	8.0	0
Oleoresin paprika	0.05	0
Onion powder	0.1	0.3
Garlic powder	0.1	0.35
Xanthan gum	0.15	0.15
Calcium disodium EDTA	0.0075	0
Potassium sorbate	0	0.1
Sodium benzoate	0	0.1
Parsley	0	0.075
Black pepper	0	0.025

[a]Anonymous (2009).

and inoculated with this microorganism did not spoil during 90 days storage at ambient temperature, whereas products adjusted to pH 4.0 or 4.2 spoiled within 30 days. None of the products at the three pH values spoiled within 90 days storage when Nisaplin™ (as 0.02% nisin) was added to the formulations. In spoiled samples without Nisaplin™, the initial inoculum of 20,000/g *L. brevis* subsp. *lindneri* quickly declined to levels of 100/g or less before growth began, a further demonstration of the microbiocidal properties of the acidified specialty products (Muriana & Kanach, 1995).

The antimicrobial properties of acidified specialty products are usually provided by acetic acid added as vinegar. However, even without vinegar, product stability can be provided. Flores, Palomar, Roh, and Bullerman (1988) tested the microbiological stability of restaurant-style Mexican hot sauces. The test sauce was composed of 51.2% water, 41.7% tomato puree, 6.0% canned Jalapeño peppers, and 1.1% salt, yielding a product with pH range of 4.0–4.2. The principal acidulant was citric acid (from the tomato puree). The authors did not mention if vinegar had been used in canning the Jalapeño peppers. The peppers may also contribute an antimicrobial effect. Nonetheless, control formulations without added preservatives were readily spoiled by yeasts within 10 days of storage at 5°C. The use of 0.05% potassium sorbate was sufficient to prevent yeast spoilage under the same conditions, whereas 0.03% potassium sorbate was not sufficient.

Ketchup

Ketchup, also called catsup, is a flavorful, widely used condiment for many foods. Typically produced from tomato paste, sugar, water, vinegar, salt, and spices, it has a pH range of 3.5–4.0 with an acetic acid content of 0.8–1.0%. It is usually preserved by pasteurization at 85–90°C and hot filled into retail containers (Michels & Koning, 2000).

Bacillus coagulans is a common spoilage microorganism for tomato-based products. However, its growth is completely inhibited even by low concentrations of acetic acid (Islam, Inoue, Igura, Shimoda, & Hayakawa, 2006). Ketchup containing 0.8% acetic acid can be spoiled by several *Saccharomyces* spp. Their mechanism of resistance to acetic acid is an inducible energy-requiring transport system that removes acetate ions from the cells (Sorrells & Leonard, 1988).

The author has occasionally noticed yeast spoilage in restaurant squeeze bottles of ketchup. The spoilage is evidenced by a pronounced off-odor and slight gassiness. Discussions with restaurant personnel typically revealed the absence of a satisfactory cleaning schedule for the reusable bottles, or ignorance of the need to clean the bottles. On a vastly larger scale, the lack of adequate cleaning and sanitation procedures for processing equipment can lead to product contamination during packaging and subsequent spoilage for most types of acidified specialty products.

Mustard

Mustard is another highly flavored, widely used condiment. Mustard preparations are generally composed of the following range of ingredients: 17–30% mustard seeds, 1.5–2.5% acetic acid, 2.5–6.5% salt, and 4–8% oil, producing products within a pH range of 3.8–4.3. Its most common spoilage microorganisms are *L. plantarum* and *Z. bailii*. Microbiological spoilage of mustard can usually be prevented by the use of 0.1% sorbic acid or benzoic acid, or 0.025% sodium bisulfite. *Z. bailii*, however, can spoil mustard in the presence of 0.15% benzoic acid. Mustard seeds produce several highly antimicrobial compounds, including *p*-hydroxybenzyl isothiocyanate and allyl isothiocyanate (Buchta, Sláviková, Vadkartiová, Alt, & Jilek, 1996; Michels & Koning, 2000).

Examination of 12 samples of commercially produced mustards revealed the absence of yeasts, enterococci, staphylococci, and enterobacteria, while numerous bacterial spore formers and seven *Lactobacillus* species were isolated. Examination of mustard ingredients revealed that most of the product microflora originated with the spices. That microflora is usually not involved in spoilage, however, because bacterial sporeformers cannot grow in mustard and the vegetative cells are killed by the other mustard ingredients (Kneifel & Slama, 1992). The addition of 0.3–1.5% mustard to mayonnaise accelerates death of the microorganisms that are present (Radford & Board, 1993). Modern mustard preparations designed to mimic the texture and color of mayonnaise can be created by diluting mustard in modified starch and gum suspensions and using artificial or natural colors.

Vinegar

One of the most essential ingredients in nearly all of the products described in this chapter, vinegar itself is manufactured for sale as a food product. It is also subject to microbiological spoilage. Vinegar is produced by the oxidation of ethanol to acetic acid by *Acetobacter aceti*. Yeast-fermented fruit juices are a common source of ethanol for vinegar production. Their further fermentation by *A. aceti* produces vinegars of different flavors and final acetic acid levels of about 4–5%. *A. aceti* is an obligately aerobic, Gram-negative bacterium that grows as a pellicle on the surface of juices, forming the "mother" of vinegar. In different settings it is itself a spoilage microorganism in fermented beverages such as wine (Krieg & Holt, 1984).

While few microorganisms can grow in large concentrations of acetic acid, several molds, yeasts, and bacteria are capable of spoiling vinegar, some being capable of growth at acetic acid concentrations as high as 9% (w/w) (Table 5). *Lactobacillus acetotolerans*, a homofermentative lactic acid bacterium, can grow in vinegars containing 4–5% acetic acid at pH 3.5 (Entani, Masai, & Suzuke, 1986). In contrast, vinegar preserves – vegetables such as beets, cucumbers, and onions that are preserved by vinegar – contain acetic acid at concentrations as low as 0.6% and are typically spoiled by *L. fructivorans* (Dakin & Radwell, 1971).

Table 5 Upper limit of acetic acid concentration (% w/w) permitting the growth of aceto-tolerant spoilage microorganisms in the pH range 3.5–4.0[a]

Microorganism	Acetic acid (% w/w)
Molds	
Moniliella acetoabutans	9.0
Cladosporium suaveolens	8.0
Monascus ruber	1.8
Penicillium glaucum	1.4
Yeasts	
Saccharomyces acidifaciens	3.5
Pichia membranaefaciens	3.0
Bacteria	
Lactobacillus acetotolerans	5.0
Lactobacillus buchneri	2.5

[a]Tuynenburg Muys, van Gils, and de Vogel (1966); Tuynenburg Muys, 1971; Entani et al. (1986).

Methods for Detection of Spoilage Microorganisms

There are relatively few microbiological methods for detecting, enumerating, and identifying the microorganisms that can spoil acidified specialty products because of the very narrow range of growth parameters in the products and the limited types and range of spoilage microorganisms. As is the case with many microbiological investigations, a simple microscopic examination to establish the predominance of bacteria, yeasts, or molds can be helpful (Fleming, McFeeters, & Breidt, 2001). Further clues to aid the investigation of spoilage incidents can be gathered by examining samples from the processing equipment, particularly product buildups that may harbor spoilage microorganisms and allow their growth to high levels. The acclimation of microorganisms to product residues on equipment may enable them to more readily spoil the product should cross-contamination occur during packaging. Microorganisms isolated from equipment swab samples may be useful for research in challenge tests and process validation studies (Smittle & Cirigliano, 2001).

Tests for the detection and identification of yeasts have been compiled by Deak and Beuchat (1996). A test to enumerate acid-resistant yeasts is most helpful to study microbiological spoilage in this category of products. A long-used test involves the use of Malt Extract Agar (MEA) or Tryptone Glucose Yeast Extract Agar (TGYA) to differentiate acid-tolerant yeasts that could cause spoilage from non-acid-tolerant yeasts that cannot spoil products in this category. MEA or TGYA are sterilized according to instructions. After tempering in a water bath, sufficient glacial acetic acid (16 N) is added to yield 0.5% acetic acid in the medium. The acidified media – MEA will be about pH 3.0, TGYA about pH 3.5 – must be used relatively quickly before the agar is acid hydrolyzed. The glacial acetic acid is used without any sterilization treatment.

Routine tests for the detection and enumeration of lactic acid bacteria, yeasts, and molds are described in the citations immediately above.

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Microbiological Spoilage of High-Sugar Products

Sterling Thompson

Introduction

The high-sugar products discussed in this chapter are referred to as chocolate, sugar confectionery (non-chocolate), liquid sugars, sugar syrups, and honey. Products grouped in the sugar confectionery category include hard candy, soft/gummy candy, caramel, toffee, licorice, marzipan, creams, jellies, and nougats. A common intrinsic parameter associated with high-sugar products is their low water activity (a_w), which is known to inhibit the growth of most spoilage and pathogenic bacteria. However, spoilage can occur as a result of the growth of osmophilic yeasts and xerophilic molds (Von Richter, 1912; Anand & Brown, 1968; Brown, 1976). The a_w range for high-sugar products is between 0.20 and 0.80 (Banwart, 1979; Richardson, 1987; Lenovich & Konkel, 1992; ICMSF, 1998; Jay, Loessner, & Golden, 2005). Spoilage of products, such as chocolate-covered cherries, results from the presence of yeasts in the liquid sugar brine or the cherry. Generally, the spoiled product will develop leakers. The chocolate covering the cherry would not likely be a source of yeast contamination.

In addition to a_w, the stability of high-sugar products is influenced by pH, processing and storage temperatures, and the presence of preservatives (Deak & Beuchat, 1996).

To consumers, the term sugar is usually associated with sucrose, which is widely distributed in nature and used extensively in products throughout the food industry. However, dextrose, lactose, and fructose are also used. More recently, polyols such as sorbitol, mannitol, xylitol, and maltitol have become replacements for sugar in sugar-free confectionery and chocolate products, as well as many other food products.

S. Thompson (✉)
The Hershey Company, Hershey, PA 17033, USA
e-mail: sthompson@hersheys.com

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Chocolates

Chocolate products originate from the processing of cacao beans. Cacao is grown in several tropical countries in Africa, South America, Central America, Asia, and the Caribbean. Cocoa nibs, the product obtained following winnowing and roasting, are processed to produce a liquid cocoa mass (liquor). Under pressure, this cocoa mass will yield cocoa butter and cocoa press cake. The finished chocolate product is produced by combining several ingredients in various ratios based on the type of chocolate desired. A finished chocolate product may consist of 12–58% cocoa butter, 20–35% cocoa solids, 0.5–7% milk fat, 3–14% fat-free milk solids, and up to 55% sugar (0% for unsweetened chocolate) (Cordier, 2000). A description and essential composition of chocolate products is listed in the Codex Standard 87 – 1981, (Codex Alimentarius, 1994). A milk chocolate product consists of milk fat, milk solids, cocoa solids, cocoa butter, lecithin, and flavors. Dark chocolate does not contain the milk components and white chocolate does not contain cocoa solids. Chocolate confectionery products include sugar confections covered with chocolate, as well as solid bars and solid pieces (Lenovich & Konkel, 1992). Other products on the market that are enrobed in chocolate include granola, nuts, wafers, raisins, other fruits, caramel, and flavored cremes. Chocolate is not susceptible to microbial growth or spoilage because of its high percentage of solids which provides an a_w range of 0.40–0.50 (Lenovich & Konkel, 1992).

In order to develop the chocolate flavor that is associated with the finished chocolate product, cacao beans must be fermented to develop the desired flavor precursors. The cacao bean becomes contaminated with microorganisms as soon as the pod is opened and the beans come in contact with the hands and tools of the workers, leaves, insects, animals, soil, bags, and the wood of the fermentation boxes. The fermentation consists of a succession of microorganisms: yeasts, acetic acid, and lactic acid bacteria. Once the fermentation is complete, the beans are sun-dried or mechanically dried to a moisture content of 6–8%. The microorganisms associated with the dried beans primarily consist of mesophilic and thermophilic sporeformers, *Enterobacter* spp., *Micrococcus* spp., *Flavobacterium* spp., yeasts, and molds (Barrile, Ostovar, & Keeney, 1971). Once the desired moisture range is obtained, proper handling, transporting, and storage of the dried beans will prevent the outgrowth of molds on the surface of the beans.

Most microorganisms associated with the intact bean are located on the bean's outer shell surface. A pathogen such as *Salmonella* can be introduced to the bean's outer surface through any of the early handling steps from removal from the cocoa pad to packing in burlap bags. The roasting step, a thermal process that is unique to each chocolate manufacturer, is the critical control point (CCP) step that will inactivate *Salmonella* and other vegetative bacterial pathogens. Roasting serves a twofold purpose; it kills bacterial pathogens such as *Salmonella* and modifies the flavor precursors that developed during fermentation. Roasting initiates chemical reactions that will influence the flavor of chocolate (Zak, 1988); whole beans, nibs, or cocoa liquor may be roasted. The temperature and time used for roasting will depend on the original bean type and the flavor profile desired by the chocolate manufacturer.

Temperatures from 105 to 150°C (221–302°F) will be sufficient to destroy vegetative microorganisms, including pathogens such as *Salmonella* (Williams, Clavero, Silliker, & Flowers, 2006). Microorganisms that have been detected on roasted beans included sporeformers such as *Bacillus stearothermophilus*, *B. licheniformis*, *B. subtilis*, *B. coagulans*, *B. circulans*, *B. brevis*, and *B. megaterium* (Barrile et al., 1971; Ostovar & Keeney, 1973).

The production of chocolate from roasted beans, nibs, or liquor involves several processing steps: milling and refining; mixing with sugar, milk powder, and cocoa butter; conching to obtain the desired plasticity and flavor development; tempering to obtain proper fat crystallization; and molding. None of these processing steps will affect the original microbial population (Collins-Thompson, Weiss, Riedel, & Cushing, 1981). However, changes in the microbial population will be influenced with the addition of ingredients such as milk powder, sugar, nuts, or dried fruits. The presence of vegetative bacteria such as indicator or pathogenic microorganisms is associated with post-thermal processing (post-roasting) contamination from ingredients, the environment, or from a failure to inactivate these microorganisms during roasting. The international confectionery industry has a good food safety record. Between 1999 and 2003, 125 (11%) of the 1,146 Food and Drug Administration food recalls were associated with chocolate and non-chocolate candy products. Six (5%) of these were caused by microorganisms (Williams et al., 2006). However, several salmonellosis outbreaks have been caused by the consumption of contaminated chocolate products (Craven et al., 1975; Gastrin et al., 1972; Gill et al., 1983; Hockin et al., 1989; Kapperud et al., 1990). Examples of outbreaks of salmonellosis that occurred between 1970 and 2001 are shown in Table 1. Considering the age of the candy implicated in these outbreaks, it appears that *Salmonella* can survive in chocolate for varying periods of time. Laboratory studies have revealed that *Salmonella* survives for extended periods in chocolate (Tamminga, 1979; Tamminga, Beumer, Kampelmacher, & Leusden, 1976).

