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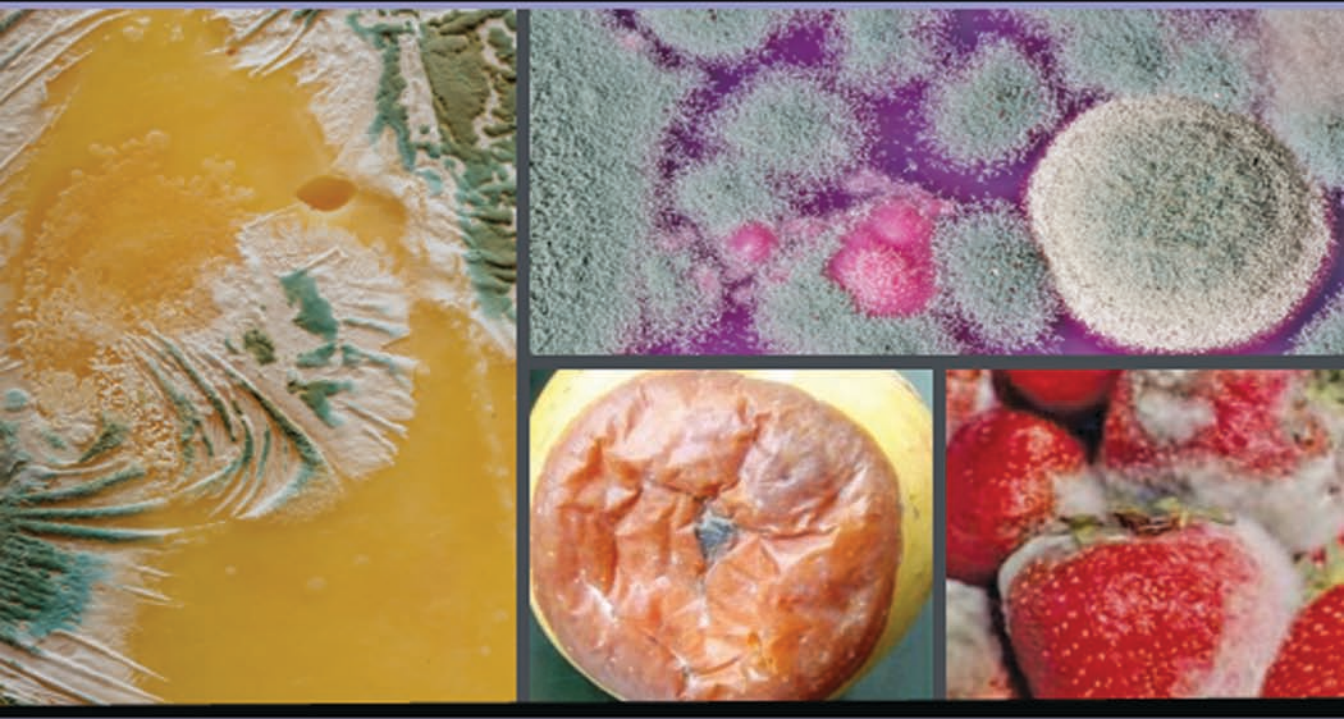

Edited by
Yanbo Wang • Wangang Zhang
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CRC Press
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CRC Press
Taylor & Francis Group
Boca Raton London New York

CRC Press is an imprint of the
Taylor & Francis Group, an **informa** business

CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

Printed on acid-free paper
Version Date: 20130717

International Standard Book Number-13: 978-1-4987-4458-4 (Hardback)

Library of Congress Cataloging-in-Publication Data

Names: Wang, Yanbo, 1978 editor.
Title: Food spoilage microorganisms : ecology and control / editor, Yanbo Wang.
Other titles: Food spoilage microorganisms (Wang) | Food microbiology series.
Description: Boca Raton : Taylor & Francis, 2017. | Series: Food microbiology series | Includes bibliographical references and index.
Identifiers: LCCN 2016034984 | ISBN 9781498744584 (hardback : alk. paper)
Subjects: | MESH: Food Microbiology | Food Analysis--methods | Food-Processing Industry--standards | Food Contamination--prevention & control | Foodborne Diseases--etiology
Classification: LCC QR115 | NLM QW 85 | DDC 664.001/579--dc23
LC record available at https://lccn.loc.gov/2016034984

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and the CRC Press Web site at
http://www.crcpress.com

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Preface

Food spoilage can occur in any segment of the whole food chain and thus threatens both public health and food quality. Therefore, food spoilage significantly affects food supply and our daily life. Microorganisms involved in food spoilage include a wide variety of bacteria, yeasts, and molds. Studying the ecology of these microorganisms and how food spoilage occurs are crucial to develop proper measures to prevent and control food spoilage. This book covers the occurrence, outbreak, important consequences, control, and evaluation of spoilage microorganisms in food.

This book contains nine chapters and each chapter is organized based on a food category so readers may easily understand and access the relevant knowledge. In all given food categories, the authors discuss the taxonomy, characteristics, and possible mechanisms of spoilage microorganisms, specific methods for detection and evaluation, corresponding control, prevention, and management options. In addition, current opinion and future research needs related to food spoilage microorganisms are discussed. Section I (Chapters 1 through 4) covers spoilage microorganisms in foods of plant origin including cereals, legumes, fruits, and vegetables. Section II (Chapters 5 through 9) tackles spoilage microorganisms in foods of animal origin including meat, poultry product, sea food, powdered milk, and egg products.

This book is intended for both food scientists/engineers and nonspecialists, especially for those responsible for food quality, safety, and regulation. This book also provides readers with the necessary basic knowledge in food spoilage consequences and control so as to ensure food safety, and in particular, in developing countries where postharvest food hygiene requires special care.

We would like to thank all the contributing authors for their excellent contribution. We also thank the editorial and production team for their work. We hope that readers will benefit from reading this book.

Junshi Chen
Senior Research Professor
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Editors

Prof. Yanbo Wang is a professor of Food Science and Technology at Zhejiang Gongshang University, Hangzhou, China. He earned his PhD degree from Zhejiang University, Hangzhou, China in June 2006. In February 2008, he was invited to the Marine Research Institute, Florida Institute of Technology (FIT), as a visiting scholar and then to the Institute of Food and Agricultural Sciences, University of Florida (UF) for 2-year postdoctoral research. Dr. Wang is currently an associate editor of the *Journal of Fisheries and Aquaculture*. Since 2008, he has received extensive research funding from the National Natural Science Foundation of China (NNSFC), the Ministry of Science and Technology of the People's Republic of China, and other related agencies. He has published 40 papers in international academic journals contained in the Science Citation Index (SCI). These papers are cited 739 times in total and 719 times by the other authors in SCI journals. Dr. Wang has published two papers as the corresponding author, which has been contained in the database of highly cited papers through Essential Science Indicators (ESI) based on the web of science. Additionally he has published four international academic books and has obtained four authorized national patents. Based on his achievements, Dr. Wang has obtained recognition as the Excellent Young Teacher in the Colleges and Universities of Zhejiang Province and the Zhejiang Provincial Natural Science Foundation for Distinguished Young Scientists. He also has been elected to the New Century Training Program (151) for Talent from Zhejiang Province and the Academic Discipline Leaders for the Young and Middle-Aged Teachers in the Colleges and Universities of Zhejiang Province.

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Prof. Linglin Fu is a professor of Food Science and Technology at Zhejiang Gongshang University, Hangzhou, China. She earned her PhD degree from Zhejiang University, Hangzhou, China in June 2008, and received an offer from Zhejiang Gongshang University to join the faculty to teach and conduct research. In April 2013, she was invited to Harvard University as a postdoctoral fellow for a one-year research period. Dr. Fu is currently an associate editor of the *Journal of Fisheries and Aquaculture*. Since 2009, she has received financial funding from NNSFC, Ministry of Science and Technology of the People's Republic of China, and other related agencies. She has published more than 30 papers in international academic journals contained in the SCI. She has published two international academic books and been awarded one authorized national patent. Based on her achievements, Dr. Fu has been named the Excellent Young Teacher in the Colleges and Universities of Zhejiang Province. She also has been elected to the New Century Training Program (151) for Talent from Zhejiang province.

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Section I

1 Spoilage Microorganisms in Cereal Products

Wenjian Yang, Dapeng Li, and Alfred Mugambi Mariga

CONTENTS

1.1 INTRODUCTION

Cereal grains are the most important food commodity for the world population and represent up to 80% of the diet in some cultures. Grains are often contaminated with microorganisms during harvest, transport, and storage, and the safety and quality of grains also decrease. Immediately after harvesting, cereal grains contain microbial contaminants from several sources, such as dust, water, ill plants, insects, soil, fertilizers, biofilm on surface of equipment, humans, and animal feces (Butscher et al., 2015).

A wide range of cereal products, including bakery items, frozen dough, fresh pasta products, dried cereal products, snack foods, and bakery mixes, are manufactured for food consumption. These

products are also subject to microbial spoilage that affects the taste, aroma, leavening, appearance, and overall quality of the end product. Microorganisms are ubiquitous in nature and have the potential to cause 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 the low moisture content of the final product contributes to product stability (Cook and Johnson, 2009). Nevertheless, microbial spoilage of these products occurs, resulting in substantial economic losses.

Many cereal products are often contaminated with spoilage or pathogenic microorganisms making them nonedible or affecting their taste by the production of undesirable flavors. For example, lactic and coliform bacteria can make wet mash of grains suffering acid fermentation. Molds are the most important spoilage organisms in cereal grains as they can reduce the nutritional value, properties, cause dry matter loss, heating of grain, off-odors, and in the worst case, form mycotoxins and allergenic spores. These microorganisms can sometimes produce toxic substances causing a serious hazard in human and animal health besides a very high economic loss (Gupta and Srivastava, 2014). Food poisoning can arise either through the ingestion of food containing toxigenic microorganisms or by the ingestion of food containing only toxins that are formed by the microorganisms. In particular, during the postharvest period many toxin-producing microorganisms can grow heavily on several food products (Magan and Aldred, 2007). Microbes involved in spoilage of cereal products are shown in Table 1.1.

The quality and the safety of cereal products are of major concern to producers, quality control authorities, and end product consumers. In addition to the economic losses incurred because of spoilage, possible foodborne illnesses could cost billions of dollars to the industry due to costly adverse health effects, the loss of productivity, medical expenses, and most importantly, adverse publicity for the 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 is lost annually because of the presence of fungi and mycotoxin alone.

TABLE 1.1
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

1.2 TAXONOMY OF SPOILAGE MICROORGANISMS IN CEREAL PRODUCTS

The microbial composition of the cereals is of great importance for the storage of cereal and product because at high moisture levels microorganisms can grow and alter the properties of the product. The moisture content of 13% is considered to be the maximum value for the storage of wheat, corn, barley, and rice during short periods, though temperature and oxygen concentration also play an important role. A wet mash of grains is likely to suffer acid fermentation due to the action of lactic and coliform bacteria. This may be followed by an alcoholic fermentation by yeast and finally, molds. Yeast films can be developed on the surface, although if acetic acid bacteria are present, they may oxidize alcohol to acetic acid, inhibiting mold growth (Laca et al., 2006). The initial microflora and grain conditions are the determining factors for the type of microorganisms developed. Mycotoxins produced by molds pose the most serious health risk in cereals, some of them being so resistant that they cannot be eliminated during food processing (Hill and Lacey, 1983).

Bacteria found in grains mainly belong to the families *Pseudomonadaceae*, *Micrococcaceae*, *Lactobacillaceae*, and *Bacillaceae*. Molds are mostly *Alternaria*, *Fusarium*, *Helminthosporium*, and *Cladosporium*, although other genus can also be present (Focht and Lockhart, 1965). Rice fungal flora study and evolution suggest that mold on the national paddy 41 genera, 117 species dominant fungi decrease, such as *Aspergillus flavus, A. glaucum, and Aspergillus* white, which gradually are replaced by storage fungi with the increase of storage time. Succession of fungal flora under different storage conditions has been studied and found that grain storage stability is related to the amount of mold.

1.3 CHARACTERISTICS AND POSSIBLE MECHANISMS OF SPOILAGE MICROORGANISMS

Fungi colonizing cereal has been classified into field and storage fungi. Field fungi, which include species of *Alternaria*, *Cladosporium*, and *Fusarium*, characteristically colonize cereal before harvest and seldom grow during storage. In contrast, storage fungi including species of *Aspergillus* and *Penicillium* are often widespread but in small numbers before harvest and develop during storage only when environmental conditions are suitable. Grain may also be contaminated with storage fungi during harvest and when it is placed in contaminated stores (Logrieco et al., 2003). An intermediate group is sometimes recognized to include *Cladosporium* and *Fusarium* spp., which may continue to develop in storage under certain conditions.

Growth and sporulation of fungi, both on the standing crop and in stored grain, is largely dependent on environmental factors. However, factors that determine why field fungi primarily develop on the standing crop while storage species become dominant in store are not well understood. The most important environmental determinants are probably water activity (a_w) and temperature, but hydrogen ion concentration (pH) and the gaseous composition of the atmosphere may also affect fungal growth (Gock et al., 2003; Marin et al., 1995).

Manipulating several environmental factors together may possibly change the nature and extent of fungal growth in the stored grain ecosystem synergistically so that spoilage and mycotoxin production are decreased or prevented more than additively. Most previous studies of the water relations of microorganisms have been carried out with this as the sole variable, other factors having been held close to their optimum levels for the organism concerned. For instance, the water relations of spore germination and linear growth have usually been studied at constant temperatures (Ayerst, 1969). Only occasionally have temperature and a_w been varied together. Although the effect of temperature on a_w is considerable there is little detailed information on the effect of interactions between these factors on the growth of individual field and storage fungi (Magan and Lacey, 1984).

pH in the substrate can influence a_w–temperature interactions through its effects on metabolic processes, for example, those concerned in sporulation and morphogenesis. Storage species become dominant in store are not that *Aspergillus* spp. grew at lower a_w at pH 7 than at

pH 3 or 5. pH limits of 1.5–9.8 for *A. niger* van Tiegh.; 2.1–7.7 for *A. candidus*; and 1.8–8.5 for *A. repens* at high a_w have been reported but no information is given for low a_w. However, pH values of 4 and 6.5 were equally suitable for growth of several molds at low a_w. Otherwise, there is little information on the interactions of pH with temperature and a_w on the growth of field and storage fungi (Magan and Lacey, 1984).

Mycotoxins include trichothecenes, deoxynivalenol (DON), 3-acetyl deoxynivalenol (3-A-OON), 15-acetyldeoxynivalenol (15-A-DON), DON-3-glucoside, nivalenol (NIV), fusarenon-x (FX), diacetoxyscirpenol (DAS), T-2 and HT-2, in addition to zearalenone (ZEN), fumonisin, aflatoxins, ochrotoxins, and ergot alkaloids. Aflatoxins (*A. flavus* and *A. parasiticus*) are widely recognized as major mycotoxin producers to cause health problem, especially in hot, humid countries (De Kuppler et al., 2011). This is a particular serious problem in such crops as maize, rice, peanuts, tree nuts, and dried fruits. Aflatoxin production normally occurs in the field, particularly when stimulated by drought, stress, and high temperatures or during prolonged drying. Aflatoxin-producing molds grow exponentially in conventional multi-month storage as a result of a combination of heat and high humidity (Cotty and Jaime-Garcia, 2007).

1.3.1 Spoilage Microorganisms' Characteristics and Possible Mechanisms in Bakery Products

There are many types of cereal products such as bakery products, refrigerated dough and pasta products, dried cereal products, and compound cereal products. 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. Since 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. Ambient temperatures, product pH levels between 5.4 and 7.5, and a_w 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 with levels above 0.94. Although relatively harmless, their visible presence deters customers and can result in substantial economic losses to wholesale bakeries (Cook and Johnson, 2009).

1.3.1.1 Mold and Yeast Spoilage of Bakery Products

Bakery products are the important staple foods in most country and cultures. Together with cereals, they are a valuable source of nutrients in our diet providing us with most of our food calories and approximately half of our protein requirements. Nevertheless, 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 (Fleet, 1992).

Although shelf life of bakery products such as bread is limited by the physicochemical deterioration called "staling," 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. Surface yeast growth can create white or pink areas (Sperber and Doyle, 2009). Typical shelf life lengths for various cereal products are shown in Table 1.2.

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

TABLE 1.2
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

Note: NA, not applicable.

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 (Kabak et al., 2006). 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 *A. flavus*, *A. parasiticus*, and other closely related fungi; fumonisins, produced by *Fusarium verticillioides*; and DON (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. The public health effects of mycotoxins are complex. Some mycotoxins are carcinogenic or tumor-evoking, some are vasoactive, and some cause central nervous system damage (D'Mello, 2000). 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*, *Eurotium*, and *Mucor.* In bakery products, yeast problems can be divided into two types: (1) visible yeast that grows on the surface of the bread in white or pinkish patches and (2) fermentative spoilage yeast associated with alcoholic and essence odors hence osmophilic yeasts. Products contamination by osmophilic yeasts normally results from dirty equipments. Thus, maintaining good manufacturing practices will minimize the contamination by osmophilic yeasts (Saranraj, 2012).

Yeasts that can cause surface spoilage of bakery products include *Saccharomyces*, *Debaryomyces*, *Kluyveromyces*, *Pichia*, *Candida*, *Trichosporon*, and *Zygosaccharomyces*. These species produce white spots leading to the term chalk bread. A number of strains of *Saccharomyces* spp. are used to produce millions of tons of bakery products each year, and some yeasts such as *Saccharomyces*, *Pichia*, *Candida*, and *Torulopsis* are used in the manufacture of sourdough breads.

1.3.1.2 Microbial Sources and Effects of Bakery Products

Typically, postprocess 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 (Guynot et al., 2002). 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 (Legan and Voysey, 1991). 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. Unsanitary equipment including utensils can be a source of contamination by osmotolerant yeast.

1.3.1.3 Factors Influencing Spoilage of Bakery Products

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 a_w is critical for the microbial stability of bakery products. In general, molds and yeasts are more tolerant to lower a_w 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. Most molds will not grow significantly below a_w 0.80, but xerophilic molds 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 (Slade et al., 1991). 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.

1.3.1.4 Rope Spoilage of Bakery Products

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, sticky, and slightly discolored. When broken into 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 (Sperber and Doyle, 2009).

Ropiness is bacterial spoilage caused primarily by *Bacillus subtilis*, sometimes by *Bacillus licheniformis*, *B. pumilus*, and *B. cereus*, and also by *B. clausii* and *B. firmus*. 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 a_w, 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 an a_w value of 0.95 or above, at ambient temperatures or higher. It occurs mostly in summer when the climatic conditions favor growth of bacteria. These conditions enable the rope *Bacilli* to grow faster and to produce higher levels of amylase and protease activity, resulting in enzymatic degradation of the crumb and stickiness because of the production of extracellular

mucilaginous polysaccharides by the *Bacilli*. Spoilage of bread by rope formation may constitute a health risk, since elevated numbers of *B. subtilis* and *B. licheniformis* in foods may cause a mild form of food illness. Consumption of ropy bread has been associated with food-borne illness in reports from Canada and the United Kingdom (Pepe et al., 2003). The types and numbers of microorganisms initially present in bakery products are dependent on the microbial 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. 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, and water tanks during dough production and cooling racks, conveyor belts, slicing blades, and wrapping materials after baking (Thompson et al., 1998).

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. Other effective measures that can contribute to the control of rope spoilage include use of certificates of analysis (COAs) 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.

1.3.1.5 Sour Spoilage of Bakery Products

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 sour dough 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 and Savolainen, 1984). Conversely, the same type of bacterial growth in other bakery products is highly undesirable and constitutes products 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 and 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. 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 postbaking contaminants from growing (Oliver et al., 1997).

1.3.2 Spoilage Microorganisms Characteristics and Possible Mechanisms in Refrigerated Dough and Pasta Products

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. 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 (Hesseltine et al., 1969). 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. 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. They are highly perishable and must be consumed within a few days after manufacture (Deibel and Swanson, 2001). 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.

1.3.2.1 Spoilage and Influential Factors of Refrigerated Dough and Pasta Products

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 microbial 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 microbial spoilage problems in refrigerated dough products with high sugar contents, such as cookie doughs or fruit-filled products, is fermentation by osmophilic yeasts that are capable of growing at a_w 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. 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 *Hansenula anomala*. This yeast spoilage is characterized by the production of CO_2 gas, which causes bubbling or expansion of flexible packaging (Membré et al., 1999).

Spoilage of refrigerated doughs can also be caused by *Lactobacilli* and *Leuconostocs* especially under temperature abuse conditions. 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 (Hesseltine et al., 1969). This spoilage defect will occur whether or not the lactic acid bacterium is homofermentative (nongas producing) or heterofermentative (gas producing).

Some researchers found that the microflora of spoiled doughs subjected to higher temperature consisted of such bacteria as *Leuconostoc dextranicum*, *L. mesenteroides*, *Lactobacillus*, *Streptococcus*, *Micrococcus*, and *Bacillus* species. The research indicated 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 and 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.

1.3.3 Spoilage Microorganisms' Characteristics and Possible Mechanisms in Dried Cereal Products

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. Many types of ready-to-eat grain-based products are considered convenient 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. 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, pan cake mixes were available to the U.S. 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.

1.3.3.1 Spoilage and Influential Factors of Dried Cereal Products

Manufacturing of breakfast cereals generally occur through three differing procedures called "flaking," "puffing," or "extrusion." For each of these procedures, moisture is introduced into the cereal formulation, thus providing a higher a_w by which microbial growth can be sustained (Deibel and Swanson, 2001). 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 a_w 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 a_w, 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 animals, 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. 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 (Graves and Hesseltine, 1966).

Spoilage is rarely seen in dry cake mixes. This is due to the low a_w 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*, *Penicillium*, *Absidia*, *Mucor*, and *Eurotium* species, because of "clumping" of the mix, whereby a pocket of moisture may become trapped in the packaged product (Boyer and McKinney, 2013). 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. 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 a_w so low even the most osmotolerant yeast and molds cannot grow.

1.3.4 Spoilage Microorganisms' Characteristics and Possible Mechanisms in Compound Cereal Products

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.

1.3.4.1 Spoilage and Influential Factors of Compound Cereal Products

Because of their general inability to grow at reduced a_w 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 a_w 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 spore formers that survive the cooking process. Meat- and vegetable-filled cereal products with high a_w (>0.94) and neutral pH generally require time and temperature control because the baking process can activate and permit growth of spore formers such as *Clostridium botulinum* that are present in these ingredients. 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 et al., 1995). For example, cream- or custard-filled baked products have been implicated in foodborne illness because of the growth of *B. cereus* and *S. aureus*.

1.4 DETECTION AND EVALUATION METHODS FOR SPOILAGE MICROORGANISMS

Aerobic plate counts (APCs), coliform counts, and *Escherichia 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 and N.A.M.A.M.W. Group, 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 (Sperber and Doyle, 2009).

The traditional cultural detection methodology is performed by growing the microorganisms on selective media. This method requires several days from isolation to identification and is time consuming and expensive. For these reasons, there is currently a strong demand for a faster and suitable sensitive microorganism detection method that can reduce the time taken to achieve results from days to a few hours or even minutes.

Today, many rapid methods are commercially available including deoxyribonucleic acid (DNA) probes, the polymerase chain reaction (PCR), latex agglutination tests, direct epifluorescent filter techniques, enzyme-linked immunosorbent assay (ELISA), conductance, impedance, bioluminescence, immune magnetic beads, and biochemical assay, such as API 20E and Micro ID. Other currently available methods for measuring mold contamination in food include: microscopic examination, culture on agar, electrical measurements of conductance and other changes in electrical properties of the contaminated food substratum, and detection of fungal metabolites, such as chitin, ergosterol, or adenosine triphosphate (ATP). However, all these methods are not always applicable both for the cost and labor time to analyze one sample, and new rapid methods are always being researched and developed for application for detection of microorganisms (Saranraj and Sivasakthivelan, 2015).

Recently, some biosensors have been developed and in particular, a proposed methodology is the detection of the production of volatiles and odors by microorganisms. It is well known that microorganisms produce a wide range of volatiles, such as alcohols, ketones, aldehydes, esters, carboxylic acids, lactones, terpenes, sulfur, and nitrogen compounds (Ortíz-Castro et al., 2009). The volatiles produced arise in foods due to decomposition caused by many endogenous enzymes, microbial contamination, or chemical oxidation and many factors such as substrate temperature, pH, oxygen concentration, age of culture, and microbial species can affect the composition of volatiles. Moreover,

previous studies have shown the positive correlation between fungal volatile organic metabolites with some parameters of micelial development on cereal grains, such as ergosterol, CO_2 production, and mycotoxin production (Olsson, 2000). The volatile organic patterns results are particularly useful not only to detect the early stages of grain spoilage but also to distinguish between presence of toxigenic and nontoxigenic strains of fungi such as *F. verticillioides*. Many works have also reported bacterial and fungal volatiles and a large number of components of naturally occurring odors.

It is well known that flavors are constituted by a large number of components that are perceived as integrated response of the olfactory system to the complex mixture. The human olfactory system can discriminate aromas without separating mixtures into individual compounds. From this point of view, electronic nose parallels the human olfactory system. Olfactory receptors are represented by a group of chemical sensors, which produce a time-dependent electrical signal in response to an odor. Signal-processing techniques can be used to reduce any noise and sensor drift. One of the most important uses of the electronic nose regarding the employment of this technology is to obtain an early and rapid detection of fungal and bacterial activity, and thus, it is a useful tool to distinguish between good and poor quality grain. *Penicillium chrysogenum* or *F. verticillioides*, two widely spread fungi on wheat seeds, at different a_w in order to correlate a chemically volatile profile with the presence of a specific fungal species and with a specific a_w value with the aim to improve rapid methodologies for the detection of food spoilage microorganisms. The electronic nose demonstrated its ability to follow the variation of grain sample headspaces due to the fungal contamination. This feature is particularly promising for the future exploitation of this instrument as a rapid and noninvasive method for the detection of fungal contamination of grain seeds (Paolesse et al., 2006).

1.5 CONTROL, PREVENTION, AND MANAGEMENT OPTIONS FOR SPOILAGE MICROORGANISMS

Several methods including ozone, antimicrobial agents, yeast, pulsed light, and gamma and infrared radiation have been used to reduce microorganisms and molds in grain. However, limited effects, residual of chemicals, and irradiation are main problems to prevent them from usage.

In the past, the control of food safety has been carried out by product testing of both raw materials and processed products, without considering that the damage can occur during the production process. In the recent years, the hazard analysis critical control point (HACCP) system is generally considered the method of choice for ensuring the safety of food. By applying HACCP, it is important to identify the step of the production processes where hazards could occur in order to implement monitoring procedures in place to prevent these hazards from occurring (Hulebak and Schlosser, 2002).

Milling can reduce most of the microbial contamination, but part of the microorganisms and molds can still remain in flour and have strong influence on the ultimate quality of milling end products. Moreover, when whole wheat flour products are getting more and more popular, a huge amount of microorganisms and molds existing in the bran make the whole grain flour deteriorated easily. In order to acquire clean wheat flour to meet the demand of consumers and industries and to improve the storage quality of wheat flour and its products, the microbial decontamination process of wheat grain is needed in many cases.

Superheated steam (SS) pasteurization as an emerging technology has recently attracted a lot of attention for its advantages. During superheated steam processing, large amount of heat transferred to food when steam condenses on food surfaces, which rapidly increases the surface temperature. And the reverse process of moisture transfer that condensation followed by evaporation of moisture on the materials produces characteristic food-processing property of superheated steam. Superheated steam processing is more efficient than saturated steam and hot air processing as superheated steam has a higher enthalpy. The treatment generates and performs quickly in an oxygen-free environment, in which the food stuff can maintain its natural physicochemical properties.

1.5.1 Prevention and Control Measures of Bakery Products Spoilage

Excellent postbaking hygiene, aseptic conditions (including sterile air, packaging, and equipment contact surfaces), destruction of contamination by a postbaking 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 such as flours where the dust raised during unloading can enter the bakery atmosphere and cause contamination. Hygienic measures that 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 (Kotsianis et al., 2002).

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 postbaking 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. Nylon or polypropylene containers may be used for steamed products because of the lower processing temperatures (Saranraj, 2012).

Packaging in sterile atmosphere can also be used to prevent postbaking 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).

Postbaking 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. 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 (Snyder and Poland, 1995). 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 in. 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.

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, *S. aureus*, *Campylobacter jejuni*, *Listeria monocytogenes*, or *E. coli* O157:H7 (Grolichova et al., 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 a_w 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. Humectants such as sugars or glycerin can be used to reduce a_w (Stapelfeldt et al., 1997).

Although a_w is the most important factor affecting the type and rate of spoilage in bakery products, alteration of the a_w 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). 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 (Jay, 2012). Potassium sorbate is 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 increasing shelf life; however, this often results in only an extension of 1 or 2 days. 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. 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. Many studies illustrate the increase in mold-free shelf life obtained in various bakery products packaged in different concentrations of carbon dioxide (Vermeiren et al., 1999). 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. Ethanol acts by reducing the a_w 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 (Vora and Sidhu, 1987). 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 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°C–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 microbial 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 h) because of the fact that breads stored in this manner may demonstrate discoloration of the crumb after 48 h. The bread should also be allowed to cool slowly from this short-term storage. Any method of storage, whether deep freezing, refrigeration, and/or hot storage can result in loss of product quality if storage of the bakery product is prolonged.

1.5.2 Spoilage Prevention and Control of Refrigerated Dough and Pasta Products

The microbial stability of refrigerated doughs is provided by controlled refrigeration temperature and by the reduction of a_w caused by leavening salts and other solutes. The a_w 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 lactic acid bacteria 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.

Refrigerated cookie dough is usually chub-packed in impermeable films. The low a_w 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. 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. Very careful control over microbial 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 (Anonymous, 2005; Deibel and Swanson, 2001).

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 *C. botulinum*, thereby exacerbating the process of eliminating spoilage microorganisms.

1.5.3 Spoilage Prevention and Control of Dried Cereal Products

Microbial spoilage rarely occurs in dried cereal products primarily because of their low a_w (below 0.60), which prevents the growth of all bacteria, molds, and yeasts. Reduced a_w 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.

1.5.4 Spoilage Prevention and Control of Compound Cereal Products

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, for example, humectants, such as glycerol, sorbitol, salt, or sugar, can be used to reduce a_w 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.

1.6 CONCLUSION AND PERSPECTIVES

Nowadays, the detection and evaluation methods for spoilage microorganisms are more complex and most of them are not suitable to quickly get the detected results, especially for the instant cereal products. Some safe, rapid, convenient, and effective detection methods should be developed. Lots of process methods were needed to control the spoilage microorganisms in cereal product. However, these methods were also required to bring little loss of nutrition and destruction of texture. In order for any conventional or molecular-based detection format to be a feasible tool in the food industry for hygiene monitoring or quality control, it must demonstrate reproducible sensitivity (ability to detect target cells or molecules at very low levels), marked specificity (ability to exhibit positive results in a high background of nontarget molecules or cells), speed in obtaining results, low cost per assay, acceptability and ease of use by the scientific community and food microbiology laboratory staff, and standardized protocols and data interpretation. Since no single approach satisfies all or even most of these criteria, the user must prioritize the features of each available format against the needs of the facility and implement the most practical method(s) accordingly.

Improvements in the field of immunology, molecular biology, automation, and computer technology continue to have a positive effect on the development of faster, more sensitive, and more convenient methods in food microbiology. Further development of "on-line" microbiology, including ATP bioluminescence and cell counting methods, is important for rapid monitoring of cleanliness in HACCP programs. One of the most challenging problems is sample preparation. More research is needed on techniques for separating microorganisms from the food matrix and for

concentrating them before detection by immunological or nucleic acid-based assays (Zhou, 2003). The possibilities of combining different rapid methods, including immunological and DNA methods, should be further exploited. Antibodies can be used for capture of target cells that are then detected by a genetic method. On the other hand, amplified PCR sequences can be quantified using immunoassays. Further developments in immunoassays and PCR protocols should result in quantitative detection of microorganisms and the simultaneous detection of more than one pathogen or toxin. For immunoassays, further research on the application of sensor chips may result in multianalyte assays. As positive results of PCR tests are not indicated if the virulence or toxin gene was actually expressed, future studies will focus on the development of assays, which measure biological activity.

Molecular detection and typing methods are largely based on gel electrophoresis, which is a labor-intensive expensive technique and difficult to automate. A new development, which combines semiconductor manufacturing technology with molecular biology to build so called "DNA chips," promises to be one of the major molecular diagnostic breakthroughs for the future. With this technique, DNA sequences can be analyzed quickly and cheaply. DNA chips consist of large arrays of oligonucleotides on a solid support. The array is exposed to labeled sample DNA and hybridized. The detection of the probe–target hybrid is achieved by direct fluorescence scanning, or through enzyme-mediated detection. DNA chip technology also makes it possible to simultaneously detect diverse individual sequences in complex DNA samples. Therefore, it will be possible to detect and type different bacterial species in a single food sample. The development of this approach is continuing at a rapid pace and for the microbiologist, the DNA chip technology will be one of the major tools for the future. There are still many problems to solve, such as sample preparation, eliminating the effects of nonspecific binding and cross-hybridization, and increasing the sensitivity of the system.

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2 Spoilage Microorganisms in Bean Products

Hui Zhou, Yun Tian, and Rongrong Wang

CONTENTS

2.1 INTRODUCTION

Soybean (*Glycine max*) is a species of legume that originated from Asia and was introduced to the United States in 1765 by Samuel Bowen. Soybeans offer high-quality and affordable protein and oil for humans and animals.

Fermentation of soybean is one of the techniques resulting in novel foods (i.e., fermented soybeans) with unique features. Other advantages include an extension of the product's shelf life. Fermented soybean is a traditional and popularly consumed food in many parts of the world, especially in Asia. The best-known examples are Japanese *natto*, the Korean *chongkukjiang*, Indian *kinema*, Thai *thua nao,* and the *tempeh* from Indonesia. During the fermentation of soybean, the steamed and/or boiled soybeans are sprayed with an inoculum solution of *Bacillus subtilis* or *Rhizopus oligosporus*, and incubated at 30°C–40°C for 24–48 h.

During the course of soybean domestication, the Chinese gradually discovered that it was possible to transform soybeans into various forms of soy foods, such as tofu, soy sauce, soy paste, and soy sprouts, making soy-based foods more versatile, more flavorful, and more digestible.

Soy foods including tofu and soymilk have traditionally been the daily foods in many parts of Asia and their consumption in Western countries has been increasing due to their potential health benefits.

Tofu, a fundamental part of Asian food culture, is a traditional oriental soybean food composed principally of protein and lipid. Currently, tofu is one of the most popular soy-based foods, and the relatively high concentrations of bioactive compounds existing in soybeans, such as isoflavones, add to the claim of soy as a healthful food (Lee et al., 2003). This highly nutritious plant-protein based food can be used in soups, salads, pastries, sandwiches, and so forth. It is easy to digest and it is substituted for meat, cheese, and certain other dairy products for dairy-sensitive individuals, vegans, and the elderly. Based on the coagulant type, tofu can be a good source of calcium added to its inherent B vitamins, isoflavones, minerals, fiber, and unsaturated fat content (Rollins and Joseph, 2000). The history of tofu can be traced back to the Han dynasty 2000 years ago, and the two traditional types of tofu include northern and southern tofu, which were named after the places where they are popular in China.

The manufacturing process of tofu is complex—involving specific stages (Figure 2.1). The first step is to obtain soymilk, which is also a product for final consumption. The soymilk is then heated

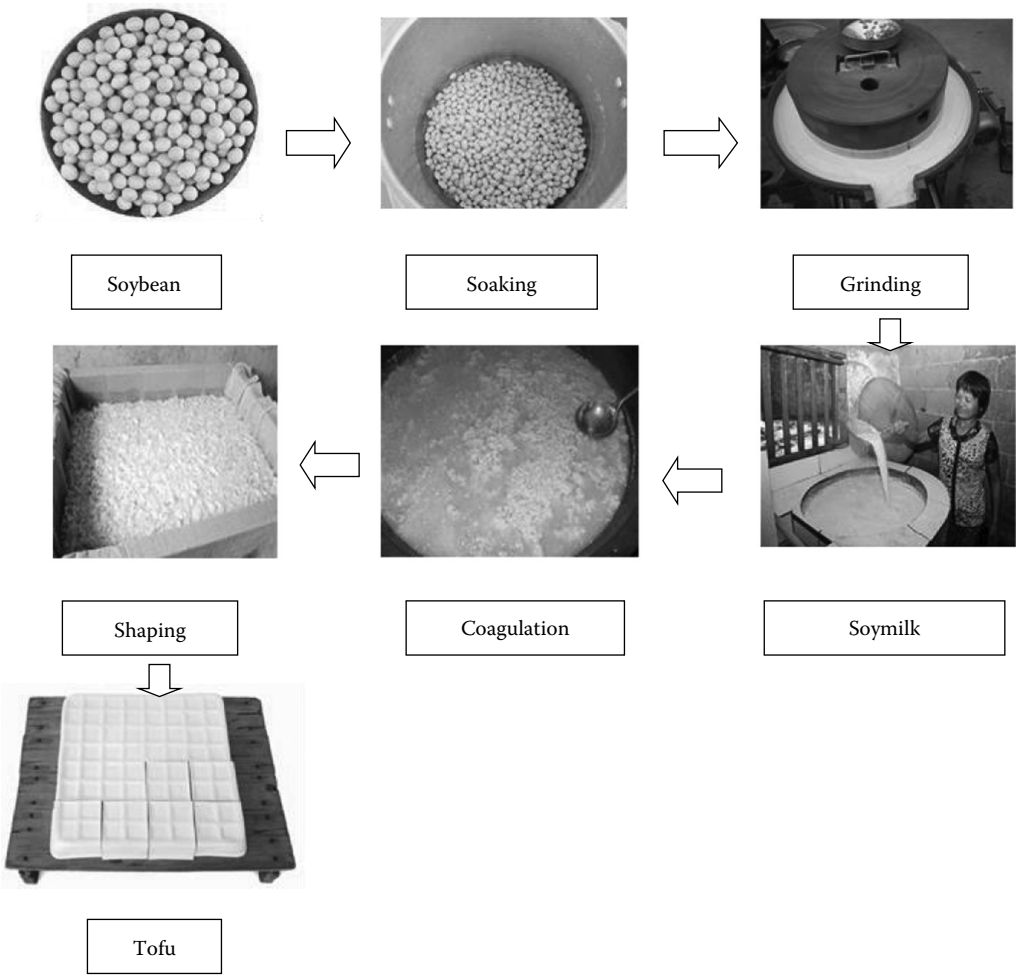

FIGURE 2.1 Traditional manufacturing process of tofu.

up to 90°C for more than 10 min. For filled tofu, coagulant was added to cooled soybean milk, followed by heating without mixing to initiate and finish the coagulation of the proteins to form into curd in the package (Liu et al. 2010). Soymilk coagulation is the most critical step for obtaining good tofu texture and yield. The coagulants used can be of slow action ($CaSO_4$ and GDL or glucono-δ-lactone) and quick action ($CaCl_2$, $MgCl_2$, and $MgSO_4$) and result in silken, filled, and firm (momen) tofu, respectively.

Thermal treatment is typically used for making tofu to dissociate, denature, and aggregate the soy protein; inhibit the microbial growth; reduce the beany flavor; and inactivate undesirable biological compounds such as trypsin inhibitors and lipoxygenase (Kumar et al., 2003). The main difference between northern and southern tofu of China is the type of coagulant used for their processing; the former is induced by magnesium chloride and the latter by calcium sulfate. Despite wide consumption of tofu by Chinese, processing of tofu in China remains largely dependent on workers' experience. As tofu is a gel-like protein matrix containing a high amount of water, a rapid method must be established to study the dynamic states of water and the morphology of tofu to improve its production. It is generally known that tofu is susceptible to microbial spoilage because of its high nutrient and moisture content (>75%), and its relatively neutral pH (5.8–6.2)

(Champagne et al., 1991; Kim et al., 2003). Thus, tofu has a very short shelf life, and it is usually consumed freshly. The growth of contaminated spoilage microorganisms, which leads to the spoilage of the product, has become a great barrier for producer during production, transportation, and preservation.