Implementing proper and effective process and sanitation controls can control access to the processing environment by microorganisms. Such steps will prevent pathogenic and spoilage microorganisms from becoming established in the

Table 1 Examples of salmonellosis associated with chocolate[1]

Year	Country	Serotype	Product	Source
1970	Sweden	*S. durham*	Confectionery	Cocoa powder
1973	US/Canada	*S. eastbourne*	Chocolate candy	Environment
1982–1983	United Kingdom	*S. napoli*	Chocolate bars	Water
1986	Canada	*S. nima*	Chocolate coins	Cross-contamination
1987	Norway	*S. typhimurium*	Chocolate	Unknown
2001	Germany, Denmark, and other European Union Nations	*S. oranienburg*	Chocolate/chocolate bars	Inadequate thermal processing suspected

[1]Adapted from Williams et al. (2006).

processing environment where they may survive and cross-contaminate the product. Contamination of chocolate products can be controlled by using validated roasting conditions, selecting ingredient suppliers with the desired food quality/safety programs in place to prevent contamination of the ingredients, using ingredients that meet the company's microbiological specifications, testing ingredients for spoilage and pathogenic microbes, controlling the production environment to prevent the introduction of pathogens, and preventing post-processing contamination.

Sugars

Sucrose is derived from two primary agricultural sources: sugarcane (*Saccharum officinarum*) which is grown in subtropical and tropical climates and sugar beets (*Beta vulgaris*) which are grown in moderate climates. These two sources represent 99% of the world's sucrose production. Smaller amounts of sucrose are produced from sugar palms (1%), sweet sorghum (0.05%), and maple trees (0.01%) (FAO, 1998). Purified crystallized sucrose contains less than 0.1% moisture, whereas the moisture content of brown or soft sugar ranges from 2 – 4.5%. Invert sugar, a hydroscopic sugar, consists of equivalent percentages of dextrose and fructose and residual sucrose, and has a moisture content of 23–27%. Sucrose liquid sugars have a moisture content of 32–33%. Dextrose or D-glucose is used in the food industry as crystallized dry powder in anhydrous or monohydrated form containing 2 and 10% moisture, respectively. Fructose is also supplied to the food industry as a dry powder with about 0.5% moisture. The polyols are commercialized as crystalline powder with up to 6% moisture. The powder and granulated forms of sugars are more microbiologically stable than the liquid sugars. The large and diverse microbial populations associated with sugarcane and sugar beets originate from the soil and decaying plant materials. Microorganisms detected in sugarcane include *Pseudomonas*, *Bacillus*, Enterobacteriaceae, *Lactobacillus*, *Erwinia*, *Leuconostoc*, *Flavobacterium*, *Xanthomonas*, *Corynebacterium*, yeasts, and molds (Nunez & Colmer, 1968; Shehata, 1960). Microorganisms isolated from sugar beets include *Pseudomonas*, *Arthrobacter*, *Erwinia*, *Streptomyces*, *Bacillus*, *Clostridium*, *Flavobacterium*, and yeasts (Allen, Cooper, Cairns, & Maxwell, 1946; Bungee, Cole, & Nielsen, 1975). In each of the processing steps associated with refining sucrose from sugar beets and sugarcane, from the raw agricultural product to the finished product, some level of microbial contamination originating from these diverse populations typically occurs. The microbial populations change during processing; however, some processing steps have a greater influence on the microbial population than others. Overall, the microbial population decreases as the product flows from the initial processing step to the final step. Some of the microbes occurring at the various sugarcane processing steps are listed in Table 2. Changes in the mesophilic and thermophilic populations of sugarcane at different stages during the processing of cane sugar are shown in Table 3.

Table 2 Microorganisms associated with the major steps in cane sugar processing

Process step	Microorganisms
Pre-harvest	*Flavobacterium, Lactobacillus, Xanthomonas, Enterobacter,*[1] *Pseudomonas, Erwinia, Leuconostoc, Bacillus, Corynebacterium*; yeasts, molds[2]
Post-harvest	*Leuconostoc*
Crushing and extraction	*Leuconostoc, Enterobacter,* yeasts
Clarification	None
Evaporation/crystallization/centrifugation	Thermophilic sporeformers
Refining	Thermophilic sporeformers

[1] Nunez and Colmer (1968).
[2] Shehata (1960), Tilbury (1970).

Table 3 Bacterial content (range) of sugarcane at different stages during processing[1]

Product	Mesophiles (CFU/ml or g)	Thermophiles (CFU/ml or g)
Raw juice	$8 \times 10^6 - 8 \times 10^8$	$1 \times 10^1 - 1 \times 10^2$
Clarified effluent	0–11	0–8
Press juice	$0–5 \times 10^4$	$3 \times 10^3 - 2 \times 10^5$
Evaporator	$2 \times 10^2 - 3 \times 10^4$	$2 \times 10^2 - 2 \times 10^3$
Crystallizer	$1 \times 10^3 - 4 \times 10^4$	$3 \times 10^2 - 2 \times 10^4$
Raw sugar	$3 \times 10^2 - 5 \times 10^3$	$2 \times 10^2 - 2 \times 10^3$

[1] Adapted from Owen (1977).

Populations of *Leuconostoc mesenteroides* which may increase during the harvesting of sugarcane may result from contact with contaminated harvesting tools and insects (Bevan & Bond, 1971; Egan, 1971). *Leuconostoc mesenteroides* can hydrolyze sucrose to dextran, a slimy, gummy glucose polymer. An effective cleaning and sanitation program for the mill equipment will control the presence of microorganisms and reduce recontamination from the raw juice. Heating the juice from 80 to 100°C (176–212°F) will kill vegetative bacteria and yeasts. Only bacterial spores, typically *Bacillus* spp. and *Clostridium* spp., will remain in the raw juice. If dextran is formed during the earlier stages of the process, it will clog pipes and lines, and affect evaporation and crystallization of the juice. The flow diagram for processing sugarcane is shown in Fig. 1 (Cordier, 2000).

After sugar beets are harvested, they are stored in covered, ventilated piles for several days to months before processing. The piles will maintain a temperature in the range of 1.5–5.0°C (34.7–41°F) even if the external temperature decreases below 0°C (32°F) (Cordier, 2000; ICMSF, 1998). Similar to the process used to produce sugar from sugarcane, there are several processing steps required to obtain the final sugar product from sugar beets (Fig. 2; Cordier, 2000). Generally, sugar beets will not spoil during storage unless the storage time is extended or the beets become damaged from freezing and thawing, or overheating 50°C (122°F). Under

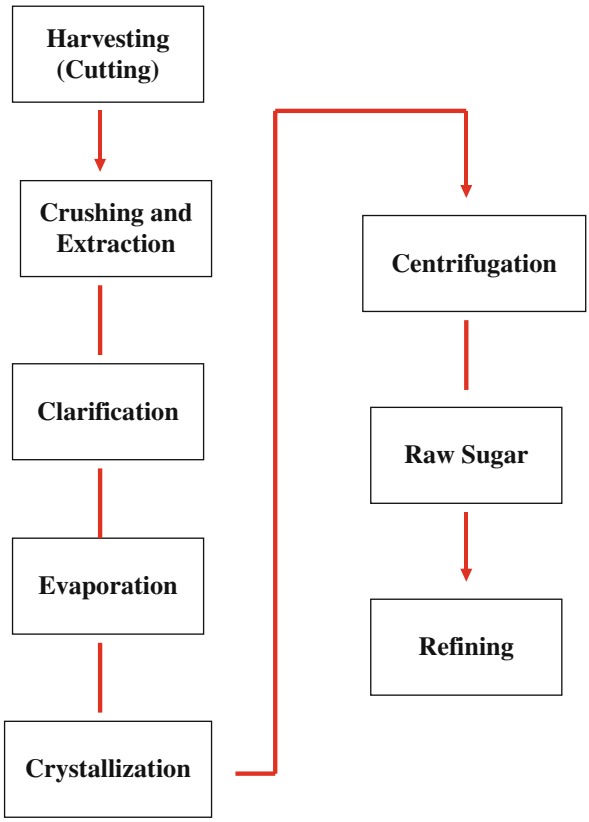

Fig. 1 Flow diagram of cane sugar processing

these conditions, dextran, levan, or inverted sugars are produced (Cole & Bugbee, 1976; Oldfield, Dutton, & Teague, 1971).

Soil and microorganisms are removed as the sugar beets move through the first process step (washing). Steps must be taken to ensure that clean water replaces the heavily contaminated water or the raw juice will become highly contaminated. As the beets move through the process, the microbial population will be influenced by the different ecosystems created at each processing step.

The types of microorganisms occurring at the various processing steps are listed in Table 4. Heating the water to between 65 and 70°C (149–158°F) will favor the growth of spore-forming thermophiles, such as *Bacillus* spp. and *Clostridium* spp. (Belamri, Douiri, Fakhereddine, & Tantaoui-Elaraki, 1993; Belamri, Mekkaoui, & Tantaoui-Elaraki, 1991; Klaushofer, Hollaus, & Pollach, 1971). Microorganisms that survive or that are introduced during the process and remain viable through the final refining step may grow during refining. Bacteria that have been detected in refined product include *Clostridium thermosaccharolyticum*, *Leuconostoc*, *Lactobacillus*, and *Streptococcus* (Belamri et al., 1993; Tilbury, 1975; Tilbury,

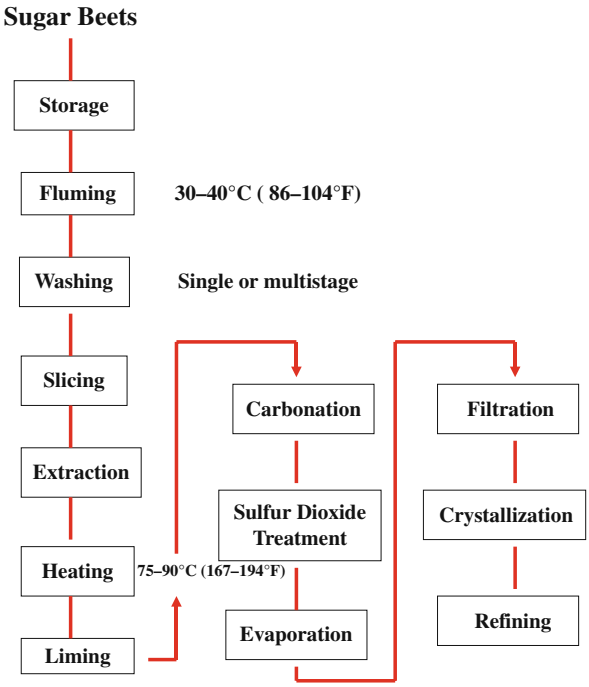

Fig. 2 Flow diagram of beet sugar processing

Table 4 Microorganisms associated with the major steps in beet sugar processing

Process step	Microorganisms
Preharvest	*Pseudomonas, Bacillus, Arthrobacter, Erwinia, Flavobacterium, Streptomyces,* yeast,[1] *Clostridium*[2]
Post-harvest storage	*Bacillus, Leuconostoc*
Fluming	*Pseudomonas, Flavobacterium*
Washing	*Pseudomonas, Flavobacterium*
Extraction	*B. stearothermophilus, Clostridium,* thermophilic cocci
Crystallization	Thermophilic sporeformers

[1]Bugbee et al. (1975).
[2]Allen et al. (1946).

Orbell, Owen, & Hutchenson, 1976). The yeasts most frequently detected include *Torulopsis*, *Zygosaccharomyces*, and *Hansenula*. Growth of thermophiles involves metabolism of sucrose, creating losses from 0.1 to 0.5% (Carruthers & Oldfield, 1955; Klaushofer & Parkkinen, 1966; Stark, Goodban, & Owens, 1953). *Bacillus stearothermophilus* and *B. coagulans* produce lactic acid and other acids from sucrose (McMaster & Ravnö, 1977). The most noticeable spoilage that occurs in sugar beet juice is slime (dextran) formation. The slime will clog pipes, lines, filters, and processing equipment. Dextran is formed by *L. mesenteroides*,

Leuconostoc dextranicum, and heterofermentative *Lactobacillus* spp. (Perquin, 1940). Several *Bacillus* spp. produce levan from sucrose. Some strains of *B. stearothermophilus* reduce nitrate to nitrite. The nitrite that is formed reacts with other chemicals and reduces the quality and yield of sugar. Nitrite combines with bisulfate and affects its ability to prevent browning and inhibit thermophiles (Carruthers, Gallagher, & Oldfield 1958; Oldfield, Dutton, & Shore, 1974) and forms imidodisulfonate. Imidodisulfonate cocrystallizes with sucrose to increase the ash content and cause malformed crystal formation. In its dry granular form, refined sugar will not support microbial growth. When water is combined with the granular form to produce a liquid sugar or if environmental conditions, such as high relative humidity cause the uptake of moisture, the sugar will become more susceptible to microbial growth.

Generally, refined sugar is not a source of spoilage yeasts or molds. However, refined sugar may be a source of bacteria, including *B. stearothermophilus*, *B. coagulans*, *C. thermosaccharolyticum*, *Desulfotomaculum nigrificans*, and mesophilic bacteria. The presence of any one of these bacteria in sugar that is used in the formulation of products such as bottled drinks or canned foods could spoil the products. Specific microbiological specifications were established for granulated and liquid sugars by bottles and canners to limit the maximum levels of mesophilic and thermophilic spores (Horwitz, 1975; NFPA, 1972; National Soft Drink Assoc. 1975).

Microbiological specifications for different types of sugars include the following:

1. Sugar (National Canners Association)

   A. *Total thermophilic spore count*: Of five samples from a lot of sugar, none shall contain more than 150 spores per 10 g, and the average for all samples shall not exceed 125 spores per 10 g.
   B. *Flat-sour spores*: Of five samples, none shall contain more than 75 spores per 10 g, and the average for all samples shall not exceed 50 spores per 10 g.
   C. *Thermophilic anaerobe spores*: Not more than three (60%) of five samples shall contain these spores, and in any one sample, not more than four (65%) of six tubes shall be positive.
   D. *Sulfide spoilage spores*: Not more than two (40%) of five samples shall contain these spores, and in any one sample, there shall be no more than five colonies per 10 g (equivalent to two colonies in six tubes).

2. "Bottlers" Granulated Sugar, Effective July 1, 1953 (American Bottlers of Carbonated Beverages)

   A. *Mesophilic bacteria*: Not more than 200 per 10 g.
   B. *Yeasts*: Not more than 10 per 10 g.
   C. *Molds*: Not more than 10 per 10 g.

3. "Bottlers" Liquid Sugar, Effective in 1959 (American Bottlers of Carbonated Beverages). All figures based on dry-sugar equivalent (DSE)

A. *Mesophilic bacteria* (a) Last 20 samples should average 100 organisms or less per 10 g of DSE; (b) 95% of the last 20 counts should be 200 or less per 10 g; (c) 1 of 20 samples may be greater than 200; other counts should be the same as in (a) or (b).
B. *Yeasts*: (a) The last 20 samples should average 10 organisms or less per 10 g of DSE; (b) 95% of the last 20 counts should be 18 or less per 10 g; (c) 1 of 20 samples may be greater than 18; other counts should be the same as in (a) and (b).
C. *Molds*: Specifications are the same as those for yeasts.

Syrups

Sugar syrups are categorized into three categories: liquid sugar; starch-based syrups, and naturally occurring tree saps, e.g., maple syrup or starches of multiple origins (potato, corn, wheat). Sugar syrups are used in a variety of products throughout the food industry (confectionery, beverages, bakery, ice cream, jams, jellies, and meat products). Glucose syrup should consist of a minimum of 20% dextrose equivalents and 70% total solids (Codex Alimentarius, 1994). Liquid sugar manufactured from cane or beet sugar is produced by mixing crystalline refined sugar with water. Maple syrups are collected from the sugar maple tree, *Acer saccharum* or *Acer rubrum*, after the tree is drilled and tapped. Maple syrup is composed of 88–99% sucrose, 0–11% glucose and fructose, and several organic acids, minerals, and vitamins (Morselli & Feldheim, 1988). Maple sap must be concentrated, typically by evaporation or reverse osmosis, to prepare maple syrup and sugar. Syrups made from starches are concentrated solutions consisting of glucose and maltooligosaccharides. The starch is hydrolyzed using an acid process, an acid-enzyme or a multi-enzyme process.

The addition of water to refined sugar to produce liquid sugar produces an ingredient that will be more susceptible to microbial spoilage. The a_w of liquid sugar is at a level that makes it prone to yeast and mold growth. *Zygosaccharomyces rouxii*, *Saccharomyces cerevisiae*, *Saccharomyces mellis*, and molds may grow in liquid sugar. Whether these microorganisms will grow depends on the level of contamination, the availability of non-sucrose nutrients, and gradients with high water activities within this substrate material. Gradients develop when water does not readily mix with high concentrations of sugar without effective agitation. Gradients with varying water activities will also occur in inadequately washed storage tanks, pipes, valves, and damp equipment. These pockets of water provide a favorable environment for the growth of yeasts. Yeasts have been detected in liquid sugar samples obtained directly from tankers used to transport liquid sugar (S. Thompson, unpublished data). Conditions for proliferation of these microorganisms may have resulted from moisture gradients developing in tankers or from uncleaned or an inadequately cleaned tanker or equipment at the processing facility in which a niche was created for yeast growth. Contaminants can be controlled by thoroughly cleaning and

sanitizing tanks, pipes, and valves. Liquid sugar can be filtered, heated, or passed over ultraviolet lamps to remove or inactivate microbial contaminants. The development of condensate in storage tanks can be prevented by pumping UV-treated filtered air over the surface of liquid sugar.

Maple sap in the tree is usually sterile; however, sap obtained using traditional harvesting practices becomes contaminated with yeasts and bacteria (Naghski & Willis, 1955). The most prevalent bacterium in the sap is *Pseudomonas fluorescens* (Morselli & Feldheim, 1988). A variety of bacteria, including *Pseudomonas*, *Aerobacter*, *Leuconostoc*, and *Bacillus* spp., have been isolated from late-season raw sap (Kissinger, 1974). Some spoilage defects associated with sap include "green sap" caused by fluorescent pseudomonads, "red sap" caused by red yeasts and some bacteria, "milk sap" caused by bacilli, and "ropy sap" caused by exopolysaccharides excreted by *Enterobacter agglomerans* (Britten & Movin, 1995).

Concentrating maple sap by evaporation pasteurizes the sap (ICMSF, 1998). However, environmental monitoring has revealed that a facility's processing environment can be a source of *Penicillium* spp., *Aspergillus* spp., and yeast contamination. The low a_w of maple syrup will inhibit bacterial growth. Molds may grow on the surface of the syrups and produce flavor and color defects (Hayward, 1946; Whalen & Morselli, 1984). With an a_w range between 0.83 and 0.86, maple syrup will not support the growth of bacterial pathogens. Microbial growth during processing can be controlled by processing the sap quickly, holding the sap at 40°C (104°F) until ready to process, or using UV light to sterilize the headspace and surface of the tank. All equipments should be cleaned on a regular frequency.

Generally, maple syrup is packaged at temperatures from 99.4 to 103.3°C (211–218°F). As with other products that are packaged using a hot fill, the bottle should be inverted or laid on its side in order to pasteurize the headspace and cap. *Saccharomyces acerissacchari*, *Saccharomyces behrensianus*, *Saccharomyces monocensis*, *Zygosaccharomyces mellis*, *Zygosaccharomyces barkeri*, *Zygosaccharomyces japonicus*, and *Zygosaccharomyces nussbaumeri* have been isolated from spoiled maple syrup (Smittle, Krysinski, & Richter, 1992). Microorganisms may be killed by sanitizing processing equipment or heating the product before packaging (Dumont, Saucier, Allard, & Aurouze, 1993). Molds can grow under aerobic conditions and cause visible spoilage of maple and other syrups (Walker & Ayres, 1970). Maple syrup without an added preservative such as potassium sorbate may support mold growth on its surface, especially after the package has been opened. Refrigerating the container of opened syrup can prevent mold growth. If the syrup is not refrigerated, it should be used quickly to avoid product spoilage.

Invert syrup is produced from sucrose solutions containing 60–75% total solids. There are three techniques used to achieve inversion: enzymatic inversion using microbial invertase at 65°C (149°F), acid inversion using HCl at 90°C (194°F), and ion-exchange inversion using acidic cationic exchange resins (Pancoast & Junk, 1980).

Several flavored syrups are available at retail. Some of these have a simple formulation of flavor, color, sugar, gum, water, and preservative. A few of the syrups may be acidified, having a pH of less than 4.6. In addition, some may contain a

preservative to inhibit the growth of yeasts and molds. The combination of low a_w, acidic pH, and the presence of a preservative makes syrups less susceptible to microbial spoilage. If the microbial population in syrup is high, microorganisms likely contaminate the product's post-thermal processing. The syrups that receive a thermal process are hot filled and cooled in the primary package before being placed into cases for shipment. The thermal process usually inactivates vegetative bacteria, yeasts, and molds that may have been present in one or more of the ingredients used in the formulation. A possible source of microbial contamination of syrup is cooling water that is sprayed on the containers to cool the product so that it does not become a heat sink. Occasionally, a cap may not be properly screwed onto the plastic bottle, "cocked cap," allowing water to enter the container as the container moves through the cooling tunnel, thereby introducing microorganisms. However, if the cooling water is properly chlorinated the microbial population will be controlled. Another potential cause of water entry is the presence of pinholes in the primary package. Contaminated syrup that is stored for an extended period of time at conditions that will allow microbial growth will typically show visible spoilage either as a swollen package or as surface mold growth on the product. Syrups that are packaged in glass containers can present an explosion hazard if yeast spoilage occurs.

Corn starch processing consists of the steps depicted in Fig. 3 (Petersen, 1975; Smith, 1981). Steeping is a controlled enzymatic degradation step wherein corn is soaked in 45–50°C (113–122°F) water containing 0.1–0.2% SO_2 for 24 – 48 hrs at a pH of ~4.0 (the acid pH and SO_2 is used to control microbial contamination). Wet milling is conducted in a liquid cyclone used to crack open the corn kernels to release the germ. Prior to washing, the germ is pressed to remove oil. During washing, the remaining starch–gluten mixture is separated and washed chemically and/or physically modified. The germ is dried to 10–17% moisture using flash, belt, or drum dryers (Whistler & Paschall, 1967).

Starch is used to produce glucose, fructose, and maltose syrups. These syrups can be used to produce crystallized sugars. Starch-based syrups are made by hydrolyzing corn starch or another starch using one of the three processes. The acid process uses acids, such as sulfuric acid, to reduce the pH to about 2.0 to facilitate hydrolysis. The hydrolysis is terminated by adding calcium carbonate, a neutralizing agent, when the desired degree of hydrolysis, i.e., dextrose equivalent (DE) is achieved. The acid-enzyme process combines the former acid hydrolysis with an enzymatic hydrolysis step using amyloglucosidases to achieve the desired DE. The multi-enzyme process combines amylases with amyloglucosidase to achieve the desired DE. This reaction is stopped by heating. If necessary, the mixtures should be neutralized, clarified, and evaporated.

High maltose syrup is produced by adding β-amylase or pullulanase to a partially hydrolyzed starch slurry. High fructose syrup is produced by adding isomerase to a partially hydrolyzed starch slurry. High glucose syrup can also serve as an intermediate product in the preparation of high fructose syrup. Maltodextrins are produced by stopping the conversion at an early step in the process. Polyols are produced by hydrogenation of different sugar syrups (Le Bot & Gouy, 1995). After the various

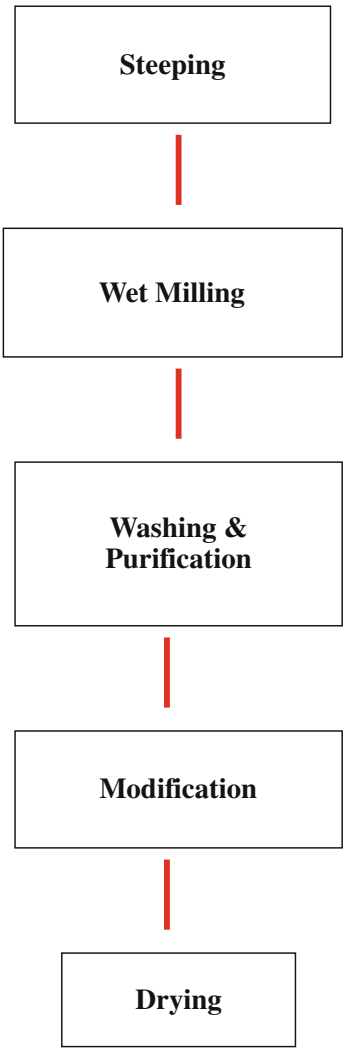

Fig. 3 Steps associated with product of sugars from starch

processes, the syrups can be filtered, decolorized, concentrated, and purified using an ion-exchange column.

Generally, the water activities of syrups range from 0.70 to 0.85, making them susceptible to spoilage by *Zygosaccharomyces bailii* and *Z. rouxii*. The presence of these yeasts in syrups that may be incorporated into formulations to produce other products may cause spoilage of those products. Inclusion of contaminated syrup as an ingredient into a formulation of a confectionery product can result in fermentation, gas production, leakage, and product destruction (Pitt & Hocking, 1997).

Table 5 Examples of spoilage microorganisms detected in sugars and syrups

Product	Microorganisms
Maple syrup	*Zygosaccharomyces rouxii, Saccharomyces cerevisiae, S. mellis*
Honey	*Z. japonicus, Z. barkeri, Z. mellis, Z. prioriano, Z. nussbaumeri, Z. richteri*
Moistened white crystalline sucrose	*Torulopsis apicola*
Raw sugar	*S. rouxii, T. candida*
Brown and white sugar syrups	*Z. rouxii, Z. bailii* var. *osmophilus, Saccharomyces* spp., *C. valida, Hansenula anomala* var. *anomala, Kloeckera apiculata, S. cerevisiae*
Canned syrup	*S. zsopfi*
Table syrups	Molds

ICMSF (1980); Tilbury (1976); Troller (1979); Walker and Ayres (1970).

Examples of spoilage microorganisms detected in sugars and syrups are listed in Table 5.

Bacterial growth and spoilage of syrups is prevented by low water activity. Sugar syrups have not been associated with a foodborne disease outbreak in part because bacterial pathogens will not grow in these products. Several studies have been conducted to determine if *Clostridium botulinum* spores concentrate sugar syrups. Kautter, Lilly, Solomon, and Lynt (1982) detected *C. botulinum* spores in 1.3% of corn syrup samples tested at a contamination level of ca. 50 spores/g. Later studies did not detect *C. botulinum* spores in 43 samples of different syrups (Hauschild, Hilsheimer, Weiss, & Burke, 1988) or in 738 samples of corn syrup or products in which corn syrup was an ingredient (Lilly, Rhodehamel, Kautter, & Solomon, 1991).

Microbial spoilage of sugar syrups can be controlled by removing or inactivating microorganisms capable of growing in syrups, properly cleaning and sanitizing equipment, and preventing the development of condensate (ICMSF, 1998). Kelly (1967) concluded that the microorganisms associated with equipment (tanks, pipes, valves) could be controlled with heat or chlorine and their growth prevented by applying persistent iodophores (no rinse sanitizers) to equipment. Liquid sugar may be filtered to remove microorganisms and heated or exposed to ultraviolet lamps to kill microorganisms that may be present. UV lamps may be placed in the upper areas of tanks to kill yeast and molds that may be on the surface of the sugar syrup. However, a UV lamp has only a limited distance of effectiveness above the surface of the ingredient. Microbial contamination of condensation is controlled by treating filtered air with UV irradiation; the air is blown over the surface of the sugar syrup. UV irradiation will not significantly affect the physicochemical quality characteristics of sugar syrups (Giorgi & Gontier, 1980).

Osmophilic yeasts and molds, if present, can grow slowly in sugar syrups. The lag times and generation times are influenced by the water activity of the syrup. Growth of yeasts and molds is also influenced by the size of the inoculum, the availability of non-sucrose nutrients, and gradients with increasing water activity. Bacteria are inhibited from growing in sugar syrups.