As a consequence, research on the prevalence, characterization, evolution, and interactions of different microorganisms during tofu processing and storage has been intensified in recent years. Research has shown that from the great variety of microbial contaminants on soybean, only a fraction develops and eventually dominates the so-called spoilage association, especially the spores-forming *Bacillus* spp. This selection depends on the combined effect of intrinsic, extrinsic, and processing factors affecting microbial survival, growth, and competition in tofu. They include, among others, the heat, pH, composition and texture of raw or processed tofu, and mainly the storage temperature and the packaging atmosphere.

During food process, the thermal treatment delays spoilage of perishable foods, such as soymilk, by killing the cells of microorganisms, while packaging acts as a barrier to protect foods from microorganisms, and physical or chemical changes in their surrounding environment. Recent advances in the development of modified atmosphere packaging technologies have led to food products of increased shelf life, and to the evolution of fresh and minimally processed food preservation techniques. However, in the typical tofu making process, the heat-resistant microorganisms such as *Bacillus* sp. spores can survive after the thermal treatment, which is the main source of spoilage organisms of the tofu products (Wang et al., 2006).

This chapter aims at (1) providing an overview of current knowledge on the microbial ecology of fresh soymilk and tofu products, and the range of microorganisms involved in different types of spoilage; and (2) emphasizing the sources of spoilage microorganisms, and methods of detection and evaluation for spoilage microorganisms.

2.2 TAXONOMY OF SPOILAGE MICROORGANISMS IN TOFU

The range of spoilage microorganisms is wide. The increasing variety of consumer products together with the need to preserve flavor and texture throughout minimum processing means that the susceptibility to spoilage has increased, as has the diversity of spoilage species.

A large number of bacteria have been implicated as contributing spoilers of tofu, and there have been a few reports on their successive spoilage patterns and the contaminants associated in the production process. The bacterial colonizers include *Bacillus* sp., *Pseudomonas* sp., *Enterobacter* sp., and *Enterococcus* sp. (Fouad and Hegeman, 1993; Dong et al., 2003; Wang et al., 2006; Stoops et al., 2011). (Table 2.1).

Among the aforementioned bacteria, the *Bacillus* sp. and *Pseudomonas* sp. are the main spoilage bacteria of the tofu. Heat-resistant microorganisms such as *B. subtilis* spores derived from soil may exist in soybean milk and can reduce the shelf life of tofu produced from conventional processing of soybean milk. It is worth noting that there are several opportunistic pathogens in the spoilage bacteria of tofu, such as the Gram-positive bacteria *Bacillus cereus*. *B. cereus* is a facultative anaerobic and spore-forming bacterium, and is widely distributed in the environment due to its ability to resist hostile conditions (Carlin et al., 2010). *B. cereus* is a common food contaminant, it can be found in different types of raw food such as rice, meat, vegetables, raw milk, dairy products, as well as cooked dishes (Eglezos et al., 2010; Fangio et al., 2010; Altayar et al., 2006; Ankolekar et al., 2009; Chang et al., 2011; Samapundo et al., 2011). The presence of *B. cereus* in food is usually associated with food spoilage as well as food poisoning that usually occur in two types of illness.

Pseudomonas sp. were isolated from the endogenous microflora of tofu, which was capable of growing in atmospheres containing no or limited amounts of oxygen and increased amounts of carbon dioxide (Stoops et al., 2011). *Pseudomonas* sp. belong to the most important spoilage organisms in this product. Also in aerobically stored meat, fish, eggs, milk, and vegetables, *Pseudomonas* sp. represent the dominant spoilage flora (Masson et al., 2002). Members of the genus *Pseudomonas*

TABLE 2.1
Summary of Main Spoilage Microorganisms Isolated from Soybean Post-Harvest, Soybean Products

Gram-Positive Bacteria	Reference	Gram-Negative Bacteria	Reference
Bacillus subtilis	Zhao et al. (2011); Ou et al. (2012); Guan et al. (2013); Yuan et al. (2014)	*Pseudomonas aeruginosa*	Tuitemwong et al. (1991); Guan et al. (2013)
Bacillus cereus	Nout et al. (1987); Zhou et al. (2014); Peng et al. (2015)	*Pseudomonas putida*	Tuitemwong et al. (1991)
Bacillus pumilus	Wang et al. (2006)	Enterobacter agglomerans	Tuitemwong et al. (1991); Wang et al. (2006)
Bacillus firmus	Li et al. (2001)	*Enterobacter cloacae*	Tuitemwong et al. (1991)
Bacillus megaterium	Wang et al. (2006)	*Enterobacter aerogenes*	Liu et al. (2015)
Lactobacillus sp.	Tuitemwong et al. (1991); Yang et al. (2014)	–	–
Leuconostoc sp.	Yang et al. (2014)	Yeast	–
Enterococcus sp.	Tuitemwong et al. (1991); Li et al. (2001)	*Pichia membranaefacien*	Zhao et al. (2011)
Pediococcus sp.	Tuitemwong et al. (1991); Ou et al. (2012)	*Pichia guilliermondii*	Ou et al. (2012)
Fungi	–	*Trichosporon cutaneum*	Ou et al. (2012)
Penicillium cyclopium	Ou et al. (2012)	–	–
Penicillium toxicarum Miyake	Ou et al. (2012)	–	–

(sensu stricto) are fluorescent, motile, and nutritionally versatile. They can be found abundantly in soils, waters, and many other habitats. *Pseudomonas* sp. are globally active in aerobic decomposition and biodegradation, and hence play a key role in balancing nature and in the economy of human affairs. The genus *Pseudomonas* sp. consist of five phylogenetic groups based on rRNA similarity studies (Palleroni, 1993).

2.3 SPOILAGE MICROORGANISMS CHARACTERISTICS AND POSSIBLE MECHANISMS

Soil is a direct source of contamination into soybean products. Soil is regarded as a major habitat of spore-forming bacteria. The major spore-forming bacteria, such as *B. subtilis*, have been detected worldwide in soil samples. Sporulation of endospore-forming bacteria has also been demonstrated in a variety of environments other than soil and its multiple components. Soybean might be contaminated by *B. subtilis* from harvest in the farm.

The quality of any food and its shelf life are mostly dependent on the production processes and conditions as well as its chemical (nutritional) composition and associated microflora (Dainty, 1971). During processing, tofu can be easily contaminated by microorganisms in the stage of storage, packaging, transportation, and sales process. After soaking the soybean, the water, the workers' hand, the air, and the packing bag also can cause the microorganism pollution (Mulyowidarso et al., 1989; Pirttijärvi et al., 1996). Different production processes and facilities cause the number and variety of contaminated microorganisms. Also the successful colonization of a food material by an initial microbial population or community, subsequent development of several and climax communities that leads to the ultimate alteration of food quality is determined by several intrinsic and extrinsic

factors including food type, nutritional composition, pH, water activity, storage temperature, and possession of suitable enzymes (Hueck, 1968; Tucker, 2007). Among these, temperature is considered the most important factor (Dotsom et al., 1977). Severe thermal stress is a widely employed treatment to inactivate bacteria, in particular bacterial spores. The latter are survival structures of *Bacilli* and *Clostridia* with a high to very high resistance to heat stress. In particular, certain food spoilage *B. subtilis* isolates are described as producing spores of extreme high thermotolerance.

The spores are common in many ingredients and thus form a significant burden to the manufacturing process for instance, dairy-based products, composed food products such as soups and sauces as well as ready-to-eat meals in the fresh chain. Wild-type isolates of *B. subtilis* as well as various strains from *Bacillus sporothermodurans* are causative agents of many cases of food spoilage in which products containing herbs, spices, milk powder, and other dry ingredients of manufactured food are involved. The general observation is that the heat resistance of spores isolated from spoilage isolates is higher than that observed for laboratory strains. In the manufacturing process of tofu, the soymilk is heated up to 90°C for more than 10 min, which cannot efficiently inactivate the spores of *Bacillus* sp. Furthermore, it is well known that sporulation conditions of *Bacilli* may even further enhance the thermal resistance of their spores.

Pathogenic microorganisms are the major safety concern for the food industry. As they are generally undetectable by the unaided human senses and capable of rapid growth under favorable storage conditions, much time and effort have been spent in controlling and/or eliminating them. As for an opportunistic pathogen in the spore-forming bacteria, *B. cereus* is a large (1.0–1.2 μM by 3.0–5.0 μm) Gram-positive rod-shaped bacterium, which grows on common agar media to large colonies (3–8 mm diameter) with a rather flat. *B. cereus* was originally described as a mesophilic organism, growing between 10°C and 50°C and with an optimum temperature between 35°C and 40°C (Johnson, 1984; Claus and Berkeley, 1986). On blood agar, the colonies are surrounded by zones of β-hemolysis (Kramer and Gilbert, 1989), the size of which is often large, but can vary depending on culturing conditions. Most strains will form endospores within a few days on commonly used agar media. *B. cereus* spores are ellipsoidal, centrally or paracentrally placed, and do not distend the cell (Gilbert and Kramer, 1986). Employing phase-contrast microscopy or spore-staining techniques, the placement and morphology of the spores are much used criteria to distinguish the species of the genus *Bacillus* (Fritze, 2002). Other commonly used features for identification are motility, hemolysis, carbohydrate fermentation (*B. cereus* does not ferment mannitol), and the very active lecithinase (phospholipase) production (Johnson, 1984). Various plating media are used for the isolation, detection, and enumeration of *B. cereus* from foods, including MYP (mannitol-egg yolk-phenol red-polymyxin-agar) and PEMBA (polymyxinpyruvate-egg yolk-mannitol-bromthymol blue-agar). In addition to selective compounds like polymyxin, these media utilize the bacterium's lecithinase production (egg-yolk reaction giving precipitate zones) and lack of mannitol fermentation. A thorough description of these media is found in Kramer and Gilbert (1989).

There is no doubt that microbiological activity is by far the most important factor influencing the changes that cause spoilage in tofu. However, it should be clarified that, it is the microbial activity per se, rather than the activity of microbial enzymes and as a consequence, it is the accumulation of metabolic by-products that characterizes food spoilage. These deteriogens no doubt have had their colonizing and degrading potential enhanced by the possession and action of various extracellular enzymes (Fapohunda and Olajuyigbe, 2006), although there is a paucity of information as regarding the screening of tofu deteriogens for extracellular enzymes.

Pseudomonas members are adapted to various conditions and therefore are found in a wide range of niches as soil, water, plants, animal tissues, foods, and so forth (Franzetti and Scarpellini, 2007) thanks to their complex enzymatic systems. It is important to notice (1) that many enzymes produced by *Pseudomonas* members, in particular the proteases, are heat-resistant; and (2) that results in consolidation of activity after heat treatment processes are used to eradicate microorganisms in certain matrices. The mechanisms of these spoilage processes are not completely known but are strain dependent and related to temperature and environmental conditions (Chabeaud et al., 2001;

Woods et al., 2001; Nicodème et al., 2005). However, it is known that an important protease responsible for spoilage is an extracellular alkaline metallo-protease belonging to the AprX protein family, which has been extensively studied especially in *Pseudomonas aeruginosa* (Duong et al., 2001; Blevesa et al., 2010).

2.4 DETECTION AND EVALUATION METHODS FOR SPOILAGE MICROORGANISMS

B. cereus is increasingly recognized as the etiological agent of foodborne diseases especially, foodborne outbreaks caused by the emetic toxin cereulide, including ones with fatal outcomes. Thus, *B. cereus* is a special challenge for public health and for the food industry. Conventional detection methods for *B. cereus* generally relies on selective medium and serological studies, which may include homogenization, enrichment, enumeration, antibiotic susceptibility, and toxicity studies of flagellar antigens have been used extensively (Drobniewski, 1993). Nevertheless, these are all time-consuming and laborious processes.

More recently, newer molecular methods based on DNA hybridization and polymerase chain reaction (PCR) have been proposed for the rapid and sensitive detection of *Bacillus* spp. The current information on the 16S rRNA genes as well as forthcoming genotyping data of food spoilage spore formers is relevant. The tools have also been translated to probes that can detect single-nucleotide polymorphisms using the oligonucleotide ligation amplification technology. Thus, primers targeted to the 16S rRNA gene have been used to identify *Bacillus* spp. by conventional PCR (Wattiau et al., 2001). This technique has also been combined with amplified rDNA restriction analysis (ARDRA) for identification purposes (Wu et al., 2006). More recently, a large number of real-time polymerase chain reaction (RTi-PCR) procedures are currently available for the specific detection and quantification of foodborne bacteria. Fernández-No et al. (2011) developed a new primer and probe set targeted to 16S rRNA sequences for detecting the presence and determining the concentration of *B. cereus*, *B. licheniformis*, and *B. subtilis* by RTi-PCR. And this method allows the microbiological control of such pathogenic and spoilage sporoformers not only in fresh but also in pasteurized food products (Fernández-No et al., 2011).

PCR is a rapid and sensitive technique for pathogenic bacterium detection and identification in food (Wang et al., 2009). Several molecular methods have been developed for the detection of *B. cereus* (Martínez-Blanch et al., 2009; Fernández-No et al., 2011; Dzieciol et al., 2013). These tools either targeted a limited number of toxin producing genes or species-specific genes. Forghani developed a highly sensitive pentaplex RTi-PCR high-resolution melt curve assay for simultaneous detection of four major enterotoxin genes (*cytK*, *entFM*, *hblD*, *nheA*) and emetic toxin gene (*ces*). The detection limit in food samples was approximately 10^3 CFU/g without enrichment and 10^1 CFU/g was observed following 7 h enrichment. Thus, the developed multiplex RTi-PCR approach can be a reliable tool for the identification of emetic and enterotoxic strains of *B. cereus* present in food and food-related samples (Forghani et al., 2016). The PCR method also has the ability to discriminate between *B. anthracis* and other *Bacillus* species. However, the relative lengthy time in analysis for PCR method makes it difficult for situations requiring quick identification of the bacterial spores. There is an increasing need to develop rapid and sensitive methods for the detection of the bacterial spores.

Conventional PCR cannot distinguish viable from dead cells (Wang and Levin, 2006). More recently, ethidium monoazide (EMA) and propidium monoazide (PMA), which eliminate positive signals from dead bacteria have been developed. Recent reports confirmed that EMA can also penetrate the membranes of viable cells of some bacterial species and induced degradation of a portion of the genomic DNA (Chang et al., 2009; Clayton et al., 1992; Nocker et al., 2006). Therefore, PMA was selected in combination with multiplex PCR (mPCR) for the specific detection of emetic and nonemetic *B. cereus* (Zhang et al., 2014; Forghani et al., 2015), and this novel PMA–mPCR–IAC (internal amplification control) assay is rapid and reliable, providing an efficient diagnostic tool with promising application in monitoring food samples.

Identification of *P. aeruginosa* in the clinical laboratory is generally performed by growing the bacteria on either cetrimide agar or nalidixic acid–cetrimide (NAC) agar. Although this method is reliable, the time required for performing it is up to 48 h. PCR methods, which allow for more rapid identification of *P. aeruginosa* by DNA amplification, have been reported (Spilker et al., 2004; Xu et al., 2004; Lavenir et al., 2007). However, the amplification specificity of PCR is highly influenced by the primer design and reaction conditions, and the gel electrophoretic analysis of the DNA product is required for confirming DNA amplification.

Loop-mediated isothermal amplification (LAMP), auto-cycling, and strand displacement DNA synthesis have been reported as possible replacements for PCR, and used for the detection of specific gene sequences (Notomi et al., 2000). The LAMP reaction is carried out with a set of four oligonucleotide primers, which recognize six distinct regions on the target DNA, at a constant temperature ranging from 60°C to 65°C (Nagamine et al., 2002). The LAMP assay showed 100% specificity for the serogroup and other bacteria, and the sensitivity was tenfold higher than the PCR assays, and the assay was completed within 2 h from DNA extraction (Goto et al., 2010).

Although these methods are fast, highly specific, and sensitive, the application of molecular-based techniques in the control of food safety is still relatively limited as they suffer from some serious drawbacks. The development of methods can be uniformly applied is particularly hampered by the fact that all different produce and food products contain their own interfering components. The development of such methods becomes even more difficult due to a constant introduction of new food matrices. While molecular methods are very sensitive, low DNA copy numbers are difficult to detect when the sample size is very small. Introduction of an enrichment step preceding DNA detection is a solution, but this makes results qualitative, rather than quantitative. Sample preparation needs close attention. Preferably, such sampling needs to be rapid and as homogeneous as possible. Innovative strategies focus on the use of magnetic beads coated with cell-recognizing molecules, on physical methods such as floatation, and on lysis of whole food matrices (Wagner and Dahl, 2008).

Among different methods for detecting bacterial spores, spectroscopic methods have been increasingly demonstrated as powerful tools for rapid and sensitive chemical or biological detection, especially with the use of metal nanoparticles as detection probe. For example, the use of DNA-anchored gold nanoparticles has been demonstrated for colorimetric detection of the DNA from the infecting organism *B. anthracis* (Park et al., 2002). Because of nanoparticle-based enhancement effect on Raman scattering intensity, surface-enhanced Raman scattering (SERS) technique has been widely utilized for the detection of biomolecules including proteins, DNAs, and bacteria. Cheng described SERS detection of *B. subtilis* spores by using gold nanoparticle (Au NP)–based substrates as the spectroscopic probe. The SERS substrates are shown to be highly sensitive for the detection of *B. subtilis* spores, which release calcium dipicolinate (CADPA) as a biomarker (Cheng et al., 2011).

2.5 CONTROL, PREVENTION, AND MANAGEMENT OPTIONS FOR SPOILAGE MICROORGANISMS

Spoilage organisms either can be naturally present or can gain access to food. While not a food safety concern, increased levels of spoilage organisms will usually mean a reduction in the length of time that the food remains fit to eat. This can affect product quality and so also influence the consumer's perception of the product.

Spore-former species causing food spoilage have major financial consequences in the food industry, therefore, control of spores is one of the main problems in food preservation and complete inactivation is often impossible without affecting food quality and structure. Spore-forming bacteria belonging to the genus *Bacillus* are widespread in the environment and can be isolated from a large variety of foods and ingredients used for food production. The spores can survive a wide range of treatments including wet and dry heat, high hydrostatic pressure, desiccation, ultraviolet (UV) and γ-radiation, and antimicrobial compounds rapidly kill vegetative the cells. The general observation

is that the heat resistance of spores isolated from spoilage isolates is higher than that observed for laboratory strains. Thus, the inactivation of spores is an important step in food processing.

Therefore, a more intense treatment in terms of higher temperatures and longer temperature-holding times is required to guarantee the inactivation and the microbial safety. However, the high heat load causes a quality loss of the food regarding the functional, nutritional, and sensory value, such as color and taste.

The ohmic heating method is older than microwave heating and was reported to inactivate microorganisms in milk in 1919 (Anderson and Finkestein, 1919). Today, ohmic heating using a frequency of around 20 kHz has been used to process fish cake since 1990 because of the increased stability and increased energy efficiency. For a long time, it was believed that microorganisms in food were inactivated by the electrical effects of ohmic heating. Uemura et al. (2010) developed radio-frequency flash heating (RF-FH) to soybean milk through a Teflon film to process *B. subtilis* spores in milk and examined the effectiveness of inactivation (Uemura et al., 2010). The results showed that RF-FH processing reduced *B. subtilis* spores in soybean milk by four-logarithmic orders at an outlet temperature of 115°C. Tofu made from RF-FH–treated soybean milk had a higher breaking strength than tofu made from conventionally heated soybean milk.

Infrared radiation (IR) is easily absorbed by water and organic materials, which are the main components of food, and has been widely applied to the heating and drying of food (Van Zuilichem et al., 1986). The application of IR radiative heating during thermal processing is expected to improve the hygiene of working environments by eliminating the need for a heating medium. IR heating is known to increase the shelf life of foodstuffs (Muramatsu et al., 1989; Rosenthal et al., 1996). Compared with thermal conductive heating, far-infrared (FIR, 3–1000 μm) heating (under identical bacterial suspension bulk temperature) was more effective for inactivating the vegetative bacteria cells. Moreover, FIR irradiation caused heat activation and death of *B. subtilis* spores over a temperature range in which thermal conductive heating had no effect on spore viability (Sawai et al., 1997; Hamanaka et al., 2003). FIR irradiation can be an effective and easy method for the promotion of rapid germination at low temperatures. Therefore, this technology is widely applicable for the effective sterilization of spores on food surfaces, as well as on the surface of polymer materials with low heat resistance (Sawai et al., 2009).

Pulsed light (PL) consists of a successive repetition of short duration and high-power flashes of broadband emission light (190–1000 nm) with approximately 40% of the emitted light corresponding to the UV region. This technology appears as a promising alternative to conventional heat preservation processes to ensure the microbial quality and safety of food products. It has been shown to be effective in inactivating a wide range of microorganisms involved in food spoilage and foodborne pathogens including bacteria, fungi, viruses, and protozoa (Elmnasser et al., 2010; Gómez-López et al., 2005; Huffman et al., 2000). However, one of the main challenges for the application of nonthermal technologies in the food industry is the inactivation of bacterial spores. The inactivation effectiveness of PL would not only depend on the physiological state of the cells, but also on their exposure to the incident light, which could be influenced by cell population density among other factors. Future research should be conducted to investigate the behavior of bacterial spores in complex food systems, in particular in fluids with limited light transmittance, to determine the effectiveness of PL for the inactivation of these microbial forms at cell densities currently found in liquid food products (Artíguez and de Marañón, 2015a). Exposure to sublethal treatments of PL sensitizes *B. subtilis* spores to subsequent thermal treatments, and was the most effective combination in reducing *B. subtilis* counts (Artíguez and de Marañón, 2015b).

High-pressure (HP) treatment is an emerging mild food preservation technique that is increasingly finding its way into industrial processes. In its most common application, it is based on the exposure of the food to an extremely high pressure (200–600 MPa) at ambient or moderately reduced temperature (0°C–25°C). This treatment inactivates vegetative bacteria, fungi, and viruses as well as most enzymes, and thereby increases the safety and extends the shelf life of the food (Considine et al., 2008).

It is believed that pressures well above 1000 MPa may be needed to inactivate dormant bacterial spores, because the dehydrated state of the spore core protects vital spore proteins and membranes from HP denaturation or disruption. Nevertheless, despite this extreme resistance, a certain degree of inactivation can be achieved by HP treatment for most types of spores because HP treatment induces spore germination and, once germinated, the spores lose their resistance to HP and heat. Spores of *Bacillus* species can initiate and go through the process of germination under relatively low pressure (i.e., 100–150 MPa) (Black et al., 2005). Treatments of 150 MPa for 7 min at 37°C (Black et al., 2005) or 100 MPa for 30 min at 40°C (Wuytack and Michiels, 2001) are sufficient to trigger germination of ~90% of the spore population without further incubation. Several studies suggest that pressure inactivates bacterial spores by a three-step model of inactivation, which involves a germination step followed by an inactivation step that compromises the spore's inner membrane (Mathys et al., 2007, 2009).

A more recent development in HP processing is the use of elevated process temperatures (high-pressure-and-high-temperature [HPHT]), as this was found to allow more efficient spore inactivation. Several studies have demonstrated effective inactivation of different spore types. The spores of *B. cereu*s can be efficiently inactivated at lower temperature and/or shorter time by HPHT treatment than by HT treatment (Luu-Thi et al., 2014).

Previous studies have been carried out to develop different protocols for the preservation period extension of tofu. Physical methods include low-temperature storage, high-pressure processing (Préstamo et al., 2000), and microwave treatments (Wu and Salunkhe, 1977). Currently, biopreservation has substantially attracted attentions to improve the shelf life and the safety of food. The studies and applications of bacteriocinogenic lactic acid bacteria (LAB) and their antimicrobial metabolites have received a huge interest in recent years. Chen et al. (2014) had developed an innovative tofu, which is coagulated by both acid and enzyme together through the using of bacteriocinogenic *Weissella hellenica* D1501 combined with microbial transglutaminase (MTGase). Compared with tofu prepared with traditional coagulants, *W. hellenica* D1501 in LAB could significantly suppress the microbial spoilage and extend the shelf life based on microbiological analyses (Chen et al., 2014).

Strategy to control contamination into foods must include good hygienic practices. The hygienic demands on food factories, often led by product labelling, brand protection, and food safety requirements, continue to grow and are leading to new design and refurbishment considerations. In the process of tofu, an important source of pollution is the water. Food factories must have an adequate supply of potable (hot and cold) water, which is to be used whenever necessary to ensure foodstuffs are not contaminated. Where appropriate, facilities for water storage, distribution, and temperature control shall be adequately designed and constructed, shall be covered, and shall have air vents that are insect and rodent proof.

In the process of tofu, hand washing with both soap and water, which act as emulsifying agents to solubilize grease and oils on the hands, will remove transient bacteria. Increased friction through rubbing the hands together or using a scrubbing brush reduces the number of both transient and resident bacteria.

2.6 CONCLUSION AND FUTURE TRENDS

Until now, many studies have reported the isolation and identification of spoilage bacteria in the process of tofu production. However, there is a lack of in-depth research on the source and growth characteristics of the spoilage microorganism. Risk analysis has emerged over the past decade as the internationally recognized framework for improving food control systems with the objectives of producing safer food, reducing the number of foodborne illnesses, and facilitating the international trade of foods. More and more research will strengthen the analysis of the risk in tofu processing, especially the microbial risk.

Bacterial spoilage continues to be the most limiting factor in extending the shelf life of tofu products. Control and elimination of typical spores-forming *Bacillus* spp. contaminants in the processing environment have led to product's shelf life improvement. In addition to inactivating

microorganisms, high-pressure treatment offers the advantage of only minimally affecting flavor characteristics. The spores of *Bacillus* spp. can be efficiently inactivated at lower temperature and/or shorter time by HPHT treatment. However, there is no further study in expanding the scope of application of that technology. As an enterprise, the production process of tofu should be standardized, and should strengthen the management of water, facility, and personal hygiene of employees.

Predictive (food) microbiology is a key area of food microbiology where the behavior of microorganisms is quantitatively described at the level of cell growth, lag-time and cell death with the aid of mathematical models. The ultimate goal of predictive food microbiology is to help the food technologist choose upfront the most appropriate combination of stress conditions to come to a stable product formulation. The final key to success in such cases is the need to have failsafe models, which are refractive to slight model parameter variation and are valid at low numbers of starting microbes. Many of the models relevant to food microbiology look at cellular behavior under a continuous preservative or temperature stress. The growth model of spoilage microorganisms in tofu processing should be established.

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3 Spoilage Microorganisms in Fruit Products

Liang Gong, Yueming Jiang, and Xuewu Duan

CONTENTS

3.1 INTRODUCTION

Fresh fruits are indispensable food for people's daily life, which are important sources of nutrients, vitamins, and fiber for humans. The consumption of fruits increased to an average of 4.5% yearly between 1990 and 2004 (EU, 2007). However, due to poor storage and transportation conditions, it is estimated that the annual loss of fruits is more than 15%. Especially in developing countries the losses are well over the figure because of the lacking facilities and incorrect technologies for fresh-keeping and preservative storage (FAO, 2011). Fruits with high water content and nutrients are favorable for the development of plant pathogens and foodborne pathogens, which are the major reasons for the losses during the supply chain, and even cause hazards for humans and other animals (Blackburn, 2006). The spoilage microorganisms of postharvest fruits, including virus, fungi, and bacteria, are generally taken along from preharvest and developed under favorable conditions after harvest. Of the microorganisms, fungi not only lead to postharvest decay, but also produce mycotoxins that can cause disease and death in humans and animals (Marin et al., 2013). In this chapter, we will describe the characteristics of pathogens in fruits to develop decay-control measurements and maintain the quality of fruits, as well as to reduce the postharvest losses.

Currently, it is well established that the control of postharvest disease for fresh horticulture produces involves cultural practice and fungicide application. Synthetic fungicides are widely used for controlling postharvest diseases. However, the long-time use of chemical synthetic fungicides results in the residues that affect human health and environment, as well as the possible development of resistant-pathogens (Berger and von Holst, 2001). Hence, scientists focus on alternative control strategies, via integrated application of physical and biological methods to protect fruits against postharvest disease, such as controlled atmosphere and antagonistic yeasts (Liu et al., 2013; Pareek et al., 2014). In addition, inorganic fungicides, such as sodium hypochlorite (NaClO), ozone (O_3), and chlorine dioxide, are widely used as disinfectant for cleaning pathogens on the surface of fruits (Fukuzaki, 2006).

Biological control is an environmentally friendly means to reduce plant disease by using natural antagonists or metabolites. Natural microbial antagonistic yeasts and bacteria that exist on fruit surfaces could suppress *Penicillium* disease development (Wilson and Chalutz, 1989). Recently, the use of an antagonistic yeast, *Candida diversa*, combined with harpin treatment is very efficient in preventing infection of *Botrytis cinerea* or *Penicillium expansum* in kiwifruit fruit (Tang et al., 2015). Essential oils (EOs), aromatic oily liquids derived from plant materials, have showed high antimicrobial activity against plant pathogens (Antunes et al., 2012; Gong et al., 2016). Moreover, EOs could provide a possible alternative used in food industry, instead of chemical-based preservatives. Studies have demonstrated that plant extracts with diverse bioactive components can efficiently control foodborne pathogens, such as *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, and *Shigella dysenteriae* (Burt, 2004). EOs are accord with a popular concept of "green" consumerism, which desire fewer chemical synthetic food additives and products with a smaller impact on environment (Prakash et al., 2015).

Although there is little knowledge on the mechanism of plant pathogen being given pathogenicity on fruits, plant pathogens secrete effector molecules that are generally considered to be the key factors for the host colonization (Rovenich et al., 2014). The effector molecules were defined as all pathogen proteins and small molecules that have the ability to change host cell structure and function (Hogenhout and Bos, 2011). However, it was difficult to identify the pathogen effector molecules before the development of sequencing technology. Recently, the development of dual-transcriptome technology opened a door to analyze simultaneously the occurrence and expression of genes from fruit and their pathogens. There are two breakthrough studies on the interaction of pathogen with fruits. Alkan et al. (2015) conducted dual-transcriptome analysis of *Colletotrichum gloeosporioides* in tomato during infecting course and found that pathogenicity factors, such as pectate lyase and polygalacturonase, are upregulated. In another study, a slice of virulence factors in *P. expansum* are identified by means of a transcriptomic analysis of infected apple fruit (Ballester et al., 2015). In addition, mycotoxins are poisonous secondary metabolites produced by fungi, which is considered in relation to pathogenicity of plant pathogen. There is evidence that mutation or downregulation of genes or their regulators involved in biological synthesis of mycotoxins, such as trichothecenes affected pathogenicity in *Fusarium graminearum* (Geng et al., 2014). However, it is a controversial issue as knockout mutants clearly demonstrated that neither patulin nor citrinin are required by *P. expansum* to successfully colonize in apples (Ballester et al., 2015). Anyway, mycotoxins cause disease and death in humans and animals, which raise an issue of food safety, so the detection and characterization of mycotoxins and their producing pathogens in the food chain have been largely reviewed (Gong et al., 2015).

In this chapter, an overview is provided for the detection, classification, and characteristics of fruit pathogenic fungi and foodborne pathogens, including the spoilage microbial species, current spoilage hazards status, evaluation techniques, management systems, prevention tools, and related mechanisms in the fruit product.

3.2 TAXONOMY OF SPOILAGE MICROORGANISMS IN FRUIT

Postharvest decays of fruit caused majorly by fungi and bacteria, for example, citrus fruits are suffered from green and blue mildew caused, respectively, by *Penicillium digitatum* and *Penicillium italicum*, followed with sour rot caused by *Geotrichum citri-aurantii* (Caccioni et al., 1998; Palou et al., 2001). There is another very important bacterial disease named huanglongbing, caused by *Candidatus liberibacter* a genus of Gram-negative bacteria in the rhizobiaceae family (Teixeira et al., 2005). Banana anthracnose, caused by *Colletorichum musae* is a major postharvest disease worldwide, which is in preference to infect ripe bananas and represent the symptoms of black and sunken lesions (Zakaria et al., 2009). Banana *Xanthomonas* wilt, caused by the bacterium *Xanthomonas campestris* pv. *musacearum*, was considered to be one of the greatest threats to banana productivity, infecting not only banana fruit, but also banana stem and leaf

(Tripathi et al., 2009). Fruit diseases can originate from latent infections during the proharvest stage in the orchard. They are commonly named according to the apparent symptoms, such as black rot caused by *Botryosphaeria obtusa* or *Physalospora cydoniae* in apple and pear, brown rot caused by *Monilinia fructicola,* in plums and peaches, anthracnose caused by *Neonectria galligena* in apple, and powdery mildew caused by *Erysiphe necator* in grape. Each fruit has some special pathogens, which cause disease in this fruit, but maybe not in others. For example, stored tomato suffered from the common diseases including gray mold, caused by *B. cinerea* and *Boreyotina fuckeliana*; soft rot, caused by *Rhizopus stolonifer*, as well as black spot, caused by *Alternaria alternata* and *Stemphylium botryosum* (Chen et al., 2008; Nowicki et al., 2012). Pitahaya is a cactus fruit crop with high economic value in national and international level, and it is cultivated in more than ten countries worldwide. The causal agent of the stem spots in Pitahaya is identified as fungus species, *Botryosphaeria dothidea*, and anthracnose has become an aggressive fungal disease for fruit and stem of Pitahaya, which is caused by a fungus *Colletotrichum gloesporioides* (Valencia-Botín et al., 2013). As shown in Table 3.1, the main postharvest diseases from fruits are listed, which are identified basically according to morphology and structure of hyphae and spores of these organisms.

TABLE 3.1
Main Postharvest Diseases from Fruits

Fruit	Name of the Disease	Pathogens
	Fungal diseases	
Litchi	Downy blight	*Peronphythora litchii* Chen ex Ko et al.
	Sour rot	*Geotrichum candidum* Link.
	Anthracnose	*Colletotrichum gloeosporioides* Penz.
		Glomerella cingulata (Stonem.) Schr. Et spauld.
		Colletotrichum acutatum Simmonds
	Fungal diseases	
Longan	Sour rot	*G. candidum* L. K. ex Pers.
	Downy blight	*P. litchii* Chen ex Ko et al.
	Anthracnose	*C. gloeosporioides* Penz.
		G. cingulata (Stonem.) Schr. et spauld.
	Fungal diseases	
Mango	Anthracnose	*C. gloeosporioides* Penz.
		G. cingulata (Stonem.) Spauld. et Schrenk.
		C. acutatum Simmonds
	Stem-end rot	*Botryodiplodia theobromae* Pat.
		Variant name: *Lasiodiplodia theobromae* (Pat.) Criff. et Maubl. = *Diplodia natalensis* Pole-Evans
		Botryosphaeria rhodina (Cke.) Arx.
		Dothiorella dominicana Pet. et Cif
		Variant name: *Dothiorella aromatica* (Sacc.) Petr. and Syd. = *Fusicoccum asculi* Corda
	Leaf spot	*Alternaria alternata* (Fr.) Keissl
		A. tenuissima (Ness ex Fr.) Wiltsh.
	Bacterial diseases	
	Bacterial angular leaf spot	*Xanthomonas campestris* pv. *mangiferae-indicae* (Patel et al.) Robbs et al.
	Bacterial soft rot	*Erwinia carotovora* subsp. *carotovora* (Jones) Bergey et al.
		Erwinia herbicola (Lohnis) Dye

(*Continued*)

TABLE 3.1 (*CONTINUED*)
Main Postharvest Diseases from Fruits

Fruit	Name of the Disease	Pathogens
	Fungal diseases	
Citrus	Blue mold	*Penicillium italicum* Wehmer
	Green mold	*Penicillium digitatum* (Pers. Ex Fr.) Sacc.
	Sour rot	*G. candidum* Link.
		Variant name: *Galactomyces geotrichum* (Butl. and Pet.) Redh. and Mall.
		G. citri-aurantii (Fer.) Butler
		Variant name: *G. citri-aurantii* Butler
	Bacterial diseases	
	Bacterial leaf spot	*X. campestris* pv. *citri* Dye
	Phomopsis stem-end rot	*Pseudomonas syringae* van Hall
	Fungal diseases	
Banana	Anthracnose	*C. musae* (Berk. and M. A. Curt.) Arx
		Variant name: *Gloeosporium musarum* (Cook. et Mass.)
	Botryodiplodia fruit rot	*A. alternata* (Fr.) Keissl.
		A. triticina Prasada et Prabbu
		Bipolaris sp.
	Collar rot	*B. ribis* Grossenb. and Duggar
		B. theobromae Pat.
	Bacterial diseases	
	Bacterial soft rot	*E. carotovora* subsp. *carotovora* (Jones) Bergey et al.
		Pseudomonas aeruginosa (Schroeter) Migula
		Pseudomonas cichorii (Swingle) Stapp
	Fungal diseases	
Pineapple	Black rot	*Thielaviopsis paradoxa* (de Seynes) V. Hohnel
		Ceratocystis paradoxa (Dade) C. Moreau
	Heart rot	*Phytophthora cinnamomi* Rands
		Phytophthora parasitica Dast
		Phytophthora nicotianae Breda de haan.
	Botryodiplodia fruit rot	*B. theobromae* Pat.
		Variant name: *B. rhodina* (Cke.) Arx. *D. natalensis* Pole-Evans
		B. rhodina (Cke.) Arx.
	Bacterial diseases	
	Crabapple rot	*Pantoea citrea*
		Acetobacter aceti
		E. herbicola
	Bacterial canker rot	*E. chrysanthemi* Burkholder
	Soft rot	*E. carotovora* subsp. *carotovora* (Jones) Bergey et al.
	Virus disease	
	Rind blotch	Tomato spotted wilt virus
	Fungal diseases	
Apple	Black rot	*Physalospora obtuse* Schw. Cooke
		Sphaeropsis malorum Peck
	Anthracnose	*C. gloeosporioides* (Penz.) Penz. et Sacc.
		G. cingulata Stonem.
	Scab	*Fusicladium dendriticum* (wallr.) Fuck.
		Variant name: *Spilocaea pomi* (Fr.)

(*Continued*)

TABLE 3.1 (*CONTINUED*)
Main Postharvest Diseases from Fruits

Fruit	Name of the Disease	Pathogens
	Fungal diseases	
Pear	Anthracnose	*C. gloeosporioides* Penz.
		G. cingulate (Stonem.) Spauld.et Schrenk
	Scab	*Venturia nashicola* Tanak et Yamamota
		Fusicladium plrinum (Lib.) Fuck.
	Leaf spot	*Physalospora piricola* Nose.
		Macrophoma kuwatsukai Hara.
	Fungal diseases	
Peach	Brown rot	*Monilia fructicola*
		Monilia cinerea
		Monilia fructigena
	Soft rot	*Rhizopus nigricans*
	Fungal diseases	
Grape	Gray mold	*Botrytis cinerea*
		Boreyotina fuckeliana
	Anthracnose	*C. gloeosporioides*
		Glomerella cingulata
	White rot	*Coniella diplodiella*
		Charrinia diplodiella

3.3 SPOILAGE MICROORGANISMS CHARACTERISTICS AND POSSIBLE MECHANISMS

The majority of fruit phytopathogenic fungi belong to the Ascomycetes and the Basidiomycetes, which can reproduce spores or other structures to complete their life cycle. When spores land on wounded fruit tissue, the pathogens start the infection process, but in another case, the pathogens may forcibly touch on the unripe fruit cuticle and then remain on the surface of fruit for months until fruit ripens and then cause major losses. It has been reported that the postharvest fungal pathogens can secrete organic acids or ammonia that acidify or alkalinize the host ambient surroundings in order to increase fungal pathogenicity (Alkan et al., 2013). Pathogenicity refers to the ability of a microorganism to cause disease, but the process of plant pathogenic fungi infecting on the host is very complex, including the following steps: (1) body surface attachment; (2) cell wall penetration stage; and (3) in vivo colonization and symptom apparent stage (Meng et al., 2009). Among them, the appressorium of the pathogenic fungi plays a key role during the step of adsorption and penetration into the host cell, which is special shaped structure like a sucker, forming by swelling in the top of the mycelium or germ tube (Emmett and Parbery, 1975). The appressorium is highly specialized infection cell, which is induced by specific physical or chemical factor provided by their host plant (Deising et al., 2000). During the process of infection, at first, the appressorium secretes sticky substance that is used to firmly adhere to the surface of the host, then, accumulation of glycerol in the cytoplasm of appressorium that produces huge expansion pressure to supply mechanical power for the hyphae penetrating into the host tissues (Howard et al., 1991). For example, *Magnaporthe oryzae* is the most destructive disease for rice, whose pathogenic mechanisms have been studied extensively. Infection of *M. oryzae* toward rice begins when a three-celled spore lands on the surface of a leaf, which then germinate and develop to specialized infection structure called the appressorium. A high internal turgor is formed from the mature appressorium, which acts on a thin penetration peg and force it through the rice cuticle into the underlying epidermal

cells. At the last stage, the invasive hyphae of *M. oryzae* grow asymptomatically from one living rice cell to another till host cells begin to die, and the symptom of necrotic lesions present on the surface of the leaf, from which the spores are produced to continue another life cycle (Fernandez and Wilson, 2014). On the other hand, plant pathogenic fungi produce cell wall–degrading enzymes (CWDE) that can degrade the cell wall components of plants. Plant pathogen does not only digest plant cell wall polymers to obtain an important nutrient source but also degrade the cell wall for enabling cell penetration and spreading through plant tissues (Kikot et al., 2009). For example, Phalip et al. (2005) analyzed the diversity of extracellular proteome of *F. graminearum* that was grown on plant cell wall, and identify 24 different enzymes, such as cutinases, which are necessary to digest the complete plant cell wall for the penetration of plant surfaces. In the cases of fruit pathogens, *C. gloeosporioides* is the causal agent of anthracnose disease on fruit crops, such as avocado, which use the surface wax of the host, mainly including fatty alcohol, to induce germination and appressorium formation (Podila et al., 1993). In addition, pH can modulate the pathogenicity of *C. gloeosporioides* via alkalization as a result of secretion of ammonium that is due to enhanced fungal nitrogen metabolism during the host colonization, in which the expression of two types of ammonium transporter-encoding genes, *AMET* and *MEP* have been induced (Shnaiderman et al., 2013).

Recent findings emphasized the role of pathogen's effector molecules that support host colonization and deregulate host immunity response (Mukhtar et al., 2011). In addition, they differ in the action sites, one group of effectors act in the host apoplast, another group is delivered into the host cytoplasm to interfere with immune responses, or protect the pathogen from host-derived antimicrobials (Okmen and Doehlemann, 2014, Figure 3.1). For example, tomato fungal pathogen *Cladosporium fulvum* abundantly secretes the LysM-containing effector or protein Ecp6 to suppress its host induction of pattern-triggered immunity (PTI) in apoplast (de Jonge et al., 2010), which is because of Ecp6 outcompeting host receptors to bind fungal chitin with a unprecedented ultrahigh affinity through intrachain LysM dimerization (Sánchez-Vallet et al., 2013). Effector protein Pit2 secreted by *Ustilago maydis*, a pathogen of corn smut, are identified with

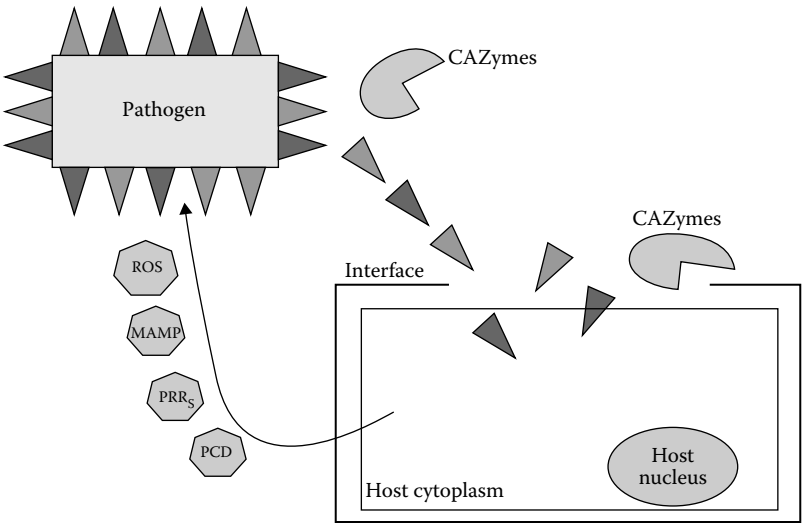

FIGURE 3.1 Filamentous pathogen secretes effector molecules to deregulate host immunity in various host subcellular compartments. There are two groups of pathogenic effector molecules. One group is incomplete circle shapes that interact with host immune activated targets (pentagonal shapes), another group is trilateral shapes that act in self-defence to protect the pathogen from host-derived antimicrobials. (From Rovenich, H., *Curr. Opin. Plant Biol.,* 20, 2014.)

roles in inhibiting cysteine proteases in apoplastic maize, whose activity is directly associated with salicylic acid–induced plant defenses (Mueller et al., 2013). Pathogen effectors translocate into the host cytoplasm, which is a complex biological process involving numerous steps (Petre and Kamoun, 2014). For example, Irish potato famine pathogen *Phytophthora infestans* delivers effector protein PexRD2 to plant cells to perturb plant immunity-derived signaling, through its interaction with the kinase domain of MAPKKKε that is a cell death positive regulator (King et al., 2014).

On the one hand, when pathogen successfully enters to the plant apoplast, the enzymes, such as chitinases, can target fungal cell walls to release chitin fragments that hamper the microbial colonization. On the other hand, the fungal pathogens secrete effector molecular, such as chitin-binding effectors, to protect their cell walls (Kombrink et al., 2011). Although the effector molecules secreted by postharvest pathogen have not yet been directly identified, 1954 putative secreted proteins are found in the *C. gloeosporioides* genome, of which, 767 can be induced the expression and classified as expressed "small secreted proteins" (Alkan et al., 2015). Molecular basis of the compatible interaction between tomato and *Verticillium dahlia*, a necrotrophic fungus leading to *Verticillium* wilt in tomato, has been revealed by RNA sequencing, in which 1953 significantly differentially expressed genes have been found, including 1281 upregulated and 672 downregulated genes (Tan et al., 2015). *Penicillium* is a genus within ascomycetous fungi with many well-known plant postharvest pathogens, such as *P. expansum*, *P. italicum*, and *P. digitatum*. *P. expansum* can infect a wide range of fruit and vegetable hosts, except citrus fruit, and produce many mycotoxins, such as patulin (Andersen et al., 2004). However, *P. digitatum* and *P. italicum* are restricted to citrus fruit, not others. The genome of *P. expansum*, *P. italicum*, and *P. digitatum* have been sequenced recently, in which a large number of cysteine-rich effector proteins and carbohydrate-active enzymes, known to exhibit diverse biological functions including adherence (Wosten, 2001), virulence (Stergiopoulos et al., 2009) or antimicrobiosis (Marx et al., 2004), have been predicted (Marcet-Houben et al., 2012; Li et al., 2015).

3.4 DETECTION AND EVALUATION METHODS FOR SPOILAGE MICROORGANISMS

The detection and evaluation of plant pathogens, such as plant pathogenic fungi, traditionally is based on the morphological characteristics and refer to their phylogeny as well. However, this method is disturbed to some extent by factors from personal experiences and environmental conditions that lead to uncover the genetic relationship among populations, insufficiently (Whittaker, 1970). Molecular identification has enormous potential to improve our understanding of fungal biodiversity and morphological study of fungi (Begerow et al., 2010). Recombinant DNA (rDNA) is a gene included in all organisms except the virus, and its nucleotide sequence can be used as a strong reference for system evolution analysis (Table 3.2). At present, fungal rDNA sequences are with special conserved regions that promise to design the primers that are used to identify the species and calculate the evolutionary speed. The internal transcribed spacer (ITS) region is special conservative, but is differed in the levels of family, genus, and species. So, the specific primers are designed according to the ITS region, and widely used in molecular detection of plant pathogenic fungi (Kõljalg et al., 2005). Because this molecular detection method is fast, accurate, and simple, it has become a powerful way to study the evolution and diagnosis of pathogenic fungi. In addition, polymerase chain reactions (PCRs) with the primers RST31/RST33 were used to detect citrus variegated chlorosis (CVC), caused by the xylem-limited bacterium *Xylella fastidiosa* subsp. pauca (Xfp), which is a damaging disease of sweet oranges (Cordeiro et al., 2014). Two *Erwinia* species (*Erwinia amylovora* and *Erwinia pyrifoliae*), which cause fire blight in pear and apple, have been detected using mass spectroscopy with PCR primers designed from the pstS–glmS regions (Wensing et al., 2012).

TABLE 3.2
PCR Primers Used for Detection of Plant Pathogenic Fungi

Primer	Sequence	Site	Reference
ITS1	TCCGTAGGTGAACCTGCGC	18S	Gardes and Bruns (1993)
ITS1-F	CTTGGTCATTTAGAGGAAGTAA	18S	Gardes and Bruns (1993)
ITS4-B	CAGGAGACTTGTACACGGTCCAG	28S	Gardes and Bruns (1993)
ITS2	GCTGCGTTCTTCATCGATGC	5.8S	Borneman and Hartin (2000)
ITS3	GCATCGATGAAGAACGCAGC	5.8S	Borneman and Hartin (2000)
ITS4	TCCTCCGCTTATTGATATGC	28S	Borneman and Hartin (2000)
ITS5	GGAAGTAAAAGTCGTAACAAGG	18S	Borneman and Hartin (2000)
ITS6	GAAGGTGAAGTCGTAACAAGG	18S	Gardes and Bruns (1996)
ITS7	AGCGTTCTTCATCGATGTGC	5.8S	Gardes and Bruns (1996)
ITS1-R	(TA)TGGT(CT)(AGT)(TC)(TC)TAGAGGAAGTAA	18S	Gardes and Bruns (1996)
ITS4-R	CAGACTT(GA)TA(CT)ATGGTCCAG	28S	Gardes and Bruns (1996)
5.8S	CGCTGCGTTCTTCATCG	5.8S	Cullings and Vogler (1998)
5.8SR	TCGATGAAGAACGCAGCG	5.8S	Cullings and Vogler (1998)
SR6R	AAGWAAAAGTCGTAACAAGG	18S	Cullings and Vogler (1998)

On the other hand, another kind of spoilage microorganism, which are foodborne pathogens, such as *E. coli* O157:H7, can cause severe disease and death to humans. *L. monocytogenes* and *Salmonella* spp. are foodborne pathogens frequently associated with foods, such as fruits and vegetables. So, rapid detection and toxicology evaluation of foodborne pathogens have developed very quickly in recent years. For example, *E. coli* O157:H7 is one of the leading bacterial pathogens causing foodborne illness, which can infect even in a low dose to cause human diarrhea and enteritis (Karch et al., 2005). Because a rapid and sensitive detection technology is highly desirable for specific detection of *E. coli* O157:H7, it was reported recently that the rapid detection of *E. coli* O157:H7 by using calcium signaling of the B cell demonstrated that the developed B cell based on biosensor was able to specifically detect *E. coli* O157:H7 in pure culture samples at a low concentration within 10 min. The molecular methods are already indispensable tools for diagnostic and typing studies in food safety. A nucleic acid lateral flow assay plus PCR was explored to detect *E. coli* (Shiga toxin (Stx) -producing Escherichia coli [STEC]) with the detection limit for the STEC strains ranged from 0.1 to 1 pg of genomic DNA (about 20–200 CFU) per test (Yoshitaka et al., 2015). *Salmonella* is the second foodborne disease worldwide that can cause diarrhea, fever, and in the severe cases can increase human morbidity and mortality (Crump et al., 2015). Recently, a rapid molecular detection assay for *Salmonella* in dry pet food and dark chocolate has been developed (Ryan et al., 2015). Rapid detection of multiple foodborne pathogens by using a single-walled carbon nanotube (SWCNT)-based multijunction sensor was designed for potential multiplexed detection of foodborne pathogens, which has been developed by Yamada et al. (2015). This method was observed with high sensitivity for both the *E. coli* and *Staphylococcus aureus* functionalized array sensors, $R^2 = 0.978$ and $R^2 = 0.992$, respectively, in range of 10^2–10^5 CFU/mL (Yamada et al., 2015). *Streptococcus* is the most common pathogen for human dysentery—just a small amount of this pathogen in the body can cause disease and lead to severe sepsis (Kim et al., 2013). Wang et al. (2015) developed the primer sets for loop-mediated isothermal amplification, which was designed according to 16S rRNA gene and 16S–23S rRNA intergenic spacers and it enables to rapid and specific detection of *Streptococcus dysgalactiae*, *Streptococcus uberis,* and *Streptococcus agalactiae*, with a detection limit of 0.1 pg DNA template per reaction (Wang and Liu, 2015). *L. monocytogenes* can survive under adverse conditions, such as low pH and/or low temperature, and cause high lethality (30%) for infected individuals, so it is one of the most significant foodborne pathogens. Garrido-Maestu

et al. (2015) developed a method for detection and enumeration of *L. monocytogenes* by using most-probable-number method combined with a real-time PCR. The development of robust and efficient sample preparation techniques is crucial to improve detection sensitivity and workflow. Immunomagnetic separation using magnetic nanoparticles (MNPs) is attractive, as it can efficiently capture target cells (Malic et al., 2015).

New methods for detection and evaluation of spoilage microorganisms include thermal imaging, which is a technique for converting the invisible radiation pattern of an object into the visible images used for feature extraction and analysis (Vadivambal and Jayas, 2011). Thermal imaging using infrared thermography was used to visualize the development of downy mildew in cucumber leaves, which is caused by plant pathogen *Pseudoperonospora cubensis* (Oerke et al., 2006). Hahn et al. (2006) demonstrated the possibility of application of thermal imaging in determining *E. coli* at their earlier stage. Current developments in nanotechnology have explored the nanomaterials with the ability to detect many bacterial strains, which have an important advantage of separating the bacteria from biological samples by using magnets (Bohara and Pawar, 2015). Lin et al. (2005) reported that iron oxide nanoparticles are immobilized by vancomycin, which can be used to detect bacteria such as *Staphylococcus saprophyticus, S. aureus*, and *Enterococcus faecalis.*

3.5 CONTROL, PREVENTION, AND MANAGEMENT OPTIONS FOR SPOILAGE MICROORGANISMS

Chemical fungicides are the most widely used treatment for fresh fruits and fruit produce in order to avoid microorganisms' contamination. Timing of application and type of fungicide used depend primarily on the target pathogen and when infection occurs. The benzimidazole group of fungicides are very useful for the control of many important postharvest pathogens such as *Penicillium* and *Colletotrichum* species, for citrus fruit, which can be coated with food-grade vegetable, beeswax, and/or lac-based wax or resin to maintain freshness and also be treated with one or more of the following fungicides: thiabendazole, orthophenyl phenol, and imazalil.

It is generally accepted that environmental conditions such as temperature, humidity, and atmosphere can significantly impact the quality of fresh fruits. Low temperature is the most effective way to preserve quality and nutrition of the postharvest fruit, and can be combined with other control measures in order to achieve better results, such as storage before heat treatment, precooling treatment, cleaning and coating, and so forth. For example, tomato is refrigerated in combination with appropriate humidity environment, which can better maintain the quality of the fruit, and usually the humidity must be controlled between 85% and 90%. The mechanism of low temperature for preservation is mainly included in reducing respiration intensity and transpiration in a low-temperature environment and the internal metabolism of fruit are reduced. Besides, low temperature can obviously inhibit microbial growth and reproduction, as well as reducing the speed of the oxidation and senescence of the fruit (Nunes, 2008). There are several major preservative measures for postharvest fruit, which are outlined in the following sections.

3.5.1 Modified Atmosphere Packaging

Modified atmosphere packaging (MAP) involves actively controlling or modifying the atmosphere to produce a packaging surrounding that consists of various types of gas, and combining the use of films (Parry, 1993). Oxygen, CO_2, and N_2 are most often used in MAP. Other gases such as nitrous and nitric oxides, sulfur dioxide, ethylene, chlorine, as well as O_3 and propylene oxide have been suggested and investigated experimentally (Phillips, 1996). The combined use of passive modified atmosphere and aqueous ClO_2 at concentrations between 16 and 20 mg/L has potential to maintain the quality of sweet cherry, showing good features such as total soluble solid contents and firmness (Colgecen and Aday, 2015). When pomegranate arils were packaged in polyester film with high

O_2 or 100 kPa N_2, the respiration rate and quality of pomegranate arils is significantly affected (Banda et al., 2015). Changing the gas composition can reduce fruit respiration, inhibit ethylene production, and delay senescence of fruit, but also can change microbial environment, inhibiting the growth of aerobic microorganisms. The package can be used to maintain the dynamic equilibrium of atmosphere by balancing respiration or gas (carbon dioxide and oxygen) penetration, simultaneously. Mangaraj et al. (2014) designed MAP packages used for guava preservation. The results show that the modified atmosphere with 5% O_2 and 4% CO_2 is suitable for keeping the guava fresh till 26, 19, 13, and 7 days of storage at 10°C, 15°C, 20°C and 25°C, respectively.

3.5.2 Chitosan

Chitosan is of great interest because their all-round properties have great application potential in food industry and agriculture (Chien et al., 2007; Shiekh et al., 2013). The use of chitosan at the preharvest or postharvest stages has been considered as a suitable alternative to replace synthetic fungicides. Strawberries sprayed with chitosan at concentration of 0.2%, 0.4%, and 0.6% have shown decreased incidence of gray mold and maintained the quality of strawberries during storage temperatures between 3°C and 13°C (Reddy et al., 2000). Recently, postharvest chitosan treatment effected on senescence of cherry, which indicated that the chitosan treatment enhanced the activity of some antioxidant enzymes, including superoxide dismutase (SOD), ascorbate peroxidase (APX), inhibited polyphenol oxidase (PPO), and guaiacol peroxidase (POD) (Pasquariello et al., 2015). The treatments with 0.5% or 1.0% chitosan confirm about 30% reduction of decay in postharvest strawberry compared with the control groups (Feliziani et al., 2015).

3.5.3 Essential Oils

EOs are a group of aroma compounds abstracted from plants. The antimicrobial activity of EO is provided not only against plant pathogens, but also foodborne pathogens (Moghimi et al., 2016). The mechanism of EOs for control of microorganisms is attributed to target in the cell wall and cell membrane, as hydrophobicity of EO is able to permeate intolipids of bacterial cell membrane and mitochondria, leading to leakage of intracellular constituents (Diao et al., 2014). Carvone has been examined with promising efficiency against various storage diseases of potato including *Fusarium sulphureum*, *Phoma exigua* var. *foveata*, and *Helminthosporium solani* (Hartmans et al., 1995). In addition, the foodborne pathogens including *E. coli 0157*, *Salmonella Enteritidis*, *Salmonella Typhi*, *Yersinia enterocolitica,* and *L. monocytogenes* are all sensitive to the EO obtained from *Artemisia annua* (Donato et al., 2015). EOs extracted from *Agastache mexicana* ssp. *xolocotziana* and *Porophyllum linaria* have shown potent antifungal activity against *Aspergillus* and *Penicillium* species, with the MICs ranging from 0.3 to 30 μg/mL for *A. mexicana*, and 0.0069–0.92 μ g/mL for *P. linaria* (Juarez et al., 2015). Fennel seed essential oil (FSEO) has shown antifungal effects on *Trichophyton rubrum*, *Trichophyton tonsurans*, *Microsporum gypseum*, and *Trichophyton mentagrophytes* in relation to the antifungal mechanism of damaging the plasma membrane and intracellular organelles (Zeng et al., 2015).

3.5.4 Sodium Hypochlorite (NaClO)

Before the storage, fruit can be washed with water to remove dirt on the surface of fruit, such as sand, microbial contamination, and chemical pollution; this procedure also is beneficial for maintaining fruits' quality and safety. In addition, adding detergent such as NaClO, O_3, and chlorine dioxide (ClO_2) in the washing water can effectively reduce the microbial contamination, thereby enhancing the cleaning effect.

NaClO is the most commonly used sanitizer for cleaning pathogens on the surface of fruit. It can effectively inhibit or kill fungi or bacteria that cause fruit disease and human illness, but it has no effect on pathogens inside the fruit. Naturally infected mango fruits were treated with 100 ppm NaClO and the combined use of hot water and UV-C irradiation, which induce the resistance to anthracnose disease and improve the quality of harvested mangoes (Sripong et al., 2015). Alvindia (2013) reported an integrated approach that uses hot water treatment and NaClO for control of crown rot disease and preservation of quality in banana, as well as the efficacy against *Lasiodiplodia theobromae*, *Thielaviopsis paradoxa*, *C. musae,* and *Fusarium verticillioides*, is evaluated. An oxidizing compound using NaClO and hydrogen peroxide was applied to analyze its antifungal activity against *P. digitatum*, which shows the minimum inhibitory concentration (MICs) is 300 ppm for NaClO and 300 mM for H(2)O(2) when conidia suspensions were treated for 2 min (Cerioni et al., 2009). *P. expansum*, a causal agent of apple blue mold, which can be inhibited by an oxidative treatment, suggesting MICs were 50 mg/L and 400 mmol/L for NaClO and H(2)O(2), respectively (Cerioni et al., 2013).

3.5.5 Ozone (O_3)

O_3 is generated when molecular diatomic oxygen (O_2) receives an electrical discharge. Upon release of the third O_2 atom, O_3 acts as a strong oxidizing agent and was investigated as a promising alternative for food decontamination. In addition, as O_3 decomposes back to O_2, leaving no residue on food products, it has been recognized as a disinfectant reached up to safety standard in United States (generally recognized as safe, GRAS). In addition, O_3 has been used for hundreds of years. Recently, O_3 was used for preservation of fresh fruit and vegetable, the main mechanism including O_3 interaction with ethylene that inhibits respiration, and O_3 directly or indirectly uses the free radicals against pathogens. Many studies have shown that O_3 has good effect on antisepsis of fruit, such as apple (Yaseen et al., 2015), table grapes (Ozkan et al., 2011), and citrus (Palou et al., 2001). There are some limitations to the application of O_3, including O_3 toward people's eyes, skin, and respiratory tract has stimulative effect. The use of the O_3 concentration undeserved may damage plasma membrane of epidemic cells on the surface of fruit and vegetable. The increasing permeability and leaking cellular contents are also presented. In addition, the incorrect operational approach will decline the quality and accelerate the senescence and decay (Horvitz and Cantalejo, 2014). In addition, Tanou et al. (2015) reported that O_3 (0·3 μL/L) can induce ripening inhibition in kiwi fruits, in relation to depression in the biosynthetic mechanism of ethylene.

3.5.6 Chlorine Dioxide (ClO_2)

ClO_2 is a new type of disinfectant, and due to its disinfection, preservation, and safety properties it has been recognized by the World Health Organization (WHO) as safe disinfectant at A1 level. ClO_2 can effectively kill microorganisms on the surface of fruit; for example, strawberries are applied to ClO_2 by using a controlled release pad, indicating that the ClO_2 treatments are able to induce closing of stomata, which markedly slowed weight loss and softening and reduced decay incidence of strawberry fruit at 10°C or lower temperatures (Wang et al., 2014). Ray et al. (2013) reported that ClO_2-releasing packaging films can release ClO_2 to reduce *Salmonella* spp. and *E. coli* O157:H7 inoculated on the tomatoes, and the film-treated tomatoes do not show visible changes in color and texture compared with control groups during storage at 10°C for 21 days. Harvested longan fruits have problem of pericarp browning that reduces both the shelf life and market value. A study investigated the efficiency of ClO_2 fumigation at reducing pericarp browning of longan, which suggested that ClO_2 fumigation at a concentration of 10 mg/L can be the most effective treatment to reduce pericarp browning of longan, and simultaneously maintain the fruit quality (Saengnil et al., 2014).

3.6 CONCLUSION AND PERSPECTIVES

Fresh keeping of fruits should be integrated with all kinds of preservative and antioxidative measures in order to exert their respective advantages and achieve the best effect of fresh keeping, such as low temperature, controlled atmosphere, packaging, biological preservative, low-dose irradiation, and so forth. In the future, the use of natural biological preservation combined with new packaging and sterilization technology will be a primary trend in reducing fruit disease. Biological preservation agent because of its natural, safe, and efficient attributes may reach a research hotspot in the field of food preservation and will eventually replace the chemical preservatives. But single biological preservative often cannot effectively inhibit and kill all microorganisms, which limits the application in food preservation. Hence, comprehensive utilization of fresh agents that can be a synergy effect not only enhances their antibacterial effect but also reduces the costs. Therefore, comprehensive use of various techniques is one of the main directions of the future research in the field of fresh food. DNA-based diagnostic and typing methods for the detection and evaluation of plant pathogens and their producing-mycotoxin, as well as foodborne pathogens will be increasingly improved in the future, but further important indicators, such as cost, time effectiveness, sensitivity, and specificity, have to be compatible with the internationally standardized official methods. Some pathogens can cause disease in fruit; however, they have to encounter other microorganisms including competitors or cooperators, which play important roles with antagonistic and symbiotic relationships, respectively. These microorganisms share the decayed fruit with the plant pathogenic microbes, but as we know little on them, an experimental way should be explored to understand the interaction between the fruit pathogenic microbes and the organisms they encounter in their niches.

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4 Spoilage Microorganisms in Vegetables

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CONTENTS

4.1 INTRODUCTION

Vegetables have been associated with outbreaks of foodborne disease in many countries. Organisms involved in these diseases include bacteria, viruses, and parasites. These outbreaks vary in size from a few affected persons to many thousands. Contamination of vegetables may take place at all stages during pre- and postharvest procedures. Cultivation and operation or preparation of vegetables is responsible for this contamination. Unsafe water used for rinsing the vegetables and sprinkling to keep them fresh is also a source of contamination. Other possible sources of microorganisms include soil, feces (human and animal origin), water (irrigation, cleaning), ice, animals (including insects and birds), handling of the product, harvesting and processing equipment, and transport (Johannessen et al., 2002). Microorganisms, normally present on the surface of raw vegetables, may consist of chance contaminants from the soil or dust. These include bacteria or fungi that have grown and colonized by utilizing nutrients exuded from plant tissues. Among the groups of bacteria commonly found on plant vegetation are mainly coliforms or precisely fecal coliforms, such as *Klebsiella* and *Enterobacter*. Microorganisms capable of causing human illness and others whose foodborne disease potential is uncertain, include *Aeromonas hydrophila*, *Citrobacter freundii*, *Enterobacter cloacae*, and *Klebsiella* sp. and these have been isolated in lettuce and salad vegetables. Plate count of aerobic mesophilic microorganisms found in food is one of the microbiological indicators for food quality (Aycicek et al., 2006). These organisms reflect the exposure of the sample

to any contaminant and in general, the existence of favorable conditions for the multiplication of microorganisms. For various reasons, this parameter is useful to indicate whether cleaning, disinfection, and temperature control during industrial processing, transportation, and storage have been performed sufficiently.

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. Carriers for disseminating these microbes include soil particles, airborne spores, and irrigation water. 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 soilborne 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 (GAPs) will provide dramatic reductions in yield loss due to spoilage at all subsequent steps in the food-to-fork continuum (Eckert and 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. In 1998, the Food and Drug Administration (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:

1. Water
2. Manure and municipal biosolids
3. Worker health and hygiene
4. Sanitary facilities
5. Field sanitation
6. Packing facilities sanitation
7. Transportation
8. Trackback

In addition, the 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, the 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.

4.2 TAXONOMY OF SPOILAGE MICROORGANISMS IN VEGETABLES

The bacteria *Escherichia coli* are the species associated with fecal contamination and naturally found in the intestines of humans and warm-blooded animals. The presence of these bacteria poses a serious threat to public health with outbreaks arising from food and water that has been contaminated by human or animal feces or sewage. *Staphylococcus aureus* is the third most common cause of confirmed food poisoning in the world and the illness is due to the ingestion of preformed enterotoxins produced in foods (Andrews and Harris, 2000). 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 less than 2% O_2 and more than 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 adenosine triphosphate (ATP) by creation of a proton gradient. The lactics can only obtain ATP by fermentation, usually of sugars. Because 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.

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 acetyl methylcarbinol and diacetyl, responsible for 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 (Jacxsens et al., 2003). The most common and important spoilage microorganisms of refrigerated fresh-cut vegetables are the fluorescent *Pseudomonas* species of which *Pseudomonas marginalis* is an example. *Pseudomonas* sp. is a Gram-negative rod and strict aerobe. These species can be divided into four groups based on ribonucleic acid (RNA) homology and nine groups based on cellular fatty acid composition. *Pseudomonas* is widely distributed in nature and is 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 maltose. Some *Pseudomonas* species produce pyoverdine or fluorescein that are water soluble, fluorescent pigments and can be observed in spoiled foods under ultraviolet (UV) light. They are usually yellow-green but may appear blue or orange depending on the species and environmental factors. *Pseudomonas* produce catalase, oxidase, and enzymes that catalyze proteolytic and lipolytic reactions that contributes to spoilage of refrigerated fresh animal products, and pectinolytic enzymes that can cause soft rot of fleshy vegetables.

Erwinia sp. 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* sp. consists of three species or subspecies, including *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica*, and *Erwinia chrysanthemi*. *Erwinia* sp. are the major single cause of microbial spoilage of whole vegetables (Liao and Well, 2007).

The bacterium *E. carotovora* subsp. *carotovora* is a highly effective spoilage microbe that causes soft rot across a broad host range of vegetables and some fruits (Lund et al., 2000). One of the six known genera of soft-rot bacteria (including *Xanthomonas*, *Pseudomonas*, *Clostridium*, *Cytophaga*, and *Bacillus*), *E. carotovora* subsp. *carotovora* is one of the several species of *Erwinia* that infect and destroy plant tissues both pre- and postharvest and 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 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 et al., 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. Probably this is one of the reasons for the high prevalence of these bacteria on decayed vegetables at wholesale and retail markets (Liao, 2005).

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 UV light (Munsch et al., 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.

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 fungus formation 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.

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, botrytis rot, and brown rot. Like yeasts, mold populations have been reported in various types of fresh-cut fruits and vegetables and visible molds have resulted in inedible fresh-cut fruits, such as strawberry, honeydew, pineapple, and cantaloupe (Tournas, 2005; Ukuku and Sapers, 2005). 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 reported for contamination of fresh-cut produce.

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*, *Rhodotorula glutinis*, *Zygosaccharomyces bailii*, *Zygosaccharomyces bisporus*, and *Zygosaccharomyces rouxii* and have been isolated widely from fresh-cut fruits and vegetables, and salad mixes, even though the specific species were rarely identified (Jacxsens et al., 2003). Yeasts have a slightly higher growth rate than molds, ferment sugars into alcohols, and are responsible for off-flavors and off-odors. Yeast growth is responsible for shelf life failure of grated celery stored under modified atmosphere packaging (MAP) at 4°C and shredded chicory endives packed in high-oxygen atmosphere with barrier film.

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 or inadequate culling, an expanding infestation of spoilage microorganisms can destroy a substantial portion of a stored lot of fruits. *P. expansum* and *B. cinerea* are pathogens of apples, pears, and a number of other pectin-rich fruits (Miedes and Lorences, 2004). *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).

4.2.1 Salmonella

Salmonella spp. are the most commonly identified etiological agent associated with fresh produce-related infection, isolated in 48% of cases between 1973 and 1997 in the United States and in 41% of cases during 1992–2000 in the United Kingdom. During 2006, two major tomato-related *Salmonella*

outbreaks in the United States accounted for 23.2% of reported *Salmonella* cases (CDC, 2007). A range of fresh fruit and vegetable products have been implicated in *Salmonella* infection, most commonly lettuce, sprouted seeds, melon, and tomatoes. *Salmonella* spp. are often isolated from produce sampled in routine surveys, including lettuce, cauliflower, sprouts, mustard cress, endive and spinach, and mushrooms.

4.2.2 Escherichia coli O157:H7

E. coli O157:H7 can be isolated from the feces of livestock; therefore, its presence in animal manures and slurries is inevitable. In addition to farm animals, *E. coli* O157:H7 may be present in the feces of wild birds, for example, starlings and gulls. Leafy vegetables are most commonly linked to *E. coli* infection, but apple juice (cider in the United States) is an interesting vehicle, as the acidity of the product is considered inhibitory to bacterial proliferation. *E. coli* O157:H7 is commonly recovered from the feces of ruminants; therefore, livestock grazing in orchards may contaminate fallen apples with feces and, as *E. coli* O157:H7 can proliferate in damaged apple tissue, this can result in the contamination of unpasteurized fruit juices/ciders. Janisiewicz et al. (1999) demonstrated that fruit flies were an important vector in the contamination of apples with *E. coli* O157:H7, both preharvest and in packing houses.

4.2.3 Listeria monocytogenes

Listeria spp. are ubiquitous in the environment and can be isolated from soil, water, vegetation, the feces of livestock, and vegetation irrigated with contaminated water. The potential for environmental *Listeria* to contaminate fresh produce and lead to enteric infection has long been recognized and Harvey and Gilmour (1993) suggested this most probably occurred during processing. The predominant *Listeria monocytogenes* serotype isolated from salad vegetables has been shown to be serogroup 1 (Harvey and Gilmour, 1993). Beuchat (1998) reports a number of surveys documenting the presence of *L. monocytogenes* on cucumber, peppers, potato, radish, leafy vegetables, beansprout, broccoli, tomato, and cabbage at point of sale (POS). These surveys show variation in prevalence on different types of produce and between countries. Arumugaswamy et al. (1994) isolated *L. monocytogenes* from beansprout (85%), leafy vegetables (22%), and cucumber (80%) in Malaysia. In comparison, only 6.7% of cucumbers sampled in Pakistan yielded *L. monocytogenes* (Beuchat, 1998). Two surveys of cabbage in Canada reported a prevalence of 2.2% and 6.7%, respectively, but a larger study in the United States reported only 1.1% positive samples (Heisick et al., 1989). An US survey of potatoes reported a higher incidence (27.1%) than a survey conducted in Spain (16.7%) (Beuchat, 1998) and two separate studies of radish in the United States reported results of 14.4% and 36.8% prevalence. Generally, contamination is higher on root vegetables and Heisick et al. (1989) suggest that this is due to increased contact with soil. Variations in sample size, sampling regimes, and laboratory protocols will also impact on the likelihood of isolation and therefore prevalence data. Szabo et al. (2000) surveyed 120 bagged lettuce samples in Australia and reported isolation of *L. monocytogenes* in 2.5% samples. Thunberg et al. (2002) sampled a range of fresh produce and isolated *L. monocytogenes* only from potatoes (50%) and field cress (18%) purchased at farmers' markets. These varied results are likely to represent differences in production practices between countries, within countries, and between outlets. This indicates the huge natural variability in the microbial quality of produce. Crépet et al. (2007) analyzed 165 studies and reported that prevalence on salad vegetables is usually under 5%, with lower numbers isolated from leafy salad vegetables than from sprouted seeds and other vegetables (e.g., carrots, cabbage, celery, and spinach). This paper also showed that surveys conducted after 2000 reported lower instances of *L. monocytogenes* isolation, suggesting that increased knowledge of the behavior of the pathogen in the food processing environment and more effective sanitization procedures have led to improved product control.

L. monocytogenes will grow at refrigeration temperatures; therefore, it is likely to multiply during storage if present on fresh produce. Beuchat and Brackett (1990) showed that *L. monocytogenes* is capable of growth on lettuce when exposed to processing conditions, although carrot juice

seemed inhibitory. Farber et al. (1998) demonstrated that *L. monocytogenes* populations declined on grated carrot by 2-logs over nine days. Although *Listeria* contamination of fresh produce and survival up to POS seems likely, outbreaks linked to fresh produce are infrequent and tend to be limited to vulnerable groups. The two documented outbreaks which have occurred, in 1979 and 1981, respectively, were attributed to cabbage (in coleslaw) and salad items (celery, lettuce, and tomatoes) served as part of hospital meals. The high infective dose of *L. monocytogenes*, ~10^6 cells, variation in susceptibility in the population and long incubation period may also explain the scarcity of recorded fresh produce-related outbreaks.

4.2.4 Campylobacter jejuni

Campylobacter jejuni is the most common cause of gastrointestinal illness worldwide, affecting over two million people in the United States and 50,000 throughout England and Wales annually, but cases are usually sporadic. Outbreaks are most commonly linked to poultry or cross-contamination from poultry products. As *Campylobacter* cannot grow outside of a warm-blooded host, survival on fresh produce is limited, especially if not protected from UV light. Epidemiological evidence suggests that salad vegetables are the second highest risk factor for *Campylobacter* infection after poultry, and outbreaks have been linked to lettuce, sweet potatoes, cucumber, melon, and strawberries. Kumar et al. (2001) isolated *C. jejuni* from spinach, fenugreek, lettuce, radish, parsley, green onions, potatoes, and mushrooms and Park and Sanders (1992) reported *Campylobacter* spp. on 1.6%–3.3% vegetables tested, but other extensive studies of raw organic and prepack salad vegetables failed to isolate *Campylobacter*. It is suggested that outbreaks linked to fresh produce may be due to cross-contamination in the kitchen.

4.2.5 Aeromonas

Aeromonas spp. are ubiquitous in water, soil, feces, and on vegetation. *Aeromonas* has been isolated from a wide range of fresh produce including sprouted seeds, asparagus, broccoli, cauliflower, carrot, celery, cherry tomatoes, courgette, cucumber, lettuce, mushroom, pepper, turnip, and watercress. Watercress was found to yield higher numbers of *Aeromonas* than lettuce or escarole (chicory) samples. Thirty-four percent of organic vegetables were found to be contaminated with *Aeromonas* compared with 26% of conventionally cultivated vegetables. Growth of *Aeromonas* will occur on shredded lettuce, chicory, and tomatoes but not on carrots or Brussels sprouts. Callister and Aggar (1987) found that 48% *Aeromonas* isolates from fresh vegetables were *A. hydrophila*, the species most often linked to disease in humans. Food isolates of *Aeromonas* have been shown to tolerate low pH and to grow at the refrigeration temperatures relied on for preservation throughout the fresh produce supply chain. It has been suggested that *Aeromonas* may be responsible for the many gastrointestinal infections (~40%) for which no etiological agent is found. However, results from volunteer studies are inconclusive and investigations of foodborne outbreaks fail to indisputably establish *Aeromonas* as the causative agent. A link has been suggested between the increased incidence of *Aeromonas* in human stools and on fresh vegetable samples during the summer months. Neyts et al. (2001) suggest that *Aeromonas* may only pose a risk to immuno-compromised groups.

4.3 SPOILAGE MICROORGANISMS CHARACTERISTICS AND POSSIBLE MECHANISMS

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. Some spoilage microbes are capable of colonizing and creating lesions on healthy, undamaged plant tissue. 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. 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) use more strategies to achieve irreversible attachment to the plant surface, and (3) initiate steps leading to internalization of the tissue. 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. However, the fruit of the plant lacks many of these structures, requiring the spoilage microbe to employ other methods to become internalized. Table 4.1 may partially explain the rather limited success of bacteria to spoil fruits and Table 4.2 shows an improved ability to spoil vegetables that are not the fruit of the plant. The natural acidity of most fruits also serves as a barrier to many spoilage microbes, especially bacteria. As seen in Tables 4.3 and 4.4, by contrast, spoilage fungi that typically produce more diverse and greater amounts of extracellular depolymerases successfully attack and spoil both fruits and vegetables.

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 on

TABLE 4.1
Bacterial Fruit Pathogens[a]

	2004 Annual U.S. Per Capita Consumption (LBS)[b]	*Acidovorax*	*Pseudomonas*	*Xanthomonas*	*Erwinia*
Apples	18.8	–	–	–	+
Pineapples	4.4	–	–	–	–
Berries	6.1	–	–	–	–
Citrus	22.7	–	+	+	–
Peaches	5.1	–	–	–	–
Melons	14.7	+	–	–	–
Grapes	7.9	–	–	–	–
Pears	3.1	–	–	–	+
Bananas	25.8	–	–	–	–

[a] Important postharvest diseases retrieved from Sholberg et al. (2004).

[b] 85.2% of all fresh fruits consumed per capita in the United States in 2004 (ERS, 2007).