As indicated above, there are methods that can be used to prevent contamination, recontamination, or growth of yeasts. Preventive measures include good manufacturing practices (GMP) and good hygienic practices (GHP), sanitation and sterilization of processing equipment, and/or the product to destroy yeasts. During storage, recontamination can be prevented by adding air filters and UV lamps to tanks (Fiedler, 1994; Pancoast & Junk, 1980). Without proper aeration of filtered air, condensation will develop causing water activity gradients to form. The presence of these gradients will allow multiplication of microorganisms in pockets or surfaces where the water activity has increased.

Honey

Honey is used widely throughout the food industry in such products as condiments, salad dressings, barbecue sauce, peanut butter, dairy products, meats, beverages, snacks, bread, cereal products, and candy. The composition of honey is primarily influenced by the composition of the nectar harvested by bees. Honey generally contains 15 – 21% water, 30 – 35% glucose, 35 – 45% fructose, 1 – 3% sucrose, 10 – 12% maltose, organic acids, minerals, proteins, amino acids, and enzymes (Cordier, 2000; ICMSF, 1998). The a_w of honey ranges between 0.54 and 0.75 (Banwart, 1979; Fett, 1973). As with other high-sugar products, the moisture content of honey will have a major influence on its susceptibility to spoilage. If honey, which is hygroscopic, is not stored under appropriate conditions, moisture will be absorbed, increasing the likelihood of spoilage. Honey is commercially available in two predominant forms: liquid and crystallized (granulated) (ICMFS, 1998). Other forms which exist include spreadable creamed honey, comb honey, and chunk honey (Cordier, 2000).

The microorganisms of primary concern to the honey industry are osmophilic yeasts, which can grow and cause spoilage; *C. botulinum* types A and B, which have been associated with infant botulism (Aureli & Accorti, 1981; Hazzard & Murrell, 1989; Midura, 1996); and *Bacillus larvae*, which causes the "foul brood" or "American plague" disease of bees. The sporadic contamination of honey by *C. botulinum* may not be preventable. The conditions normally used to process honey will not inactivate bacterial spores. Sterilization of diluted honey may be the only effective control treatment. To reduce the threat of infant botulism, the American Academy of Pediatrics has recommended that infants up to 12 months of age not be fed products containing honey (Cordier, 2000).

In addition to these sporeformers and osmophilic yeasts, a wide range of other bacteria, yeasts, and molds have been detected in honey (Table 6). Tysset, Brisou, Durand, and Malaussene (1970) identified *Bacteridium*, *Bacterium*, *Bacillus*, *Brevibacterium*, *Enterobacter*, *Flavobacterium*, *Micrococcus*, *Neisseria*, *Pseudomonas*, and *Xanthomonas* in French honey (Kokubo, Jinbo, Kaneko, & Matsumato, 1984). The genus *Bacillus* was predominant, with *B. cereus* and *B. pumilus* the dominant species (Kokubo et al., 1984). Smaller populations of

Table 6 Microorganisms isolated from honey[1]

Bacteria	Fungi Yeasts	Molds
Alcaligenes	*Ascosphaera*	*Aspergillus*
Bacillus	*Debaryomyces*	*Atichia*
Bacteridium (sic)	*Hansenula*	*Bettsia alvei*
Bacterium (sic)	*Lipomyces*	*Cephalosporium*
Brevibacterium	*Nematospora*	*Chaetomium*
Clostridium	*Oosporidium*	*Coniothecium*
Enterobacter	*Pichia*	*Hormiscium*
Flavobacterium	*Rhodotorula*	*Penicillium*
Klebsiella	*Saccharomyces*	Peronosporaceae
Micrococcus	*Schizosaccharomyces*	*Peyronelia*
Neisseria	*Schwanniomyces*	*Triposporium*
Proteus	*Trichosporon*	Uredianceae
Pseudomonas	*Torula*	Ustilaginaceae
Xanthomonas	*Torulopsis*	
	Zygosaccharomyces	

[1]Snowdon and Cliver (1996).

B. coagulans, *B. megaterium*, and *B. alvei* were also observed. *Micrococcus*, *Pseudomonas*, and *Staphylococcus* have been isolated from honey produced in the United States (Snowdon & Cliver, 1996). Several researchers reported aerobic plate counts of honey ranged from 0 to 9,500 CFU/g (Nakano, Okabe, Hashimoto, & Sakoguchi, 1989; Piana et al., 1991; Tysset, Durand, & Taliergio, 1970; Tysset & Rousseau, 1981).

The presence of *C. botulinum* spores in honey has been reported by several researchers (Aureli, Ferrini, & Negri, 1985; Du, Cheng, Lai, & Chen, 1991; Flemming & Stojanowic, 1980; Guilfoyle & Yager, 1983; Hartgen 1980, Hauschild et al., 1988; Huhtanen, Knox, & Shimawki, 1981; Kautter et al., 1982; Kokubo, Jinko, Kanekop, Matsumoto, 1984; Nakano et al, 1989; Nakano & Sakaguchi, 1991; Midura, Snowden, Wood, & Arnon, 1979; Sakaguchi et al., 1987; Stier, Ito, & Stevenson, 1982). In these studies, 104 of 2,033 (5.1%) honey samples had detectable levels of *C. botulinum* spores which ranged from <1 to 80 spores/g. Kokubo et al. (1984) observed that the *B. cereus*, *C. perfringens*, and *C. botulinum* spore counts remained unchanged in honey stored at 25°C (77°F) for 4 months. The *C. botulinum* spore count remained unchanged for 1 year at 3.9°C (39°F) (Nakano et al., 1989). The *C. botulinum* spore count began to decrease after 100 days when honey was stored at 25°C (77°F), and no *C. botulinum* spores were detected after 5 days in honey stored at 47.2°C (117°F). No studies have revealed that *C. botulinum* can grow in honey. Similarly, there is no evidence to suggest that any other vegetative or spore-forming bacteria detected can grow in honey. *Salmonella* spp. can survive in commercial honey. Tysset and Durand (1976) observed that at 10°C (50°F) *S. dublin* survived for over 28 months, *S. enteritidis* for over 23 months, *S. typhi* for over 4 months, *S. typhimurium* for over 28 months, and *S. derby* for slightly over 6 months.

Osmophilic yeasts can grow in honey. Growth of these microorganisms in honey will result in fermentation of the sugars to alcohol and carbon dioxide. Tokuoka, Ishitani, Gotto, and Komagata (1985) reported that *Z. bailii* caused spoilage of honey that had a water activity between 0.65 and 0.68. *Saccharomyces* spp. are the predominant yeasts in honey (Tysset & Rousseau, 1981). Other yeast genera that have been detected include *Rhodotorula*, *Debaryomyces*, *Hansenula*, *Lipomyces*, *Oosporidium*, *Pichia*, *Torulopsis*, *Trichospora*, *Nematospora*, *Schizosaccharomyces*, *Schwanniomyces*, *Torula*, and *Zygosaccharomyces* (Furuta & Okimoto, 1978; Tysset de Rautlin de la Roy, 1974; Crane, 1979).

The level of yeast and osmophilic yeast contamination of honey varies depending on the source. Cell numbers of 0–300 CFU/g and 0–10,000 CFU/g have been reported (Nakano & Sakaguchi, 1991; Tysset et al., 1970). In Italian honey, 1–3,500 yeasts/g, with osmophilic yeasts in 34 of the 50 samples, have been reported by Piana et al. (1991). Osmophilic yeast counts ranging from 0 to 1.1×10^4 CFU/g in 175 honey samples (Tysset & Rousseau, 1981) and from 0.1 to 10^6 CFU/g in 320 Canadian samples (Root, 1983) have been reported.

Generally, molds are present in honey but at low levels, with reported counts of 0–2,500/g (Tyssett, Durand et al., 1970). The genera of molds present included *Ascosphaera*, *Aspergillus*, *Cephalosporium*, and *Penicillium*. Mold counts of 1–100 CFU/g were found in Italian honey samples by Piana, Poda, Cesaroni, Cuetti, Bucci, and Gotti, (1991) concluded that molds can survive in honey but will not grow. The presence of high mold counts in honey suggests recent contamination from equipment or other uncleaned sources (Snowdon & Cliver, 1996). Several examples of microbiological purchase specifications for honey used by different companies in the food industry are provided in Table 7.

Table 7 Examples of microbiological specifications (CFU or MPN/g) for honey used in the food industry[1]

Company	Standard plate count	Coliforms	*E. coli*	Yeasts	Yeasts and molds	Molds	*Staphylococcus*	*Salmonella*	Rope-forming sporeformers (sic)
1	1,000	10	Neg.	100		100	Neg.	Neg.	50
2	5,000	10	<10	100		100	100		
3[a]	10,000	<3	<0.3	100		100	<3	Neg.	
4	10,000	10	Neg.				Neg.	Neg.	
5	10,000	10	Neg.		10		Neg.	Neg.	
6	10,000	100	Neg.		200		Neg.	Neg.	
7	10,00	10	10	100		100	100		
8	10,000	10	Neg.	100		100	Neg.	Neg.	50
9	10,000	10	Neg.	100		100	Neg.	Neg.	50
10[b]	50,00	10	Neg.	10		10			

[1] Adapted from Snowdon and Cliver (1996).
[a] Also negative for *C. botulinum* and *C. perfringens*.
[b] Also 10 CFU/g mesophilic and thermophilic aerobes and anaerobes.

Several investigators have reported bacteriostatic and bactericidal factors in honey (Molan, 1992; Tysset & de Rautlin de la Roy, 1974). Molan (1992) suggested that the honey-associated antibacterial activity included acidity, osmolarity, hydrogen peroxide, pinocembrin, lysozyme, acids, terpenes, benzyl alcohol, and volatile substances. Tysset and de Rautlin de la Roy (1974) proposed that honey's antibacterial activity was also influenced by its low protein content and high carbon-to-nitrogen ratio, acidity, low redox potential, limited dissolved oxygen, and high osmotic pressure. Hydrogen peroxide was identified as an inhibitory factor by White, Subers, and Schepartz (1962), who determined that peroxide was formed by the glucose oxidase system, which is an enzymatic reaction that is active in unripe or diluted honey. In addition to hydrogen peroxide, gluconic acid was also produced. White, Subers, and Schepartz (1962) also determined that yeasts and molds were not as sensitive to hydrogen peroxide as bacteria.

Low microbial counts in most honey samples may be the result of a specific processing step, e.g., honey is heated at 71.1°C (160°F) for 30 min, which should kill yeasts and most vegetative bacteria. In addition, strained honey flows through a 150-μm screen and filtered honey flows through a 1-μm filter. These processing steps may reduce or eliminate most microorganisms (Snowdon & Cliver, 1996).

Yeasts and molds can survive in honey, and yeasts may grow. Some vegetative and spore-forming bacteria can survive in honey for varying periods of time under certain conditions; however, no bacteria are known to grow in honey. Honey contains some intrinsic antimicrobial factors that inhibit the growth of bacteria. Avoiding the introduction of moisture maintains the shelf stability of honey. Furthermore, it is important to limit post-process contamination of honey. Processing equipment that comes in contact with honey should be cleaned and sanitized effectively and the production environment must be maintained under conditions that limit the introduction of microorganisms into the product.

Sugar Confectionery Products

Products that comprise this category include hard candy, soft/chewy candy, caramel, jellies, creme-centered candy, licorice, mints, toffee, and shell-covered/panned candy. These products rarely undergo microbial spoilage if properly formulated with high-quality ingredients, produced on equipment that is adequately cleaned and sanitized, and processed, packaged, and stored under conditions that will prevent the uptake of moisture. Water activity is the principal product characteristic that controls microbial growth in sugar confectionery products. The a_w range for products in this category is from 0.20 for hard candy to 0.80 for mints (Banwart, 1979; ICMSF, 1998; Mossel & Sand, 1968). Examples of water activities of other products are listed in Table 8.

Products with water activities ranging from 0.60 to 0.83 are susceptible to spoilage by osmophilic yeasts and xerophilic molds. The observable spoilage that results from yeast growth is bursting/fracturing of products, resulting in leakers,

Table 8 Water activities of selected high-sugar confectionery products

Product	a_w	Sources
Toffee	0.60–0.65	Fett (1973)
Caramels	0.40–0.50	Richardson (1987)
Licorice	0.60–0.65	Mossel and Sand (1968)
Marzipan	0.65–0.75	Mossel and Sand (1968)
Boiled Sweets	<0.60	Mossel and Sand (1968)
Fondant Creams	0.75–0.80	Mossel and Sand (1968)
Jellies	0.65–0.75	Richardson (1987)
Nougat	0.40–0.70	Richardson (1987)
Marshmallow	0.60–0.75	Richardson (1987)

slime formation, off-flavors, and off-odors. Mold growth is obvious with visible mycelia on the surface of the product or liquefaction of the product (Mossel & Sand, 1968; Pitt & Hocking, 1985). The a_w of a product is the property that mostly influences the type of microorganisms that will be associated with sugar confectionery products (Lenovich & Konkel, 1992).

Other factors that serve as hurdles to provide product stability include pH and preservatives. The pH range for sugar confectionery products is 2.0–8.0. There are several preservatives that may be used, but the most common are potassium sorbate and sodium benzoate. In addition, many of the sugar confections are thermally processed at temperatures greater than 93.3°C (200°F), which will kill vegetative bacteria, yeasts, and molds. Thermally processing fondants at 60°C (140°F) for 20 min will kill yeasts that may be present (Gibson, 1973). Both batch as well as high temperature, short time (HTST) heat processing conditions are used to process sugar confectionery products. Hard candies and toffees are least likely to undergo microbial spoilage. Licorice will show visible mold growth if the moisture content is above 18% and mold is present. Occasionally, products with caramel, creams, or fondant centers will undergo microbial spoilage. Caramels are heat processed at temperatures above 101.7°C (215°F) to produce specific characteristics in the finished product. During cooking, moisture is evaporated, thereby reducing the available water in the finished product and the high temperature will kill vegetative bacteria, yeasts, and molds. Microorganisms that can be found in caramels include *Bacillus* spp. and *Clostridium* spp. Other microorganisms that may be observed are introduced post-processing. However, the low a_w of caramels will inhibit the growth of these microorganisms because they will not grow at a_w <0.90. Water activities less than 0.90 will prevent the growth of most bacteria in sugar confections, thereby preventing bacterial spoilage (Troller, 1979).

The primary microorganisms associated with the spoilage of sugar confectionery products are yeasts. For spoilage to occur, yeasts must be present and capable of growing in the product, storage conditions must permit growth, pH of the product must allow growth, and the a_w must be ≥0.60. *Zygosaccharomyces rouxii* has been the most frequently detected spoilage yeast. *Brettanomyces bruxellensis* has also been detected but at a lower frequency (Lenovich & Konkel, 1992).

Mold spoilage will be obvious due to the visible appearance of mycelia on the surface of the product. Genera that have been detected include *Aspergillus*, *Penicillium*, *Verticillium*, *Rhizopus*, *Mucor*, and *Trichothecium* (Hopko, 1979; Windisch & Newman, 1965a, 1965b). Controlling the moisture content will prevent the spoilage of sugar confectionery products most susceptible to mold growth. Moisture is created when product that is thermally processed is packaged before the product is properly cooled. If a product such as licorice is not dried properly, the potential for mold growth is high, provided mold spores are present.

The composition of sugar confectionery products, processing conditions, and the product's physical and chemical characteristics make it less likely that pathogens will grow to sufficient levels to cause foodborne disease (Lenovich & Konkel, 1992). No reported food poisoning case has been associated with the consumption of sugar confectionery products.

The source of microorganisms found in sugar confectionery products can be the raw ingredients, such as liquid sugar. Other ingredient sources include colors, nuts, dairy products, gelatin, starch, and processed eggs. The production environment provides another source of contamination such as air, equipment, workers (Dragoni, Balzaretti, & Ravaretto, 1989; Mossel & Sands, 1968; Walker & Ayres, 1970), and moisture. Moisture can originate from leaky roofs, condensation, steam leaks, inadequately dried equipment and improper cleaning. Improperly cleaned and sanitized equipment plus the presence of product residue will allow microorganisms that are present to establish microbial niches.

The control of microbial contaminants in finished sugar confectionery products will be achieved by the use of quality ingredients, implementation of a microbiological ingredient monitoring program, establishment of steps to control microbial contamination in the processing environment, application of a documented cleaning and sanitation program, implementation of an effective environmental monitoring program, application of Good Manufacturing Practices (GMP), and implementation of a Hazard Analysis and Critical Control Point (HACCP) program.

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Microbiological Spoilage of Spices, Nuts, Cocoa, and Coffee

Joan M. Pinkas, Karen Battista, and Theodora Morille-Hinds

Introduction

Spices, nuts, cocoa, and coffee are raw materials that may be used alone or as ingredients in the manufacture of processed food products. The control of microbiological spoilage of these raw materials at the ingredient stage will enable the food processor to better assure the production of high-quality foods with an acceptable shelf life. While this chapter is limited to four materials, many of the spoilage control procedures recommended can also be applied to other raw materials of a similar nature.

Types of Food

Spices

The American Spice Trade Association (2001a) defines spices as "dried plant products used primarily for culinary purposes." This category includes many familiar items such as black pepper, cinnamon, and cloves as well as the herbs and dehydrated vegetables such as onion and garlic.

Spices are derived from an assortment of plant parts. For example, black pepper, coriander, and allspice are fruits, cinnamon is bark, turmeric and ginger are rhizomes, fennel and caraway are seeds, cloves are unopened flower buds, and basil, marjoram, and sage are leaves. In some cases, the same plant is the source of two spices. Coriander is the fruit of the plant whose leaves are cilantro. Likewise, nutmeg and mace originate from the same plant. Nutmeg is the seed and mace is the delicate, membranous tissue that surrounds the nutmeg.

J.M. Pinkas (✉)
McCormick & Co., Inc., Hunt Valley, MD 21031, USA
email: joan_pinkas@mccormick.com

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Spice consumption in the United States has grown rapidly in the past 25 years and currently exceeds one billion pounds per year (American Spice Trade Association, 2001a). In 2000, the per capita consumption was 3.7 pounds (American Spice Trade Association, 2001a). While many spices such as onion, garlic, and chili peppers are grown in the United States, the majority of spices are sourced from other countries. Listed in Table 1 are the top 12 spices consumed in the United States and the supplying nations. The sources are not static and may change rapidly as a result of natural disasters and shifts in the political climate of the area.

Table 1 Top twelve spices consumed in the United States in 2000 (Reprinted with permission, American Spice Trade Association, 2001a)

Spice	Pounds	Major sources
Dehydrated onion/garlic	321,171,000	USA, China
Mustard seed	172,494,000	Canada, USA
Red pepper	109,416,000	USA, India, Mexico, China
Sesame seed	108,133,000	Guatemala, India, Mexico, Venezuela
Black pepper	102,495,000	Indonesia, India, Brazil
Paprika	52,771,000	USA, Spain, Chile
Cinnamon	37,022,000	Indonesia, Sri Lanka, Vietnam
Cumin seed	17,234,000	Syria, Turkey, India
White pepper	16,113,000	Indonesia, Malaysia, China
Oregano	14,522,000	Turkey, Mexico, Greece
Poppy seed	11,682,000	Australia, the Netherlands, Turkey
Ginger	10,894,000	China, India, Nigeria
Total	973,947,000	

Nuts

Tree nuts and peanuts comprise the nut category. Tree nuts, as the name indicates, grow on trees and include almonds, Brazil nuts, cashews, filberts, macadamia nuts, walnuts, and pecans. Peanuts, often termed ground nuts, are technically classified as a legume and grow from a plant stalk.

Nuts are cultivated in various regions throughout the world. Macadamia nuts are primarily grown in Australia and Hawaii, whereas Brazil nuts are indigenous to the Amazon. Turkey is the main supplier of hazelnuts, but the United States, Italy, Spain, Korea, and Russia contribute to the world crop as well (Kinderlerer & Phillips-Jones, 1992). Walnuts, ranking second to almonds in tree nut production, grow well in California's mild temperate climate as well as in the Mediterranean, Eastern Asia, Indo-China, Canada, Central America, and the Andes (Sze-Tao, Schrimpf, Teuber, Roux, & Sathe, 2001). India, Africa, and South America are countries of origin for the cashew nut (American Whole Health Inc., 2000). Pecans are associated with North America and pistachios with California. Peanuts grow well in various parts of the United States and China.

A successful crop, or lack of, can greatly impact a country's economy depending on the extent to which the nut contributes to the national income. For instance, Turkey produces approximately 550,000 tons of hazelnuts per year, exporting 83%, with the United States as one importer purchasing one billion dollars of that product alone (Ozdemir & Devres, 1999). Turkey's hazelnut production involves 370,000 families, or 8 million people (Ozdemir & Devres, 1999). Many countries are seeking ways to enhance the growing, harvesting, and production process in order to improve quality, minimize spoilage, maximize the use of labor, and, therefore, monetary return (Ozdemir & Devres, 1999; Cunningham, 1989; Liang, Meng, & Ji, 1996).

Nuts are globally recognized as a nutritionally important food, being high in proteins, vitamins, and minerals such as potassium, iron, vitamin E, thiamin, niacin, riboflavin, and unsaturated fats. Providing such value, they are of greater dietary importance in countries where other food groups are not available. Additional benefits include the inherent phytochemicals which may contribute to protection against coronary disease and cancer (American Whole Health Inc., 2000).

Cocoa

The cocoa tree belongs to the genus *Theobroma* (Chittenden, 1951), a group of small trees that grow in the wild in the Amazon basin and other tropical areas of South and Central America. Cocoa trees produce large numbers of flowers at certain times of the year; however, only 1–5% of the flowers are successfully pollinated to produce a pod (Posnette & Entwistle, 1958). Cocoa beans are in pods surrounded by sterile pulp. The beans must be removed from the pods, fermented, dried, roasted, ground, and pressed to be converted to cocoa.

The pulp is the white, or sometimes pale pink, mucilaginous mass in which the beans are embedded. The pulp of ripe pods consists of a mass of parenchyma cells derived from the endocarp and is composed of 80–90% water, 6–13% sugar, of which about one-third is sucrose, and the remaining being invert sugars, 0.5–1% citric acid, and small amounts of aspartic acid, asparagines, and glutamic acid (Forsythe & Quesnel, 1963; Rohan, 1963).

The bean consists of testa (shell or skin) and cotyledons. The cotyledons contain about one-third water and one-third fat. The remainder is composed of phenolic compounds, starch, sugar, theobromine, nonvolatile acids, and many other components in small concentrations (Forsythe & Quesnel, 1963).

Coffee

Coffee is the world's most popular beverage after water. Geographically, coffee grows only between the Tropic of Cancer and the Tropic of Capricorn. Coffee plants will not survive outside of this area due to the occurrence of frost (Franco, 1958).

There are two major species of coffee grown for commercial use: robusto (sometimes called canephora) and arabica (Arunga, 1982). The robusto plant grows at lower elevations, has a higher bean yield per plant, and is more disease resistant than its arabica relative. Robusto beans are noteworthy for their harsh, dirty flavor and contain twice as much caffeine when compared to the arabica bean. The coffee shrub can grow to 4.572 m in height with thick branches and broad, waxy green leaves. The fruits, called cherries, are comprised of the pulp (colored skin and a fleshy mesocarp, known as mucilage), the parchment or the endocarp (thin, crumbly paper-like covering on the bean), the silver skin (seed coat), and the coffee bean. Each cherry usually contains two coffee beans; if there is only one bean, it assumes a rounder shape and is known as a peaberry.

Types of Spoilage Microorganisms

Spices

Spices are cultivated in many countries around the world, with most of the world's spice production occurring in India, Egypt, China, Indonesia, Malaysia, Mexico, Turkey, and Brazil (Kithu, 2002). In the source country, the spices are harvested, dried, cleaned, graded, and packaged. Additional processing may also occur at the source. Drying is a key stage in the process and is usually accomplished naturally by the sun and wind. The spices are spread out on mats in the field, on raised platforms, or on concrete pads in areas that may or may not be shaded. The final moisture values for a particular spice can vary because this is a natural process. The spice industry has traditionally used moisture levels as an indicator of the potential for microbial growth. While water activity would be a better indicator, moisture limits are already firmly entrenched in the industry and serve a similar purpose. Typically, the maximum moisture values for spices range from 8 to 12%, but they can be higher or lower as is the situation with white pepper, cinnamon, and sesame seed (Table 2). It is critical that the spices are dry enough to prevent the growth of microorganisms but not too dry to adversely affect the shelf life, color, and hardness attributes. For example, ground spices with very low moisture tend to lose their flavor more rapidly than those with higher moisture (Tainter & Grenis, 2001). Also, paprika is especially prone to fading when the moisture content is too low (Tainter & Grenis, 2001).

To achieve the correct moisture range, the farmer relies on experience to determine, during the drying stage, the appropriate thickness of the wet material and the total drying time. The quicker the spices are dried, the less opportunity there is for microbial growth. Properly dried and stored spices are resistant to bacterial and fungal spoilage due to their low water activity. However, improper handling, drying, transporting, and storing the spices can increase the opportunities for postharvest contamination and mold spoilage.

Molds associated with spices can be divided into two categories based on their growth characteristics: those associated with the field and those that dominate during

Table 2 Guidelines for maximum moisture levels in spices (Reprinted with permission, American Spice Trade Association, 2000)

Spice	Moisture contents (%)
Allspice	12
Anise	10
Basil	12
Bay leaves	9
Caraway seed	11
Cardamom	12
Cassia	12
Celery seed	10
Chilies	11
Cinnamon	14
Cloves	8
Coriander seed	9
Cumin seed, whole	9
Dill seed, whole	10
Fennel seed	10
Ginger, whole and split	12
Mace	8
Marjoram	10
Mustard seed	11
Nutmeg, broken and whole	8
Oregano	10
Pepper, black	12
Pepper, white	14
Poppy seed	9
Rosemary	10
Sage	10
Sesame, natural and hulled	6
Tarragon	10
Thyme	10
Turmeric	10

storage. The primary sources of field fungi are the soil, air, and surrounding vegetation so there is ample opportunity for natural contamination of spices prior to harvest. Field fungi invade the living plant structure through sites that may have been damaged by insects and other means or by attacking and invading healthy areas of the plant. The results are "leaf spot diseases, dry rot, decomposed and discolored tissue of stems and roots, or decay in seeds and fruits" (U.S. Food and Drug Administration, 1998a). *Verticillium dahliae* causes wilting, stunting, and early dying of paprika plants (Tsror, Erlich, Amitai, & Hazanovsky, 1998), whereas *Pestalotiopsis palmarum* causes leaf spot disease on nutmeg/mace plants (Naseema & Sulochana, 1994). *Fusarium oxysporum* causes wilting of cumin (Gaetan & Madia, 1993) and basil plants (Guirado Moya et al., 2004) as well as wilting of clove seedlings (Joshi & Raut, 1994). Storage molds dominate after the spices are dried. Their presence can reflect inadequate drying, the storage of respiring spices in a poorly ventilated area, or, most often, the storage of adequately dried spices in warm, humid environments

or environments with large temperature gradients. For example, the storage of spices in areas where warm, humid days are followed by very cool nights can result in the formation of condensation on the inside top surface of the container which then drips into the product. This scenario provides the perfect environment for mold growth. Pockets of moisture may also develop in otherwise dry spices as a result of insect activity. The observation of *Aspergillus flavus* conidia arising from insect larvae suggests that they act as vectors of this mold (Chourasia, 1995). The storage molds produce a range of defects from obvious surface growth on black pepper fruits and cinnamon bark to internal mold growth in nutmeg and other products. In addition to those molds mentioned above, *Alternaria* sp., *Helminthosporium* sp., and *Cladosporium* sp. are typically considered field fungi, whereas *Aspergillus* sp. and *Penicillium* sp. are categorized as storage molds (Aziz, Youssef, El-Fouly, & Moussa, 1998). A recent survey by Mandeel (2005) revealed that the predominant fungal genera occurring in spices included *Aspergillus, Penicillium, Rhizopus, Cladosporium*, and *Trichoderma*. No particular species was common to all spice samples or restricted to a certain spice. Black pepper had the widest spectrum of fungal species (10), followed by green cardamom (9), and black cumin (8). Coriander, caraway, and cloves had the narrowest species diversity (1) among all spice samples.