TABLE 4.2
Bacterial Vegetable Pathogens[a]

	2004 Annual U.S. Per Capita Consumption (LBS)[b]	*Erwinia*	*Xanthomonas*	*Bacillus*	*Clostrilium*	*Pseudomonas*	LAB
Cucumbers	6.3	+	–	+	–	–	–
Spinach	2.1	–	–	–	–	–	–
Carrots	8.9	+	–	+	–	+	–
Corn, sweet	9.6	–	–	–	–	–	–
Onions	21.7	+	–	+	–	–	–
Lettuce, head	22.5	+	+	–	–	+	–
Lettuce, leaf	12.0	+	+	–	–	+	–
Mushrooms	2.6	–	–	–	–	+	–
Cabbages	8.3	+	+	–	–	+	–
Potatoes	46.5	+	–	+	+	+	–
Tomatoes	19.3	+	+	+	–	+	+
Broccoli	5.9	+	+	–	–	+	–

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

TABLE 4.3
Fungal Fruit Pathogens[a]

	2004 Annual U.S. Per Capita Consumption (LBS)[b]	*Penicillium*	*Geotrichum*	*Fusarium*	*Botrytis*	*Colletotrichum*	*Mucor*	*Monilinia*	*Rhizopus*	*Phtyophthora*
Citrus	22.7	+	+	–	–	+	–	–	–	+
Peaches	5.1	+	–	–	+	–	–	+	+	
Berries	6.1	+	–	–	+	+	+	+	–	+
Apples	18.8	+	–	–	+	+	+	+	–	
Pineapples	4.4	–	–	+	–	–	–	–	–	
Melons	14.7	–	–	–	–	–	–	–	–	
Bananas	25.8	–	–	+	–	+	–	–	–	
Pears	3.1	+	–	–	+	–	+	–	–	
Grapes	7.9	+	–	–	+	–	–	–	+	

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

storage conditions and storage time (longer 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.

Two wound pathogens, *P. expansum* and *B. 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

TABLE 4.4
Fungal Vegetable Pathogens[a]

	2004 Annual U.S. Per Capita Consumption (LBS)[b]	*Botrytis*	*Rhizopus*	*Phytophthora*	*Fusarium*	*Pythium*	*Alternaria*	*Colletotrichum*	*Geotrichum*	*Sclerotinia*
Lettuce, leaf	12.0	+	–	–	–	–	–	–	+	+
Corn, sweet	9.6	–	–	–	+	–	–	–	–	–
Carrots	8.9	–	–	–	–	–	–	–	+	+
Spinach	2.1	–	–	–	–	–	–	–	–	–
Cucumbers	6.3	–	+	–	+	+	–	+	–	–
Lettuce, head	22.5	+	–	–	–	–	–	–	+	+
Cabbages	8.3	–	+	–	–	–	+	–	–	+
Broccoli	5.9	+	+	–	–	–	+	–	–	+
Onions	21.7	+	–	–	+		+	+	+	–
Potatoes	46.5	–	+	+	+	+	–	–	–	–
Mushrooms	2.6	–	–	–	–	–	–	–	–	–
Tomatoes	19.3	+	+	+	+	–	+	+	+	+

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

cross-contaminate adjacent fruits. As shown in Figures 4.1 and 4.2, 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. *P. expansum* and *B. cinerea* 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.

Table 4.4 showed that the bacterium *E. carotovora* subsp. *carotovora* is a highly effective spoilage microbe to cause soft rot across a broad host range of vegetables and some fruits. One of the six known genera of soft-rot bacteria (including *Xanthomonas*, *Pseudomonas*, *Clostridium*, *Cytophaga*, and *Bacillus*), *E. carotovora* subsp. *carotovora* is one of the 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 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. 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., *P. fluorescens* and *P. 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 et al. (1988) revealed through several *P. viridiflava* mutants defective in pectate lyase expression that

FIGURE 4.1 Extensive blue mold infestation on apples.

FIGURE 4.2 Visible mold growth on cut strawberry.

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.

P. 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, *A. bisporus*. Similar to *P. fluorescens* and *P. viridiflava*, *P. tolaasii* produces siderophores that fluoresce under UV light. 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 et al. (1996) identified three pathotypes of mushrooms based on pathology and fatty acid analysis: *P. tolaasii* and *Pseudomonas gingeri*, which cause severe and yellowed lesions and *Pseudomonas reactans*, which causes a mild discoloration of the infected area.

4.3.1 Organic Fertilizers

Use of organic fertilizers, such as animal manures and slurries, abattoir wastes and sewage sludge introduce pathogens directly to the field, and run-off can contaminate irrigation water. Over 90 million tons of animal waste is put to land annually in the United Kingdom. There are comprehensive guidelines available to growers that advise on sufficient treatment of wastes and correct timing of application, with the aim of limiting contamination of crops. In the United Kingdom, these guidelines are set out in *The Safe Sludge Matrix* (ADAS) and the *Codes of Good Agricultural Practice* (Department of the Environment, Food and Rural Affairs). The Safe Sludge Matrix, for example, states that even when enhanced-treated sludge is applied to land, a 10-month harvest interval is necessary and the use of conventionally treated sludge requires a 30-month harvest interval for salad crops. These intervals should be sufficient to ensure the microbial quality of produce at harvest. Similar recommendations are set out in the U.S. Environment Protection Agency's Part 503 Biosolids Rule and Canadian Council of Ministers of the Environment guidelines.

4.3.2 Irrigation Water Quality

Fecal material, soil, and other inputs like sewage overflow introduce enteropathogens directly to watercourses from which irrigation water may be extracted. In the United Kingdom, 71% of irrigation water is obtained from surface waters, which receive treated sewage effluent. The potential for contamination via irrigation water is increased in the developing world, as untreated wastewater is used for irrigation of around 10% of crops. Wastewater irrigated crops show an increased incidence of enteropathogens. Wachtel et al. (2002) describe *E. coli* contamination of the roots of cabbage irrigated with sewage-contaminated stream water, although the edible part of the plant was unaffected. Islam et al. (2004) demonstrated that a single application of *Salmonella typhimurium* inoculated irrigation water resulted in contamination of carrot and radish at harvest, with *Salmonella* surviving for 203 days in soil postapplication. Lettuce plants irrigated with a single application of *E. coli* O157:H7 contaminated water tested positive for presence of *E. coli* O157:H7 at harvest (30 days postinoculation), and plants contaminated at days 7 and 14 of the study were shown to yield increased populations. Quantitative risk assessment models for the use of reclaimed water show that risk varies between crops, with lettuce found to pose a higher risk than cucumber, but comparable to that of broccoli and cabbage. The interval between irrigation and harvest will affect the likelihood of pathogenic bacteria surviving to reach the consumer. A survey of UK-based salad vegetable producers showed that over 50% growers will harvest baby-leaf crops within 24 h of the last irrigation.

A number of outbreaks have been traced to the use of contaminated water in irrigation. Iceberg lettuce imported from Spain during 2005 caused cases of *S. typhimurium* throughout the United Kingdom and Finland, after wastewater was used to irrigate the crop. Cases of *E. coli* O157:H7 in Sweden in 2005 were traced back to lettuce irrigated with water from a stream contaminated with cattle feces. Water may also act as a vehicle for the dissemination of viral particles. Beuchat (1996) reports an atypical outbreak of norovirus linked to celery and irrigation with sewage-contaminated water has resulted in the outbreaks of hepatitis A linked to lettuce consumption and spring onions. Hilborn et al. (1999) describe an outbreak of *E. coli* O157:H7 attributed to mesclun lettuce, assumed to be irrigated with water contaminated by cattle grazing a nearby field. Solomon et al. (2002) showed that *E. coli* O157:H7 in contaminated water can enter the vascular system of lettuce and reach the edible parts of the plant, although the authors point out that unrealistic inoculum concentrations were used.

4.3.3 Soil

Pathogens may be naturally present in soil, for example, *Listeria* spp., or may become incorporated in the soil matrix from organic wastes added as fertilizer. Pathogens within soil may contaminate crops directly when heavy rain or water gun irrigation causes leaf splash. The ability of the

pathogen to survive in the environment will impact on the likelihood of crop contamination and pathogen viability at harvest and through to consumption. Initially, the pathogen must survive in the propagation environment until crops are planted out, or in organic wastes applied to the land. Table 4.5 lists survival times for each enteropathogen from a number of studies.

Survival times are often inconsistent and reflect the variability in propagation environments and organic waste treatments. Kudva et al. (1998) demonstrated that aeration of ovine manure decreased survival of *E. coli* O157:H7 from >365 to 120 days. The application method used for organic wastes may increase survival time: clumping of material applied above ground, and injection application of liquid manures can protect bacteria from desiccation and high temperatures. Stresses encountered during passage through the gut, for example, the acidity of the environment, may increase survival by inducing entry to survival stages. *E. coli* and *Salmonella* will exhibit the general stress response, producing a range of stress proteins that can confer cross-resistance to a range of stresses. Cross-protection mechanisms may extend bacterial survival in the environment, by reducing the impact of abiotic factors. Leyer and Johnson (1993) report that after acid adaptation, *S. typhimurium* displayed increased tolerance of heat and osmotic stress, while Hartke et al. (1995) demonstrated that preir-radiation of *Lactococcus lactis* increased resistance to lethal challenges of acid. The stress response of *L. monocytogenes* is similar to that of *E. coli*, but is regulated by the sigma factor r B, which has been suggested to increase virulence. *Campylobacter* may enter a viable but nonculturable stage, but the main mechanism of survival is production of large numbers of cells within the host. Seasonal variation in shedding of pathogens can result in higher than expected microbial loads in fecal material: *Campylobacter* shedding increases in spring and autumn and *E. coli* levels during spring and summer. If increased pathogen loads are present, then simply following guidelines may not be sufficient for preventing crop contamination.

TABLE 4.5
Survival Time of Enteropathogens in the Field Environment

Pathogen	Environment	Survival (day)	References
Escherichia coli O157:H7	Soil + animal manure	30	Nicholson et al. (2005)
E.coli O157:H7	Soil + animal manure	99	Nicholson et al. (2005)
E.coli O157:H7	Animal manure	60	Avery et al. (2005)
E.coli O157:H7	Slurries	60	Avery et al. (2005)
E.coli O157:H7	Abattoir waste	60	Avery et al. (2005)
E.coli O157:H7	Sewage sludge	60	Avery et al. (2005)
E.coli O157:H7	Nonaerated ovine manure	>365	Kudva et al. (1998)
E.coli O157:H7	Aerated ovine manure	120	Kudva et al. (1998)
E.coli O157:H7	Nonaerated slurry	600	Kudva et al. (1998)
E.coli O157:H7	Aerated slurry	30	Kudva et al. (1998)
E.coli	Slurry + dirty water	90	Nicholson et al. (2005)
Salmonella	Soil	968	Nicholson et al. (2005)
Salmonella	Soil + bovine slurry	300	Nicholson et al. (2005)
Salmonella	Soil + animal manure	30	Nicholson et al. (2005)
Salmonella	Slurry + dirty water	90	Nicholson et al. (2005)
Campylobacter	Soil + animal manure	30	Nicholson et al. (2005)
Campylobacter	Slurry + dirty water	90	Nicholson et al. (2005)
Listeria	Soil + animal manure	30	Nicholson et al. (2005)
Listeria	Slurry + dirty water	180	Nicholson et al. (2005)
L. monocytogenes	Soil + sewage sludge	56	Everis (2004)
Hepatitis A	Water	>365	Seymour and Appleton (2001)
Hepatitis A	Soil	96	Seymour and Appleton (2001)

4.3.4 Survival in the Phyllosphere

Interest is shifting toward the fitness of the enteropathogen on the leaf surface (phylloplane): if a pathogen can persist on the phylloplane, then the chance of an infectious dose remaining at consumption is increased. Beuchat (1999) showed that *E. coli* O157:H7 contained in bovine feces and inoculated onto lettuce could be isolated from lettuce up to 15 days after inoculation. Fett (2000) suggested that transient occupants of the leaf, such as enteropathogens, may become incorporated into phylloplane biofilms.

Biofilms are complex structures composed of many species of bacteria, filamentous fungi, and yeasts, with 10^6–10^8 cells/fresh weight/g. Cells are enclosed within an exopolymeric matrix, which can buffer environmental changes such as nutrient stress and desiccation; therefore, bacteria within biofilms will have an increased survival rate. Between 30% and 80% of the total bacterial population on a leaf surface will be contained in these aggregates, which tend to be associated with sources of nutrients such as leaf veins and glandular trichomes. Fett (2000) showed that biofilms were present on the cotyledons, hypocotyls, and roots of alfalfa, broccoli, sunflower, and clover sprouts, by 2-day postgermination.

Enteropathogens can adapt to the phyllosphere environment, but may be outcompeted by epiphytic bacteria, especially if both species compete for the same carbon source. Interactions between immigrant bacteria and epiphytes are diverse: *Salmonella enterica* has been demonstrated to aggregate with *Pantoea agglomerans* on the leaf surface of cilantro and *Wausteria paucula* was shown to support actively the survival of *E. coli* O157:H7 in the rhizosphere and leaf surface of lettuce. Barak et al. (2002) demonstrated that *Salmonella newport* attached to alfalfa sprouts as efficiently as the plant-associated bacteria *Pseudomonas putida, P. Agglomerans*, and *Rhanella aquatilis*, and significantly better than *E. coli* O157:H7. However, epiphytes may also limit survival of immigrant bacteria; Cooley et al. (2006) demonstrated that *S. newport* and *E. coli* O157:H7 could be outcompeted on lettuce by *Enterobacter asburiae*, repressing growth of the enteropathogens 10-fold. Carlin et al. (1996) demonstrated that the background flora present on endive prevented the growth of *L. monocytogenes*. These results suggest that there is potential for the naturally occurring microflora to be used as a biocontrol agent, to prevent enteropathogenic bacteria becoming established on the leaf.

Solomon and Matthews (2006) showed that bacterial processes, such as gene expression, motility, or production of extracellular compounds, were not necessary for initial attachment but are likely to be important in further colonization and survival on the leaf. Plant-associated bacteria produce acyl-homoserine lactones (AHLs) for communication via quorum sensing, and Brandl (2006) hypothesizes that AHLs may help upregulate factors in enteropathogens beneficial to their survival on the leaf, such as expression of *rpoS* which increases resistance to stresses commonly encountered on the leaf, for example, desiccation.

A major factor in limiting bacterial survival in the phyllosphere is UV radiation. Ecologically successful phylloplane bacteria are efficient in UV-induced deoxyribonucleic acid (DNA) damage repair or preferentially colonize sites that are protected from UV, such as within the interior of a leaf (phytopathogens) or at the base of structures such as trichomes (saprophytes). The biofilm matrix also shields against the damaging effects of UV irradiation. Phyllosphere communities exhibit a marked shift toward UV-tolerant phenotypes, for example, pigmented bacteria, as the growing season progresses. In *Pseudomonas syringae*, expression of the gene *rulAB* confers DNA repair capabilities and therefore increased UV tolerance. *E. coli* and *S. enterica* possess homologues of *rulAB*, suggesting an ability to withstand UV irradiation. Enteropathogens encounter osmotic stress frequently when passing through the host gut, and consequently display a number of stress-avoidance mechanisms, mediated by *rpoS*, which may induce cross-resistance to stresses encountered on the leaf (Brandl, 2006). For this to impact on survival, pathogens would have to become established on the leaf surface relatively and quickly after excretion from the host. Exposure to plant-produced antimicrobials upregulates a homologue of the sap operon in *E. chrysanthemi*. In *S. enterica*,

induction of the sap operon promotes acid resistance and therefore survival in the acidic conditions of the gut; Brandl (2006) suggests that, therefore, a period of residence in the phyllosphere may lead to increased virulence of enteropathogens.

Further protection from environmental stresses may be afforded by movement into the internal tissue of the plant. This is normally a passive process, unlike the destructive entry of many phytopathogens: enteropathogens in irrigation water can be taken up by the root systems and enter the edible portion of the crop, for example, lettuce, apple, and tomato. Enteropathogens may also gain entry via wounds, or structures such as lenticels and stomata. Infiltration of enteropathogenic bacteria through these structures can occur when bacteria are present in water on the surface of fruits and can be increased during processing if wash water is of a lower temperature than the fruit, creating a negative temperature differential.

The presence of phytopathogens may increase the penetration and growth of enteropathogenic bacteria, because of disruption of the cuticle and increased release of nutrients. Richards and Beuchat (2005) report that coinoculation of wound sites on cantaloupe with *Salmonella Poona* and the phytopathogens *Cladosporium cladosporiodes* or *P. expansum* increased penetration of *Salmonella* into the internal tissues of the fruit, because of the tissue breakdown caused by the fungi. Oron et al. (1995) demonstrated that poliovirus applied to the roots of tomato plants can be recovered from the leaves, but not the fruit. This was attributed to the presence of antiviral substances and not to an inability of the virus to reach the fruit. Dingman (2000) analyzed the proliferation of *E. coli* O157:H7 in bruised apple tissue and observed that growth was suppressed in McIntosh apples, unlike the other cultivars used. This was thought to be due to production of an unstable or volatile inhibiting factor, as the effect was reduced during storage. Reinders et al. (2001) studied the effect of caffeic acid on *E. coli* O157:H7 survival in a model apple juice medium and demonstrated a reduction in *E. coli* populations, suggesting that phenolic acids play an important role in limiting the bacterial survival in planta. A range of phenolic acids, including caffeic acid, were shown to inhibit the survival of *L. monocytogenes* in vitro and Delaquis et al. (2006) report evidence that an antilisterial factor, thought to be of a phenolic nature, is produced by wounding (shredding) of iceberg lettuce and therefore is likely to play a role in limiting *L. monocytogenes* growth in bagged salads.

The interaction between the host plant and epiphytes, symbionts and phytopathogens has been extensively studied. However, the role of plant–microbe interactions in limiting the colonization by enteropathogenic bacteria is not so well described. Production of antimicrobial factors may be a direct response to the presence of pathogenic bacteria. Barak et al. (2004) showed that virulence factors, including aggregative fimbriae and expression of *rpoS*, were involved in *S. enterica* attachment to alfalfa. Interestingly, these virulence genes are essential for infection in an animal host. There are a number of similarities between the systems employed by plant and animal pathogens. The type III secretion system (TTSS), which enables the delivery of pathogenicity proteins to the host cell, is conserved across the Gram-negative plant and animal pathogens, although the proteins secreted differ. Recognition of elements of the TTSS in phytopathogens causes induction of host plant defense mechanisms. The conserved nature of these factors in human pathogens suggests that plants may also respond to the presence of enteropathogenic bacteria in the phyllosphere. Iniguez et al. (2005) describe the role of host defenses in limiting endophytic colonization by the enteric bacterium, *S. typhimurium*. Addition of ethylene, the signal molecule which induces systemic resistance, decreased *Salmonella* colonization of the roots of the legume *Medicago trunculata*. Use of *Salmonella* mutants, deficient in structural components of the TTSS or flagella, showed increased colonization of the roots of alfalfa, and ethylene production was reduced, suggesting that the plant did not recognize the pathogen. Further colonization studies using an *Arabidopsis thaliana* mutant (*npr1*) provided evidence that recognition of TTSS components induces salicylic acid-mediated defense signaling. The authors suggest that overexpression of defense-related genes in crop plants may present a novel method to control enteropathogen colonization in the field.

4.4 DETECTION AND EVALUATION METHODS FOR SPOILAGE MICROORGANISMS

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 or vegetables. 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 handshaking 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, and reportedly provides a high rate of recovery of viable microbes from the sample (Wu et al., 2003).

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. During a comparison of total viable bacterial cell and total coliform, recovered from samples of 30 different fresh vegetables, with the Stomacher and with the Pulsifier, there were apparently no differences in viable recovery between the two methods (Vishwanathan and Kaur, 2001). 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 qualitative analysis.

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. A plethora of literature exists that describe routine diagnosis of fungal and bacterial diseases of fresh fruits and vegetables. Many spoilage microbes develop very distinctive lesions depending on 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* is a two-volume set of text that provides a comprehensive review of the biology, epidemiology, and physical appearance of a large number of fruit and vegetable spoilage microorganisms (Snowdon, 1990). Included are several photomicrographs of the pathogens described, illustrating their appearance as single cells, mycelia, or fruiting bodies. "*Basic Plant Pathology Methods*" is a very comprehensive reference text that

includes not only methods for enrichment, isolation, and identification of most plant pathogens, but also provides information 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 (Dhingra and Sinclair, 1985).

Lactic acid bacteria, yeasts, and 48 strains of pectinolytic bacteria of the genera *Pseudomonas*, *Erwinia*, *Bacillus*, *Xanthomonas*, and *Flavobacterium* has been reportedly isolated from baby carrots (Liao and Wells, 2007). Bacteria isolated from "Nam Dokmai" mango cubes were predominantly Gram-negative rods of which about 60% were *Enterobacteriaceae*, including the genera *Klebsiella* and *Pantoea* (Poubol and Izumi, 2005). Phytopathogenic bacteria that cause rot in vegetables such as *P. 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*. One of the most common types of spoilage microbes associated with cut melon was *Pseudomonas* sp., *E. coli*, *Enterobacter* sp., and *Micrococcus* sp. were the predominant microflora on sliced watermelon (Ukuku and Sapers, 2005). 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.

During an assessment of microbiological quality of fresh vegetables collected from several regions, the microbiological quality of fresh vegetables ranged from 4.3 to 10.4 $\log_{10}$ CFU/g (aerobic bacteria); 2.0 to 0.71 $\log_{10}$ CFU/g (coliforms); 1.0 to 8.77 $\log_{10}$ CFU/g (*E. coli*); and 1.47 to 8.77 $\log_{10}$ CFU/g (*S. aureus*) (Halablab et al., 2010). Lettuce samples had significantly higher microbial loads including coliforms, *E. coli*, and *S. aureus* than parsley samples collected from different locations in Bekaa Valley. Neither *E. coli* nor *S. aureus* had been detected on Malva samples. In addition, Bar Elias had higher microbial loads, coliform, *E. coli* and *S. aureus* than any other location investigated in Bekaa Valley. Moreover, *E. coli* was significantly higher in lettuce samples (42.30%) than in parsley samples (13.8%) and *S. aureus* was significantly more often detected in lettuce samples (51.5%) than in parsley samples (38%).

4.5 CONTROL, PREVENTION, AND MANAGEMENT OPTIONS FOR SPOILAGE MICROORGANISMS

4.5.1 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. 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.

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 like high temperature, provides the three components required for disease expression such as host, environment, and pathogen. Therefore, spoilage management should begin in the field using an integrated strategy of GAPs. Balanced crop nutrition influences susceptibility to spoilage. For example, Sugar et al. (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. 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%. Other preharvest fungicides are also available and several new fungicides are under development. 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.

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. The developing appressorium ramifies through these protective layers and into the pulp through a combination of mechanical pressure and tissue destruction by extracellular enzymes. 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.

4.5.2 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. 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 like 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. 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.

4.6 CONCLUSION AND FUTURE TRENDS

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 held at 10°C and fresh-cut celery 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°C versus 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 food service 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. A purified ethanolic extract of peeled and shredded carrots had an antimicrobial effect against a wide range of microorganisms, including *Leuconostoc 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. 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.

ACKNOWLEDGMENT

This work was supported by the National Science and Technology Support Program of China (2015BAD16B06).

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Section II

5 Spoilage Microorganisms in Meat Products

Wangang Zhang and Yimin Zhang

CONTENTS

5.1 INTRODUCTION

Current food microbiology research often focuses on hazards caused by pathogenic microorganisms to humans while neglecting food spoilage (Mohareb et al., 2015). Spoilage of chilled raw meat remains a major challenge to the meat industry, because meat spoilage causes large losses every year. The original muscle tissue of healthy animals is sterile; however, a large number of microorganisms exist in cuts that occur during a series of processing procedures from muscle to meat after slaughter (Sofos, 1994). Subsequent storage and sales processes are

also associated with the reproduction of spoilage microorganisms until the end of the shelf life of the meat. Therefore, the common goals of the meat industry and meat microbiologists are to determine the origin, the classification, and the distribution characteristics of those meat spoilage-related microorganisms and the factors affecting their growth are also considered. Then, the ultimate purpose is to achieve the accurate and rapid identification, and effective control of these microorganisms.

Numerous studies have been done on microbial contamination in large-scale cattle, hog, and sheep slaughterhouses in developed countries (Gill, 2005; Gill and Bryant, 1992; Sofos et al., 1999). However, in some developing countries such as China, although some of the hog slaughtering houses are developed well to automation, most cattle slaughtering houses are small-scale operations, and no more than 100 cattle slaughtering houses have a daily throughput of more than 200 cattle (Luo and Cao, 2011). Here, the contamination and distribution of spoilage microorganisms in the animal slaughter environment and during subsequent meat sales and distribution might differ from previous findings. Therefore, this chapter focuses on the current status of microbial contamination, the type of spoilage organisms involved, and the corresponding countermeasures adopted in red meat producers in developing countries. On the basis of the existing literature, a further review is given to summarize the characteristics of red meat spoilage, possible spoilage mechanisms, and the methods used to detect and quantify spoilage microorganisms.

At the same time, we should pay attention to the development of the meat microbiology. Research on this area has continued to broaden, and great progress has been made since the community first began to express concern about meat spoilage and associated microorganisms in the 1970s. For instance, only some types of microorganisms are known to occur in meat in the early days, whereas hundreds of microbial species are currently identified (Zhao et al., 2015), and the detection of the spoilage microorganisms at strain level has become an important issue recently (Doulgeraki et al., 2012). Research on the spoilage mechanisms of microorganisms has extended from the metabolic activity of individual organisms to the synergism, antagonism, and quorum sensing exhibited by multiple microorganisms (Nychas et al. 2008, 2007). Concerning the detection and evaluation of spoilage microorganisms, researchers previously have used a laborious plate count technique to isolate and screen spoilage organisms from meat; however, polymerase chain reaction (PCR)–based molecular and spectroscopic techniques are currently available for the rapid detection of them and allow for tracking of microbial origins (Ercolini et al., 2007; Pennacchia et al., 2009; Doulgeraki et al., 2012; Kamruzzaman et al., 2015). Furthermore, researchers have also explored spoilage gene markers in spoilage organisms (Mohareb et al., 2015). For the control of spoilage microorganisms, antimicrobial measures by single chemical or physical means have been evolved to the present establishment of management control systems including Good Manufacturing Practices (GMP), Sanitation Standard Operating Procedures (SSOP), Hazard Analysis and Critical Control Points (HACCP), and Food Safety Objectives (FSO) (Koutsoumanis et al., 2006). All the aforementioned issues are also summarized and discussed in Sections 5.2, 5.3, 5.4, and 5.5.

5.2 CLASSIFICATION OF SPOILAGE MICROORGANISMS IN MEAT PRODUCTS

5.2.1 Source of Microbial Contamination

As known, the animal hides and contaminants attached are two major origins of microorganisms in the slaughterhouse, and consequently contaminated the carcasses (Gill, 2004, 2005; Koutsoumanis et al. 2006; Byrne et al., 2000; Bell, 1997). The level of visible dirt on cattle hair can affect the number of microorganisms on the carcass (Byrne et al., 2000). The contaminants include feces, soil, waste, and others that can be transferred onto the animal hide during animal feeding, transportation, lairage, and so on (Biss and Hathaway, 1996), and then have an effect

on the hygienic conditions of the carcasses. Researchers have investigated visual cattle cleanliness and correlation to hide and carcass microbial contamination, and data obtained from some Chinese cattle slaughtering houses showed that the numbers of the microbial load on the hide and on the corresponding carcasses were significantly affected by the degree of visible dirt of the cattle hide (Table 5.1). As the visible dirt degree increased from 1 to 5, the total number of bacterial colonies on the cattle hides and carcasses increased. The number increased from 4.88 to 5.74 $\log_{10}$ CFU/cm^2 for the cattle hide and from 2.85 to 3.60 $\log_{10}$ CFU/cm^2 for the corresponding carcasses. Coliforms have been considered as the indicator organisms of carcass contamination in some countries. The numbers of coliforms on the carcass and cattle hide also increased as the degree of visible dirt on cattle hides increased, which was in agreement with the former research on cattle and sheep carcasses (Byrne et al., 2000; Hadley et al., 1997; Gill and Landers, 2004). Therefore, microbial contamination of the carcass begins when the knife could be cutting through the microorganism-bearing hide for bloodletting. Subsequently, almost each of the following steps of the slaughtering procedure would give a further contamination to the carcass (Koutsoumanis et al., 2006; Zhang et al., 2011).

The legs, buttocks, chest, and abdomen are contaminated successively with the removal of the hooves and the preskinning of the rear and front legs, as knife incisions for the removal of animal hides introduces bacteria from the hide onto the underlying tissue (Koutsoumanis et al., 2006). At this point, the skinning knife and operating hands become the greatest sources of microbial contamination. The number of microorganisms reaches as high as 7.34 $\log_{10}$ CFU/cm^2 on skinning knives in certain slaughterhouses (Yu, 2012). As the completion of dressing, the entire carcass is exposed to the air, which makes it more vulnerable to contamination. In some Chinese cattle slaughterhouses, the total visible counts of the carcass is 2.7–4.0 $\log_{10}$ CFU/cm^2 (Zhang et al., 2011) and the coliforms is in the range of 0.57–2.37 $\log_{10}$ CFU/cm^2 (Zhang, 2011). In the Northern America, most animal slaughterhouses applied water/steam/organic acid spraying to wash the entire carcass after dressing and before eviscerating resulting in a significant bacteria reduction (Gill and Bryant, 1997; Gill and Landers, 2003; Sofos, 2002). Currently, the automatic spraying equipment is not sufficient in China. Most of the cattle slaughterhouses apply the spray procedure with lactic acids solutions or apply water spraying alone at the end of the slaughter line. As for the hog, the average bacteria distribution on the carcass is 4.2 $\log_{10}$ CFU/cm^2 during the slaughter while the number of coliforms is 2.06 $\log_{10}$ CFU/50 cm^2 in dressing (Wang, 2006). And dressing is rarely used before slaughter for hog; however, the depilation machine is a major source of microbial contamination (Gill and Jones, 1995). Li (2006) found that the microbial diversity of the hog carcasses decreased after scalding.

Eviscerating after dressing is also a critical operation. Leakage of the gastrointestinal contents will contaminate the carcass greatly. In this stage, both cutting tools and operating hands are major sources of microbial contamination, and the total number of bacterial colonies has been reported to be approximately 5 $\log_{10}$ CFU/cm^2 in some Chinese slaughterhouses. As mentioned, in the developed countries, the carcass is often washed with high-pressure water, hot steam, and an organic acid spray between dressing and evisceration (Sofos, 2002). At this point, microorganisms from the air and other origins have not yet firmly attached to the carcass, and the bacteria-reducing effect will be evident (Hamby et al., 1987; Cabedo et al., 1996; Dickson and Anderson, 1992). In China, spraying immediately after skinning is not used and it is more common to spray the carcasses with clean water before the carcass enters the chilling room. Few slaughterhouses use the clean water, steam, and organic acid spray device in China. Prior to spraying with water, the total number of bacterial colonies on beef (chuck, brisket, back, and buttocks) is reported to range from 2.6 to 4.4 $\log_{10}$ CFU/cm^2. Spraying with clean water alone does not significantly reduce the number of microorganisms on the carcass surface; instead, it redistributes the existing microorganisms on the carcass surface. After spraying with clean water, the total number of bacterial colonies counted on meat at the above four positions is reportedly to reach 3.0 $\log_{10}$ CFU/cm^2 (Xu, 2013).

TABLE 5.1
Visual Evaluation of Cattle Cleanliness and Correlation to Hide and Carcass Microbial Contamination

Pollution Level	Cattle Hides						Carcasses					
	TVC			Coliforms			TVC			Coliforms		
	Minimum	Means	Maximum	Minimum	Means	Maximum	Minimum	Means	Maximum	Minimum	Means	Maximum
1	3.79	4.88 ± 0.50^{c}	5.60	1.30	2.28 ± 0.43^{c}	3.23	2.14	2.85 ± 0.29^{c}	3.32	0.00	0.23 ± 0.28^{c}	0.88
2	4.70	5.57 ± 0.41^{a}	6.22	1.89	2.68 ± 0.46^{b}	3.57	2.77	3.28 ± 0.28^{b}	3.81	0.08	0.51 ± 030^{bc}	0.91
3	4.00	5.13 ± 0.58^{bc}	5.99	1.47	2.32 ± 0.52^{c}	3.60	2.00	2.95 ± 0.44^{c}	3.53	0.00	0.26 ± 0.42^{c}	1.26
4	4.48	5.42 ± 0.57^{ab}	6.47	2.26	2.87 ± 0.54^{b}	4.08	2.76	3.18 ± 0.45^{b}	4.12	0.25	0.79 ± 0.33^{b}	1.32
5	4.00	5.74 ± 0.69^{a}	7.33	2.32	3.40 ± 0.58^{a}	4.52	2.81	3.60 ± 0.36^{a}	4.30	0.23	1.28 ± 0.63^{a}	2.95

Source: Data from Xu, 2013.

Notes: Carcass sampling parts were foreleg, buttock, abdomen, chest, hind leg, neck. Means within the columns with different letters differ at $p < .05$.

After chilling, both the number and diversity of contaminant microorganisms on the carcass can decrease. However, during the cutting stage, work-contact surfaces, cutting tools, and operating hands become the major sources of microbial contamination; this is particularly true of the conveyor belt that carries meat obtained from various parts of the carcass (Gill and Jones, 1995, Gill et al., 1999). Available data have revealed the contamination of work-contact surfaces at various steps in the cutting department of three medium-sized cattle slaughterhouses in China (Table 5.2). The knives and hands related with boning and trimming are the seriously contaminated area with the total viable counts at the range from 4.69 to 6.92 $\log_{10}$ CFU/cm^2 (Yu, 2012). And the number on those surfaces is increasing as the processing time extends allowing the work-contact to be a contaminated source (Xu, 2013). The data obtained from the hog cutting room show the number of the total bacteria on the meat cuts is above 5.70 $\log_{10}$ CFU/cm^2 and the coliforms is above 2.70 $\log_{10}$ CFU/cm^2 (Wang, 2006).

Meat cuts are packaged and delivered to the consumer market. Thereafter, meat products will not suffer further contamination if the meat is not subcut further or the packaging remains intact. However, various factors, including the packing conditions and storage temperature can affect the shelf life and spoilage level of the meat.

In summary, contaminant microorganisms on meat products as received by consumers originate from the animal hides and the work-contact surface during various processing procedures in the slaughterhouses.

5.2.2 Classification of Spoilage Microorganisms in Meat Products

The spoilage of meat includes enzymatic and nonenzymatic changes that occur in the composition and sensory quality of meat. These changes are due mainly to microorganisms and other factors and result in lower quality or inedible meat. Essentially, meat spoilage is a process in which spoilage microorganisms grow and reproduce during extended storage times, and the organisms metabolize protein, fat, carbohydrates, and other nutrients in the meat produce many metabolites.

The types of spoilage microorganisms present in meat are closely associated with the processing environment as muscle tissue is processed to food and subsequent storage and sales environments. Furthermore, the types of spoilage microorganisms present are also relevant to the types and chemical components of meat. The spoilage of cooked meat products is related to processing and cooking conditions as well as storage conditions.

TABLE 5.2
Total Viable Counts of the Environments in Three Different Beef Slaughter Plants

	TVC log (CFU/cm^2)		
Sampling Points	**Plant A**	**Plant B**	**Plant C**
Boning hand	6.63 ± 0.113[a]	5.60 ± 0.007[b]	5.58 ± 0.064[b]
Boning knife	4.69 ± 0.021[c]	6.54 ± 0.021[b]	6.92 ± 0.078[a]
Boning apron	4.69 ± 0.021[a]	4.41 ± 0.057[b]	4.22 ± 0.092[b]
Boning chopping board	4.52 ± 0.078[a]	4.53 ± 0.028[a]	4.46 ± 0.042[a]
Trimming hand	6.49 ± 0.057[a]	5.82 ± 0.049[b]	5.31 ± 0.092[c]
Trimming knife	5.79 ± 0.021[b]	5.62 ± 0.028[c]	6.11 ± 0.057[a]
Trimming apron	5.86 ± 0.035[a]	3.50 ± 0.028[b]	2.71 ± 0.078[c]
Trimming chopping board	5.09 ± 0.035[a]	4.54 ± 0.007[b]	3.10 ± 0.042[c]
Packaging contact surfaces	5.39 ± 0.049[a]	2.97 ± 0.127[c]	4.32 ± 0.057[b]
Belt conveyor	4.01 ± 0.134[a]	4.30 ± 0.120[a]	3.33 ± 0.163[b]

Source: Data from Yu, 2012.

[a, b, c] The right side of the same list shows a significant difference between samples ($p < .05$).

Spoilage microorganisms associated with red meat spoilage mainly include bacteria, molds, and yeasts. Bacteria are responsible for some of the most rapid and evident spoilage of the proteinaceous meat (Huis in 't Veld, 1996). Molds and yeasts preferentially grow on raw meat during late storage and on cooked meat products with low moisture contents. Therefore, bacteria are considered the main organisms that cause the spoilage of fresh raw meat. Various bacteria exist in meat, and more than 200 species of bacteria have been found in vacuum-packed pork (Zhao et al., 2015). However, not all bacteria that are present in meat can cause spoilage. Under normal conditions, it is believed that approximately 10% of initial contaminant bacteria can survive in chilled meat during storage, and only a small fraction of these play a role in spoilage (Borch et al., 1996). The main spoilage bacteria are also known as specific spoilage organisms (SSO) or ephemeral spoilage organisms (ESO) (Nychas et al., 2008) and those SSOs and ESOs play the same role in the spoilage of meat no matter the scales and/or the locations of the producing plants.

Spoilage bacteria in red meats include Gram-negative *Pseudomonas*, *Acinetobacter*, *Psychrobacter*, *Aeromonas*, *Shewanella putrefaciens*, and Enterobacteriaceae and Gram-positive lactic acid bacteria (LAB) and *Brochothrix thermosphacta* (Ercolini et al., 2011; Borch et al., 1996; Barakat et al., 2000; Ercolini et al., 2006, 2009). The growth of these spoilage microorganisms is closely associated with storage conditions. *Acinetobacter*, *Psychrobacter*, and *Moraxella* grow well under aerobic conditions while *Pseudomonas* spp. are dominant spoilage bacteria (Molin and Ternstrom, 1986; García-Lopez et al., 1998), whereas LAB and *B. thermosphacta* commonly occur under anaerobic or modified atmosphere packaging conditions (Rattanasomboon et al., 1999; Barakat et al., 2000). Casaburi et al. (2015) have listed the microorganisms that are associated with red meat spoilage (Table 5.3). Detailed reviews on the origin, classification, and characteristics of the listed microorganisms are available at the genus level in many books and research reports. Here, the discussion focuses on meat-related bacterial species in relevant genera.

5.2.2.1 *Pseudomonas*

Pseudomonas is a genus of strictly aerobic Gram-negative, motile, straight, or curved bacilli. Members of this genus are characterized by the ability to reproduce rapidly, grow at low temperatures, and produce large amounts of ammonia and other spoilage products. This genus widely occurs in water, humans, soil, and on hides, mouth, and intestines of animals. And they exist in lots of food products.

Pseudomonas species are typically psychrophiles. Since the 1980s, this group of bacteria has frequently been isolated from chilled meat (Molin and Ternstrom, 1982, 1986; Shaw and Latty, 1982; Labadie, 1999; Gill, 2003; Stanbridge and Davies 1998). The genus is subdivided into five rRNA similarity groups and the most relevant species involved in meat spoilage are located in group I including *Pseudomonas fragi*, *Pseudomonas lundensis*, *Pseudomonas fluorescens*, and *Pseudomonas putida* (Nychas et al., 2007a, 2007b, 2008). Among those species, *Ps. fragi* is the dominant spoilage bacterium in chilled meat under aerobic storage conditions with a great incidence on spoilt meat in the range from 56.7% to 79.0% (Nychas et al., 2008), while the isolation rate of *Ps. lundensis* is up to 40% sometimes (Liao, 2006). *Pseudomonas* can fully use carbon and energy sources in meat and produce a series of metabolites to cause the meat spoilage (Casaburi et al., 2015). *Pseudomonas* contamination of meat and meat products often leads to surface spoilage, forming slime, and an unpleasant odor, especially, *Ps. fragi* can develop a "fruity sour smell" in meat (Liao, 2006). Due to the spoilage potential of this genus, it is used as target bacteria to establish the shelf-life prediction model of chilled meat (Zhang et al., 2011).

5.2.2.2 Lactic Acid Bacteria

LAB comprise a class of Gram-positive, facultative anaerobic bacteria that can metabolize fermentable carbohydrates and produce a large amount of lactic acid. LAB are extremely widely distributed in nature and exhibit great species diversity. The growth temperature of LAB covers a broad range from lower than 4°C to 45°C (Schillinger and Holzapfel, 2006). This group of bacteria is complex and includes at least 18 genera and more than 200 species, but few LAB types can cause meat spoilage.