Every effort is made by the spice grower and processor to minimize the risk of mold growth and spoilage through the use of Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs), and attention to the storage environment. The end user must also consider the potential for spoilage of their finished product when using spices as ingredients. The primary factors to consider are the composition of the food, the water activity, the pH, the processing parameters, the storage temperature of the finished product, and the microbial load of the ingredients. It is not unusual for untreated spices such as parsley, black pepper, basil, fennel, dill seed, celery seed, coriander, white pepper, cinnamon, sage, thyme, and others to have mold counts of 10^4 CFU/g or higher. Conversely, counts less than 10^2 CFU/g can be found in many spices. It is important to consider the mold counts of spices when using them as ingredients in foods because of their ability to grow at lower pH and water activity values compared to bacteria.

Along with Molds, yeasts can also be found in spices, particularly basil, marjoram, parsley, thyme, dill weed, coriander, celery seed, and fennel. Untreated fennel, parsley, basil, and dill weed can have yeast counts greater than 10^5 CFU/g. Yeast counts can be even higher in untreated celery seed and coriander. Manufacturers of ambient processed, low-pH condiments and sauces often use spices with small numbers of yeasts in order to reduce the risk of spoilage if the products are intended to be shelf stable.

Bacterial spoilage of spices is usually not a concern. Although the water activity of the dry material may be elevated if improperly dried or stored, it is usually still low enough to prevent the growth of the bacteria that are residing on the spice. The bacterial profile of spices can vary greatly depending on the environment in which they are grown, the surface structure, their proximity to the soil, the way they are handled, processed, and stored, and the chemical constituents of the spice. Untreated spices can contain high numbers of bacteria. Root and berry spices as well as herbs

tend to have the highest counts, whereas bark and seed items generally have the lowest counts. Untreated black pepper and basil are examples of spices that can contain 10^7–10^8 CFU of aerobic plate count bacteria per gram. Heredia, Galvan, Garcia, and Iracheta (2001) found 10^5–10^7 CFU of aerobic mesophilic microorganisms per gram in most samples of garlic powder, cumin seed, and black pepper. APCs of less than 10^2 CFU/g were determined in samples of oregano and bay leaves. Candlish et al. (2001) reported a range of total viable counts from 10^3 to 10^7 CFU/g in the samples that they surveyed. Spices with antimicrobial constituents in the essential oil fraction, such as eugenol in cloves and allyl isothiocyanate in mustard seed, generally have lower numbers of microorganisms. The pH values of spices do not appear to influence the microbial load. Chili peppers are a good example in that they can have a pH of 4.4, yet still may harbor a moderate to large number of bacteria and fungi.

The predominant bacteria in spices are mesophilic aerobic sporeformers of the genus *Bacillus*. These sporeformers will not spoil spices because they cannot grow in the dry spices; however, they can spoil the foods to which they are added if conditions are adequate for growth. Baxter and Holzapfel (1982) determined that aerobic sporeformers accounted for 50–95% of all the microorganisms isolated from spice samples obtained from two different wholesalers. They included *Bacillus subtilis, B. brevis, B. firmus, B. cereus, B. licheniformis, B. megaterium*, and others. Many of the sporeformers isolated from black pepper, white pepper, paprika, marjoram, coriander, allspice, and onion powder were proteolytic. Amylolytic microorganisms were also found in significant numbers in black and white pepper, onion powder, cinnamon, coriander, and allspice. Julseth and Deibel (1974) reported similar counts of proteolytic and amylolytic microorganisms in spices. They noted that samples with high total plate counts also contained large numbers of proteolytic, amylolytic, and thermophilic bacteria. The microbial data from their survey of imported and domestic spices in the United States are summarized in Table 3.

The incorporation of spices in hermetically sealed low-acid foods is usually not an issue because the thermal process applied to these foods inactivates mesophilic spores. Thermophilic sporeformers, both aerobic and anaerobic, are also in spices but at much lower numbers than the mesophilic sporeformers. Proper cooling of thermally processed, low-acid foods in hermetically sealed containers and storage of these foods at a temperature below 43°C are important to prevent spoilage by thermophilic flat sour sporeformers and thermophilic anaerobes that may be introduced with spices (Compendium of Methods for the Microbiological Examination of Foods, 2001).

Lactic acid bacteria are another group of potential spoilage microorganisms that can occur in some spices such as onion powder. Choosing spices with small numbers of lactic acid bacteria, particularly the heterofermentative variety, is important for manufacturers of shelf-stable, ambient processed, low-pH condiments and sauces.

It is not appropriate to have industry standards for maximum bacterial and fungal counts because the application dictates the specification for a spice. A spice that is acceptable as an ingredient in one type of food may be unsatisfactory for another. For example, there would be no spoilage risk to a dry snack food if a

Table 3 Microbial profile of selected imported and domestic spices (count per gram of spice) (Reprinted with permission, Julseth & Deibel, 1974)

Spice	Number of samples tested	Mean (M) and range (R) of counts	Standard plate count	Bacterial spore count	Thermophilic anaerobes	Proteolytic organisms	Amylolytic organisms	Yeasts and molds
Black pepper	12	M	3.2×10^{7}	3.2×10^{7}	2.0×10^{4}	2.0×10^{7}	8.5×10^{5}	1.9×10^{4}
		R	5.5×10^{6}–5.0×10^{7}	5.5×10^{6}–5.0×10^{7}	5.0×10^{2}–3.0×10^{4}	7.5×10^{4}–5.0×10^{7}	3.0×10^{4}–5.0×10^{7}	10^{1}–1.5×10^{5}
Cassia	12	M	2.8×10^{5}	4.8×10^{5}	2.5×10^{2}	5.1×10^{4}	7.1×10^{4}	5.3×10^{4}
		R	1.0×10^{3}–3.0×10^{7}	1.0×10^{3}–5.5×10^{6}	10^{1}–5.0×10^{2}	1.0×10^{3}–1.5×10^{5}	1.0×10^{3}–4.0×10^{5}	10^{1}–3.5×10^{5}
Celery seed	10	M	1.5×10^{5}	1.3×10^{6}	4.8×10^{3}	6.8×10^{5}	1.2×10^{5}	10^{1}
		R	7.5×10^{4}–7.5×10^{5}	7.5×10^{4}–7.5×10^{5}	10^{1}–3.0×10^{4}	1.0×10^{3}–5.5×10^{6}	5.5×10^{3}–4.0×10^{5}	0–9
Ginger	6	M	2.7×10^{6}	1.8×10^{6}	1.0×10^{3}	5.9×10^{6}	1.0×10^{5}	1.0×10^{4}
		R	5.5×10^{3}–5.5×10^{6}	5.5×10^{3}–5.5×10^{6}	10^{1}–5.5×10^{3}	1.0×10^{3}–3.0×10^{7}	1.0×10^{3}–5.5×10^{6}	10^{1}–3.0×10^{4}
Mace	6	M	3.2×10^{4}	3.6×10^{4}	1.1×10^{3}	2.1×10^{4}	1.3×10^{4}	1
		R	5.5×10^{3}–7.5×10^{4}	5.5×10^{3}–7.5×10^{4}	10^{1}–5.5×10^{3}	5.5×10^{3}–3.0×10^{4}	5.5×10^{3}–3.0×10^{4}	0–9

(Continued)

Table 3 (Continued)

Spice	Number of samples tested	Mean (M) and range (R) of counts	Standard plate count	Bacterial spore count	Thermophilic anaerobes	Proteolytic organisms	Amylolytic organisms	Yeasts and molds
Mustard seed	9	M	9.8×10^4	6.7×10^3	1.7×10^2	2.1×10^4	9.4×10^3	1
		R	1.0×10^3–7.5×10^5	1.0×10^3–3.0×10^4	10^1–5.1×10^2	1.0×10^3–7.5×10^4	1.0×10^3–3.0×10^4	0–9
Nutmeg	10	M	7.5×10^3	4.6×10^3	6.0×10^2	8.6×10^3	4.1×10^3	4.9×10^1
		R	1.0×10^3–3.0×10^4	1.0×10^3–5.5×10^3	10^1–5.5×10^3	1.0×10^3–3.0×10^4	1.0×10^3–5.5×10^3	10^1–5.1×10^2
Oregano	13	M	4.7×10^4	2.6×10^4	6.0×10^2	9.4×10^3	1.1×10^4	1.2×10^3
		R	5.5×10^3–1.5×10^5	1.0×10^3–7.5×10^4	10^1–5.5×10^3	1.0×10^3–3.0×10^4	1.0×10^3–3.0×10^4	10^1–5.0×10^3
Paprika (domestic)	16	M	8.6×10^5	7.9×10^5	4.1×10^3	7.9×10^4	1.6×10^5	7.7×10^1
		R	3.0×10^4–5.5×10^6	5.5×10^3–5.5×10^6	10^1–5.5×10^3	5.5×10^3–4.0×10^5	5.5×10^3–7.5×10^5	10^1–5.1×10^2
Paprika (imported)	10	M	4.9×10^6	7.9×10^6	2.4×10^4	1.0×10^7	3.9×10^6	5.9×10^1
		R	5.5×10^6–3.0×10^7	5.5×10^6–3.0×10^7	10^1–3.0×10^4	5.5×10^6–3.0×10^7	1.5×10^5–5.5×10^6	10^1–5.1×10^2
Rosemary	10	M	9.2×10^4	1.3×10^4	6.5×10^2	2.3×10^4	5.7×10^3	5.1×10^3
		R	5.5×10^2–1.5×10^5	5.5×10^3–3.0×10^4	10^1–5.5×10^3	1.0×10^3–1.5×10^5	1.0×10^3–3.0×10^4	10^1–3.0×10^4

seasoning blend that has an aerobic plate count of 10^6 CFU/g and a fungal count of 10^3 CFU/g is topically applied to the snack. However, the same blend introduced into a high-moisture food may shorten the shelf life and impose a spoilage risk. For this reason, there are general quality standards for spices in the United States (American Spice Trade Association, 2001b; U.S. Food and Drug Administration, 2005), but not specific limits for aerobic plate count bacteria, fungi, and other categories of microorganisms. Various treatment options such as ethylene oxide gassing, steam treatment, and irradiation exist to reduce the bacterial and fungal counts of spices in order to make them suitable for use in a variety of sensitive applications.

Nuts

The outer shell of a nut affords natural protection against most contaminating agents such as insects, prey, rodents, and microorganisms, making the nutmeat virtually microbe-free (International Commission on Microbiological Specifications for Foods, 1998). If the shell is damaged or removed, the nutmeat is at risk for contamination. This can occur in the field during maturation or harvesting, during the shelling operation, while stored and transported, or during plant processing. In the field and in storage, insects and other prey can physically damage the shell and nut, and by doing so make the nut susceptible to contamination by microorganisms by creating routes for exposure.

Field contamination often occurs when the nut contacts soil either by falling from the tree, contacting the ground during mechanical harvesting, or during the drying stage. While on the ground, the nut is vulnerable to a wide range of contaminants in the soil. The intrinsic characteristics of the nut make it most susceptible to mold contamination, although to a much lesser degree, bacterial contamination has also been documented. Nuts are typically harvested and then left to dry in fields naturally on tarpaulins or are subjected to mechanical methods of drying. This is an important processing control step that is necessary to reduce the moisture level which can be as high as 30% when nuts are harvested. After drying, but before further processing, nuts contain approximately 8% moisture and have an approximate water activity of 0.70. This low-moisture content and water activity minimizes spoilage concerns due to bacteria. However, the low moisture and water activity of nuts, along with low-soluble carbohydrate levels, high oil levels, near-neutral pH, and hygroscopic properties, creates an ideal environment for the survival of molds.

Shelling and other processing practices such as chopping and slicing can increase the risk of contaminating the nutmeat. For example, mechanical abrasion and washing during the shelling process may expose the nutmeat to mold if it is present in the environment (International Commission on Microbiological Specifications for Foods, 1998).

The exposure of nuts to moisture at any stage in processing will subsequently increase their water activity to a level that could be sufficient for mold growth

(Hocking, 1988; Adebajo & Diyaolu, 2003). Mold growth results in physical decomposition which is a natural process for any agricultural product. Once infected, decomposition of the nut can be accelerated by the mold's enzymatic activity, which alters the nut's appearance, texture, taste, and overall quality, and ultimately shortens the shelf life. The visible change in a nut due to its physiological breakdown varies. Nuts may become discolored, shriveled, split, smaller in size, lighter in weight, or gummy. Because some shells provide greater protection than do others, nuts such as almonds, Brazil nuts, and walnuts have a lower risk of mold decomposition. Cashews have a low spoilage rate as well, not only because of their thick shell but also due to the presence of a caustic liquid that is toxic to most microbial contaminants (International Commission on Microbiological Specifications for Foods, 1998).

The resident mycoflora on a nut changes throughout the various stages of processing. The field molds are present during harvest and include *Alternaria* spp., *Fusarium* spp., *Cladosporium* spp., *Penicillium* spp., and some yeasts (Hocking, 1988). Once nuts are dried and in storage, conditions are less favorable for the survival of field molds. Molds such as *Eurotium* spp., *Aspergillus* spp., *Penicillium* spp., and *Wallemia* spp. subsequently dominate. Pitt and Hocking (1999) reported that any increase in a nut's moisture content may substantially increase its water activity to allow for the growth of molds which thrive on the nut's low-soluble carbohydrate level. *Eurotium* sp. is the predominant spoilage mold if there is a marginal increase in moisture. Larger shifts in moisture content can result in the growth of *A. flavus*. Peanuts, almonds, pecans, pistachios, and sunflower seeds are often associated with *Aspergillus* spp. infections. Hazelnuts are most susceptible to *Rhizopus* spp., *Penicillium* spp., and *Aspergillus* spp. contamination during harvest and postharvest treatments and storage (Ozdemir & Devres, 1999).

Cocoa

Cocoa beans are raw agricultural products that are exposed to numerous microorganisms during harvesting and subsequent fermentation. After the pods are harvested, they are split with a hammer or a machete to expose the beans covered in pulp. Although the beans within the cocoa pods are sterile, the pulp is an excellent medium for microbial growth. The workers' hands, the machete, the transport baskets, and fermentation containers are all potential vectors for contamination.