TABLE 5.3
Genera of Bacteria Commonly Found in Raw Meat Stored in Different Conditions

	Storage Conditions				Storage Conditions		
Gram-Positive	**Air**	**MAP**	**VP**	**Gram-Negative**	**Air**	**MAP**	**VP**
Bacillus	+		+	*Achromobacter*	+		
Brochothrix	+	+	+	*Acinetobacter*	+	+	+
Carnobacterium	+	+	+	*Aeromonas*	+		+
Corynebactenum	+			*Alcaligenes*	+	+	+
Clostridium			+	*Alteromonas*	+	+	+
Enterococcus	+	+		*Campylobacter*	+		
Kocuria	+			*Chromobacterium*	+		
Kurthia	+			*Citrobacter*	+	+	
Lactobacillus	+	+	+	*Enterobacter*	+	+	
Lactococcus	+			*Escherichia*	+		
Leuconostoc	+	+	+	*Flavobacterium*	+		
Listeria	+	+		*Hafnia*	+	+	+
Microbacterium	+	+	+	*Klebsiella*	+		
Micrococcus	+	+		*Kluyvera*	+		
Paenibacillus	+			*Moraxella*	+		
Staphylococcus	+	+	+	*Pantoea*	+		+
Streptococcus	+	+		*Proteus*	+	+	
Weissella	+	+	+	*Providencia*	+	+	+
				Pseudomonas	+	+	+
				Serratia	+	+	+
				Shewanella	+		
				Vibrio	+		
				Yersinia	+		+
				Moraxella	+		

Source: Adapted from Casaburi, A. et al., *Food Microbiol*., 45(Pt A), 83–102, 2015.
Note: VP, vacuum packaging; MAP, modified atmosphere packaging.

LAB are dominant spoilage bacteria under anaerobic conditions and are more commonly found in meat stored at low temperatures and in vacuum and modified atmosphere packaging. LAB cannot only resist the antimicrobial effects of nitrite and smoke in processed meat products but also tolerate high concentrations of salt solution (Korkeala et al., 1992). These bacteria can ferment carbohydrates in meat, resulting in sour, cheesy, and liver-like smells, which are sometimes accompanied by the generation of carbon dioxide. Gas production easily slackens vacuum packaging or causes "blown pack," negatively affecting the appearance of meat products. Various LAB have been associated with meat spoilage, including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Carnobacterium*, *Weissella*, *Pediococcus*, and *Enterococcus* (Schillinger and Holzapfel, 2006; Pothakos et al., 2015). *Carnobacterium* spp., *Lactobacillus* spp., and *Leuconostoc* spp. are common LAB in meat and meat products and the grown number can be up to densities of 10^7 CFU/cm^2 (Shaw and Harding, 1984; Borch and Molin, 1988). *Lactobacillus sakei*, as a dominant spoilage bacterium in vacuum-packed processed meat products (Mäkelä et al., 1992; Schillinger and Holzapfel, 2003), can generate hydrogen sulfide and thereby cause the greening of meat products (Eagan et al., 1989). *Lactobacilli* and *leuconostocs* can produce gas through heterofermentation (Korkeala and Lindroth, 1987; Ahvenainen et al., 1990; von Holy et al., 1991). Within the genus *Lactobacillus*, both *Lactobacillus algidus* (Kato et al., 2000) and *Lactobacillus fuchuensis* (Sakala et al., 2002) are associated with the spoilage of meat

products. *Leuconostoc carnosum* is the dominant spoilage bacterium in vacuum-packed cooked ham (Bjorkroth et al., 1998). *Leuconostoc* sp. (e.g., *Leuconostoc gelidum*, *L. carnosum*, and *Leuconostoc mesenteroides*) can produce organic acids (acetic acid), resulting in a cheesy smell and the formation of slime in meat, which are accompanied by gas production and greening (Diez et al., 2009; Nieminen et al., 2011). *Carnobacterium* sp. (e.g., *Carnobacterium divergens* and *Carnobacterium maltaromaticum*) can cause red meat spoilage in low-oxygen environments (Casaburi et al., 2011; Rieder et al., 2012). *Weissella* (e.g., *Weissella viridescens* and other *Weissella* spp.) often leads to the spoilage of vacuum-packed minced meat and meat products (Diez et al., 2009; Nieminen et al., 2011; Samelis et al., 2006). In addition, *Lactococcus* (e.g., *Lactococcus piscium* and *Lactococcus raffinolactis*) and *Enterococcus* (e.g., *Enterococcus viikkiensis* and *Enterococcus hermanniensis*) are common spoilage bacteria in meat (Pothakos et al., 2015). Great concern is raised by *L. gelidum* as a spoilage microorganism in meat in recent years (Chaillou et al., 2014; Pothakos et al., 2014). The processing environment is the main source of contamination, and LAB can be isolated from the carcass during dressing, chilling, and deboning and from contact with work surfaces during slaughter.

5.2.2.3 Enterobacteriaceae

Enterobacteriaceae is a family of Gram-negative, nonspore-forming, facultative anaerobic bacteria. Members of this family are extensively distributed in water, soil, and animal feces in nature. Thus far, 34 genera, 149 species, and 21 subspecies are identified (Baylis, 2006). These bacteria include a large number of pathogenic bacteria such as *Escherichia* O157: H7, as well as some *Salmonella* and *Yersinia*. Those with spoilage potential are generally psychrophilic, such as *Enterobacter*, *Serratia*, *Hafnia*, and *Rahnella*, as well as *Serratia proteamaculans* and *Hafnia alvei* (Brightwell et al., 2007). *Pantoea agglomerans*, *Escherichia coli*, and *Serratia liquefaciens* are the major spoilage bacteria in minced beef. Moreover, this group of bacteria can cause the rapid spoilage of vacuum-packed dark, firm, and dry beef (Gribble et al., 2014). Putrid odors are caused by the Enterobacteriaceae: aerobic spoilage results in a sulfide odor, discoloration, slime formation, and an ammonia odor, whereas anaerobic spoilage is associated with a sulfide odor and surface greening (Gill and Badoni, 2004; Labadie, 1999).

Coliform bacteria are commonly regarded as indicators of hygiene quality in meat and meat products. This group of bacteria can be used to determine the freshness of meat and reflect the hygiene condition of meat during production, transport, and sale, thereby providing reference data for the timely adoption of effective control measures. Dressing devices are the main source of carcass contamination with bacteria, particularly Enterobacteriaceae (Gustavsson and Borch, 1993). An investigation of a medium-sized cattle slaughtering enterprise in China reveals that bacterial contamination with Enterobacteriaceae during dressing: the total number of Enterobacteriaceae could reach 2.37 $\log_{10}$ CFU/cm^2 in certain parts of the carcass (Xu, 2013), within the range of critical values (2001/471/EC, 1.5–2.5 $\log_{10}$ CFU/cm^2) that has been specified in a few countries. Depilation devices are a major source of Enterobacteriaceae contamination in hog slaughter (Baylis, 2006).

5.2.2.4 *Brochothrix*

Brochothrix are widely present in water, soil, and animal gastrointestinal tracts. This genus of bacteria comprises Gram-positive, facultative anaerobic, nonpigmented bacilli that can produce lipase and protease. Two species are known in the genus *Brochothrix*, *Brochothrix campestris* and *B. thermosphacta*. *B. thermosphacta*, a common bacterium in chilled meat, is first isolated from pork sausage in 1951 and has recently been found in pork, beef, mutton, and cured meat (Gill, 1996; Stackebrandt and Jones, 2006). *B. thermosphacta* has been reported to cause the spoilage of vacuum-packed mutton at temperatures lower than −1.5°C (Gribble and Brightwell, 2013). Li et al. (2006) has noted that *Lactobacillus* and *B. thermosphacta* are the major spoilage bacteria in vacuum-packed pork at the end stage of storage. Meat spoilage caused by *B. thermosphacta* is characterized by an unpleasant odor of cheese or dairy products, gas production, and noticeable discoloration (Gill, 2004), with greening and production of a green slime (Gribble and Brightwell, 2013; Gribble et al., 2014). The proportion of *B. thermosphacta* in pork is reported to be 44.3% after four days of storage.

5.2.2.5 Other Bacteria Associated with Meat Spoilage

Achromobacter, another group of spoilage bacteria, is present in water, soil, and animal gastrointestinal tracts. The genus *Achromobacter* comprises six species and two subspecies, which are nonpigmented and nonmotile Gram-negative bacteria. *Achromobacter* species are the dominant bacteria in pork sausage. Thus far, 15 species of *Achromobacter* are known and many of these bacteria are able to grow at low temperatures. *Achromobacter johnsonnii* and *Achromobacter lwoffii* can be detected in spoiled bacon (Kampfer, 2000).

Moraxella occur in the marine environment and in animal mucus. The genus *Moraxella*, together with *Acinetobacter* and *Psychrobacter*, belongs to the family Moraxellaceae. *Moraxella* species are aerobic. In accordance with standard naming, 19 species of *Moraxella* occur in prokaryotes (Euzéby, 2005). Within the *Moraxella* group, bacteria associated with meat and meat products have mostly been identified by genus. This genus accounts for a small portion of spoilage bacteria (rather than being dominant spoilage bacteria) in meat and mainly occurs in tray- and vacuum-packed pork (Li et al., 2006). The spoilage mechanism of *Moraxella* species remains unclear.

Psychrobacter is a genus of Gram-negative bacteria. Most species of *Psychrobacter* can grow at low temperatures (5°C). This genus currently comprises 26 species (Anon 2005; Euzéby, 2005) and cannot degrade proteins or produce hydrogen sulfide (Gennari et al., 1992).

The spore-forming genus *clostridium* is another meat spoilage-related bacteria, which is often detected from a "blown pack" vacuum-packaged meat sample. Some strains could survive under refrigerator conditions. *Clostridium estertheticum*, *Clostridium algidicarnis*, *Clostridium difficile*, *Clostridium Beijerinckii*, *Clostridium lituseburense*, *Clostridium gasigenes*, *Clostridium frigidicarnis*, and *Clostridium algidixylanolyticum* have been discovered successively presented in beef, pork, or lamb (Lawson et al., 1994; Broda et al., 1996, 1999, 2000a, 2000b; Kalchayanand et al., 1993).

5.2.2.6 Molds and Yeasts Associated with Meat Spoilage

Not much attention has been paid to yeast and mold spoilage in meat and processed meat products, as yeast and mold spoilage phenomenon occurs very rarely. Most of the yeasts and molds are more resistant than bacteria to low water activity and low pH environments, and they contribute a minor to the spoilage.

Cryptococcus laurentii var *laurentii* has been found predominated in lamb at −5°C (Lowry and Gill, 1984), and *Candida lipolytica*, *Candida zeylanoides*, and *Yarrowia lipolytica* have been found in spoiled beef and retail meats, which may play a role in spoilage (Hsieh and Jay 1984; Chabela et al., 1999). It has also been observed that yeasts become the main spoilage agents only in cured meat products preserved by sulfite such as fresh British sausage (Dalton et al., 1984) or when products are chill-stored aerobically (Samelis et al., 2000). Nielsen et al. (2008) has found that the yeast microflora is complex with 4–12 different species isolated from bacon, ham, salami, and liver pates, and *C. zeylanoides*, *Debaryomyces hansenii*, and *Candida alimentaria* are the dominant species.

5.3 CHARACTERISTICS OF POSSIBLE MECHANISMS ASSOCIATED WITH SPOILAGE MICROORGANISMS

5.3.1 The Apparent Phenomenon of Meat Spoilage

5.3.1.1 Slime Formation

The massive reproduction of microorganisms on the surface of meat can lead to the formation of a slime comprising metabolic products of the reproduced colonies or microorganisms (Nychas et al., 2008). When the slime is examined, it appears filiform and is accompanied by a strong off-odor. This phenomenon is caused mainly by Gram-negative bacteria, LAB, and yeasts. When the surface of the meat appears slimy and filiform, the total number of microbial colonies is approximately 7 $\log_{10}$ CFU/cm^2 (Nychas et al., 2008).

5.3.1.2 Discoloration

Various color changes often occur on the surface of meat during spoilage. The most common such color is green, which is caused when sulfide (resulting from protein degradation) binds to hemoglobin in meat, and the resultant sulfhemoglobin accumulates on the surface of muscle and fat tissues showing a dark green color. In addition, *Serratia marcescens* forms red stains on the surface of the meat, whereas *Flavobacterium* produce yellow color (Nychas et al., 2008).

5.3.1.3 Off-Odors

Meat spoilage is commonly associated with abnormal or unpleasant odors. Putrid odors are generally produced when the total number of colonies on the surface of the meat reaches 7 $\log_{10}$ CFU/cm^2. An off-odor can be noted with 5–6 $\log_{10}$ CFU/cm^2 Gram-negative bacteria. The odors are produced mainly due to highly alkaline metabolic by-products of protein breakdown by bacterial enzymes. The odorous substances include ammonia, amines, hydrogen sulfide, and other sulfur-containing compounds (e.g., dimethyl sulfide ether). Certain species of the genus *Pseudomonas* first utilize oxygen and glucose in meat as energy sources; when glucose is depleted, the bacteria begin to metabolize protein as a carbon source. *Ps. fluorescens* can degrade sulfur-containing amino acids including methionine and cysteine. The highly alkaline metabolic by-products produced by the bacteria can increase the pH of the meat to 6.5 or higher in a short period leading to final spoilage of the meat (Ercolini et al., 2011)

5.3.1.4 Mildew Stain

Molds growing on the surface of the meat often form mildew stains. This phenomenon is more common in dry-cured meat products. For instance, *Thamnidium elegans* and *Thamnidium chactocladioides* produce feathery hypha on the surface of the meat. *Sporotrichum album* and *Geotrichum candidum* form white mildew stains. *Penicillium expansum* and *Penicillium oxalicum* form green mildew stains, and *Cladosporium herbarum* forms black stains (Samelis, 2006).

5.3.2 Spoilage Mechanisms

As stated previously, the spoilage of meat and meat products is caused by a small fraction of bacteria including SSO and ESO. Various bacterial populations utilize the substrates in meat in different orders (Table 5.4).

Glucose is a preferential substrate of most spoilage microorganisms in meat. When glucose is depleted, other substances including lactic acid, gluconic acid, pyruvic acid, propionic acid, formic acid, ethanol, acetic acid, amino acids, nucleotides, and water-soluble proteins serve as subsequent substrates of most spoilage bacteria (Nychas et al., 2007; Pothakos et al., 2015). Under aerobic conditions, the leading spoilage microorganisms in meat are *Pseudomonas*, followed by *B. thermosphacta*; LAB, and Enterobacteriaceae are also present (Koutsoumanis et al., 2008).

Pseudomonas can fully use carbon and energy sources in meat following the indicated order (Table 5.4). Under aerobic conditions, *Pseudomonas* spp. preferentially use glucose in meat through the Entner-Doudoroff pathway, producing gluconic acid and Z-oxo-gluconate. The two acid products accumulate outside the cells and are further metabolized by *Pseudomonas*; however, competing bacteria are unable to use these two acids. When the bacterial density reaches 8 $\log_{10}$ CFU/cm^2, the glucose supply can no longer meet bacterial growth needs, and *Pseudomonas* can begin to use amino acids as a growth substrate, thereby producing odorous sulfur compounds, esters, and acids. In addition, glucose is considered a major internal factor that can describe or predict the level of spoilage (Koutsoumanis et al., 2006). This component plays an important role in the level and type of meat spoilage (Nychas, 1998). The initial signs of spoilage are evident when the glucose concentration becomes very low and the limitation of glucose promotes the shift of *Pseudomonas* from

TABLE 5.4
Substrates Used by Meat Spoilage Bacteria During Growth in Aerobic Storage

	Pseudomonas spp.	*Enterobacteriaceae*	*Brochothrix thermosphacta*	Lactic Acid Bacteria	*Clostridium* spp.
Substrates[a]	**AVP and MAP**	**AVP and MAP**	**AVP and MAP**	**AVP and MAP**	**AVP and MAP**
Glucose	1 1	1 1	1 1	1 1	1
Glucose-6-P	2 2	2 2	2 2	2 2	2
Lactic acid	3	3	–	–	–
Pyruvic acid	4 3	–	–	–	–
Gluconic acid	5 3	–	–	–	–
Glunonate-6-P	6	–	–	–	–
Acetic acid	3	3	–	–	–
Amino acids	7 3	4	3	3	–
Ribose	–	–	4	–	–
Glycerol	–	–	5	–	–

Source: Adapted from Casaburi, A. et al., *Food Microbiol*., 45(Pt A), 83–102, 2015.

[a] The numbers reported indicate the order of substrate utilization.

Note: A, aerobic storage; VP, vacuum packaging; MAP, modified atmosphere packaging.

carbohydrate to amino acid catabolism. Conversely, the symptoms of spoilage, including hydrolysis and slime formation or the production of unpleasant odors, are delayed with increasing glucose availability. This is due to the great impact (positive or negative) played by the physiological behavior (metabolite use or uptake) of *Pseudomonas*. Moreover, studies have shown that *Pseudomonas* can degrade proteins. This group of bacteria can therefore penetrate deeply into meat to better utilize new nutrients than other bacteria. Under aerobic conditions, more free amino acids are present. This fact is consistent with the finding that *Pseudomonas* preferentially use amino acids as substrates after glucose depletion, thereby causing spoilage under aerobic conditions (Gill, 1986; Nychas, 2008).

LAB in meat are obligate or facultative heterofermentative species. The former type of bacteria produces lactic acid, acetic acid, carbon dioxide, and ethanol and the later type of bacteria breaks down glucose into two molecules of lactic acid. In the presence of pentose, LAB can produce lactic and acetic acids through heterofermentation without gas production. In the presence of low concentrations of glucose, *Lactobacilli* that can degrade ribose in meat can transform their metabolism from homofermentation to heterofermentation and produce substantial quantities of acetic acid (Borch et al., 1996). When glucose is limited, spoilage LAB can metabolize lactic and pyruvic acids to produce acetic acid during aerobic storage (Samelis, 2006). High concentrations of acetic acid can endow the meat with a strong acid smell. Other carbon sources and amino acids also support the growth of LAB when glucose is insufficient. For example, *Lb. sakei* can metabolize arginine to ammonia and biogenic amines such as putrescine and spermine (Labadie, 1999). *Leuconostoc* spp. can produce lactic and fatty acids by using glycogen and protein matrix as substrates. *L. gelidum* subsp. *Gasicomitatum* can generate diacetyl, which produces a cheesy odor in meat through heme-dependent respiration or by using ribose and inosine as carbon sources (Jaaskelainen et al., 2013, 2015). In short, LAB can degrade carbohydrates to lactic, isobutyric, isovaleric, and acetic acids. However, LAB-induced meat spoilage is slower than aerobic spoilage and therefore extends the shelf life of anaerobically packaged meat.

As for the Enterobacteriaceae, they also preferentially utilize glucose prior to degrading amino acids and then release the amines, sulfides, and H_2S. They have the ability to produce H_2S not dimethyl

sulfide, which significantly increases the severity of spoilage. Under anaerobiosis, *S. liquefaciens*, *H. alvei*, and other enterobacteria may become the main spoilage agents in dark, firm, and dry meat by producing H_2S and greening such as sulfmyoglobin (Dainty and Mackey, 1992). *Sh. putrefaciens* can lead to the release of large amounts of ammonia contributing to spoilage off-odors (Dainty and Mackey, 1992), while *S. liquefaciens* produces acetic acid. Generally, Enterobacteriaceae cause the spoilage at the microbial load number at a level of 7 $\log_{10}$ CFU/cm^2. Ammonia is also produced by most pseudomonads in air-stored meat (Dainty and Mackey, 1992). However, not like *Pseudomonas* spp., which produces ethyl esters as one of their main spoilage by-products, Enterobacteriaceae may produce acids, alcohols, and acetoin/diacetyl (Nychas et al., 1998).

In addition to the role of individual microorganisms, we should also consider the interaction between different microbial populations: the "metabiotic spoilage association" (Pothakos et al., 2015; Gram et al., 2002). Various microorganisms can competitively consume nutrients, oxygen, and carbon sources in meat and produce various metabolites including organic acids, bacteriocins, and volatile compounds, which all mutually affect microbial growth. *Pseudomonas* can produce siderophores and maintain high levels of glucose utilization; thus, this group of bacteria can suppress the growth of *Sh. putrefaciens*, one of the main species promoting meat spoilage (Nychas et al., 2007; Nychas et al., 2008). LAB exhibit an antagonistic effect on *B. thermosphacta*, when these two groups of bacteria coexist, the number of LAB remains higher than that of *B. thermosphacta,* and the effect is highly significant, especially after 48 h and the number (log value) of *B. thermosphacta* decreases by 2 h after LAB inoculation (Russo et al., 2006). Communication between cells is known as "quorum sensing" and refers to the fact that certain bacteria synthesize and release signal molecules termed autoinducers (AIs). The extracellular concentrations increase with increasing bacterial density and AI can trigger the expression of relevant genes in bacteria when a critical concentration is reached regulating their behavior. For example, it can regulate the production of toxins, biofilms, antibiotics, spores, and fluorescence in order to adapt to environmental changes. Signal molecules include acylated homoserine lactones (AHLs) and autoinducer-2 (AI-2). AHLs and AI-2 have been found in beef at various storage temperatures and under various packaging conditions. The former are produced when the numbers of *Pseudomonas* and Enterobacteriaceae reach 10^7 CFU/g, whereas the latter remain at low concentrations in beef during storage (Blana and Nychas, 2014).

5.4 METHODS USED TO DETECT AND EVALUATE SPOILAGE MICROORGANISMS

As stated by "Bibek Ray," due to the development of modern microbiology, microbial detection techniques have evolved from "the dominance of plate count and coliform test" to "the combined development of molecular biology and food safety." Many researchers have reviewed the methods used to detect and evaluate spoilage microorganisms (Nychas et al., 2008; Ercolini et al., 2011). In addition to the traditional plate count method, many approaches have been developed that are based on the detection of pathogenic microorganisms. These include several PCR-based fingerprinting methods including the repetitive-sequence-based PCR (rep-PCR) of bacterial genomes, pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) analysis, and random amplified polymorphic D. Moreover, high-throughput sequencing technology has developed rapidly in recent years, and a simple spectroscopic microbial nondestructive test also has been developed.

5.4.1 Repetitive Sequence-Based PCR

Versalovic has first described the rep-PCR fingerprinting of bacterial genomes in 1996. Rep-PCR is used to amplify short repetitive sequences that are widely distributed throughout the bacterial genome. This technique reveals the differences between genomes through the comparative analysis

of electrophoretic bands. Repetitive sequences in the bacterial genome include commonly used repetitive extragenic palindromic (REP) sequence, enterobacterial repetitive intergenic consensus (ERIC) sequence, and 154-bp BOX element. The distribution of REP, ERIC, and BOX elements differs according to strain, species, and genus and is relatively conserved during evolution. Rep-PCR is being developed rapidly and has been used for the gene classification of various bacteria. The procedure is simple to apply and is well suited for large samples. Rep-PCR exhibits high resolution and repeatability and is superior to RFLP, biochemical typing, and ribotyping. Presently, Rep-PCR is available for automated typing and the establishment of a standard database for use in the REP and ERIC-PCR typing of various bacteria.

5.4.2 Quantitative Real-Time PCR (RT-PCR)

RT-PCR measures the amount of products after each cycle of a PCR reaction using a fluorescent chemical included in the deoxyribonucleic acid (DNA) amplification reactions. This technique quantifies a specific DNA sequence by comparing it with internal or external standards. The progress of PCR amplification is subject to real-time monitoring through fluorescence signals. During the exponential stage of PCR amplification, a linear relationship exists between the Ct value of the template and its initial copy number, which forms the basis of the quantitative analysis. RT-PCR has been used to identify *B. thermosphacta* in beef (Pennacchia et al., 2011); however, the result of quantization is unsatisfactory (Pennacchia et al., 2009).

5.4.3 RAPD-PCR

The RAPD technique has been developed in the 1990s. This PCR-based molecular technique is used to perform polymorphism analysis on the entire genome of an unknown sequence. PCR amplification is conducted using a thermostable Taq DNA polymerase with genomic DNA as a template and a single synthetic random polymorphic nucleotide sequence (typically 10 bp) as the primer. The PCR products are separated using agarose or polyacrylamide electrophoresis and then stained using ethidium bromide. The polymorphism is then examined using an ultraviolet (UV) visualization system. The polymorphism of PCR products reflects the polymorphism of the genome. The RAPD technique has been widely used for species identification, pedigree analysis, and the phylogenic analysis of biological samples.

5.4.4 Pulsed-Field Gel Electrophoresis

PFGE is a method of separating large DNA molecules. In conventional gel electrophoresis, the migration rates of large DNA molecules (>10 kb) are similar; thus, resolution of the DNA bands is difficult. In PFGE, the electric field is continually shifted in two directions (at a certain angle, rather than in the opposite direction). Negatively charged DNA molecules can migrate toward the positive electrode. After the electronic field is altered, the direction of migration of smaller molecules is easily altered, whereas that of larger molecules is altered with more difficulty. Consequently, smaller molecules migrate faster than larger molecules on the PFGE gel. PFGE can resolve DNA molecules ranging from 10 kb to 10 Mb.

5.4.5 High-Throughput Sequencing

A novel enzyme-linked cascade sequencing technology, 454-pyrosequencing, has been developed by 454 Life Sciences. In 454-pyrosequencing, DNA is amplified in water droplets that are dispersed in a water-in-oil emulsion such as emulsion PCR. Each water droplet initially contains only a magnetic microbead wrapped in a large quantity of primers and a DNA template molecule linked to

the microbead (control high-probability event DNA concentrations). The products of emulsion PCR are loaded onto a special pico titre plate (PTP) that has been drilled with millions of wells and each microwell can only accept one microbead. DNA polymerase polymerizes one deoxynucleotide (dNTP) onto the template, and one pyrophosphate molecule (PPi) is released; adenosine triphosphate (ATP)-sulfurylase catalyzes the reaction of PPi and APS to form an ATP molecule; the ATP molecule is then used as a substrate by luciferase to convert luciferin into oxyluciferin. The produced visible light is then captured by a CCD optical system, and a specific detection signal is acquired. The signal intensity is proportional to the corresponding base number. Four types of dNTP are added sequentially and cyclically, and the signal intensity and reaction time are recorded to realize DNA sequencing. High-throughput sequencing enables the detailed analysis and full description of the transcriptome and genome of a species. Therefore, this technology is also known as deep sequencing. Using high-throughput sequencing, Ercolini et al. (2011) have detected 403 types of bacteria coexisting in beef under vacuum, modified-atmosphere, active, and air-packaging conditions. Various bacterial species have been found to be present in vacuum-packed beef including *B. thermosphacta*, *Pseudomonas* sp., *Streptococcus* sp., *Lactobacillus* sp., *Lactococcus* sp., *C. divergens*, and *Carnobacterium* sp. (Ercolini et al., 2011). Zhao et al. (2015) has found more than 200 types of bacteria in vacuum-packed pork at 21 days of storage. The phylotypes of microorganisms in meat were found to be more complex than previously reported (De Filippis et al., 2013; Xiao et al., 2013).

Furthermore, biological sensor, electronic nose, and headspace analyses can be applied to microbial typing by identifying specific metabolites (Balasubramanian et al., 2009). High-performance liquid chromatography has been used to evaluate microorganisms in meat by determining the organic acid content in beef and applying a specific mathematical analysis (Argyri et al., 2011). Proton nuclear magnetic resonance spectroscopy and solid phase microextraction–gas chromatography–mass spectrometry have been applied to test and evaluate spoilage microorganisms in meat by identifying metabolic end products (Ercolini et al., 2011). Fourier transform infrared spectroscopy can be used to identify microorganisms at densities of less than 5 $\log_{10}$ CFU/g in beef.

5.5 CONTROL, PREVENTION, AND MANAGEMENT OPTIONS FOR SPOILAGE MICROORGANISMS

As mentioned, the initial contamination of muscles started from slaughter, and this cannot be completely avoided in practice. Thus, one of the critical tasks for the meat industry is to control this contamination to a minimum, as the initial number of contaminated microorganism is the most important factor affecting the shelf life of fresh meat. Under this circumstance, GMP and HACCP programs are the best way to minimize the access of microorganisms to the meat. However, GMP may not always provide sufficient control, and HACCP is not fully implemented for all the meat producers in developing countries, and additional procedures, therefore, are alternative applied in some slaughterhouses.

5.5.1 Cleaning of Hides and Carcasses

As the main initial contamination originates from the animal hides, preslaughter animal washing is supposed to be an applicable procedure to reduce the microbes counts. Although the effectiveness is not as expected (Koutsoumanis et al., 2006), this procedure has been applied in New Zealand, Australia, and United States (Gill and Bryant, 1992). While chemical dehairing is found to be a potential way to reduce bacterial levels of beef carcasses (Nou et al., 2003), in some Asian country, it is seldom to do the animal washing or hair removal before slaughter. After the carcass exposure, steam-vacuuming is useful to remove the visible contamination on carcasses, and shows significant reduction of aerobic bacteria and total coliform counts (Kochevar et al., 1997). Such technique is very popular in U.S. slaughterhouses (Sofos and Smith, 1998), but in China it needs to be promoted,

where knife-trimming is still used as a promising way to decontaminate the fecal and ingesta contamination spots. After dressing, water, steam, and organic acid spraying are usually used to wash the whole carcass before the bacteria firmly attaches to the muscle (Cabedo et al., 1996). The effectiveness of this spray more depends on the pressure of water, the temperature of hot water or steam, and the type of organic acid, while hot water at 74°C or higher (80°C– 85°C) is reported to have a significant microbial reduction (Graves-Delmore et al., 1998; Gorman et al., 1995) and 1%–2% lactic acid and acetic acid are applied successfully in practice (Kochevar et al., 1997). The spray procedure is also used at the end of the slaughter line before the carcasses are transferred into the chilling room, but at that moment, the bacteria are much more firmly attached to the carcasses than that before evisceration resulting in a discount of the spraying effectiveness.

5.5.2 Cleaning of Equipment Surfaces and The Surrounding Environment

Equipment surfaces and the surrounding environment are very common niches for microorganisms in slaughterhouses. Cleaning, sanitizing, and rinsing are the typical steps to decontaminate the microbes on the surfaces of processing equipment and surrounding environment. The cleaning involves the use of water and cleaning aids to remove the visible contaminants and disperse insoluble materials. The detergents currently used in the plants include sodium carbonate, citric acids, surface-active agents, and sequestering agents being not corrosive to the equipment surfaces (Forsythe and Hayes, 1998). The sanitizing is a necessary follow-up step involving the use of antimicrobial agents to spray those surfaces, which can rapidly inactivate microorganisms and prevent the biofilm formation to some extent (Forsythe and Hayes, 1998; Koutsoumanis et al., 2006). Quaternary ammonium compounds, iodophors, and amphoteric compounds are applicable options for the sanitation. Those sanitizers are ideally able to inhibit the bacteria, be stable in the presence of aforementioned detergents, and also be easily rinsed off (Koutsoumanis et al., 2006).

5.5.3 Preservative Technologies

Preservation technologies mostly rely on the control of those factors affecting microbial growth, such as temperature, growth atmosphere, water activity, pH, and antimicrobials (Koutsoumanis et al., 2006). Chilled storage for fresh and meat products can inhibit the growth of the bacteria and largely reduce the bacteria diversity on the meat. However, most of the meat spoilage–related bacteria belong to the group of psychrophilic microorganisms, such as *Pseudomonas* spp., LAB, *B. thermosphacta* and so forth. The water activity and pH values of fresh meat and specific meat products drop into a very narrow range, and are hard to be adjusted by manual intervention. Therefore, the bacterial growth atmosphere becomes a critical factor influencing the shelf life of the meat.

5.5.3.1 Preservative Packaging

Oxygen is one of the most important factors affecting the growth of aerobic bacteria. Consequently, removal of oxygen from the pack environment (vacuum package) is an effective way to inhibit the growth of those bacteria. Alternatively, introduction of CO_2 or CO to the in-pack surrounding atmosphere can slow down their growth. In general, there are several modified atmosphere packaging (MAP) applied in commercial. Vacuum packaging with low oxygen permeability is most widely used for primal cuts of chilled meat. The shelf life of vacuum packaged beef stored at 0°C–1°C can be extended to 10–12 weeks (Koutsoumanis et al., 2006), while those of aerobic-packed ones is only 1–2 weeks. *Pseudomonas* species, such as *Ps. fragi*, *Ps. lundensis*, and *Ps. putida* are inhibited well by vacuum package. Vacuum package is the most stable packaging systems for ground beef (Lavieri and Williams, 2014) as the protein and lipid oxidations are retarded under this condition, although the color is not as attractive as other MAPs. The gas combination with 80% O_2 and 20% CO_2 is typically used as a high oxygen MAP in promoting fresh beef color (Resconi et al., 2012), while such concentration of CO_2 is sufficient to lower the counts of the aerobic bacteria, although their growth

are not inhibited completely. The concentration of O_2 can be adjusted to 50%–60% with 50%–40% of CO_2, or part of CO_2 can be replaced by N_2. The diversity of bacteria is lowered in MAP than that in aerobic package, with *C. divergens*, *Carnobacterium* spp., and *Staphylococcus xylosus* are dominated under 60% O_2 and 40% CO_2 MAP. MAP with a high concentration of oxygen may cause quality deterioration through lipid and protein oxidation negatively affecting flavor stability, drip loss, and tenderness (Kim et al., 2010). A recent study found that steaks stored in packages containing 50% oxygen are well accepted compared with other oxygen levels especially with respect to flavor and texture (Zakrys et al., 2008). The number of aerobic *Pseudomonas*, total visible counts in 50% O_2-MAP beef steak are less than that in 80% O_2-MAP over time, while there is no significant difference of *Enterobacteriaceae* counts, LAB, and *B. thermosphacta* between those two aerobic MAP under aerobic conditions within 12-day chilled storage (Yang et al., 2016). Carbon monoxide (CO) is also introduced to the MAP that can result in the formation of carboxymyoglobin possessing a very stable and bright-red meat color (Cornforth and Hunt, 2008). Although CO is a potential hazardous gas that might induce a negative image by consumers, the U.S. Food and Drug Administration (FDA) has approved the use of CO as a color fixative in case-ready meats and CO concentration not exceeding 0.4% is generally regarded as safe (FDA, 2004). Rogers et al. (2014) has found that CO-MAP could reduce lipid oxidation and microbial spoilage of 19% fat ground beef than aerobic packaging. Yang et al. (2016) has shown CO-MAP could inhibit the growth of *B. thermosphacta* completely and result in lower numbers of total visible counts of *Pseudomonas* and LAB than that in aerobic MAPs. Active packaging is also an effective packaging to inhibit the growth of bacteria on meat surface, including the use of substances that absorb oxygen or flavors/odors, and that release carbon dioxide, antimicrobial agents, and antioxidants (Koutsoumanis et al., 2006). Those substances are used as an ingredient of packaging films or coatings, which could sustain release into the meat, or be used in the forms of spraying and dipping, both showing a satisfactory inhibition against spoilage and pathogenic bacteria (Quintavalla and Vicini, 2002; Cutter, 1999; Ercolini et al., 2010).

5.5.3.2 Other Preservative Technologies

Irradiation, high hydrostatic pressure (HHP), use of natural antimicrobials, and application of protective cultures are currently potential preservative technologies that can be used in the meat industry. Meat irradiation is defined as the process in which meat is exposed to ionizing energy from radioactive sources (cobalt 60) or with machine sources (high-energy electron beams) or x-rays at the last step of the processing (Koutsoumanis et al., 2006). Irradiation treatments can kill the nonspore forming spoilage and pathogenic bacteria in meat and meat products (Farkas, 1998; Lee, 2011). Its application in frozen and nonfrozen red meat has been approved by FDA and Food Safety and Inspection Service (FSIS) with a limitation of maximum dose in United States, and the national standard of using irradiation in food has been implemented in China since 1994. However, the change of the sensory characteristics of the irradiated meat is still a limitation for applying in meat industry (Koutsoumanis et al., 2006).

HHP is a nonthermal processing technology whereby foods are subjected to 100–600 MPa hydrostatic pressure at low or moderate temperatures (Koutsoumanis et al., 2006). It has a promising preservative effect toward cooked and cured meat products. Unlike irradiation, HHP can reduce microbial contamination without resulting in a significant alteration of the meat taste and flavor (Carlez et al., 1994; Hayes et al., 2014; Sampedro et al., 2009; Zhou et al., 2010. The reduction of the spoilage bacteria such as *Pseudomonas* could be up to 5 log units after the treatments at 200–600 MPa and 10°C–25°C for 10–20 minutes (Carlez et al., 1994) and the aerobic total count could be above 4.5 log after 600 MPa and 31°C for 6 minutes. However, HHP may be not widely applied due to its very high cost and capacity limitation (Koutsoumanis et al., 2006).

Addition of organic acids or other natural antimicrobials is another preservative technology that has been applied in meat and meat products extensively (Samelis and Sofos, 2003; Theron and

Lues, 2007). The addition of antimicrobials is through the way that dipping or spraying meat or meat products with organic acid or in form of their salts such as sodium lactate, acetate, diacetate, and potassium sorbate or benzoate, and so forth, and the application medium include bologna (Samelis et al., 2001; Mbandi and Shelef, 2002), frankfurters (Samelis et al., 2002; Samelis and Kakouri, 2004), and other ready-to-eat meat products (Theron and Lues, 2007). This preservative technology in meat products has been used for inhibiting pathogenic bacteria at the very beginning, while it shows promising reduction of the spoilage bacteria (Samelis et al., 2001; FSIS, 2003). The 2% sodium or potassium lactate and 0.1%–0.15% sodium diacetate are most widely used agents in meat industry (Tompkin, 2002). In addition, some LAB are used as biopreservation to extend meat storage life due to their production of some inhibitory metabolic substances such as organic acids, acetoin, diacetyl, and bacteriocins (Pothakos et al., 2015). However, in some countries, the application of antimicrobials and biopreservations cannot be added in fresh meat, thus more natural preservative agents and more mild preservative methods are still needed to be explored for the meat industry.

5.6 CONCLUSION AND FUTURE TRENDS

Meat spoilage is an ecological phenomenon involved in several very specific spoilage organisms. Those microorganisms lead to the changes of the available substances whose growth depends largely on meat storage temperatures and packing atmosphere. The detection of spoilage-related bacteria and the early signs of incipient spoilage for meat are critical for the spoilage control. Currently, the development of rapid detection methods is relying on the development of biotechnology with a highlight on high-throughput sequencing at the present time. Also, the genomics, transcriptomics, and metabolomics should be introduced to the meat spoilage field, and the better understanding of spoilage compounds through those omics can effectively develop technologies that can prevent meat spoilage. While the control of the spoilage bacteria in meat still has a long way to go, milder and more effective preservation technologies or the combination of preservative ways with effective HACCP procedures are demanded by the meat industry.

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6 Spoilage Microorganisms in Poultry Products

Qiuqin Zhang, Jinxuan Cao, and Lingzhi Cheong*

CONTENTS

6.1 INTRODUCTION

The feathers, skin, feet, and alimentary tract of live birds entering commercial processing facilities are heavily contaminated with diverse native microbial flora. Commercial broiler processing consists of several operations including scalding, defeathering (picking), evisceration, and chilling. Although processing generally reduces microbial contamination of broiler carcasses, cross-contamination between carcasses, processing water, and equipment may actually increase the level of carcass contamination following processing. Bacteria colonizing feathers and skin of live broilers are removed during the scalding and picking operations. Nevertheless, scalding and picking operations that remove epidermis of the broiler skin will provide new surfaces for cross-contamination during the subsequent evisceration and chilling operations. These microorganisms may survive during processing operations, multiply during storage, and result in spoilage.

6.2 TAXONOMY OF SPOILAGE MICROORGANISMS IN POULTRY

6.2.1 Bacteria Involved in the Spoilage of Poultry

The spoilage bacteria in slaughtered broiler considerably differ from those of living birds. Spoilage bacteria on carcasses may originate from the feathers and feet of living birds, water supply in processing plant, and processing equipment.

The first work to identify spoilage flora in fresh chicken and other muscle foods can be dated back to the 1800s. In 1887, Forster (1887) reported that most foods are exposed to saprophytic spoilage bacteria found in air, soil, and water. Spoilage organisms predominated in poultry meat and stored aerobically under chill conditions are invariably pseudomonads and a small amount of other Gram-negative bacteria. Pseudomonads on spoiled poultry can be divided into fluorescent or pigmented strains and nonpigmented strains (Barnes and Thornley, 1966). In 1960, pseudomonas, the Gram-negative spoilage organisms in poultry meat, was the first to be described extensively at genus level using determinative schemes (Ayres, 1960). Decades later, Arnaut-Rollier et al. (1999b) found four major clusters of pseudomonads using numerical taxonomy namely *Pseudomonas fragi*, *Pseudomonas lundensis*, *Pseudomonas fluorescens* biovars, and an unidentified group resembling *P. fluorescens*. Identification of bacterial species responsible for spoiling poultry is important for better understanding of the spoilage mechanisms. The pseudomonads species indeed predominated when spoilage was obvious. Nonfluorescing species were identified mainly as *P. fragi*, while other strains belong to *P. fluorescens* biovars, *P. lundensis*, *Pseudomonas putida* biovars, and *Pseudomonas marginalis* (Table 6.1).

Acinetobacter is another primary group of spoilage bacteria in poultry carcasses (Barnes, 1976). Barnes and Impey (1968) reported that organisms most commonly found on poultry carcasses stored at 1°C are pigmented and nonpigmented strains of pseudomonads, *Shewanella putrefaciens*, and *Acinetobacter*. Growth of *Sh. putrefaciens* was much faster in the leg muscle than breast muscle. Strains of *Acinetobacter* groups B and C can be found growing mostly in the leg muscle, but not in the breast muscle. Moreover, *Acinetobacter* groups C grow much more rapidly than *Acinetobacter* groups B. These differences could partially be explained by differences in pH between the breast and leg muscles. Geornaras et al. (1996) reported that 14.8% of the bacteria found on chicken carcass following processing were *Acinetobacter*. Schefferle (1965) first found high populations of *Acinetobacter* (8 log CFU/g) on the bird feathers suggesting that they might originate from litter. Meanwhile, Akinde and Obire (2008) indicated that *Acinetobacter* might originate from poultry manure. Yeasts are also found to be involved in poultry spoilage (Viljoen et al., 1998). Using the Sherlock Microbial Identification System (Microbial Identification System: MIDI, Inc., Newark, Delaware), Hinton et al. (2002) enumerated and identified yeasts associated with broiler carcasses taken from various stages of commercial poultry processing operations and broiler carcasses stored at fridge temperature. They found a decreased amount of yeasts and changes in yeast compositions as the carcasses are moved through the processing line. Increase in the yeast population of carcasses occurred during storage at fridge temperature (4°C) for up to 14 days. The same strain of yeast was recovered from different carcasses at different points in the processing line and from carcasses processed on different days in the same processing facility. Vijoen et al. (1998) found a total of 159 representative yeast isolates from fresh and spoiled processed carcasses with conventional methods. Species of *Candida*, *Cryptococcus*, *Debaryomyces,* and *Yarrowiawere* were isolated from fresh and

TABLE 6.1
Number of Fluorescent and Non-Fluorescent Species (%) within the *Pseudomonas* Clusters from Chilled Chicken

Cluster	Fluorescent (%)	Nonfluorescent (%)
Pseudomonas fluorescens biovar	14–66	34–86
Pseudomonas marginalis	38	62
Pseudomonas aeruginosa	100	0
Pseudomonas fragi	0	100
Pseudomonas lundensis	15	85
Pseudomonas putida biovar	50–100	0–50

spoiled carcasses. Meanwhile, *Rhodotorula* and *Saccharomyces* spp. were isolated only from fresh samples; and *Trichosporon* spp. was isolated only from spoiled samples.

Other bacteria including *Sh. putrefaciens*, psychrotrophic, Enterobacteriaceae, *Staphylococcus*, *Carnobacterium*, and *Aeromonas* are sometimes present in poultry meat stored at chilling temperature (Zhang et al., 2012). *Shewanella* and psychrotrophic strains of Enterobacteriaceae commonly found in soil and water are thought to originate from live-bird environment. *Escherichia* isolates predominated on the skin sampling site, cranio-dorsal to the pygostyle, while *Staphylococcus* isolates predominated on the skin sampling site, caudal to the breastbone (Olivier et al., 1996).

6.2.2 Contamination in Poultry Processing

In order to cause spoilage, microorganisms must first be present on the poultry meat. Hence, microbial contamination of birds' carcasses is a possible result of processing from live birds to retail poultry meat. Commercial poultry processing involves complicated operations including killing, dressing, evisceration, chilling, and packing (Figure 6.1).

Microbiological contamination is a major problem affecting commercial poultry processing (Daud et al., 1979; Mcmeekin et al., 1979; Thomas and Mcmeekin, 1980a, 1980b). *Acinetobacter, Staphylococcus*, and *Enterococcus* are frequently isolated from chicken carcasses from nonautomated poultry processing plant (Olivier et al., 1996). Meanwhile, Pseudomonads, *Achromobacter*, *Flavobacterium*, *Corynebacterium*, yeasts, *Aeromonas*, Enterobacteriaceae, lactic acid bacteria, Micrococcaceae, and *Bacillus* are isolated from industrial poultry processing plants (Lahellec et al., 1975). Hinton et al. (2004) indicated that the number of spoilage bacteria recovered from processed carcasses was less than that recovered from carcasses first entering the processing line. *Acinetobacter* and *Aeromonas* are the primary isolates recovered from carcasses taken from processing line (Table 6.2). Gill et al. (2006) found similar microbes following scalding, plucking, and evisceration namely *coliforms*, *Escherichia coli*, and presumptive *Staphylococci plus listerias* on carcasses were similar. They also found that cooling water can reduce *coliforms*, *S. plus* and *E. coli*.

Cohen et al. (2007) found that the traditional slaughtering process and the hot season resulted in high levels of microbial contamination and occurrence of pathogenic bacteria. Aerobic plate counts, fecal coliforms, and *E. coli* in the hot season were higher than that in the cold season.

6.2.3 Survival of Bacteria during Storage

Poultry meat can be contaminated by a variety of microorganisms including those capable of spoiling the product during chill storage. For example, psychrotrophic bacteria population is able to multiply on broiler carcasses even at low temperature resulting in spoilage of the poultry meat. Although processing is able to decrease carcass contamination by psychrotrophic spoilage bacteria, significant levels of bacterial cross-contamination occur during processing, and the bacteria that survive processing may multiply on the carcasses during refrigerated storage. Hinton et al. (2004) reported that pseudomonads was the predominant bacteria during chill storage, while *Acinetobacter*

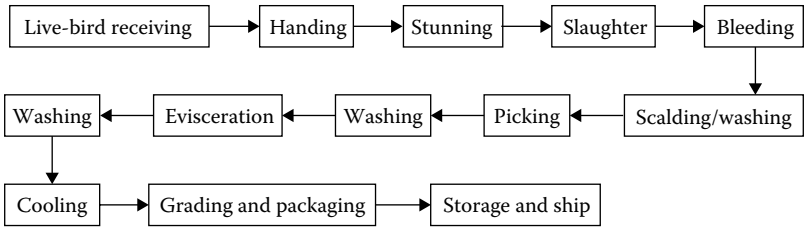

FIGURE 6.1 Generic poultry process.

TABLE 6.2
Bacteria Frequently Isolated from Commercially Processed Broiler Carcasses

Isolate	Carcass Treatment
Acinetobacter spp.	Prescalded, Picked, Eviscerated, Chilled
Aeromonas spp.	Picked, Eviscerated, Chilled, Storage
Brochotrix spp.	Eviscerated
Myroides spp.	Prescalded
Shewanella spp.	Storage

TABLE 6.3
Predominant Microorganisms in Spoilage Poultry Meat

Aerobic Package	Modified Atmosphere Package
Pseudomonas spp.	Lactic acid bacteria
Acinetobacter spp.	*Carnobacterium* spp.
Moraxella spp.	*Brochothrix* spp.
Psychrobacter spp.	*Shewanella* spp.
Arobacter spp.	*Leuconostoc* spp.
Candida spp.	Enterobacteriaceae

and *Aeromonas* spp. are commonly found from carcasses on the processing line. Dendrograms of the isolates' fatty acid profiles indicated that bacterial cross-contamination of carcasses occurs during all stages of processing. Bacteria that survived processing might have proliferated during chill storage. Berry and Maggon (1934) reported that moderately cold storage temperatures might negatively impact bacterial growth to a greater degree than storage at –20°C. The predominant microorganisms in spoilage refrigerated poultry meat under aerobic and modified atmosphere packaging (MAP) conditions were different (Table 6.3).

6.3 SPOILAGE MICROORGANISMS CHARACTERISTICS AND POSSIBLE MECHANISMS

Microorganisms appear first in damp pockets on the carcass, such as folds between the foreleg and breast. Dispersion of the microorganisms is promoted by condensation, which occurs when the cold carcass is exposed to warm, damp air. In addition, spoilage is also caused by accumulation of metabolic by-products or action of extracellular enzymes produced by psychrotrophic spoilage bacteria as they multiply on poultry surfaces at chill temperatures. Some of these by-products can be detected in the form of off-odors and slime, as bacteria utilize nutrients on the surface of meats.

Off-odor is one of the commonly used principal criterions for spoilage in poultry meat. The development of off-odors and the shelf-life of product were highly dependent on the number of spoilage organisms (Pooni and Mead, 1984). Viehweg et al. (1989) demonstrated that virtually all the odor substances found in chill-stored spoiled poultry could be attributed to microbial growth and metabolism. Sensory odor of modified atmosphere packaged chicken meat was found to highly correlated with Enterobacteriaceae, hydrogen sulfide–producing bacteria, and proteolytic bacteria (Balamatsia et al., 2006, 2007; Patsias et al., 2006; Rokka et al., 2004). The total intensity, pungency, faultiness, and sulfuric nature of the odor of modified atmosphere packaged broiler chicken cuts were clearly dependent on the storage temperature and time (Smolander et al., 2004).

Daud et al. (1979) found several psychrophilic bacteria namely pseudomonads groups I, II, and III/IV, *Acinetobacter/Moraxella*-like species, and enteric types on the skin of broiler chicken carcasses, which caused formation of sulfide and off-odors during storage at 2°C. Ayres et al. (1950) identified a characteristic rancid, sweet aromatic ester-like odor that might be described as "dirty dishrag" odor in cut-up chickens stored in a high humidity environment. Russell et al. (1995) identified the bacteria responsible for spoilage of fresh broiler chicken carcasses and to characterize the off-odors these bacteria produce. They found that *Sh. putrefaciens* A, B, and D, *P. fluorescens* A, B, and D, and *P. fragi* from spoiled broiler chicken carcasses obtained from processing plants in the northeast Georgia area, southeastern United States, Arkansas, California, and North Carolina. These bacteria were consistently producing off-odors resembling "sulfur," "dishrag," "ammonia," "wet dog," "skunk," "dirty socks," "rancid fish," or "unspecified bad odor." Numbers of spoilage bacteria can produce odors. For example, "dishraggy" odors can be produced by *Sh. putrefaciens* and the pseudomonads (Russell et al., 1995).

Initial off-odors was found not to originate from breakdown of the protein in skin and muscle of broilers as previously thought, rather from direct microbial utilization of low-molecular-weight nitrogenous compounds. Higher numbers of bacteria (7.57–9 log CFU/cm^2) were required to produce slime compared with that needed for odor to become noticeable (Pooni and Mead, 1984).

In most cases, off-odor precedes slime formation and is considered the initial sign of spoilage. Immediately after off-odors are detected, many small, translucent, moist colonies may appear on the cut surfaces and skin of the carcass. Eventually, meat surfaces become coated with tiny drop-like colonies, which coalesce to form a slimy coating (Dainty et al., 1975; Pooni and Mead, 1984). Haines (1933) found that Glage's *Aromobakterien* were similar to isolates that produced slime on meat stored at refrigeration temperatures. With the exception of some members of pseudomonads group rang a few *Proteus*, microorganisms found on lean meat stored at 0°C–4°C were found to mostly belong to the *Achromobacter* groups. At the final stage of spoilage, the meat may begin to exhibit a pungent ammonic odor in addition to the dirty dishrag odor, which may be attributed to the breakdown of protein and formation of ammonia or ammonia-like compounds. Various authors reported that degradation of meat by pseudomonads resulted in formation of slime (Dainty et al., 1975). A microbial number around 8 log CFU/cm^2 resulted in visible surface slime in poultry (Arnaut-Rollier et al., 1999a; Ayres et al., 1950; Ayres, 1960; Balamatsia et al., 2006).

6.4 DETECTION AND EVALUATION METHODS FOR SPOILAGE MICROORGANISMS

Detection, differentiation, and identification of microorganisms can be performed using numerous methods including phenotypic, biochemical, and immunological assays and, nowadays routinely applied as well, molecular techniques.

The psychrotrophic bacterial flora on freshly dressed broiler chicken carcasses is heterogenous. *Flavobacteria*, *Sh. putrefaciens*, *Acinetobacter* spp., *Corynebacteria* spp., *Moraxella* spp. and fluorescent pseudomonads are all common on aerobically stored, chilled poultry meats (Ayres, 1960; Barnes and Impey, 1968; Barnes and Thornley, 1966). In order to characterize these microorganisms, most authors have used the differentiation scheme established by Shewan et al. (1960). Stanier et al. (1966) characterized pseudomonads with particular emphasis on biochemical, physiological, and nutritional characters. Barnes and Impey (1968) reported that the spoilage microorganisms on poultry skin were predominantly pseudomonads, with *Acinetobacter* spp. and *Sh. putrefaciens* present in lower numbers. However, taxonomic research has developed over the years. Most species of *Acinetobacter* are now assigned to the family Moraxellaceae (Rossau et al., 1991). In current classification, the pseudomonads are divided into fluorescent and nonfluorescent species. The nonfluorescent species predominated on spoiled poultry skin (Barnes and Impey, 1968). Further identification of these species was not available in contrast with red meat, where numerical taxonomic analyses have been performed (Arnaut-Rollier et al., 1999a).

Microorganism identification, when exclusively based on physiological and biochemical characteristics, is intrinsically ambiguous. Currently, phenotypic characterization represents the standard method for most bacterial identifications. Phenotypic analysis is the most tedious task during the process of microbial species recognition, as it requires significant amount of time for preparation, laboratory skills, and technical standardization for an objective interpretation of results. In general, 10 or more tests may be necessary for differentiation of the species within a group, but processing of large numbers of samples is not easy. Commercial identification kits, such as the analytical profile index (API), and automated identification systems, such as the VITEK, are available for identifying many bacteria to the species level and may allow relatively rapid identification. Some strains may not fit in the commercial biochemical assays. This may be due to specific individual requirements (Settanni and Corsetti, 2007).

Genotypic (molecular) methods are useful to identify bacteria either as a complement or alternative to phenotypic methods; besides enhancing the sensitivity and specificity of the detection process, they reduce much of the subjectivity inherent to interpreting morphological and biological data. Basically, DNA is invariant throughout the microbial life cycle and after short-term environmental stress factors. Thus, molecular methods targeting genomic DNA are generally applicable.

Restriction fragment length polymorphism (RFLP) of total genomic DNA represents a technique belonging to the first generation molecular (not based on polymerase chain reaction [PCR]) methods widely applied in microbial differentiation in past years (Rossello-Mora and Amann, 2001). For the same purpose, Southern blot hybridization tests, which enhance the result of an agar gel electrophoresis by marking specific DNA sequences, have been also extensively used. The second generation molecular techniques (known as PCR-based technologies) such as PCR–RFLP and randomly amplified polymorphic DNA (RAPD) have been used for differentiation and also identification of microbial isolates. In the latter case, as the profiles generated on ethidium bromide–stained gels do not contain specific sequence information, they need to be compared with those of reference strains. If used alone, these methods do not segregate strains into a particular species since they do not rely on the appearance or absence of a specific PCR product generated from a specific primer pair. However, RAPD–PCR has been successfully applied to the identification of microorganisms from different sources (Bautista-Munoz et al., 2003; Corsetti et al., 2003; Jensen et al., 2002 Raclavský et al., 2006). Identification based on PCR amplification of targeted genes, when developed and validated for a certain species, is considered to be a reliable technique (Settanni et al., 2005); many species-specific monoplex PCR strategies have been validated and routinely used to identify microbial isolates.

One of the branches of applied molecular microbiology is particularly involved in the development of molecular methods for monitoring microorganisms in natural ecosystems. Classical technology and monoplex PCR approaches are not suitable for such studies of complex flora consisting of multiple microbial species. The current trend is toward culture-independent, PCR-based methods as these are believed to overcome problems associated with selective cultivation and isolation of microorganisms from natural samples, and because the PCR-based methods are generally characterized by their simplicity, speed, cost-effectiveness, and reliability. Perhaps the most commonly used method among the culture-independent fingerprinting techniques is PCR followed by denaturing gradient gel electrophoresis (DGGE). PCR–DGGE provides information about variation of PCR products of the same length but with different sequences upon differential mobility in an acrylamide gel matrix of increasing denaturant concentration (Muyzer et al., 1993).

To rapidly detect multiple microorganisms in a single reaction, simultaneous amplification of more than one locus is required; a methodology referred to as multiplex PCR (MPCR) (Chamberlain et al., 1988) in which several specific primer sets are combined into a single PCR assay. Hence, MPCR is undoubtedly useful to rapidly identify several isolates and, with respect to DGGE, it enables the selection of various species and represents the fastest culture-independent approach for strain-specific detection in complex matrices. Thus, MPCR might also be useful to define the structure of certain microbial communities and to evaluate community dynamics, for example,

during fermentation or in response to environmental variations. At a certain level, MPCR might be considered as a quantitative (or better yet, a semiquantitative) technique, since once it has been established, the detection limit can be retrieved and used as the minimal microbial concentration detectable. Quantification of microorganisms is sometimes of paramount importance, as in the case of toxigenic bacteria, the concentration of which depends on toxin production. With this in mind, real-time reverse-transcriptase PCR, which is based on the detection and quantification of a fluorescent reporter whose signal increases in direct proportion to the amount of PCR product, is a preferable alternative (Settanni and Corsetti, 2007).

6.5 CONTROL, PREVENTION, AND MANAGEMENT OPTIONS FOR SPOILAGE MICROORGANISMS

6.5.1 Carcass Washing

There are a variety of approaches to reduce microorganisms in retail poultry and these can be targeted at various control points, from farm to fork. High-temperature treatments have been used for many years to improve the safety of food products. The development of heat treatments that could reduce bacterial numbers on raw poultry is of public health importance. The potential for a surface decontamination technique relevant to raw poultry by using condensing atmospheric steam has been investigated previously at the University of Bristol, Bristol, United Kingdom.

Condensing steam (at 100°C) rapidly transferred heat to the surface of the product while it was in the steam environment and the product then dropped out of the vessel into a cold-water spray giving rapid chilling. This process resulted in a 2 log 10 reduction in aerobic plate counts but the occurrence of unacceptable surface changes and splitting of the poultry skin during trussing prevented the method proceeding to industrial trials. Purnell et al. (2004) found that a heat treatment regime of 70°C for 40 s did not detrimentally affect the chicken skin and reduced the numbers of aerobic bacteria, Enterobacteriaceae and *Campylobacter.*

6.5.2 Chemical Methods

Chemical and mechanical methods have been studied or practiced to improve food safety and quality. To eliminate or reduce bacteria attached to poultry carcasses or in processing water, chlorine (Mead et al., 1975; Lillard, 1980), ozone (Yang and Chen, 1979; Sheldon and Brown, 1986), and other food-grade chemicals (Thomson et al., 1976; Izat et al., 1990) have been applied in poultry processing.

Various methods especially those focusing on washing and sanitizing procedures have been developed to reduce the level of contaminating bacteria on carcasses. Spray with hot water, chlorine, and short-chain organic acids or other food-grade chemicals have been applied in poultry processing (Li et al., 1997). Effects of these compounds varies depending on the concentration of sanitizers used, temperature and contact time of the sanitizers, sensitivity of the native microflora to the specific compound, and to a certain extent the design of the specific experiments (Dickson and Anderson, 1992).

6.5.2.1 Chlorinated Water

Chlorinated water is used at frequent intervals to wash carcasses during processing. Lillard (1980) found that chilling water in a commercial broiler processing plant was treated with chlorine and chlorine dioxide. Microbiologically, water samples were significantly better when 34 ppm chlorine or 5 ppm chlorine dioxide rather than 20 ppm chlorine or 3 ppm chlorine dioxide was used. Despite differences in chill-water quality, all treatments significantly reduced bacterial counts of carcasses over those chilled in untreated water. Tsai et al. (1992) determined the disinfection efficiency of chlorine. They found the added chlorine was rapidly consumed in chill water. However,

the consumption rate quickly decreased to 1/1000 of the initial rate after about 50 min. The change of residual chlorine with time was found to fit mathematically to a two-term exponential equation. The equilibrated chill water had a chlorine demand greater than the highest chlorine dosage tested (400 ppm). No free chlorine was detected in the water after 30 min of exposure to 300 ppm chlorine. Because of the high chlorine demand, dosages of 100–150 ppm chlorine were required to reduce the number of bacteria by at least 99% within 3–5 min in laboratory tests. Increasing the time of exposure to chlorine resulted in further reduction, although at a slow rate, of surviving bacteria from treatments that gave incomplete destruction with short exposure.

6.5.2.2 Trisodium Phosphate Treatment

Trisodium phosphate treatment has been reported to reduce populations of different pathogenic and spoilage bacterial groups without affecting the products' sensory quality (Vareltzis et al., 1997; Hinton and Ingram, 2005). Carneiro et al. (1998) indicated that high trisodium phosphate concentrations (approximately 10% [w/v]) were needed for successful decontamination of Gram-negative bacteria on the surface of poultry and other foodstuffs. The Gram-negative bacteria were substantially reduced by further trisodium phosphate treatment in combination with exposure to low lysozyme or nisin concentrations. After the explosion of trisodium phosphate, the susceptibility of *Campylobacter jejuni, E. coli, P. fluorescens*, and *Salmonella enteritidis* to lysozyme and/or nisin would increase. Under optimal conditions at 37°C, reductions in viable count after 30 min were up to 6 log CFU. *C. jejuni* showed greater resistance at 4°C than 37°C, and maximal cell kills (95%) were reduced by more than 2 log CFU. Cells dried on the surface of chicken skin were more resistant than suspended cells to trisodium phosphate—lysozyme and trisodium phosphate—nisin treatments. Nevertheless, at 37°C, kills varied from approximately 95% for *S. enteritidis* cells with nisin (30 μM) or lysozyme (100 μg/mL) to >99.9% for *C. jejuni* and *E. coli* cells with nisin. Under the experimental conditions used, nisin also reduced viable counts of skin-attached *Staphylococcus aureus* by >99.9%. Del Río et al. (2007) found that chemical compounds, such as TSP, acidified sodium chlorite, and citric acid were effective in reducing microbial populations of chicken legs.

6.5.2.3 Organic Acids Treatment

Organic acids have a long history of being utilized as food additives and preservatives for preventing food deterioration and extending the shelf life of perishable food ingredients. Zeitoun and Debevere (1990) indicated that the use of buffered lactic acid systems enhances the decontaminating effect and increases shelf life of chicken legs compared with unbuffered lactic acid solutions. A reduction of 2 pH units of the chicken skin is obtained by treatment with 10% lactic acid buffer. Buffer resulted in a lower pH of the skin compared with that of untreated legs. Legs treated with 10% lactic acid buffer have a shelf life of 12 days at 6°C, which means an increase of 6 days compared with the shelf life of untreated legs. Ouattara et al. (1997) evaluated the relative ability of acetic, benzoic, citric, lactic, propionic, and sorbic acids to inhibit growth of six common spoilage bacteria (*Brochothrix thermosphacta, Carnobacterium piscicola, Lactobacillus curvatus, Lactobacillus sake, P. fluorescens,* and *Serratia liquefaciens*). Due to their low solubility in the growth media, benzoic and sorbic acids could only be used in low concentrations (below 0.15% [w/v]) and did not efficiently inhibit bacterial growth. All other acids totally inhibited growth at concentrations ranging from 0.1% to 1% (w/v). On a weight basis, acetic acid was found to be most inhibitory, followed by propionic, lactic, and citric acids, whereas the order of efficiency was reversed (citric > lactic > propionic > acetic) when the acid concentrations were expressed on a molar basis or when the acid effectiveness was evaluated relative to the concentration of undissociated molecules. Overall, the lactobacilli were the bacteria most resistant to the action of organic acids, followed by *P. fluorescens* and *S. liquefaciens*, while *B. thermosphacta* and *C. piscicola* were considerably more sensitive.

6.5.2.4 Other Sterilization

In order to get more insight in the effect of sanitizers on spoilage bacteria associated with poultry, Russell (1998) investigated this phenomenon. *P. fluorescens*, *P. putida, P. fragi*, and *Sh. putrefaciens* were collected from spoiled broiler carcasses. The following sanitizers in three replicate trials (Rep) were applied to the isolates: sodium hypochlorite, quaternary ammonium, lactic acid, trisodium phosphate, hydrogen peroxide, and timsen, a novel compound containing a quaternary ammonium derivative in combination with urea. Sodium hypochlorite eliminated growth of *P. fluorescens*, *P. putida*, and *P. fragi*, but did not consistently kill *Sh. putrefaciens* at concentrations ≤50 ppm. Quaternary ammonium did not consistently eliminate any of the spoilage bacteria analyzed. At levels of 5% or above, lactic acid eliminated or inhibited all spoilage isolates. At 0.5%, trisodium phosphate eliminated *pseudomonads*, whereas a level of 1% or higher was required to prevent *Sh. putrefaciens* growth. Hydrogen peroxide was an effective sanitizer at 0.1%. Few spoilage bacteria multiplied in the presence of 10 ppm timsen. *Sh. putrefaciens* grew when exposed to 10 ppm or 100 ppm timsen in Reps 1 and 2, respectively. All of the sanitizers tested, except quaternary ammonium, prevented growth of the *pseudomonads*. In general, *Sh. putrefaciens* was more resistant to sanitizers than were the *pseudomonads*. Another study was conducted to evaluate the effect of an acidic, copper sulfate-based commercial sanitizer on indicator, pathogenic, and spoilage bacteria associated with broiler chicken carcasses when applied at various intervention points during poultry processing. Exposure of *Salmonella typhimurium*, *Listeria monocytogenes*, *St. aureus*, *P. fluorescens*, or *Sh. putrefaciens* to the sanitizer in scalder water at 54°C for 2 min resulted in complete elimination of these bacterial species. Exposure of *E. coli* to the treated scald water resulted in a 4.9 log CFU reduction. These data suggest that this sanitizer would be effective for use in scalders. When applied during scalding in a commercial processing plant, total aerobic bacteria and *E. coli* counts were reduced on all days of sampling. The average log CFU reduction overall was 3.80 and 3.05 for total aerobic bacteria and *E. coli*, respectively. *Salmonella* prevalence was reduced by an average of 30%. For carcasses that were scalded, picked, and dipped postpick using this sanitizer, total aerobic bacteria were significantly reduced on all days of sampling by an average of 1.19 log CFU. *E. coli* counts were reduced on all but 2 days of sampling for carcasses scalded, picked, and dipped in this sanitizer, except for day 2 and day 10. Averages on these days were higher for controls, but were not significantly different. *Salmonella* prevalence was not consistently impacted overall. For the shelf-life study, odor scores were reduced for treated carcasses at day 8 through day 14 of storage. The psychrotrophic plate counts were lower in treated carcasses at day 6 through day 14 of storage. This sanitizer suppressed spoilage bacteria with a 99.99% reduction at day 10 and a 99.9% reduction at 12 days of storage. This effect could result in an extension of the shelf life of the poultry carcasses by up to 4 days (Russell, 2008).

The effect of sodium hypochlorite and monochloramine on bacterial populations, which associated with broiler chicken carcasses, was different. Nominal populations (6.5–7.5 log CFU) of *E. coli*, *L. monocytogenes*, *P. fluorescens*, *Salmonella serovars*, *Sh. putrefaciens*, and *St. aureus* were exposed to sterilized chilled water (controls) or sterilized chilled water containing 50 ppm sodium hypochlorite or monochloramine. Sodium hypochlorite at 50 ppm eliminated all (6.5–7.5 log CFU) viable *E. coli*, *L. monocytogenes*, and *S. serovars*; 1.2 log CFU of *P. fluorescens*; and 5.5 log CFU of *Sh. putrefaciens*. Monochloramine eliminated all (6.5–7.5 log CFU) viable *E. coli*, *L. monocytogenes*, *Sh. putrefaciens*, and *S. serovars* and 4.2 log CFU of *P. fluorescens*. Chicken carcasses were inoculated with *P. fluorescens* or nalidixic acid–resistant *S. serovars* or were temperature abused at 25°C for 2 h to increase the populations of naturally occurring *E. coli*. The groups of *S. serovars*–inoculated or temperature-abused *E. coli* carcasses were immersed separately in pilot-scale poultry chillers and exposed to tap water (controls) or tap water containing 20 ppm sodium hypochlorite or 20 ppm monochloramine for 1 h. The *P. fluorescens*–inoculated group was immersed in pilot-scale poultry chillers and exposed to tap water (controls) or tap water containing 50 ppm sodium hypochlorite or 50 ppm monochloramine for 1 h. Carcasses exposed to the sodium hypochlorite treatment

had nominal increases (0.22 log CFU) in *E. coli* counts compared with controls, whereas exposure to monochloramine resulted in a 0.89 log reduction. Similarly, average nalidixic acid–resistant *S. serovar* counts increased nominally by 34% compared with controls on carcasses exposed to sodium hypochlorite, whereas exposure to monochloramine resulted in an average nominal decrease of 80% (4.1–8 log CFU/mL). *P. fluorescens* decreased by 0.64 log CFU on carcasses exposed to sodium hypochlorite and decreased by 0.87 log CFU on carcasses exposed to monochloramine. Sodium hypochlorite or monochloramine was applied to the chiller in a commercial poultry processing facility. *E. coli* counts (for carcass halves emerging from both saddle and front-half chillers) and *Salmonella* prevalence were evaluated. Data from carcasses exposed to sodium hypochlorite during an 84-day historical (Hist) and a 9-day prepilot (Pre) period were evaluated. Other carcasses were exposed to monochloramine and tested during a 27-day period (Test). *E. coli* counts for samples collected from the saddle chiller were 25.7, 25.2, and 8.6 log CFU/mL for Hist, Pre, and Test, respectively. *E. coli* counts for samples collected from the front-half chiller were 6.7, 6.9, and 2.5 log CFU/mL for Hist, Pre, and Test, respectively. *Salmonella* prevalence was reduced from 8.7% (Hist + Pre) to 4% (Test). These studies indicate monochloramine is superior to sodium hypochlorite in reducing microbial populations in poultry chilled water (Russell and Axtell, 2005).

6.6 CONCLUSION AND PERSPECTIVES

Although poultry processing decreases carcass contamination by psychrotrophic spoilage bacteria, significant levels of bacterial cross-contamination occur during processing. Contamination of broiler carcasses can be minimized by good manufacturing processes, but the total elimination of foodborne spoilage microorganisms is difficult. In order to monitor and control spoilages, more rapid and useful methods should be developed based on genotypic methods.

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7 Spoilage Microorganisms in Seafood Products

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CONTENTS

7.1 INTRODUCTION

Seafood is one of the most highly perishable food products and the shelf life of such products is limited due to the presence of oxygen in the air and the growth of aerobic spoilage microorganisms (Özogul et al., 2004). Seafood contains dynamic systems where pH, atmosphere, nutrient composition, and microflora change over time. Seafood spoilage is defined as changes in the organoleptic properties (visual, taste, odor, and texture) of a seafood product rendering it unsuitable for human consumption (Grigorakis et al., 2003). Sulfurous, ammoniac, or fishy odors are some of the main organoleptic changes taking place during spoilage development (Gram and Dalgaard, 2002). Spoilage of seafood can be caused by enzymes, dehydration, oxidation, contamination, and physical damage (Harbell, 1988). However, the major cause of seafood spoilage is microbial growth and metabolic activity resulting in the formation of amines, sulfides, alcohols, aldehydes, ketones, and organic acids with unpleasant and unacceptable off-flavors (Robson et al., 2007).

Microorganisms are the major cause of spoilage of most seafood products. However, only a fraction of the initial microbiota of seafood, known as specific spoilage organisms (SSOs), which is favored by storage conditions (e.g., atmosphere, temperature), prevails over the rest of the microbiota, reaching

high populations and producing several metabolites (biochemical spoilage indices) (Parlapani et al., 2015). SSOs are not the same in every case and are dependent on the climatic and storage conditions, the type of fish, and even the place where the seafood was harvested (Tsigarida et al., 2003). Besides, the expression of many phenotypic traits in microorganisms is governed by tight gene regulation, several behavioral patterns are correlated with the density of the population, and the ability to regulate gene expression as function of cell density has been termed "quorum sensing" (QS) (Fuqua et al., 1994).

Fish and shellfish are perishable protein sources for human consumption. Shelf life of fish and shellfish can be short and variable, because the flesh has a high water activity (a_w), neutral pH, high amino acid content, and also frequently harbors psychrotolerant spoilage bacteria with the ability to grow during chilled storage (Cruz-Romero et al., 2008b). The identification of bacteria that are responsible for spoilage requires sensory, microbiological, and chemical studies (Gram and Huss, 1996). Identification of SSOs and determination of their spoilage domain and spoilage level are prerequisites to accurately predict shelf life (Koutsoumanis and Nychas, 2000). For a long time, the study of a natural microbiota ecosystem has consisted of a cultural methodology using different culture media. However, this approach does not necessarily provide reliable information about the composition of microbial communities, which may contain species that would be viable but are not cultivable on current culture media (Duthoit et al., 2003). Thus, molecular methods have been developed to analyze diversity within bacterial communities (Muyzer and Smalla, 1998). The use of molecular approach allows a more comprehensive and rapid assessment of microbiota, and provides a further insight into the dynamic evolution of the biodiversity in different seafood ecosystems.

Scientists have been constantly searching for improved methods to preserve or extend the shelf life and safety of various aquatic food products (Chang et al., 1998). Shelf life is defined as the period of time under defined conditions of storage for which a food product remains safe and fit for use. In other words, during this period, it should retain its desired sensory, chemical, physical, functional, or microbiological characteristics (IFST, 1993). The degradation of seafood quality resulted from microbial spoilage and biochemical reactions during handling and storage can be controlled via preservation techniques. A change in atmosphere, for example, by vacuum packing (VP), will inhibit the respiratory pseudomonads; further "selective pressure," for example, addition of low levels of salt to and drying of fish eventually switch the microflora in the same direction as vacuum-packed meat and meat products, thus a microflora of lactic acid bacteria (LAB), Enterobacteriaceae, and *Brochothrix* develops; increasing the preservation by a decrease in pH, an increase in the NaCl concentration, and by adding sorbate and/or benzoate eliminates the Gram-negative microflora (Gram et al., 2002).

In this chapter, the taxonomy and detection of spoilage microorganisms corresponding to spoilage characteristics will be discussed; we will list both conventional culture–dependent and molecular culture–independent methods widely/recently used to detect and identify the SSOs in seafood. Meanwhile, the possible mechanisms involved in seafood spoilage are discussed, including external factors and interactions between spoilage microbiota, the control and/or management options aiming at inhibiting or reducing the activity depending on their spoilage mechanisms will be summarized. In addition, future trends and further advices are also discussed.

7.2 TAXONOMY OF SPOILAGE MICROORGANISMS IN SEAFOOD

Bacteria inhabit a great diversity of ecological niches and show an astounding variety in physiological properties. Bacteria in food have been extensively studied and classified. By the advance of computers beginning in the 1950s, mathematical and computational methods have turned out to be indispensable tools in bacterial taxonomy (Gyllenbergi and Koski, 2001).

In the context of bacterial systematics, the word "taxonomy" comprises three basic concepts (Koneman et al., 1997). (1) Classification: Systematic division of organisms into groups. The basic taxonomic unit, that is, the smallest and most definitive level of division, is the *species*. A classification typically has a hierarchical structure with species assigned to genera, genera to *families*, families to *tribes*, and so forth. (2) Nomenclature: Naming of organisms. All organisms that have

been given the same name form a nomenspecies. The scientific name of an organism consists of two parts, the former representing the genus and the latter the species. When there is no risk of confusion the genus name is contracted to the initial. The name often reflects characteristic features of the taxon or may commemorate the microbiologist who first described it (or both). (3) Identification: The process of characterizing a given organism to determine its taxon, its relatedness to other similar, or dissimilar, organisms. The taxonomy provides a convenient framework for storing and summarizing all available information. A good and natural taxonomy has a predictive value: it allows us not only to generalize known facts, but also to make reasonably confident generalizations in cases where observations have not yet been made. In diagnostic microbiology, a bacterial strain is often characterized by a biochemical profile. This profile is obtained by tests detecting differences in the bacterial metabolisms and thus measuring phenotypic characteristics that reflect the genotype of the organism being tested (Gyllenbergi and Koski, 2001).

7.2.1 Several Modern Taxonomy Methods

Van Leeuwenhoek's observation of microscopic organisms launched centuries of classification based on morphology and physiology (Porter, 1976). The current polyphasic species definition requires a DNA–DNA hybridization (DDH) value >70%, as well as shared phenotypic characteristics, to assign two bacteria to the same species (Stackebrandt and Goebel, 1994). Phenetics classifies organisms based on similarity regardless of shared ancestry. The definition of the term "similarity" is fluid, but in its broadest sense implies a comparison of organism phenotypes, including their molecular functional capabilities. It is important to note that though both phylogeny-based taxonomy and phenetics can be used to investigate bacterial relationships, the questions that they try to answer are different. The task of phylogeny is reconstructing organismal evolutionary *history-think Tree of Life* efforts. Phenetics, on the other hand, clusters organisms into currently consistent classes on the basis of observable traits (Zhu et al., 2015a). The *16S rRNA* gene was, and still is, applied as the universal marker gene for basic evolutionary analysis of both culturable and unculturable bacteria. This is mainly due to the fact that the *16S rRNA* gene fulfils most of the requirements of a good phylogenetic marker, it occurs ubiquitously in *Bacteria* and *Archaea*, and has an indispensable function in protein synthesis (functional constancy), which is linked to its evolutionary conservation; however, the main disadvantage of the application of the *16S rRNA* gene as a phylogenetic marker is its insufficient resolution at the species level. A higher phylogenetic resolution, especially within genera, can be obtained by additionally performing phylogenetic analyses based on protein-coding genes. In contrast to the *16S rRNA* gene, protein-coding genes are supposed to evolve at a slow but constant rate and have a better resolution power, especially at the genus level or even below (Glaeser and Kämpfer, 2015). The currently applied method in prokaryotic taxonomy considering internal fragments of several protein-coding genes is called multilocus sequence analysis (MLSA) (Gevers et al., 2005). Most often, a new strain is first phylogenetically placed on the basis of a *16S rRNA* gene-based analysis at the genus level, and then MLSA is increasingly being applied to obtain a higher resolution power among species of a genus (Glaeser and Kämpfer, 2015). The MLSA approach based on the sequence analysis of the four housekeeping genes (*16S rRNA*, *gyrB*, *rpoB*, and *rpoD*) has proven reliable for species delineation and strain identification in *Pseudomonas* (Mulet et al., 2012). MLSA was considered as the most convenient method nowadays for the assessment of the phylogenetic relationships among the species of the genus *Pseudomonas* until whole genome sequences of the type strains are available (Gomila et al., 2015). Besides, the amplified fragment length polymorphism (AFLP) method, which can recognize and evaluate species of phenotypical similarity but evolutionary distinction, was shown to distinguish clearly *Photobacterium phosphoreum* and *Photobacterium iliopiscarium*, two species that were previously difficult to distinguish with other highly specialized phylogenetic studies (Jérômea et al., 2016). Moreover, a high level of correlation between AFLP results and published DNA hybridization data has been shown for many bacterial genera and species, indicating that AFLP is a valuable technique for the classification of

bacteria (Blears et al., 1998; Mougel et al., 2002). The method shows high discriminatory power and good reproducibility and has proved to be efficient for discriminating at the species level and below in various taxa (Janssen et al., 1996; Savelkoul et al., 1999). It was proposed that phenotypic information could be directly inferred from whole genome sequences as an alternative to commercial systems applied for the phenotypic characterization. Genes coding for the enzymes responsible for key phenotypic markers are currently used to identify *Photobacteriums* species, it was revealed that all genes coding for the specific proteins involved in metabolic pathways responsible for positive phenotypes were found in the majority of the *Photobacterium* genomes, and that *Photobacterium* species that were negative for a given phenotype showed the absence of at least one gene involved in the respective biochemical pathway and/or in the regulatory genes. This contributes to microbial taxonomy when genomes are available (Amaral et al., 2015).