The color and the flavor of cocoa beans are developed during fermentation of the pulp surrounding the beans. This occurs by one of two methods. The beans are either piled in a heap and covered with banana leaves or placed in a box with removable sides and transferred every 24 h to a lower box to ensure good mixing. The fermentation process begins with yeasts converting sugars in the pulp to alcohol and carbon dioxide. Bacteria then oxidize the alcohol into lactic acid and, as conditions become more aerobic, acetic acid. This produces heat and raises the temperature during the initial 24 h to about 49–50°C which kills the plant embryo. For each method, the beans are fermented for 6 days (Lehrian & Patterson, 1983; Ostovar & Keeney,

1973; Schwan, Rose, & Board, 1995). Cocoa beans may be spoiled by molds at the outer surfaces of the fermenting heap, in particular, if the beans remain unturned for 2–3 days (Roelofsen, 1958). Visible mold on the cotyledons is especially undesirable because it produces off-color and off-flavors which persist throughout the later processes. Some fungi, in particular, *A. flavus*, have remarkable lipolytic activity and are the main contributors of spoilage of fermented cocoa beans (Kavanagh, Reineccius, Keeney, & Weissberger, 1970). *Aspergillus fumigatus*, the mold most commonly present during fermentation, is particularly harmful in that it destroys testa and enables penetration of other molds such as *A. niger, A. flavus, A. tamari, Eurotium* spp., and *Penicillium* spp. (Chatt, 1953; Roelofsen, 1958). Spoilage of fermenting beans may also be due to the activity of *Acetobacter* spp. and *Pseudomonas* spp. if the pH increases above 5.0 during fermentation (Rohan, 1963; Ostovar & Keeney, 1973). This can result in the development of off-flavors.

Following fermentation, the beans have a moisture content of 55–60% which must be reduced to 7–8% during the drying process to provide safe storage before roasting. Sun drying and artificial drying are two common methods employed. With sun drying, the beans are spread onto mats, trays, or concrete floors and exposed to the sun or onto wooden drying floors with movable roofs. The beans are turned or raked to ensure uniformity of drying and minimize mold growth. With artificial drying, beans are dried in special drying units designed to provide heat and ventilation. Artificial drying is a more controllable method, but it carries a risk of tainting the beans with a smoky bacon flavor if they come into contact with smoke from the heat source. Molds may grow during the drying stage, in storage, and when the cocoa beans are transported if they are exposed to excessive moisture.

Coffee

Coffee processing involves harvesting, pulping, fermenting, washing, drying, hulling, cleaning, grading, sorting, storing, and transporting. There are two basic methods for processing coffee: the dry process (or natural process) and the wet process. The primary difference between the two methods is that the wet method uses a procedure to remove the pulp from the bean within 12–24 h of harvesting instead of allowing the cherries to air dry.

During dry processing, harvested cherries are spread over a concrete, brick, or matting surface, ideally in sunlight, and raked at regular intervals to prevent mold growth. If it rains or if there is a decrease in temperature, the cherries must be covered for protection. The process takes about 7–10 days to achieve a moisture content of 11–12%. The outer shell will become dark brown and brittle and the beans can be heard rattling around inside their husk. The husks are removed prior to sorting, grading, and shipping.

In wet processing, the cherries are presorted using different methods to separate the ripe cherries from overripe or green cherries and other materials. The cherries are then passed through a pulping machine, which removes the outer skin and the pulp, leaving the bean surrounded by parchment and a slippery layer of mucilage.

This material is then transferred to wooden or concrete fermentation tanks where the mucilage surrounding the bean is digested. The mucilage is composed of pectin materials including protopectin (33%), reducing sugars such as glucose and fructose (30%), nonreducing sugars like sucrose (20%), and cellulose, and ash (17%) (Coleman, Lenney, Coscia, & Dicarlo, 1955). The time for digestion of the mucilage can vary from 16 to 36 h (Sivetz & Desrosier, 1979) depending upon the temperature, the thickness of the mucilage layer, and the concentration of coffee enzymes present. There are conflicting reports on how the mucilage is degraded. Some reports indicate that degradation of the mucilage may be caused by the combined action of microorganisms and endogenous coffee enzymes (Frank & De la Cruz, 1964; Van Pee & Castelein, 1972; Castelein & Verachtert, 1981). However, other studies suggest that the pectin-rich mucilage is degraded neither by endogenous pectolytic enzymes nor by pectolytic bacteria but by organic by-products such as lactic acid and acetic acid that decrease the pH and alter the mucilage cell wall (Avallone, Guiraud, Guyot, Olguin, & Brillouet, 2001; Avallone, Brillouet, Guyot, Olguin, & Guiraud, 2002).

Bacteria are reported to be active during the degradation of the mucilage but are replaced by yeasts toward the end of the fermentation process. Traditionally, the end of fermentation is determined by rubbing the beans in the hands. Gritty beans indicate that the fermentation process is complete. Recent studies (Jackels & Jackels, 2005) have revealed that systematic measurement of pH alone may be used to predict when fermentation is complete and, therefore, minimize the potential of over-fermentation. Over-fermentation of the beans can lead to "stinker beans" whereby the beans germinate and die (Gibson & Butty, 1975). These dead beans rot and develop a cheesy and putrid off-odor when cracked. One or two stinker beans can contaminate an entire batch. The normal fermentation process takes about 16–36 h depending on a number of factors, including the amount of coffee fermenting, the temperature, and the humidity. Fermentation time longer than 36 h produces lactic, acetic, and propionic acids which are believed to prevent the traditional flavor from developing.

Following the fermentation process, the beans are thoroughly washed with clean water. The coffee beans, free of the mucilage layer, are known at this stage as parchment coffee. The beans are either dried in the sun or in a mechanical dryer or a combination of the two methods may be used. The goal is to reduce their moisture content from 60 to 12–12.5%. The dried parchment coffee is then hulled shortly before shipment.

The primary microorganisms relevant to spoilage of coffee beans are molds which can be present during all phases of the coffee process prior to roasting. Mold contamination can occur after harvesting if the coffee cherries are held longer than 1 day prior to depulping. Mold growth can also occur during the fermentation process as a result of over-fermentation or if the fermentation tanks are not cleaned on a frequent basis. However, growth occurs most often during the drying phase after the parchment has been washed free of mucilage and contains about 60% moisture. In addition to causing rapid deterioration, mold growth can also produce carcinogenic ochratoxin A. Ochratoxin A is a heat-stable toxin produced by a few

species of *Aspergillus* and *Penicillium*. It can withstand the coffee roasting process and presents a potential health risk.

The following are additional defects caused by microbial spoilage of coffee beans:

Fruity flavor and sour coffee: Fruity flavor is caused by over-fermentation and outgrowth of yeast. Sour flavor is the result of conversion of alcohol to vinegar due to over-fermentation.

Onion flavor: An onion flavor is caused by the conversion of lactic and acetic acids into propionic and butyric acids, respectively, by soft rot bacteria during the fermentation stage.

Earthy, musty, and phenolic taints: These defects are caused by mold contamination of clean, wet parchment coffee. Mold growth does not occur if there is sufficient mucilage to support the growth of bacteria and yeast because they do not compete well in this type of environment (Northmore, 1969).

Rio flavor: Severe fungal infection of coffee beans can lead to a defect known as "Rio flavor" which is described as hardish, phenolic, medicinal, and musty (Liardon, Braendlin, & Spadone, 1992). It is caused by the formation of trichloroanisoles in moldy beans (Liardon et al., 1992). Rio flavor most often occurs in Brazilian (Spadone, Takeoka, & Liardon, 1990) and Kenyan coffees (Holscher & Steinhart, 1995).

Factors Influencing Spoilage

Spices

Most molds on spices are of the postharvest variety, with *A. flavus* (Aziz et al., 1998; Mandeel, 2005; Chourasia, 1995) and *A. niger* (Garcia, Iracheta, Galvan, & Heredia, 2001) being the most common types. Chourasia (1995) suggested that storage in hot, humid environments favors the growth of *A. flavus* over other types of molds due to its semithermophilic and semixerophilic nature. Xerophilic *Aspergillus* spp. have minimum water activity limits for growth in the range of 0.71–0.78 (Christian, 2000). The minimum water activity required for the growth of *A. flavus* is 0.78, whereas the minimum water activity for toxin production is 0.84 (Beuchat, 2002). *A. flavus* grows within the temperature range of 10–43°C and, optimally, slightly over 30°C (Food and Agriculture Organization of the United Nations, 2003). Although *A. flavus* is a prominent species in spices, aflatoxin contamination is generally low.

The xerophilic species of *Penicillium* have minimum water activity requirements for growth ranging from 0.79 to 0.83 (Christian, 2000). The most xerophilic microorganism known, *Xeromyces bisporus*, can grow at a minimum water activity of 0.61 (Christian, 2000). Hence, spices should be maintained under conditions that keep the water activity below 0.60.

Chourasia (1995) correlated weather conditions 15 days prior to analysis with isolation frequencies of certain fungi in spices. Maximum and minimum air temperatures favored the growth of *Alternaria alternata, Aspergillus* spp., *Curvularia*

spp., *Penicillium* spp., and *Rhizopus stolonifer* (Chourasia, 1995). Infection with *F. oxysporum* and *F. moniliforme* was associated with frequent rainfalls, low temperature, and low relative humidity, whereas the occurrence of species of *Chaetomium* was favored by minimum temperature and maximum relative humidity (Chourasia, 1995).

Nuts

During maturation in the field, nuts are exposed to a variety of environmental conditions that increase the risk of fungal contamination. Excessive rain during the growing season may increase the water activity of the nut and its susceptibility to mold growth. The opposite situation, preharvest drought conditions, can also increase susceptibility to molds. Drought conditions weaken a plant's defenses and create a hostile environment for bacteria. The lack of bacterial competition coupled with poor plant resistance creates an environment that enables molds to proliferate (International Commission on Microbiological Specifications for Foods, 1998). The overall climate in the growing region also plays a role. Temperate climates, such as the tropics and subtropical regions, have higher humidity and temperature that present an ideal environment for mold growth and a need for stringent control practices (International Programme on Chemical Safety, 1979).

There are many species of insects that contribute to nut crop damage directly or indirectly. Insects such as the beet armyworm, lesser cornstalk borer, leafhoppers, wireworms, Southern corn rootworm, and thrips may infect peanuts (The Bugwood Network, 1995). The beet armyworm is an example for which direct interaction occurs between the insect and the presence of an infecting mold. These pests prefer nuts that are already infected with certain molds due to higher nutrients and lower defensive phenolic substances (Cardoza, Lait, Schmelz, Huang, & Tumlison, 2003). Tea mosquitoes and the stem and root borer attack cashews (About Cashews, 2006). The Southern green stink bug targets macadamia nuts. Hazelnuts are a niche for the bud mite and pecans for weevils (Entomological Society of America, 2004). Peanuts are more susceptible to contamination in the field than are tree nuts because they are often in direct contact with the soil in which molds reside. If common prey such as grubs, termites, millipedes, and larvae damage the peanut pod, mold infection usually results (Horn, 2005). Contamination of pistachio nuts in the field is normally associated with early splits which may occur naturally or by insect damage. Closed pistachios can become infected through the base of the fruit or the stem area (Mahoney & Molyneux, 1998). Overall, it is environmental conditions, the physiological characteristics of the nut, the prevalence of pests, and the presence of competitive microflora that determine the risk of microbial spoilage in the field.

High temperatures, humid conditions, and extreme temperature gradients during storage and transport can increase the susceptibility of nuts to spoilage (International Commission on Microbiological Specifications for Foods, 1998). Spoilage can occur whether the nuts are raw or previously treated by a thermal or nonthermal process. Nuts should optimally be maintained at approximately 1°C and 60–70%

relative humidity for greatest protection (Codex Alimentarius Commission, 1972). If storage temperatures and humidity are not held constant, or if storage containers are exposed to sunlight, there is an opportunity for moisture migration and, therefore, higher water activity levels which can lead to mold growth and spoilage (Hocking, 1988; Codex Alimentarius Commission, 1972).

Cocoa

Cocoa beans are contaminated with bacteria and fungi mainly from the soil and air and also from the surface of pods and the hands and tools of harvesters. This usually occurs immediately after cutting and breaking the pods and removing the sterile pulp (Ostovar & Keeney, 1973). After the decline in temperature and degradation of pulp residues following fermentation, mycelia can penetrate the mass if oxygen is supplied, causing compositional changes (Hansen, 1975).

Storage in jute bags or silos under unsuitable conditions can also influence the spoilage of cocoa beans. Molds, in particular, xerophilic varieties, are able to grow if beans are damaged, improperly dried, or when the moisture content increases above 8% (Maravalhas, 1966; Hansen & Welty, 1970). This ultimately can lead to off-flavors.

Coffee

Factors influencing the microbial spoilage of coffee include a long hold time (>24 h) of coffee cherries after harvest, over-fermentation, improper maintenance of the fermentation tanks, high moisture levels during drying and storage, and rewetting of the coffee. The coffee cherry is very unstable after harvest. Holding fresh cherries longer than 24 h after harvesting can lead to mold growth. Over-fermentation of the beans may lead to the production of fruity flavor, sour coffee, and stinker beans. Picking old fruits from the ground during harvesting leads to mold contamination and subsequent moldy or musty flavors in the beans. Leaving mucilage on the beans after washing will also lead to mold growth and potential ochratoxin A production.

Prevention and Control of Spoilage

Spices

The Code of Hygienic Practice for Spices and Dried Aromatic Plants put forth by the Codex Alimentarius Commission (1995) provides minimum hygienic requirements for growing, harvesting, drying, cleaning, grading, packing, transporting, and storing spices. In addition to providing measures that help to assure the safety of spices, the Code of Hygiene also includes recommendations that help to reduce or

prevent opportunities for spoilage. These consist of requirements for drying spices on raised platforms or other suitable surfaces, using packaging materials that prevent the reabsorption of ambient moisture in humid climates, transporting spices in clean, well-ventilated ships and rail cars, using processing facilities that have a designated product flow to prevent cross-contamination, and assuring adequate design of the processing facilities to prevent the accumulation of dirt, molds, and condensation on overhead structures (Codex Alimentarius Commission, 1995).

Several studies have revealed that the type of packaging has an influence on the level of mold contamination of spices. Black pepper, red chili peppers, ginger, cinnamon, and nutmeg packed in gunnysacks (coarse fabric bags) had higher fungal counts than products stored in wooden boxes and/or plastic bags according to an investigation by mandeel (2005). Chourasia (1995) also observed a significantly higher prevalence of mold flora and insects in samples from gunnysacks compared to samples obtained from metal, glass, and wooden containers. Polythene bags were also better than cotton and gunnysacks (Misra, 1981). A likely explanation for these observations is that the loose weave of the bags provides an opportunity for dust, mold spores, and insects to enter the bags and also allows for a rapid acclimation of the spices to the surrounding environment.