Due to the absence of sexual reproduction and the presence of horizontal gene transfer (HGT), speciation is not strictly defined in prokaryotes. It was emphasized that the phenotype is still irreplaceable if a new species is proposed and described as representative of a broad range of the overall properties of a new taxon and, consequently, polyphasic taxonomy should still remain the standard (Kämpfer and Glaeser, 2012). It was supposed that the goal of greater classification stability and predictability could be better achieved via phenetically clustering organisms on the basis of quantifiable similarity of their molecular function capabilities. Homology-derived secondary structure of proteins (HSSP) techniques and the National Center for Biotechnology Information (NCBI) taxonomy were used to annotate two proteins as performing the same molecular function without specifically defining the nature of this function and as a benchmark, respectively, for the first time to show that functional similarity defined via HSSP was more descriptive of pairwise organism similarity than gene sequence identity. This functional-repertoire similarity-based organism network, which grouped microorganisms according to functional similarity, was identified to provide unambiguous and consistent classification of bacteria and add a complementary point to phylogenetic clade assignment (Zhu et al., 2015a). In addition, proteomics-based approaches represent an emerging complement to traditional methods of characterizing microorganisms, enabling the elucidation of the expressed biomarkers of genome sequence information, which can be applied to "proteotyping" applications of microorganisms at all taxonomic levels. Advanced methods of tandem mass spectrometry, in which proteins and peptides generated from proteins are characterized and identified using liquid chromatography tandem mass chromatography (LC–MS/MS), provide the ability to detect hundreds or thousands of expressed microbial strain markers for high-resolution characterizations and identifications. However, the identification of the discriminative peptide fragments is highly dependent on access to a comprehensive and accurately curated database. Indeed, erroneous or missing reference data may result in peptide fragments that cannot be matched correctly to the respective references and are, thus, falsely classified as discriminative. Such errors will consequently introduce analytical "noise" with a substantially reduced accuracy (Karlsson et al., 2015).

7.2.2 Main Spoilage Microorganisms in Different Seafood and Seafood Products

Early studies of seafood microbiology acknowledged that only part of the spoilage microflora participated in the spoilage process (Gram and Dalgaard, 2002). The recent establishment of the SSO concept has contributed significantly to our understanding of seafood spoilage (Dalgaard, 1995). The growth of different SSOs depends on several parameters: food product, type of preservation, temperature, atmosphere, and salt content, among others. During storage, the microflora changes owing to different abilities of the microorganisms to tolerate the preservation conditions (Bekaert et al., 2015).

7.2.2.1 Fresh Seafood Stored in Ice or under Modified Atmosphere Packaging/VP

In newly caught marine seafood from temperate waters, microflora is formed mainly for aerobic rods-shapes, anaerobic facultative, and psychrotrophic Gram-negative bacteria, whose growth is possible at 0°C and optimal at around 25°C. The majority belongs to Gammaproteobacteria: *Pseudomonas*,

Shewanella, *Acinetobacter*, *Aeromonas*, *Vibrio*, *Moraxella*, *Psychrobacter*, *Photobacterium*, and so forth. The same bacterial genus can be found in tropical marine seafood but Gram-positive bacteria, Enterobacteriaceae, and *Vibrionacea* are often dominant (Françoise, 2010). Generally, *Pseudomonas* spp., *Shewanella putrefaciens*, *Shewanella baltica*, or *Aeromonas* spp. are common dominant spoilage bacteria in iced sea salmon (Hozbor et al., 2006; Macé et al., 2012); gutted sea bass (Parlapani et al., 2015); chilled fresh Mediterranean swordfish (Pantazi et al., 2008); tropical prawns (Chinivasagam et al., 1998; Zhu et al., 2015c); and large yellow croaker (Zhu et al., 2016). *Pseudoalteromonas* and *Vibrio* were dominant microorganisms in shucked oysters during iced storage (Madigan et al., 2014); as spoilage proceeded, *enterococci*, *lactobacilli*, and yeasts dominated at the later stages of storage (Cao et al., 2009b). This may be due to fact that the spoilage patterns of molluscan shellfish differ to most species of seafood as they contain high levels of carbohydrate in the form of glycogen (Fernandez-Piquer et al., 2012). This physiological difference results in a dominance saccharolytic and fermentative bacteria, which can ferment glycogen to organic acids. However, the exact role of *Pseudoalteromonas* and *Vibrio* in spoilage remains unclear and further work is required to isolate these bacteria and ascertain if they are SSOs or merely associated with spoilage (Madigan et al., 2014). Dominant strains isolated from spoiled squid were identified as *P. phosphoreum* (Paarup et al., 2001). Bacteria grew faster under aerobic conditions, while the increase of CO_2 and O_2 reduction of modified atmosphere packaging (MAP) inhibited the bacterial growth and changed the microbial spoilage by suppressing mostly the Gram negatives and favoring the Gram positives (Parlapani et al., 2015). *P. phosphoreum* and *Lactococcus piscium* were identified as the main bacterial groups in MAP/VP raw salmon (Macé et al., 2012). The main SSO of modified atmosphere packaged Norway lobster is *P. phosphoreum*, since *P. phosphoreum* is known to withstand high CO_2 concentrations (Gornik et al., 2013). LAB and *Brochothrix thermosphacta* were codominant with *Pseudomonas* and H_2S-producing bacteria in gutted sea bass stored at 2°C under MAP (Parlapani et al., 2015). *Carnobacterium maltaromaticum* was the organism that showed the highest resistance to CO_2 and to the lack of O_2 among the organisms responsible for spoilage in mackerel fillets packed under modified atmospheres (Alfaro et al., 2013).

7.2.2.2 Lightly Preserved Seafood

Lightly preserved seafood are uncooked or mildly cooked products with low-level preservatives, which can influence their a_w, pH, including brined/pickled/marinated seafood, cooked and peeled shrimp, and shucked shellfish stored in MAP/VP or in brine, cold-smoked fish, and so forth. Unlike fresh fish, which presents a very high a_w value (0.99), a_w is reduced to about 0.96 when salt is added to the product. As a result, aerobic Gram-negative bacteria are inhibited, which allows the growth of other organisms more resistant to reduced a_w (Françoise, 2010).

Psychrobacter spp. and *Pseudoalteromonas* spp. were the dominant microbiota of cooked brown shrimp and enhanced spoilage by breaking down lipids and hydrolyzing amino acids and proteins (Broekaert et al., 2013). The major spoilage bacterial isolates from spoiled cooked and whole tropical shrimp stored under MAP were *C. maltaromaticum* and *S. baltica* (Macé et al., 2014), as for the spoilage of cooked and peeled tropical shrimp, *B. thermosphacta* was also involved (Jaffrès et al., 2011). LAB and *Brachothrix* spp. were dominant bacteria in the latter storage period of the VP-packed cold-smoked salmon, whereas *Brachothrix* spp. rather than LAB were responsible for spoilage because of the strong sour and cheesy odors produced (Leroi et al., 1998). On the other hand, Joffraud et al. (2006) identified *Carnobacterium piscicola* as a very lightly spoilage bacteria, *B. thermosphacta* as a lightly spoilage bacteria, *Lactobacillus sakei* and *Serratia liquefaciens*–like as the most spoiling bacteria. Besides, psychrotrophic marine *Vibrio* and *Photobacterium* spp. were reported to be dominant microflora (Hansen et al., 1998). The difference of spoilage microorganisms' profiles of cold-smoked salmon may result from the different treatments and environment. In addition, *Aspergillus niger*, *Penicillium citrinum*, and *Cladosporium cladosporioides* are the primary spoilage molds of dried fishery food products (Park et al., 2014).

Gram and Dalgaard (2002) concluded that acidification or the addition of preservatives such as sorbate and benzoate, as in the so-called semipreserved seafood, allows growth of *Lactobacilli* and yeasts that may grow in heavily wet-salted fish. Drying or heavily dry-salting of fish eliminates bacterial growth and these products will spoil due to growth of filamentous fungi.

In conclusion, as reviewed by Gram et al. (2002), increasing the preservation by a decrease in PH (below 5) and an increase in the NaCl concentration (above 6%) eliminate the Gram-negative microflora. LAB and yeast are the remaining organisms in lightly preserved seafood. Thus, as the preservation profile increases in inhibitory strength, the microflora changes along the pathway of nonfermentative psychrotrophic Gram-negative bacteria to fermentative Gram-positive bacteria to LAB to yeasts, and finally to filamentous fungi.

7.3 SPOILAGE MICROORGANISMS' CHARACTERISTICS AND POSSIBLE MECHANISMS

7.3.1 Biochemical Index for Spoilage Microorganisms

Although the characteristic spoilage off-flavors and off-odors are typical of microbiological activity, the role of the microflora in spoilage of the product remains to be determined. Microbiological, biochemical, and sensory methods have been used to assess freshness and quality of fish during handling and storage, with the main attributes of freshness being aroma, taste, texture, and appearance response (Koutsoumanis et al., 2002). The most standard and accepted way of determining fish quality is by means of sensory analysis. Because it is an analysis that is closer to ordinary consumers' experience, the assessment takes advantage of the integration of more than one sense from the assessors so the sensory analysis can be very sensitive. However, there are some disadvantages such as the impossibility of automated measurements, lack of objectiveness, poor reproducibility, and questions regarding fatigue and/or adaptation. Additionally, it is not a good method for quantification and the interpretation of results is controversial and open to dispute. What's more, high precision requires several assessors and can be very expensive (Ólafsdbóttir et al., 1997; Bremner and Sakaguchi, 2000). The activity of microorganisms is the main factor limiting the shelf life of fresh fish. An estimation of the total viable counts (TVC) is used as an acceptability index in standards, guidelines, and specifications. Newly caught fish contains a diverse microflora, TVC of 10^2–10^6 CFU/g are usual on whole fish and cut fillets. During chill storage, psychrotolerant microorganisms are selected, thus, differential counting of these microorganisms was suggested as a measure of fish quality in early studies. Obviously, microbial methods can provide useful measures of fish freshness, whereas the most promising results have been achieved with relatively slow detection methods such as plate count and other growth techniques that involve a period of incubation. At the point of sensory rejection, the TVC of fish products are typically 10^7–10^8 CFU/g. Nevertheless, standards, guidelines, and specifications often use much lower TVC as indices of acceptability. Microbial criteria based on low TVC, such as 10^6 CFU/g are problematic to use because a correlation between TVC and the remaining shelf life is assumed but generally not known (Ólafsdbóttir et al., 1997). Therefore, biochemical methods based on nucleotide metabolism, production of trimethylamine (TMA), hypoxanthine (Hx), total volatile basic nitrogen (TVB-N), and biogenic amines (BAs), have been commonly used to evaluate fish quality (Hasegawa, 1987). Hx, which may cause a bitter off-flavor in the fish, can be formed by the autolytic decomposition of nucleotides as well as by bacteria; the rate of bacterial formation is higher than the autolytic (Gram and Huss, 1996). Several of the spoilage bacteria produce Hx from inosine or inosine monophosphate, including *Pseudomonas* sp. (Surette et al., 1988), *S. putrefaciens* (Jorgensen and Huss, 1989), and *P. phosphoreum* (van Spreekens, 1977). Here, the volatile compounds and BAs widely studied as main SSOs metabolism products, which are responsible for the quality and safety degradation of seafood will be concluded as characteristics of spoilage microorganisms. The typical spoilage compounds produced by spoilage microorganisms during storage of fresh and lightly preserved fish products (LPFP) are showed in Table 7.1.

TABLE 7.1
Typical Spoilage Compounds Produced by Spoilage Microorganisms during Storage of Fresh and Lightly Preserved Fish Products

	Production of Spoilage Compounds								
Substitute Compounds	**TMAO TMA**	**Cysteine H_2S**	**Methionine CH_3SH $(CH_3)_2S$**	**Other AAs Ketones, Esters, Aldehydes, NH_3**	**Histamine**	**Putrescine**	**Cadaverine**	**IMP, Inosine Hypoxanthine**	**Fish Products (References)**
Spoilage bacteria									
Shewanella putrefaciens	+	+	+	+	+	+	+	+	Iced fish (Gram and Huss, 1996; López-Caballero et al., 2001)
Pseudomonas sp.	−	−	+	+	nr	+	nr	+	Iced fish (Gram and Huss, 1996; Paleologos et al., 2004)
Photobacterim phosphoreum	+	−	−	−	+	nr	nr	+	MAP/VP fish (Gram and Huss, 1996; Emborg et al., 2005)
Enterobacteriaceae	+	+	nr	+	+	+	+	+	Lightly preserved fish (Gram and Huss, 1996; Kim et al., 2009)
LAB	−	+	nr	+	nr	nr	nr	nr	Lightly preserved fish (Gram and Huss, 1996)
Yeast	−	−	−	+	nr	nr	nr	nr	Lightly preserved fish (Gram and Huss, 1996)

Note: AAs, amino acids; nr, not reported.

7.3.1.1 Biogenic Amines

BAs are low molecular weight organic bases that possess biological activity. BAs are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones (Askar and Treprow, 1986; Brink et al., 1990). BAs can be divided into three groups according to chemical structure: aliphatic (putrescine, cadaverine, spermine, spermidine); aromatic (tyramine, phenylethylamine); and heterocyclic (histamine, tryptamine) (Bardcz et al., 1993). In seafood, BAs are formed due to the presence of decarboxylase-positive microorganisms, and conditions that allow bacterial growth, decarboxylase synthesis, and decarboxylase activity (Brink et al., 1990). In nonfermented foods, BAs above a certain level are considered as indicators of undesired microbial activity and thus of microbial spoilage (M.H. Silla Santos, 1996; Hughes et al., 2016). Mietz and Karmas (1977) proposed the biogenic amines index (BAI) to evaluate the quality of rockfish, salmon, lobster, and shrimp. Yamanaka et al. (1987) proposed agmatine as a potential index for freshness of common squid (*Tobarodespacificus*). Dawood et al. (1988) indicated that putrescine and cadaverine could be used to assess the freshness of chilled, stored rainbow trout (*Salmon irrideus*). Okozumi et al. (1990) showed that high levels of putrescine and cadaverine were detected at the spoilage stage of horse mackerel meat when *Pseudomonas* spp. were the dominant bacterial flora. Veciana-Nogues et al. (1997) proposed the use of an index calculated from the sum of histamine, tyramine, putrescine, and cadaverine for tuna quality assessment. A good correlation was found between sensory evaluation and levels of putrescine and cadaverine in skipjack tuna (Sims et al., 1992).

The profiles of BAs formed in different kinds of seafood depend on the compositions and levels of originally existing microorganisms, contamination status, and storage conditions. *S. putrefaciens* produced mainly cadaverine and putrescine, and correspondingly produced putrid off-odors when the bacteria reached 10^8–10^9 CFU/g in spoiled hake (Lopez-Caballero et al., 2001). It seemed that amine-forming bacteria were mainly psychrophilic (and/or mesophilic) enterobacteria during the storage of amberjack samples, *Enterobacter aerogenes* and another two different species of enterobacteria were identified as the strong BAs (mainly histamine, putrescine, and cadaverine) producers. Besides, it was found that BA contents increased significantly during the storage at temperature higher than 4°C, the maximum numbers of enterobacteria and amine-forming bacteria were observed at storage temperature of 10°C while no increase was observed in any of the tested microbial group stored at −20°C (Kim et al., 2009). Apart from Enterobacteriaceae (especially, *Morganella morganii*), Stratton and Taylor (1991) stated that a few strains of *Klebsiella pneumoniae* and *Hafnia alvei* have been known to be the most prolific histamine producers in fish by the storage at greater than 4°C. Histamine formation was reported to be induced by high-temperature storage of fish after harvest, and its accumulated level was affected by the combination of time and temperature. In jack mackerel stored at different temperature, *Proteus vulgaris*, *Aeromonas hydrophila*, and *Photobacterium damsel* were found to be responsible for histamine production (Bermejo et al., 2004). *M. morganii*–like bacteria and *P. phosphoreum* were observed to produce high concentration of histamine in VP tuna stored at 2.1°C (*Thunnus albacares*), 7400 ± 1050 and 4250 ± 2050 mg/kg, respectively (Emborg et al., 2005). Storage of tuna at 20°C led to rapid histamine formation to the toxic level within 1 day, whereas storage at 0°C led to a reduction in the amount of histamine produced initially through this period (Guizani et al., 2005), which was considered to attribute to histamine decomposing bacteria present within the flora, and it was reported that the histamine accumulated initially in fish stored at 5°C started decreasing and then disappearing when the count of histamine discomposing bacteria exceeded 10^6 cells/g (Sato et al., 1994). The rapid histamine accumulation at 20°C was thought to correlate with the growth of *Proteus morganii*, which was found to be the main histamine producer at 25°C–30°C (Okuzumi et al., 1984). As for *Sardina pilchardus*, however, the number of histamine-forming bacteria increased in all treatments (in air, MAP, and VP at 4°C) with increasing storage time and

lowest histamine-forming bacteria were obtained from sardines stored under MAP (Özogul et al., 2004). *P. phosphoreum* showed greater capability of producing histamine at 4°C and 12°C than *M. morganii* in dried sardines (Kanki et al., 2004), since *P. phosphoreum* has been shown to be a more dominant histamine producer than *M. morganii* in fish samples stored at temperature lower than 15°C (Lehane and Olley, 2000). *P. phosphoreum* strains were prolific histamine producers at 12°C and 20°C (Kanki et al., 2004), and was a main spoilage bacterium of fish and fish products (Gram and Huss, 1996). Despite the high levels of *P. phosphoreum* in fresh MAP salmon stored at 2°C, however, it produced only small amounts of BAs and less than 20 $mg \cdot kg^{-1}$ histamine was observed prior to sensory spoilage, *C. piscicola* dominated the spoilage microflora of thawed MAP salmon and probably produced the ca 40 $mg \cdot kg^{-1}$ tyramine detected in this product at the end of its shelf life (Emborg et al., 2002). In ice-stored whole, gutted, and filleted Mediterranean sea bass (*Dicentrarchus labrax*), putrescien, confirmed to be formed by *Pseudomonas* species, was the main BA formed and its level showed a steep increase between days 11 and 16 when respective population of *Pseudomonas* reached approximately 10^6–10^7 (whole), 10^7–10^8 (gutted), and 10^8–10^9 (filleted) CFU/g, and the levels of BAs produced correlated well with development of spoilage off-flavors (Paleologos et al., 2004). Interestingly, *Pseudomonas* was found to be the major microflora in snow crab clusters (*Chioneocetes opilio*) stored at 0°C, however, BAs were not measured. Thus, future investigation is necessary to see if formation of BAs in crab muscle during storage is an appropriate spoilage indicator (Lorentzen et al., 2016).

Just as Lakshmanan et al. (2002) reported, during the ice storage of fish and shrimp, the predominant amine-forming bacteria widely distributed were not only of Gram-negative genera, but also of a Gram-positive genus. According to the aforementioned data, for some seafood, production of BAs can reflect the spoilage course to some extent. It should be noted that different species of seafood has its specific SSOs responsible for BAs production; meanwhile, the population of SSO sufficient to cause off-odors and the main BAs profile that contributes to spoilage are also diverse from each other.

7.3.1.2 Volatile Organic Compounds Associated with Spoilage Microorganisms

Bacterial degradation of soluble, low-molecular weight components results in the formation of volatile metabolites such as alcohols, ketones, sulfur compounds, amines, esters, aldehydes, and organic acids (Gram and Dalgaard, 2002). Some of these metabolites are responsible for the unpleasant and offensive off-odors and off-flavors that lead to sensory rejection and shorten the shelf life of seafood (Broekaert et al., 2013); therefore, these volatile organic compounds (VOCs) are described as spoilage metabolites. Generally, amines were the major spoilage compounds during the early phases of aerobic storage under ice, and sulfides and esters became dominant later (Chinivasagam et al., 1998).

TVB-N values include the measurements of TMA, dimethylamine (DMA), methylmercaptan, ammonia, and other volatile basic nitrogen compounds produced by bacterial degradation of proteins and amino acids (Gram and Huss, 1996). TVB-N values have been reported as spoilage compounds and proposed as fish and shellfish spoilage indicator in many studies (Cao et al., 2009a; Fernandez-Salguero and Mackie, 1987). TMA is formed from bacterial use of trimethylamine N-oxide (TMAO), which is found in most marine fish species (Koutsoumanis and Nychas, 1999). *Pseudomonas* spp. cannot use TMAO and produce no TMA on spoiling fish (Gram et al., 1990). SSOs such as *Aeromonas* spp., *Photobacterium phosphoreum*, *Shewanella putrefaciens*–like organisms, Enterobacteriaceae, and *Vibrio* spp. are all capable of using TMAO as final acceptor of electrons and produce TMA, causing "fishy" odors associated with seafood spoilage (Gram et al., 1987; Joffraud et al., 2001; Madigan et al., 2014). This characteristic determines that these microorganisms can grow in microaerophilic or anaerobic conditions where the oxygen is poor or absent in muscle (Hobbs and Hodgkiss, 1982). *P. phosphoreum* was found to produce 30 times more TMA than *S. putrefuciens* in VP and modified atmosphere packaged cod

fillets and was proposed to be responsible for spoilage (Dalgaard, 1995). The extent of TMA was found to be significantly lower in the samples packaged MAs with O_2; in addition, TMA production also appeared to start at higher microbiological counts when O_2 was present (Noseda et al., 2012). However, in some cases, for example, the low levels of TMAO in fish from Greek waters (Koutsoumanis and Nychas, 1999) are due to the low levels of *S. putrefaciens*, since Dalgaard et al. (1993) reported that *Shewanella* population higher than 10^8 CFU/g is required to produce considerable TMA amounts. *P. phosphoreum* is not a dominant spoilage microbiota in chilled fish from Mediterranean waters (Koutsoumanis and Nychas, 1999), so TMA can no longer be an apparent spoilage metabolism. In addition, TVB-N increased at the end of storage when *Pseudomonas* reached a considerable population level of about 6 logs CFU/g, which made it unsuitable to be an index for spoilage evaluating; therefore, VOCs have been studied as potential spoilage indices for spoilage/freshness evaluation, considering that they usually vary significantly between the initial and rejection day of seafood.

Various alcohols, aldehydes, ketones, acids, and esters have been reported as products of the metabolic activity of microorganisms such as *Pseudomonas* spp., *Shewanella* spp., Enterobacteriaceae, *B. thermosphacta*, and LAB during fish spoilage (Parlapani et al., 2015). In brown shrimp, *Pseudoalteromonas* isolates, which have a high spoilage potential, produced 1,2-butanediol, 2-propanol, 2-pentanone, butanone, acetone, methyl mercaptan, sulfur hydride, dimethyl disulfide, ethyl acetate, acetic acid, and ammonia, and might be responsible for the off-odors produced during spoilage (Broekaert et al., 2013). Production of acetic acid mainly due to the metabolic activity of *B. thermosphacta* and LAB (Parlapani et al., 2015). Ethanol is produced both by LAB under reduced oxygen and by *Pseudomonas* under air storage (Casaburi et al., 2014; Olafsdottir et al., 2005). In cooked and peeled shrimp, *B. thermosphacta* produced significant 3-methyl-1-butanal, 2,3-butanedione, 2-methyl-1-butanal, 2-methyl-1-butanol, acetaldehyde, 2-methyl-1-propanal, and ethyl acetate, which led to the formation of cheese-sour off-odors; *S. liquefaciens*–like strains formed 2,3-butanedione, ethyl acetate, 1-propanol, and acetaldehyde, which were related to the cabbage-amine off-odors; *C. maltaromaticum* strains led to production of 3-methyl-1-butanal, 2-methyl-1-butanal, 2-methyl-1-butanol, ethyl acetate, 2-methyl-1-propanal, 2,3-butanedione, and acetaldehyde, causing cheese-sour-butter odors. It is noteworthy that amino acid was metabolized into 3-methyl-1-butanal or 2-methyl-1-butanal, glycogen into 2,3-butanedione or 2,3-heptanedione, and sugar was fermented into ethyl acetate. These off-odors generated were reported characteristics of the spoilage process (Jaffrès et al., 2011). Volatile compounds produced in VP cold-smoked salmon correlated well with the main spoilage bacteria activity and corresponding off-odors. *B. thermosphacta* produced 2-heptanone and 2-hexanone, which were probably responsible for the blue-cheese odor. *Lactobacillus* spp. gave off strong unpleasant odors, which were described as "sour," "acid," "pungent," "floorcloth," and "hydrogen sulfide." Besides, although not responsible for spoilage, *C. piscicola* produced 2,3-butanedione and 2,3-pentanedione, which gave off a strong butter odor (Joffraud et al., 2001). *Pseudomonas fragi* produced fruity aroma via synergistic flavor interaction involving ethyl acetate, ethyl butyrate, and ethyl hexanoate in fish during the early stages of spoilage. This characteristic odor was gradually superseded by a distinct sulfide odor resulted from marked increases in methyl mercaptan, dimethyl sulfide, and dimethyl disulfide (Miller et al., 1973), which is the same case in tropical prawns (Chinivasagam et al., 1998). Bacterial formation of sulfur compounds are the result of the breakdown of the sulfur-containing amino acids cysteine and methionine. Sulfur compounds are known to have a low olfactory thresholds (OT) which means that a small concentration increase may cause a significant sensorial deviation, and hydrogen sulfide was considered as a seafood spoilage–associated compound (Noseda et al., 2012). Methyl mercaptan production is described in *S. baltica* spoiled cooked whole shrimp and can be linked to the sulfur/cabbage odor of this sample at the end of storage (Macé et al., 2014).

7.3.2 Interactions between Spoilage Bacteria and QS

Seafood spoilage obviously involves growth of the microorganisms in large numbers (>10^6–10^7 CFU/g), and the interaction (antagonism or symbiosis) between different groups of microorganisms may influence their growth and metabolism.

When coinoculated in cooked tropical shrimp during MAP, species groups *C. maltaromaticum* and *S. baltica* imposed their spoilage characteristics, that is, *S. baltica* growth seemed to be accelerated by the presence of *C. maltaromaticum* with a bacterial concentration of about 7.4 log (CFU/g) in coculture compared with 5.6 log (CFU/g) in monoculture after 8 days, and *C. maltaromaticum* was not inhibited and still grew quite quickly on cooked tropical shrimp, reaching about 9 log (CFU/g) in 8 days. This result is supported by Jorgensen et al. (2000), who demonstrated that the spoilage activity and BA production of *H. alvei* was enhanced in the presence of LAB. The growth of *L. sakei* was shown to strongly inhibit most of the coinoculated strains, including *P. phosphoreum*, *B. thermosphacta*, *S. liquefaciens*-like and, to a lesser extent, *Vibrio* species. *S. liquefaciens*-like and *P. phosphoreum* were weakly inhibited by *C. piscicola*; conversely, the growth of *C. piscicola* seemed to be enhanced with *B. thermosphacta* and to develop earlier with *P. phosphoreum* and *Vibrio* sp. On the other hand, the spoilage activity of the nonspoiler strains *Vibrio* sp. or the moderate spoilage strains *B. thermosphacta* and *C. piscicola* was increased when they were associated together. These phenomena may be due to the formation of lactic acid and bacteriocins by LAB or by competition for nutrients and this may contribute to their selection during spoilage of lightly preserved seafood products (Gram and Dalgaard, 2002).

Despite the nutrient richness of fish muscle, the environment is iron-limited and siderophores are produced during bacterial growth. The high iron-binding capacity of the pseudomonad siderophores may cause this bacterial group to be positively selected for (Gram and Melchiorsen, 1996). During storage, the proportion of *Pseudomonas* increased significantly ($p < .05$) and reached 73% at the end of storage. It has been known that *Pseudomonas* and *Shewanella* are the most prolific microorganisms during the ice storage of fish and shellfish (Gram and Huss, 1996). From day 4 and afterward, no *Shewanella* was detected. The reason may be that *Shewanella* constituted a low initial proportion (5%) and were inhibited by *Pseudomonas*. H_2S-producing bacteria, mainly *S. putrefaciens*, and *Pseudomonas* are two strongly competitive psychrotrophic microorganisms (Koutsoumanis et al., 1999). Gram and Melchiorsen (1996) reported that *Pseudomonas* could inhibit the growth of H_2S-producing bacteria (including *S. putrefaciens*) due to the ability of the former to produce siderophores (Cao et al., 2009a).

Pseudoalteromonas has a larger biochemical potential and may create extra nutrients for the growth and metabolic activities of the other microorganisms. It was supposed that *Pseudoalteromonas* might elevate the spoilage activity of the *Psychrobacter* strains during spoilage of brown shrimp (Broekaert et al., 2013).

Many bacteria are known to regulate their cooperative activities and physiological processes through a mechanism called QS, in which bacterial cells communicate with each other by releasing, sensing, and responding to small diffusible signal molecules. The ability of bacteria to communicate and behave as a group for social interactions like a multicellular organism has provided significant benefits to bacteria in host colonization, formation of biofilms, defense against competitors, and adaptation to changing environments (Li and Tian, 2012). QS relies upon the interaction of a small diffusible signal molecule with a sensor or transcriptional activator to initiate gene expression for coordinated activities (Schauder and Bassler, 2001). QS systems in bacteria have been generally divided into at least three classes: (1) LuxI/LuxR–type QS in Gram-negative bacteria, which uses acyl-homoserine lactones (AHL) as signal molecules; (2) oligopeptide-two-component–type QS in Gram-positive bacteria, which uses small peptides as signal molecules; and (3) *luxS*-encoded

autoinducer 2 (AI-2) QS in both Gram-negative and Gram-positive bacteria. Each type of signal molecule is detected and responded by a precise sensing apparatus and regulatory network (Li and Tian, 2012). Bacterial biofilms normally consist of bacterial cells and an extracellular matrix, including a mixture of secreted proteins, polysaccharides, nucleic acids, and dead cells (Costerton et al., 2003). Recently, QS was reported to be involved in the spoilage of refrigerated *Litopenaeus vannamei*, in which *S. putrefaciens* and *S. baltica* were present as SSOs. Both of them were able to produce AI-2 in a pattern of increasing substantially until the end of the exponential phase and then declining rapidly through the stationary phase; however, results suggested that they might not be used as QS signaling molecules. In contrast, AHLs can be sensed by transcriptional regulator (LuxR), although AHLs were not detected in SSOs. *S. baltica* seemed to utilize the cyclo-(L-Pro-L-Leu)-dependent QS system, whereas *S. putrefaciens* eavesdrops on cyclo-(L-Pro-L-Leu) in light of its inability to produce any diketopiperadines (DKPs). Correspondingly, QS can regulate the secretion of biofilm and proteases via the regulation of signal molecules and promote the competitiveness of the SSO in a way of providing the competitive advantage by boosting the growth of the SSO or inhibiting the growth of competing bacteria (Zhu et al., 2016). In addition, *S. baltica* was demonstrated as SSO in spoilage of refrigerated large yellow croaker (*Pseudosciaena crocea*) with AI-2 and two DKPs including cyclo-(L-Pro-L-Leu) and cyclo-(L-Pro-L-Phe) detected in cell-free *S. baltica* culture, while the production of biofilm, TMA, and putrescine in these spoilers significantly increased in the presence of cyclo-(L-Pro-L-Leu), rather than cyclo-(L-Pro-L-Phe) and 4,5-dihydroxy-2,3-pentanedione (the AI-2 precursor, DPD). The absence of AI-2 in QS during spoilage was further proved by *S. baltica luxS* mutant. Exogenous cyclo-(L-Pro-L-Leu) shortened lag phase durations, enhanced growth rates of H_2S-producing bacteria and promoted the accumulation of metabolites on the spoilage process of homogenate; however, exogenous DPD retarded the growth of competing bacteria, such as Enterobacteriaceae. Together, *S. baltica* in *P. crocea* might be regulated by DKP-based QS (Zhu et al., 2016). The production of AHLs, AI-2, and DKPs by Gram-negative bacteria associated with seafood spoilage is listed in Table 7.2. The specific role of QS of spoilage microorganisms involved in different seafoods remains to be further studied; besides, figuring out the mechanisms will facilitate the development of techniques for controlling seafood spoilage and reduce economic loss.

TABLE 7.2
Production of Acyl-Homoserine Lactones (AHLs), Autoinducer 2 (AI-2), and DKPs by Gram-Negative Bacteria Associated with Seafood Spoilage

Bacterial Group/ Species	Seafood	Production of			References
		AHLs	AI-2	DKPs	
Shewanella	Shrimp	−	+	−	Zhu et al. (2015c)
	Large yellow croaker	−	+	+	Zhu et al. (2016)
Pseudomonas	Fish	+	nr	nr	Reviewed by Gram et al. (2002)
Photobacterium	Fish	+	nr	nr	
Aeromonas	Fish	+	nr		
Enterobacteriaceae	Fish	+	nr		

nr, not reported.

7.4 DETECTION AND EVALUATION METHOD FOR SPOILAGE MICROORGANISMS

Detection, identification, and evaluation of spoilage microorganisms are indispensable steps to get a profile of SSOs in specific seafood and have a basic understanding of the spoilage characteristics, which are essential for developing appropriate methods to control spoilage and prolong shelf life. Detection and evaluation of spoilage microorganisms and SSOs are often done via classical microbial plating techniques using either various microbiological media or biochemical identification of selected isolates from nonselective agar, whereas these cultivation-dependent methods have limitations due to the difficulty of creating the same living substrates as marine origin and thus may lead to incorrect conclusion or inadequate measures being taken to control spoilage (Bekaert et al., 2015). Besides, one disadvantage of agar-based methods is that incubation temperatures and reagents contained within culture media generate a bias toward certain bacterial groups and do not give an indication of the true microbial diversity (Amann et al., 1995). Advanced molecular methods now allow identification of noncultivable bacteria, which can be finely combined with classical plating methods to obtain a more scientific profile of microbiota present on seafood samples. Here, we will list detection and evaluation methods for some specific spoilage microorganisms responsible for seafood spoilage.

7.4.1 Culture-Dependent Identification of SSOs

Bacterial identification was carried out according to the scheme developed by Shewan et al. (1960). Gram staining, motility test, catalase production, oxidase production, and glucose fermentation were performed for initial classification (Kim et al., 2009). Before identification, marine agar (MA) (Becton Dickinson, Sparks, Maryland) or Long and Hammer agar (LH) with 1% NaCl (Van Spreekens, 1974) was used to determine total aerobic flora; plate count agar (PCA) incubated at 25°C for mesophilic aerobic bacteria count (Gram, 1992) while incubated at 5°C for psychrotrophic bacteria (Gram et al., 1990); De Man Rogosa Sharpe (MRS, pH 6.4) (AES, France) or Elliker agar (ELK, Biokar Diagnostic, Beauvais, France) was used to count total LAB; streptomycin sulfate thallous acetate agar (STAA, Oxoid, Basingstoke, England) to enumerate *Brochothrix*. The H_2S-producing bacteria were investigated in pour plates in iron agar (IA) with 0.04% of lcysteine as described by Gram et al. (1987); Enterobacteriaceae were counted in a pour plate of Caso agar (Merck, Darmstadt, Germany) overlaid by Violet Red Bile Glucose Agar (VRBGA, Biokar) called Caso/VRBG; *Pseudomonas* spp. were enumerated on cephalosporin fucidine cetrimide (CFC) medium (Oxoid code CM 559, supplemented with SR 103, Basinstoke, UK); thiosulfate-citrate-bile salts-sucrose (TCBS) selective medium for presumptive *Vibrio* spp. count (Becton Dickinson); *P. phosphoreum* was enumerated at 15°C using a conductance-based incubation method (Dalgaard et al., 1996, 1997). Yeast and mold count were estimated on Dichloran Rose Bengal Chloramphenicol agar (DRBC) (Beuchat and Cousin, 2013) or Oxytetracycline Gentamycine Yeast Extract agar (OGYE, OxoidCM545, and SR121).

CFC agar has been found to be the most appropriate medium supporting the growth of *Pseudomonas*; however, both Enterobacteriaceae and *S. putrefaciens* were able to grow on the CFC medium. Conventional methods can be used to differentiate each genera, including oxidase test, acid from glucose, reduction of TMAO, formation of H_2S, NH_3 from arginine (Tryfinopoulou et al., 2001). Colonies isolated from IA medium (H_2S-producing and H_2S-nonproducing organism) were randomly selected and their characterization were identified as follows: Gram reactions were tested by Gram technique (Koneman et al., 1999), shape by optic microscopy (Koneman et al., 1999), motility by motility test medium (ICMSF, 1983), cytochrome oxidase by Kovacs reagent (ICMSF, 1983), catalase activity (ICMSF, 1983), glucose metabolism by O/F-test (ICMSF, 1983), ornithine and lysine decarboxylase by Möeller basemedium (Cowan and Steel, 1993), arginine dihydrolase by Thornley-medium (Thornley 1960), Simmons citrate (assimilation of citrate as carbon source), β-galactosidase by *O*-nitrophenyl-β-D-galactopyranose (ONPG, Britania), ability to reduce

TMAO and produce H_2S in the TMAO-medium (Gram et al., 1987), and ability to produce off-odors at 25°C when the microorganisms slurry was inoculated in a heat-sterilized sea salmon juice as described by Gram et al. (1987). H_2S-nonproducing organisms isolated from IA medium and characterized as Gram-negative motile rods with positive catalase and oxidase reactions, oxidative glucose metabolism, and arginine dihydrolase were identified as *Pseudomonas* spp., if they neither reduce TMAO nor produce H_2S (Hozbor et al., 2006). In addition, assimilation of carbon sources was used to differentiate groups of *Pseudomonas* strains, that is, D-arabinose, arabitol, DL-carnitine, creatine, deoxycholate, D-galactonate, D-glucuronate, 4-hydroxy-benzoate, hydroxy-L-proline, inosine, meso-inositol, malonate, D-mannitol, mucate, D-quinate, D-saccharate, D-xylose, and D-glucose. All of the strains were Gram-negative, were catalase and oxidase positive, showed oxidative metabolism on Hugh-Leifson medium, could hydrolyze arginine, and could grow at 4°C. None of them decarboxylated ornithine, produced phenazine pigment, or grew at 42°C. All of the strains were able to assimilate arabitol, hydroxy-L-proline, D-mannitol, D-quinate, and D-glucose (Tryfinopoulou et al., 2002).

Shewanella spp. are Gram-negative, motile bacilli, *Shewanella algae* and *S. putrefaciens* are nonfermentative bacilli with a single polar flagellum (Holt et al., 2005). *S. algae* (formerly classified as *S. putrefaciens*) can live in a temperature range between 10°C and 40°C with an optimum temperature at 30°C between pH 6 and 9 with optimum pH 8. They can grow in the presence of 0%–10% NaCl (w/v) (Aigle et al., 2015). *S. putrefaciens* was reclassified from *P. putrefaciens* mainly because the G + C content of 43%–55% was below the range for *Pseudomonas* spp. (58%–70%) (MacDonell and Colwell, 1985). *S. algae* was found to have a G + C content of 52%–54%, compared with 46% for the type strain of *S. putrefaciens*, and DNA homology between the type strains of *S. algae* and *S. putrefaciens* was found to be <10% (Nozue et al., 1992). Other studies confirmed the significant genomic differences between the two species by ribotyping, 16S rRNA analysis, *gyrB* gene sequencing, and DDH (Venkateswaran et al., 1999). They grow well on conventional solid media, including MacConkey agar, with 1–2 mm yellowish-brown colonies after incubation for 18–24 h. Characteristic traits include production of hydrogen sulfide, decarboxylation of ornithine, and hydrolysis of gelatine. *S. putrefaciens* is the more saccharolytic of the two species, with *S. algae* producing acid only from ribose, and sometimes from glucose and fructose, *S. putrefaciens* produces acid from maltose and glucose, and sometimes sucrose and arabinose. In contrast to *S. putrefaciens*, *S. algae* shows weak β-haemolysis on sheep blood agar after incubation for 48 h, as well as growth at 42°C and in 6% NaCl. Besides, *S. algae* was distinguished from *S. putrefaciens* by a mucoid colony consistency and an ability to reduce nitrite (Holt et al., 2004). Thus, growth at various temperatures (4°C, 37°C, and 42°C), tolerance to 6% NaCl (Fonnesbech Vogel et al., 1997), and assimilation of several carbohydrates (citrate, gluconate, glucose, lactate, and sucrose) (Ziemke et al., 1997) were used to group the isolates as putative *Shewanella* spp.