Spices are typically transported to the United States and other countries by container ships. Guidelines, such as those provided by the Transport Information Service (2005) in Germany, help to minimize spoilage during shipping. Stowage in areas of a ship that are dry and have temperatures ranging from 5 to 25°C is recommended. Since the risk of mold growth is greatest in warm, damp environments, storing spices in the uppermost deck of a ship is discouraged. Exposure to seawater and rain can increase the likelihood of mold spoilage and the intense sunlight can heat the products and drive off the essential oils, which are the primary flavoring components.

Upon receipt, spices are often further processed before being released into the retail and industrial markets. The processing includes additional cleaning to remove sticks, stems, dirt, stones, string, insects, and other items. The spices may also be treated to reduce bacterial and fungal counts. In the United States, ethylene oxide fumigation and steam treatment are the methods frequently employed. Irradiation with gamma rays is also an approved option in the United States whereby the maximum dose can be 30 kGy (U.S. Food and Drug Administration, 2006).

Gassing spices with ethylene oxide is very effective in that it can reduce 99.9% or more of the molds and 99% or more of the bacteria that are present on the spice (International Commission on Microbiological Specifications for Foods, 1980). Ranges of standard plate counts for untreated and ethylene oxide-treated spices are listed in Table 4 (Weber, 1980). The process requires control of several factors including the gas concentration in the chamber, the humidity, the temperature, and the time of exposure as well as knowledge of the chemical and physical properties of the spice (Weber, 1980). It is effective against coliforms, mesophiles, and thermophiles, including flat sour sporeformers (Pruthi, 1980). The tolerance for ethylene oxide residues in whole spices is firmly established by the US Environmental Protection Agency (2005) at a maximum of 50 ppm.

Table 4 Range of standard plate counts (SPC) for ETO-treated and -untreated spices (Reprinted with permission, Weber, 1980)

	Standard plate count (CFU/g)			
Spice	ETO treated Minimum	ETO treated Maximum	Untreated Minimum	Untreated Maximum
Allspice*	3.0×10^{2}	4.7×10^{3}	1.5×10^{5}	3.4×10^{6}
Basil*	1.2×10^{3}	2.9×10^{4}	1.3×10^{4}	3.9×10^{6}
Bay leaves	6.0×10^{2}	5.9×10^{3}	7.5×10^{3}	2.0×10^{4}
Caraway*	<10	3.0×10^{2}	1.0×10^{3}	9.3×10^{5}
Cardamom*	<10	ND	3.6×10^{3}	ND
Celery seed*	<10	3.8×10^{4}	1.2×10^{3}	7.2×10^{6}
Cinnamon*	<10	2.0×10^{3}	ND	4.6×10^{4}
Cloves*	<10	2.7×10^{3}	<10	2.8×10^{3}
Coriander*	<10	1.8×10^{4}	1.3×10^{4}	3.7×10^{6}
Cumin*	<10	9.0×10^{3}	1.7×10^{3}	1.0×10^{7}
Dill seed*	5.0×10^{1}	4.4×10^{3}	5.5×10^{3}	3.4×10^{5}
Fennel*	<10	5.8×10^{3}	8.2×10^{3}	8.8×10^{4}
Fenugreek*	<10	9.5×10^{3}	2.2×10^{4}	3.8×10^{5}
Ginger*	<10	1.5×10^{4}	1.2×10^{5}	1.8×10^{7}
Marjoram*	5.0×10^{1}	1.2×10^{3}	2.6×10^{4}	2.4×10^{6}
Mace*	ND	ND	5.1×10^{3}	1.0×10^{4}
Mustard flour	ND	ND	5.7×10^{3}	ND
Oregano	<10	4.9×10^{3}	7.3×10^{3}	1.3×10^{6}
Paprika	3.0×10^{2}	8.2×10^{3}	1.8×10^{5}	2.3×10^{7}
Black pepper	2.0×10^{2}	2.6×10^{4}	5.8×10^{4}	5.3×10^{7}
Cayenne pepper	<10	1.9×10^{3}	ND	1.3×10^{7}
Red pepper	<10	3.2×10^{3}	7.3×10^{4}	6.0×10^{5}
White pepper	<10	1.5×10^{2}	9.3×10^{4}	6.6×10^{6}
Rosemary	<10	7.7×10^{2}	1.5×10^{4}	9.2×10^{5}
Sage	ND	ND	2.9×10^{3}	7.0×10^{4}
Thyme	<10	7.2×10^{3}	9.2×10^{4}	3.2×10^{6}
Turmeric	<10	1.2×10^{4}	6.6×10^{5}	7.9×10^{6}
Savory	<10	3.2×10^{2}	4.9×10^{4}	7.1×10^{4}

* In ground form
ND = not determined

Another option for reducing the microbial load of spices is treatment with high temperature steam (Hsieh, Johnson, & Dudek 1989). Conditions for steam treatment have been optimized for many spices to reduce the microbial load while preserving the color and flavor attributes.

Gamma irradiation is also an effective and safe treatment for spices and has an advantage in that products can be irradiated in their final packaging which reduces opportunities for postprocess contamination (Kiss & Farkas, 1988). Gamma irradiation is competitive with ethylene oxide treatment with respect to the reduction of the number of microorganisms without sacrificing sensory quality (Farkas & Andrassy, 1988). However, the lack of consumer acceptance of the irradiation process has limited its use in the spice industry.

Nuts

Controlling the growing conditions and storage of the product after harvesting minimizes the spoilage risk. However, this is sometimes difficult to achieve due to each country of origin having different technologies and processing practices. The Codex International Code of Hygienic Practice For Tree Nuts (Codex Alimentarius Commission, 1972) recommends measures that can be implemented to assist in minimizing the contamination risk. Maintenance of sanitary growing conditions is foremost. Irrigation water must be of "sanitary quality and pose no health risk to consumers" (Codex Alimentarius Commission, 1972). Nuts should be grown in fields segregated from livestock and grazing fields to minimize exposure to areas presenting risk of fecal runoff.

Once harvested and dried, nuts are shelled and visually inspected for the presence of foreign material and physical defects. Automated equipment is often used to complete this task. Since damage due to spoilage is sometimes seen as defects in the nut itself, the sorting processes serve as a step in reducing further processing of contaminated product.

After the nuts are harvested, appropriate conditions and materials are necessary to protect against spoilage during storage and transport. Nuts require cool and dry conditions to maintain their protective low moisture content. Therefore, the containers used to store nuts must be clean and provide a sufficient barrier against accumulation of or exposure to moisture so that the water activity does not increase above 0.70. Storage containers may be silos, metal or corrugated lined totes, vacuum-sealed packaging, or rail cars. Once in transit, appropriate storage conditions must be monitored and controlled to minimize potential risks of microbial growth (Codex Alimentarius Commission, 1972).

Processing is a critical factor in controlling nut spoilage. The most commonly used processes are dry roasting, oil roasting, and blanching. The time and temperature parameters for these processes vary and are manufacturer dependent but must be validated to achieve a defined level of kill for microorganisms. The efficacy of the thermal treatment depends on the type of molds present. For example, ascospores are more heat resistant than conidia; hence, ascospores are more difficult to inactivate. Nonthermal treatments, such as propylene oxide (PPO), are also used as an alternative to thermal treatments and are very effective. Propylene oxide gas is applied in a vacuum with minimal heat to kill microorganisms. The advantage of this treatment is that the raw characteristics of the nut are maintained. However, due to suspected health risks associated with this gas, many countries do not allow the use of PPO for foods or the import of PPO-treated products.

As in any processing, the hurdle approach is most effective. This combines the use of more than one processing technique to more effectively preserve the product. It increases the level of control and aids in the protection if one part of the process fails. Thermal and nonthermal treatments are components of the hurdle philosophy. Others may be the application of preservatives or the use of vacuum or modified atmosphere packaging (Hocking, 1988). These techniques

are especially useful in baked products that have incorporated nuts in which the water activity may be higher than in nuts alone, thus increasing susceptibility to spoilage.

The previously described preventative measures have little impact if the risk of cross-contamination is not addressed. All phases of the process have the potential to introduce contamination if appropriate control measures are not used. Equipment employed throughout processing, harvesting, and transporting should be clean and sanitary. Plants and their associated operations must use Good Manufacturing Practices (GMPs) (Codex Alimentarius Commission, 1972). This includes plant design and structure, equipment design, air handling, pest control, product handling, employee practices and hygiene, traffic control, cleaning and sanitation procedures, and separation during all stages of processing. The composite of all the preventative measures described above affords the protection needed to minimize the risk of spoilage.

Cocoa

Maintaining the moisture level below 8% can control spoilage of cocoa beans. Artificial drying of the beans is preferred because it is very quick and does not permit mold growth, whereas sun drying may take 7 days or more depending on the atmospheric conditions (Wood, 1988). Using clean tools during the breaking of pods and removal of sterile pulp can minimize the potential of mold contamination. Using liners inside a jute bag during storage can protect the dry beans from uptake of moisture and subsequent mold development.

Coffee

Mold contamination can be controlled or minimized during harvesting by the use of pick mats beneath the trees to protect the main crop from contamination by old fallen cherries. Coffee cherries that have been in contact with orchard soil for longer than 1 week should be collected and disposed. Washing the beans within 2 h of the end of fermentation can prevent the development of fruity or sour coffee. Using fermentation to limit oxygen availability and encourage the growth of harmless competitive microorganisms during fermentation or mechanical removal of the mucilage to permit immediate drying can control parchment spoilage. If parchment coffee cannot be dried due to rain, it should be soaked in clean water to prevent mold spores from germinating. During the drying phase, coffee should be layered at a depth of 4–5 cm and should be turned approximately 4–5 times per day. Coffee beans should be stored where the air is drier than the coffee (relative humidity less than 60%). If the air is more humid than the coffee (e.g., relative humidity greater than 80%), the coffee will absorb water. The storage area must be designed to prevent rewetting of the beans. Dust extraction during milling will also control mold spore levels.

Methods for Spoilage Detection

Macroanalytical Examination

It is important that spices meet the quality specifications that have been established by the US Food and Drug Administration (2005) and the American Spice Trade Association (2001b). They include limits on extraneous matter, excreta, insect infestation, dead insects, and visible mold. The US Food and Drug Administration's (2005) food defect action levels (DALs) and the American Spice Trade Association's (2001b) specifications for mold defects vary between 1 and 10% by weight of spice examined, depending on the product. The procedure involves analyzing a maximum of 10, one-half to one-pound samples per lot (American Spice Trade Association, 2001b). For most products, the entire sample is examined for visible defects. Specific methods are outlined for products such as whole and broken nutmegs and black and white pepper. For example, 100 nutmegs are selected at random from each sample, cut in half longitudinally and examined. A spice seed, fruit, leaf, or rhizome is deemed moldy if the contamination is "visible to the naked eye, exceeding one-fourth of its surface area and confirmed by the presence of mycelial filaments and spores when examined with the aid of a microscope at 40× magnification or less" (American Spice Trade Association, 2001b).

The US Food and Drug Administration (1998b) has also established procedures to detect defects of in-shell and shelled nuts. Defects are defined as insect damaged, moldy, rancid, otherwise decomposed, dirty, shriveled, or gummy. Sampling consists of the preparation of 100 nut composite subsamples of a single nut type. A 5% defect action level is the limit for in-shell and shelled almonds (US Food and Drug Administration, 2005). A 10 or 5% defect action level scheme is defined for all other nuts (US Food and Drug Administration, 2005). A minimum of 100 nuts must be examined, with increments of 50 nuts thereafter up to 500 nuts, for visual and organoleptic properties.

Defect actions levels for mold on cocoa beans and green coffee beans have also been established by the US Food and Drug Administration (2005). Greater than 4% moldy cocoa beans by count and an average of 10% or greater moldy green coffee beans by count are the established reject criteria.

Microbiological Examination

Molds and other contaminants may not be evenly distributed throughout a lot of spices, nuts, cocoa, and coffee beans which makes adequate sampling key to maximizing protection (International Programme On Chemical Safety, 1979; Food and Beverage Mycology, 1987). The most appropriate way to sample these products for microbiological attributes is to use a 2- or 3-class sampling plan as described by the International Commission on Microbiological Specifications for Foods (1986). Spices, nuts, cocoa, and coffee are routinely tested for yeasts and molds, and may

also be analyzed for Plate Count bacteria and generic *E. coli* according to the methods in the US Food and Drug Administration's Bacteriological Analytical Manual (2001) and the Compendium of Methods for the Microbiological Examination of Foods (2001). A 3-class sampling plan in which five samples are taken at random from the lot and individually tested is recommended. When screening for pathogens that are moderately to seriously hazardous such as *Salmonella*, a 2-class plan is recommended (International Commission on Microbiological Specifications for Foods, 1986). Prior to testing, whole spices and previously chopped nuts should be ground to assure a uniform sample. Care should be taken not to overheat the sample during the grinding process. Because mold contamination is usually on the outside of whole and half nuts, a buffered diluent wash is recommended for these products. When testing spices for bacteria and molds, it is important to remember that many spices contain natural antimicrobial compounds which inhibit the growth of microorganisms on lower dilution plates. These include, but are not limited to, onion, garlic, clove, allspice, cinnamon, mustard seed, and oregano. To overcome this inhibitory effect, a range of serial dilutions is recommended. Likewise, when testing for pathogens that require a preenrichment step, the spice should be diluted with preenrichment medium at a ratio of 1:9 or greater. The US Food and Drug Administration's Bacteriological Analytical Manual (2001) outlines specific preenrichment dilutions for spices that are analyzed for *Salmonella* spp.

Dichloran 18% glycerol (DG18) agar is considered a good medium for enumerating xerophilic fungi in reduced water activity foods (Akerstrand, 1992; Hocking, 1992) such as spices, nuts, cocoa, and coffee. However, a study has revealed that other agars, including tryptone glucose yeast extract chloramphenicol (TGYC) agar and plate count agar with chloramphenicol (PCAC), are better than DG18 at recovering desiccated yeasts from dry foods (Beuchat et al., 2001). Dichloran rose bengal chloramphenicol (DRBC) agar can also be successfully used to enumerate fungi in spices, nuts, coffee, and cocoa products. Regardless of the medium, the agar plates should be incubated upright at 25°C for 5–7 days.

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Note: The letters '*f*' and '*t*' following the locators refer to figures and tables respectively

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