Photobacterium-like isolates, which are Gram-negative, oxidase-negative (or late positive), and fermentative coccobacilli with sensitivity to vibriostaticum, should be further tested for metabolism of arginine and ornithine, reduction of TMAO, production of gas from glucose, growth with 0% NaCl and at 0°C and 35°C, assimilation of D-mannitol, bioluminescence, and hydrolysis of gelatin (Dalgaard et al., 1997). Voges-Proskauer reaction, indole production, arginine dihydrolase, lysine decarboxylase, lipase, gelatinase, β-galactosidase, nitrate reduction to nitrite, and utilization of D-fructose, D-mannose, D-mannitol, D-sorbitol, L-arabinose, trehalose, cellobiose, maltose, and melibiose are applied in previous studies in order to identify species of *Photobacterium* (Amaral et al., 2015).

Enterobacteriaceae-like isolates, which are Gram-negative, catalase-positive, oxidase-negative, and vibriostaticum-resistant, were further characterized using analytical profile index (API) 20E (BioMe´rieux, Marcy-l'Etoile, France) and tested for fermentation of lactose, xylose and trehalose, phenylalanine deaminase, reduction of nitrate and TMAO, and growth at 2°C, 30°C, and 37°C (Dalgaard et al., 2006).

Gram-positive and fermentative isolates were tested depending on their catalase reaction. Catalase-positive isolates, expected to be *B. thermosphacta*, were tested for growth with 8% NaCl and fermentation of rhamnose (Talon et al., 1988). For the presumptive LAB strains (Gram-positive, catalase and oxidase negative isolates), tests were performed at 30°C. Anaerobic production of gas was observed in an inverted Durham tube on a medium recommended by Dicks and van Vuuren (1987) with 0.5% NaCl instead of sodium acetate; ammonia production from arginine was tested at a low concentration of glucose (0.05%) with Nessler's reagent in the modified medium of Hitchener et al. (1982) enriched with 0.5% NaCl and 0.02% magnesium sulfate; enantiomers of lactic acid were determined by enzymatic methods (Boehringer Mannheim, Meylan, France) in a 10-fold diluted ELK culture supernatant; detection of meso-diaminopimelic acid (meso-DAP) in peptidoglycan was achieved by thin-layer chromatography according to Bousfield et al. (1985). For the characterization of *Lactobacillus* spp., production of CO_2 and growth at different temperatures (3°C, 15°C, 30°C, and 45°C), salt concentrations (8% and 10%), and pHs (3, 3.7, and 8.5) were used in combination with the API 50CH tests (Koutsoumanis and Nychas, 1999). *Carnobacterium*-like isolates were tested for fermentation of inulin, D-lactose, D-mannitol, methyl-α-D-glucoside, methyl-α-D-mannoside, and D-xylose (Collins et al., 1987).

7.4.2 Culture-Independent Molecular Methods for Identifying SSOs

In comparison with data for meat and meat products, identification and characterization of SSOs in seafood under different storage conditions have not been sufficiently studied, and the use of conventional phenotypic methods can offer only limited results. In contrast, molecular methods are powerful tools not only for identification at the species level but also for strain characterization. For the identification of *Pseudomonas* spp., molecular methods, such as DNA–rRNA hybridization (De Vos et al., 1989), DDH (Ursing, 1986), direct comparison of rRNA sequences (Ludwig et al., 1994), and comparison of macrorestriction patterns by pulsed-field gel electrophoresis (Grothues and Tummler, 1991) have been widely applied in order to establish the phylogenetic relationships between new isolates and previously defined taxa. However, no matter what methodology is used for the identification of *Pseudomonas* strains, there is always confusion. The sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) of whole-cell proteins, an advanced phenotypic method, which falls between conventional phenotypic and molecular methods, has been used with conventional phenotypic analysis to understand the diversity of *Pseudomonas* populations on fish. Indeed, the electrophoretic separation of cellular proteins is a sensitive technique, providing information on the similarity of strains at the (sub)species level (Tryfinopoulou et al., 2002).

Fingerprinting molecular methods, such as denaturing gradient gel electrophoresis (DGGE) and temporal temperature gel electrophoresis (TTGE), are powerful tools to compare structural changes in microbial communities as well as for monitoring bacterial population dynamics due to temperature variation, changes in atmosphere, and so forth. Individual bands can be identified after excision from the gel, cloning, and sequencing (Juste et al., 2008). Bands can also be assigned and identified by comparison with a comprehensive bacterial database that should include microorganisms typical of the ecosystem (Ogier et al., 2004). Polymerase chain reaction (PCR)–DGGE is a suitable technique for studying the bacterial composition of different habitats, soils, foods, and water samples. PCR–DGGE studies on fish have been performed on Atlantic mackerel (Svanevik and Lunestad, 2011), Atlantic salmon, cod, farmed halibut (Hovda et al., 2007a, b, c, 2012), tuna (Chongtao et al., 2012), and Norway lobster (Bekaert et al., 2015). However, due to comigration of the DGGE bands, not all bands in the profiles have been sequenced and analyzed, resulting in some biologically significant bacteria remaining undiscovered (Li et al., 2006). Limitations of the DGGE method include the detection limit, which varies between 10^3 and 10^8 CFU/g depending on the species and on the other members of the microbial community (Ercolini, 2004). Thus, this method should function with combination of conventional microbiological methods to detect dominant microbiota on

seafood (Bekaert et al., 2015). PCR-TTGE has been utilized in oysters (Prapaiwong et al., 2009), tropical cooked shrimp (Jaffrès et al., 2009), and raw salmon (Macé et al., 2012).

The development of multiplex PCR (MPCR) assay for *Aeromonas* spp. has been mainly focused by the earlier authors to identify the *Aeromonas* spp. alone by the presence of different genes as well as to identify *A. hydrophila* using the set of genes unique for *A. hydrophila*. Wang et al. (2003) developed a MPCR using *aerA* and *ahh1* gene specific for *A. hydrophila* and *asa1* specific for *Aeromonas sobria* from clinical isolates. Bin Kingombe et al. (2010) reported an MPCR assay to detect the presence of three enterotoxin genes in *Aeromonas* spp., which include cytotoxic (*act*), heat-liable (*alt*), and heat-stable enterotoxin (*ast*) genes, for the detection of *Aeromonas* spp. in food. Choresca et al. (2010) developed an MPCR assay to detect *A. hydrophila* by amplification of 130 bp fragment of hemolysin and 309 bp fragment of aerolysin gene from diseased *Clarias* sp. Balakrishna et al. (2010) reported another MPCR assay for the detection of *A. hydrophila* by amplification of hemolysin (*hlyA*), aerolysin (*aerA*), and glycerophospholipid-cholestrol acyl transferase (*GCAT*) along with *16S rRNA* gene. Pinto et al. (2012) developed an MPCR method for *Aeromonas* spp. using *hlyA*, *aerA*, *alt*, and *ast* genes encoding hemolysin A, aerolysin, aeromonas labile temperature cytotonic enterotoxin, and aeromonas stable temperature cytotonic enterotoxin, respectively. Hussain et al. (2014) designed the assay targeting for the amplification of three sets of genes for simultaneous detection of *A. hydrophila* and *A. sobria* along with other *Aeromonas* spp. using the primers of *ahh1*, *asa1*, and *16S rRNA* genes, respectively. What is more, compared with PCR assay, MPCR assay had high sensitivity in terms of cells and genomic DNA content and resolved the difficulties with the nonspecific amplification of target genes (Hussain et al., 2014).

In recent years, the increased sensitivity, speed, precision, and wide dynamic range of real-time PCR have proved useful attributes in its successful application in food microbiology and it could therefore be used as a substitute for culture techniques to gather quantitative and identification information rapidly. This method has been proposed for LAB such as *Carnobacterium* (Cailliez-Grimal et al., 2005) and *B. thermosphacta* (Pennacchia et al., 2009). Real-time PCR assays have been developed to quantify and detect *B. thermosphacta* from cooked shrimp and salmon, the real-time PCR with propidium monoazide (PMA), which has been used as a DNA intercalating agent to selectively remove the DNA from dead bacterial cells, via forward primer MO405 (5′-CAC AGC TGG GGA TAA CAT CGA-3′) and reverse primer MO404 (5′-CTT GAA CAT CTT ATG ATG TTC AGC-3′) designed to target a 70 bp fragment on the region 138–208 of the *16S rRNA* gene sequence of *B. thermosphacta*, was found to be a sensitive and specific method capable of giving a good evaluation of the contamination of *B. thermosphacta* with a minimum threshold of 1.9×10^2 CFU/g. It also offers the advantage of providing results in 3–4 h compared with 48 h required for the classical microbiological method. The application of this method could be of great value for the seafood industry to detect and quantify spoilage bacteria before the apparition of spoilage odors and thus avoiding some products losses (Mamlouk et al., 2012). Similarly, the forward primer MO627 (5′-TACTGTTGAAGTGGCGAT-3′) and the reverse primer MO628 (5′-TCTGCTGGGCTTTCTAAT-3′) specific to the *P. phosphoreum* species group were designed to run the real-time PCR and thus to detect and quantify viable *P. phosphoreum* (Macé et al., 2013).

DNA probe-based fluorescent *in situ* hybridization (FISH) has been widely applied for bacterial identification and localization within samples (Frickmann et al., 2013). The 16S and 23S rRNAs are often used as phylogenetic markers for species or genus identification in FISH because of their higher copy numbers in the cells and less influence by growth conditions (Amann and Fuchs, 2008). However, DNA probe-based methods are of limited use due to poor permeability of bacterial cells with restricted accessibility of probes and low sensitivity (Brehm-Stecher et al., 2005). Peptide nucleic acid (PNA) probes are synthetic DNA mimics developed in early 1990s (Egholm et al., 1993). They exhibit greater specificity and better affinity in hybridization over regular DNA probes (Peleg et al., 2009). It was concluded that peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH), using the PNA probe Vib-16S-1 containing the sequence: 5′-AGGAGCTTCGCTTGC-3′,

could be an alternative method for rapid identification of *Vibrio* species in a broad spectrum of seafood or related samples, and due to direct visualization of the bacteria, the detection time could be greatly reduced compared with culture-based methods (Zhang et al., 2015).

7.5 CONTROL, PREVENTION, AND MANAGEMENT OPTIONS FOR SPOILAGE MICROORGANISMS

Prolonging the shelf life of seafood can have an essential economic effect by reducing losses and allowing the products to reach distance and new markets, thereby, delaying or inhibiting growth of spoilage microorganisms in fresh seafood is of great importance. Various food preservation techniques, including physical, chemical, and biological applications, have been used to control spoilage microorganisms and extend the shelf life of seafood. The recent studies on shelf life of various shellfish and crustaceans under different storage temperature and treatment conditions are shown in Table 7.3.

Freezing can inactivate luminous bacteria, probably *P. phosphoreum*, and H_2S-producing organisms that occur naturally in fish, and they were effectively inactivated by frozen storage at −20°C (Emborg et al., 2002; Dalgaard et al., 2006).

Smoking besides drying is one of the oldest methods of preserving fish. There are fundamentally three stages of the cold smoke processing, which contribute to the preservative effect: salting, dehydration, and smoking. Salting and dehydration (the latter is a direct effect of smoking) lowers the a_w, thereby inhibiting the growth of bacteria and mold, which generally cannot grow at a_w lower than 0.86 in the presence of soluble salt (Sperber, 1983). In addition, the chlorine ions are toxic for some microorganisms. Cold-smoked salmon, which is a typical example of LPFPs using smoking technique, is considered a delicacy commonly consumed as a "ready-to-eat" (RTE) product without heat treatment (Gram and Huss, 1996).

High-pressure (HP) processing is a nonthermal technique recognized as a very effective process to destroy foodborne microorganisms and lengthen their shelf life without causing significant changes in appearance, texture, flavor, and nutritional constituents (Oshima et al., 1993; San Martin et al., 2002). HP destroys microorganisms via altering their morphology, biochemical reactions, and genetic systems (Hoover et al., 1989). It was reported that processing postrigor cultured abalone at 300 MPa for 5 or 10 min was sufficient to prevent BA production (Hughes et al., 2016). When applied to oysters, numbers of total aerobic bacterial counts (TABC) were significantly lower than untreated oysters at day 0 by 10–10^5 CFU/g of oyster meat, and the most common genera in HP oysters were *Shewanella*, *Vibrio*, and *Psychrobacter* (Prapaiwong et al., 2009; Cruz-Romero et al., 2008b, 2008b). LAB were not detected after HP treatment at 260, 500, or 800 MPa (Cruz-Romero et al., 2008a). *S. putrefaciens* could survive HP treatment at 260 MPa (Cruz-Romero et al., 2008a), *Pseudomonas* spp. could survive HP treatment at 500 or 800 MPa (Cruz-Romero et al., 2008a), this may due to their being only sublethally injured by HP treatment. In 300 and 400 MPa HP-treated squid, endogenous *P. phosphoreum* was not detected while LAB (*C. piscicola* and *Carnobacterium divergens*) and Enterobacteriaceae (*S. liquefaciens* and *P. vulgaris*) composed the microflora (Paarup et al., 2001). However, HP-treated Indian white prawn (*Fenneropenaeus indicus*) never breached the acceptability limit during entire duration of chilled storage, no lag phase was observed for psychrotrophic bacteria, H_2S-producing bacteria, *B. thermosphacta*, *Pseudomonas* spp., and LAB, kinetic parameter such as specific growth rate in HP-treated samples was significantly reduced in most of the bacterial groups except for psychrotrophic bacteria, Enterobacteriaceae, and LAB. Thereby, the antibacterial effect of HP may be seafood species-dependent, and it is necessary to use other hurdles to avoid or delay outgrow of sublethally injured microorganisms during storage. Modification of the atmosphere within the package by decreasing the oxygen concentration and increasing the content of carbon dioxide and/or nitrogen has been shown to significantly prolong the shelf life of perishable food products at chill temperatures (Parry, 1993). MAP and VP, along with refrigeration, have become increasingly popular preservation techniques. MAP affected not

TABLE 7.3
Effect of Packaging and Treatment Condition on the Shelf Life of Shellfish and Crustaceans

Shellfish and Crustaceans	Treatment	Storage Temperature (°C)	TVC/APC (log10 CFU/g)	Shelf Life (Days)			References
				Air	VP	MAP	
Pacific white shrimp (*Litopenaeus vannamei*)	-	4	8.34 ± 0. 34	8			Okpala et al. (2014)
Norway lobster (*Nephrops norvegicus*)	-	1	5.09			13	Gornik et al. (2013)
	-	2 ± 1	5–6	7			Bekaert et al. (2015)
Crab							
Carcinus maenas		4		13–29			Robson et al. (2007)
Necora puber	-		-				
Cancer pagurus		20		2–16			
Paralithodes camtschaticus	Cooked	4	4.6	5			Lorentzen et al. (2014)
Chioneocetes opilio	Cooked	4	5.5	10			Lorentzen et al. (2016)
		0	4.9	14			
	-	4					
		0	2.5	6			
Cancer pagurus	Live cooked	3.4 ± 0. 9	-		13		Anacleto et al. (2011)
	Dead cooked		>6		10		
Pacific oysters (*Crassostrea gigas*)	-			8–9			Cao et al. (2009a)
	Chitosan	5 ± 1	-	14–15			
	Ozonated water			10–12			Cao et al. (2010)
Cuttlefish (*Sepia officinalis*)		2 ± 2		10			Vaz-Pires et al. (2008)
Shortfin squid (*Illex coindetii*)				9			
Squid mantle (*Todaropsis eblanae*)	-	4	-		<7		Paarup et al. (2002)
	150 MPa HP		7		7		
	200 MPa HP		-		7–14		
	300 MPa HP		8		14–21		
	400 MPa HP		8		21–28		

VP, vacuum packed; MAP, modified atmosphere packed; HP, high-pressure treatment.

only the growth rate but also the final populations of spoilage bacteria (Parlapani et al., 2015). The inhibitory effect of CO_2 on the growth rate varied according to the species as well as to the temperature. In king scallop during cold storage, the dominant species under air packaging corresponded to *B. thermosphacta*, *Pseudomonas* spp., and *Shewanella* spp. *Pseudomonas* spp. and *Shewanella* spp. were affected by all conditions eliminating oxygen from packaging atmosphere, *B. thermosphacta* and *C. maltaromaticum* were slightly affected by the presence of CO_2 with an approximately 1 log unit reduction in "100% CO_2" MAP. As for *Pseudoalteromonas haloplanktis* species, it was more sensitive to high CO_2 concentrations rather than to anaerobic conditions. And the lowest counts for every studied flora as well as lowest TVB-N and TMA contents were observed for the MAP with >80% CO_2 (Cotona et al., 2013). Results showed that the shelf life of Norway lobster *Nephrops norvegicus* could be extended to 13 days when stored in MAP at 1°C (Gornik et al., 2013). The presence of O_2 in the packages clearly stimulated microbiological growth on the shrimp product, *S. proteamaculans* was reported as a considerable sensitive bacteria to the lack of O_2, being its growth rate reduced by 29% in response to the lack of O_2 (Alfaro et al., 2013). *S. putrefaciens* was inhibited in VP cold-smoked salmon (Leroi et al., 1998). However, the addition of appropriate O_2 to a CO_2-enriched atmosphere appeared to induce an additional inhibitory effect on the growth of H_2S-producing bacteria, *P. phosphoreum* and *Pseudomonas* spp. It was supposed the dominating CO_2-tolerant microorganisms were inhibited by O_2. The most significant microbial inhibition was observed by the combination of 50% CO_2 and 50% O_2. Correspondingly, a diminishing effect on the production of metabolites was observed, especially for amines and sulfur compounds, which constituted the major fraction of components causing the offensive odor (Noseda et al., 2012). MAP of 40% CO_2/30% N_2/30% O_2 was the most effective for the inhibition of *Pseudomonas* and H_2S-producing bacteria in swordfish (Pantazi et al., 2008). MAP with 40% CO_2/60% O_2 had a strong inhibitory effect on growth and histamine formation by both the psychrotolerant *M. morganii*-like bacteria and *P. phosphoreum*; meanwhile, no formation of histamine was found in naturally contaminated fresh MAP tuna with 40% CO_2/60% O_2 during 28 days of storage at 1°C (Emborg et al., 2005). The combined effect of previous freezing and MAP (40% CO_2 and 60% N_2) resulted in both a considerable shelf life extension and a pronounced reduction ($p < .01$) in the formation of histamine in garfish (Dalgaard et al., 2006). Sivertsvik and Jeksrud (2002) reported that the efficiency of CO_2 effect on microbial growth depended on the amount of gas solubilized into the tissue. Its solubility was highly temperature dependent, it seemed that CO_2 was absorbed and retained within the tissues of Norway lobster *N. norvegicus* at 4°C, yet, when the storage temperature was increased to 6°C the amount of dissolved gas was reduced and thus the bacteria grew again (Gornik et al., 2013). Besides, the growth rate of *Yersinia intermedia* decreased as the percentage of CO_2 in the atmosphere increased, and this effect was more noticeable at low temperatures (Alfaro et al., 2013). It was demonstrated that MAP with combined 80% CO_2/10% O_2/10% N_2 gases was effective in inhibiting microbial growth while minimizing oxidative and textural changes in precooked, shellless red claw crayfish tails stored at refrigerator temperature (Chen and Xiong, 2008).

Ionizing radiation is effective for extending shelf life, reducing and inactivating spoilage and pathogenic microorganisms, and improving wholesomeness of fish and shellfish in fresh, frozen, or RTE products (Molins et al., 2001; Flick, 2003). It was suggested that 600 mWs/cm^2 of ultraviolet (UV) radiation at 260 nm could potentially be applied on the surface of dried fish fillets as an antifungal agent, *A. niger*, *C. cladosporioides*, and *P. citrinum* were effectively inactivated (>90%) without any concomitant changes in the fillet color or sensory qualities (Park et al., 2014).

As a versatile biopolymer, chitosan has a wide range of applications in the food industry (Rudrapatnam and Farooqahmed, 2003). In oysters stored at 5 ± 1°C where *Pseudomonas* and *Vibrionaceae* were dominant bacteria, chitosan concentration of 5.0 g/L, which was reported to have a mean bacterial growth inhibition zone value of 14.9 mm and a decrease in aerobic plate count (APC) values as well as TVB-N values, could be utilized to extend the oyster shelf life from 8–9 days to 14–15 days (Cao et al., 2009a, 2010).

Ozone is a U.S. Food and Drug Administration (FDA)– and U.S. Department of Agriculture (USDA)–approved food contact sanitizing agent and has been used in the seafood industry in both gaseous and dissolved forms to destroy bacteria (Chawla et al., 2007). Results indicated that ozonated water treatment reduced the total microbial load of fresh oysters by about 10-fold (from 3.2×10^3 to 1.8×10^2 CFU/g) before storage and the microbial flora was different with that of raw samples. It was also indicated that the combined treatment with chitosan had a better bacterial inhibition effect than treatment alone and extended the shelf life to at least 20 days (Cao et al., 2010). In VP-shucked mussels stored under refrigeration, ozonation affected populations of bacteria namely, APC (0.7–2.1 log cycle reduction), *Pseudomonas* spp. (0.5–1.1 log cycle reduction), and H_2S-producing bacteria (1.1–2.5 log cycle reduction), *B. thermosphacta* (0.3–1.4 log cycle reduction), LAB (0.3–0.8 log cycle reduction), and Enterobacteriaceae (0.5–1.5 log cycle reduction), and attained a 35% extension in shelf life of fresh mussels (Manousaridis et al., 2005).

An enterococci bacteriocinogenic strain (*Enterococcus mundtii*) able to produce the bactericin mundticin KS was found to be exhibited a broad spectrum of activity, which can act on *Pseudomonas aeruginosa* and *S. putrefaciens* could be used as an additional hurdle in the preservation of minimally processed fish and/or other sea products. Moreover, the combination of the bacteriocin with chitosan and sodium lactate (SL) induced a synergic action on *Listeria innocua* and *S. putrefaciens* inhibition (Schelegueda et al., 2015).

As for QS, it was strongly suggested that green tea polyphenols (TP) could be developed as a new QS inhibitor for seafood preservation to enhance shelf life. Since TP at subinhibitory concentrations interfered with AI-2 and DKPs activities of *S. baltica* without inhibiting cell growth and promoted degradation of AI-2. The green TP treatment inhibited biofilm development, exopolysaccharide production, and swimming motility of *S. baltica* in a concentration-dependent manner. In addition, green TP decreased extracellular protease activities and TMA production in *S. baltica*, correspondingly, a transcriptional analysis showed that green TP repressed the *luxS* and *torA* genes in *S. baltica* (Zhu et al., 2015b).

7.6 CONCLUSION AND PERSPECTIVES

To date, the spoilage of fresh and lightly preserved seafood is widely studied; the SSOs involved in these seafood systems have been detected and identified via classical cultural methods or to some extent combined with molecular methods. The involvement of these putative SSOs has sometimes been proven by reinoculation of candidate strains into seafood model systems to reproduce spoilage. The recent development of high-throughput sequencing has led to a deeper analysis of seafood microbial ecosystems. These approaches, mostly focused on 16S rDNA, have confirmed previous studies performed with classical cultural methods about the nature of bacteria found in foods. In addition, the data obtained by direct analysis of DNA extracted from food matrixes have enabled a better description of the ecosystems at genus or even species level, and have also revealed that the involvement of some species in food spoilage might have been underestimated. Using the genomics of spoilage microorganisms and the metagenomics of seafood ecosystems combined with other "omics," particularly metabolomics, to determine the VOCs or molecules putatively responsible for spoilage should contribute to identifying the bacterial metabolic pathways involved in the phenotype of spoilage. The use of high-throughput 16S rDNA pyrosequencing or metagenomics relying on the whole bacterial seafood ecosystem, including spoilers, however, is still scarce. Meanwhile, an increasing number of articles have reported structural changes of microbial communities as well as bacterial population dynamics through DNA extraction, followed by various types of molecular identification such as DGGE or TTGE performed on *16S rRNA* gene portions amplified by PCR.

The nature and appearance of spoilage depend on the physiological state of spoilers and on their ability to resist the processing/storage conditions and flourish on the seafood matrix; besides, spoilage relies on the interactions between the microorganisms composing the ecosystems existing in

seafood. The interactive behavior of spoilage bacteria may influence their growth and metabolism. Yet, only a few studies have successfully proved the exact nature of spoilage and the bacterial functions involved in producing the spoiling molecules. It is likely that interactive behavior (metabiosis and antagonism) is important in any foods in which a mixed flora develops during storage. The detection of chemical signals, involved in bacterial QS regulation, in seafood and seafood spoilage bacteria is of significant interest. Analyses of the possible importance of QS signal compounds in spoilage reactions, and the potential development of novel preservation techniques based on QS inhibitors, deserves further studies. The recent rapid increase in publicly available bacterial genome sequences, as well as the access to high-throughput methods, should lead to a better understanding of spoiler behavior and to the possibility of decreasing or controlling seafood spoilage.

Changing in consumers' habit has led to an increase of RTE and convenient food, including both the easy-to-use aspect and an extended shelf life of the products. The nutritional aspects are also more and more taken into consideration by the consumers who want natural products, with technological treatment and level of preservatives as low as possible. LPFP, like VP or MAP fish fillets, carpaccio, cold-smoked fish, peeled and mildly cooked shrimp, and so forth meet those requirements and their production has increased dramatically those last years. In those products, the environmental conditions favor the development of LAB, explaining the high interest for this bacterial group in the last decade. The role of LAB in marine products is complex, depending on the seafood species, treatment and storage conditions, bacterial species and strains, and interaction between the bacteria. Sometimes, LAB have no particular negative effect and can control spoilage to some degree, but in certain cases they are responsible for strong sensory degradation, leading to rejection of the products. Increasing numbers of studies are aiming to exploit its bioprotective ability to control the quality and the safety of marine products. However, this technology is still in its infancy compared with dairy products. Moreover, combining bioprotection of seafood with no modification of the sensory characteristics of the product still remains a challenge.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31571913), the National Science and Technology Support Program (2015BAD17B01), Zhejiang Provincial Natural Science Foundation of China (LZ15C200001), and Science and Technology Foundation of Ningbo (2015C10042). Thanks for the remarkable instruction from Prof. Yang Zhu, Wageningen University, the Netherlands.

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8 Spoilage Microorganisms in Powdered Milk

Yingwang Ye

CONTENTS

8.1 INTRODUCTION

Cronobacter species is a group of foodborne pathogens associated with meningitis, septicemias, and necrotizing enterocolitis, especially among newborns (Muytjens et al., 1983, 1988; Biering et al., 1989; Clark et al., 1990; Lai, 2001; Van Acker et al., 2001). The International Commission for Microbiological Specification for Foods (ICMSF, 2002) has ranked *Enterobacter sakazakii* (*Cronobacter* spp.) as a "Severe hazard for restricted populations, life threatening, or substantial chronic sequelae or long duration." The epidemic investigation indicated that powdered infant formula (PIF) was considered the main transmission vehicle for *Cronobacter* infections (Muytjens et al., 1983, 1988; Biering et al., 1989; Clark et al., 1990; Van Acker et al., 2001). To control and prevent *Cronobacter* from contaminating the powdered milk, the detection, typing methods, characteristics, and its pathogenicity were of importance for assuring food microbial safety.

8.2 TAXONOMY OF SPOILAGE MICROORGANISMS IN POWDERED MILK

Cronobacter species were originally referred to as yellow pigmented *Enterobacter cloacae*, later being reclassified as *E. sakazakii* in 1980 based on pigment production, biotyping, antibiotic susceptibility patterns, and DNA relatedness (Farmer et al., 1980; Izard et al., 1983). *E. sakazakii* has recently been reclassified as a novel genus of *Cronobacter* on the extensive analyses of 16S rRNA (ribosomal RNA) sequencing, fluorescent-labelled amplified fragment length polymorphism (f-AFLP), DNA hybridization, and ribotyping. The *Cronobacter* genus consists of *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter genomospecies* 1, and *Cronobacter dublinensis* which contain three subspecies: *dublinensis*, *lactaridi*, and

lausannensis (Iversen et al., 2007, 2008). *Cronobacter condimenti* sp. nov., and *Cronobacter universalis* sp. nov., designed for *Cronobacter* genomospecies 1 as new species, were redefined based on 16S rRNA gene sequencing and multilocus sequence typing (Joseph et al., 2012a).

8.3 SPOILAGE MICROORGANISMS CHARACTERISTICS AND POSSIBLE MECHANISMS

8.3.1 Uncertainty of Origins of *Cronobacter* spp.

Cronobacter species were isolated from clinical specimen samples, environmental samples, and foods. Most of *E. sakazakii* isolates are from infected patients' clinical specimen samples including, blood, sputum, throat, nose, stool, and gut as described by Farmer et al. (1980). Three isolates in the same hospital over 11 years appeared to be in the same strain based on ribotyping (Nazarowec-White and Farber, 1999), which indicated that *Cronobacter* spp. may persist in clinical settings over an extended period of time. *Enterobacter* species have been frequently isolated from natural environments such as soil, water, animals, sewage, and human fecal samples (Sakazaki, 1974). Water, soil, and vegetables were considered as the principal environmental sources of *E. sakazakii* (Iversen and Forsythe, 2003). Kandhai et al. (2004a) reported that *E. sakazakii* was isolated from 35 out of 147 samples from a survey of nine food factories and 16 households in the Netherlands. Furthermore, 18 out of 152 dry samples of scrapings from dust, vacuum cleaner bags, and spilled products from three milk powder plants were found to be positive for *E. sakazakii* (Kandhai et al., 2004b). *E. sakazakii* has been isolated from other environmental sources including soil (Neelam et al., 1987), cutting fluids (Suliman et al., 1988), clinical materials (Tuncer and Ozsan, 1988; Janicka et al., 1999), crude oil (Assadi and Mathur, 1991), rats (Gakuya et al., 2001), rhizosphere (Emilani et al., 2001), sediment and wetlands (Espeland and Wetzel, 2001), and air in a hospital (Masaki etal., 2001). In addition, some insects such as Mexican fruit fly (Kuzina et al., 2001) and stable fly larvae (Hamilton et al., 2003) were also found to be the potential origins of *E. sakazakii*.

Furthermore, Krieg and Holt (1984) reported that *E. sakazakii* was more prevalent in foods and environmental samples than in clinical settings. *E. sakazakii* was detected in 20 of 141 (14.2%) milk-substitute infant formulae samples from 13 of the 35 countries (Muytjens et al., 1988). In a survey of infant formula products from 11 countries, Leuscher and Bew (2004) isolated *E. sakazakii* from 8 of 58 (13.8%) samples. In recent years, *Cronobacter* spp. can be isolated from a wide variety of foods including milk, cheese, dried foods, meats, water, vegetables, rice, bread, tea, herbs, spices, PIF, and dry foods (Farber, 2004; Iversen et al., 2004b; Edelson-Mammel et al., 2005; Gurtler et al., 2005; Beauchamp et al., 2006; Estuningsih et al., 2006; Friedemann, 2007; Mullane et al., 2007; Ye et al., 2012, 2014).

Although *Cronobacter* spp. was isolated from various food samples, environments, and clinical samples, the PIF has been epidemiologically implicated as the source of *Cronobacter* spp. in several cases (Muytjens et al., 1983; Biering et al., 1989; Clark et al., 1990; Van Acker et al., 2001).

8.3.2 Resistance to Adverse Environments

Recently, *Cronobacter* species contaminations in powdered milk are of great concern because they have posed severe risks on newborns. Studies indicated that *Cronobacter* had unusual abilities to survive in the thermal resistance, osmotic, and desiccation resistance. Compared with other members of Enterobacteriaceae family, *Cronobacter* spp. are more tolerant to dry stress (Caubilla-Barron et al., 2007). The effects of water availability (a_w) on *Cronobacter* spp. indicated that an initial inoculum of 2 CFU/g could survive in the infant cereals for up to 12 months with a_w ranging from 0.30 to 0.83 (Lin and Beuchat, 2007). A desiccation test indicated that the drying treatment decreases the level of *Cronobacter* from 0.69 to 1.97 log 10 CFU/mL among 18 *Cronobacter* strains from dry edible fungus (Ye et al., 2014).

Using the proteomics, Riedel and Lehner (2007) have identified differentially expressed proteins in response to two different osmotic stresses: desiccation and growth in hyperosmotic media. Results indicated that the protein pattern of NaCl-grown cultures reflect more or less a general downregulation of central metabolic pathways, whereas adaptation of (nongrowing) cells in a desiccated state represents an accumulation of proteins that serve some structural or protective role.

In addition, *Cronobacter* spp. are also more thermotolerant compared with other members of the Enterobacteriaceae (Nazarowec-White and Farber, 1997; Breeuwer et al., 2003; Asakura et al., 2007). So, Edelson-Mammel and Buchanan (2004) suggested that a rehydration temperature of 70°C would be more appropriate and added that rehydration at this temperature would virtually assure of inactivating *E. sakazakii*.

8.3.3 Possible Transmission Mechanisms

Neonatal infections have been reported to arise via contact with *Cronobacter* (*E. sakazakii*) in the birth canal or through postbirth environmental sources (Monroe and Tift, 1979; Steere et al., 1975). Although *E. sakazakii* have caused infections in newborns through the mother's birth canal, this hypothesis seems implausible based on *E. sakazakii* infections in neonates born by Cesarean section (Bar-Oz et al., 2001; Muytjens and Kollee, 1990; Muytjens et al., 1983; Urmenyi and Franklin, 1961). Reservoir for *Cronobacter* spp. might be primarily from plant materials (Mossel and Struijk, 1995). Furthermore, solubilization of mineral phosphate, production of indole acetic acid, and siderophore production indicated that plants may be the natural habitat of *Cronobacter* spp. (Schmid et al., 2009).

8.4 DETECTION AND EVALUATION METHODS FOR SPOILAGE MICROORGANISMS

8.4.1 Traditional Detection Methods

Schematic outline of traditional methods including the U.S. Food and Drug Administration (FDA) and International Organization for Standardization (ISO) methods was shown in Figure 8.1. A method was developed by the FDA in 2002 to isolate and enumerate *Cronobacter* spp. in dehydrated PIF (FDA, 2002). This assay was similar to the method described by Muytjens et al. (1988) and Nazarowec-White and Farber (1997) except that the PIF was suspended with prewarmed sterile distilled water in the FDA method rather than buffered peptone water, and enriched samples (one loop 10 μL or 100 μL) were directly streaked or spread on violet red bile glucose agar (VRBGA) rather than via the poured plate (1.0 mL). Combined with molecular method, the traditional FDA method was revised by Chen et al. (2009). They found that valid results were obtained from 360 test portions and controls, and the revised FDA method was significantly better ($P < 0.05$) than the traditional FDA method. In 2006, a novel method was developed by the ISO (ISO, 2006). A comparison of ISO method and other molecular methods was evaluated in the 243 commercial powdered milk samples. The results indicated that polymerase chain reaction (PCR) targeting *gluA* gene was sensitive and specific for detection of *Cronobacter*, while two false negative samples were also observed by the ISO method (Ye et al., 2009).

8.4.2 Chromogenic Media Methods for Detection of *Cronobacter* Species

In recent years, chromogenic media listed in Table 8.1 for detection of *Cronobacter* spp. was developed to decrease the detection time. Existence of α-glucosidase activity has been demonstrated in specific media containing 4-nitrophenyl-α-D-glucopyranoside, which was used as a supplementary test to avoid false positive result or confirm presumptive *Cronobacter* spp. (Muytjens et al., 1984). A chromogenic medium consisting of nutrient agar (NA) supplemented with 4-methyl-umbelliferyl

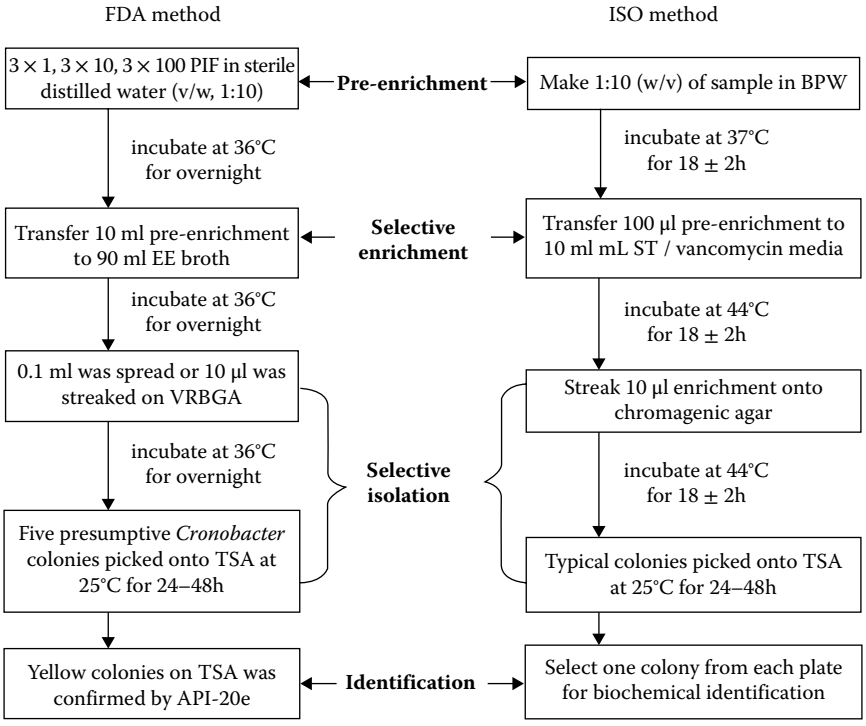

FIGURE 8.1 Schematic outline of FDA and ISO methods for isolation and identification of *Cronobacter* spp. FDA, U.S. Food and Drug Administration; ISO, International Organization for Standardization.

TABLE 8.1
Different Chromogenic Media for Isolation of *Cronobacter* spp.

Methods	Pre-enrichment	Enrichment	Primary Isolation	Presumptive Identification
FDA	BPW	EE	VRBGA	Yellow colonies on TSA
mLST	BPW	mLST	DFI	Blue-green colonies
DFI	BPW	ESE	TSA	Yellow, α-glucosidase activity
AES	ESSB	–	ESIA	Blue-green colonies
ISO	BPW	mLST	ESIA	Blue-green colonies
ESPM–ESSM	–	–	–	Blue-black colonies
CES	–	–	–	Blue-green colonies
KR medium	–	–	–	Violet-colored centers
α-MUG-NA	–	–	–	Yellow fluorescence under UV light

FDA, U.S. Food and Drug Administration; ISO, International Organization for Standardization; BPW, buffer peptone water; VRBGA, violet red bile glucose agar; TSA, trypcasein soy agar; mLST, modified lauryl sulfate tryptose medium; DFI, Druggan-Forsythe-Iversen; ESE, *Enterobacter sakazakii* enrichment; ESSE, *Enterobacter sakazakii* selective broth; ESIA, *Enterobacter sakazakii* isolation agar; ESPM–ESSM, *Enterobacter sakazakii* chromogenic plating medium–*Enterobacter sakazakii* screening medium; CES, chromogenic *Enterobacter sakazakii*; KR, Kim-Rhee; α-MUG-NA, 4-methyl-umbelliferyl alpha-D-glucoside-nutrient agar.

alpha-D-glucoside (alpha-MUG) for detection of presumptive *Cronobacter* spp. in PIF was recommended by Leuscher and Bew (2004). This assay depended on the presence of α-glucosidase which distinguished *Cronobacter* spp. from other species in the family of Enterobacteriaceae. All (56) *Cronobacter* spp. could form yellow colonies on NA and showed blue/violet fluorescence on NA + alpha-MUG plates under ultraviolet (UV) light. Although other species from infant formula also produce yellow pigment, these colonies cannot fluoresce under UV light (Leuscher and Bew, 2004). Oh and Kang (2004) used alpha-MUG as a selective marker to develop a differential medium for detection of *Cronobacter* spp. In addition, the incubation conditions (37°C for 24 h) were optimized and an optimum basal media (bile salts no. 3, 1.5 g; agar, 15.0 g; sodium thiosulfate, 1.0 g; ferric citrate, 1.0 g; and alpha-MUG , 50.0 mg per liter) and nitrogen source (tryptone, 20.0 g/L) were selected. A new chromogenic medium (Druggan-Forsythe-Iversen [DFI] medium) was described for selective detection of *Cronobacter* spp. (Iversen et al., 2004a). This method is based on the α-glucosidase reaction, which is detected using 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (XαGlc) as substrate. *Cronobacter* spp. can hydrolyze this substrate to form blue-green colonies on this specific agar. The DFI medium had 100% sensitivity for *E. sakazakii* due to the constitutive expression of α-glucosidase. Using the given range of Enterobacteriaceae, the specificity of DFI medium was 87.2% (Iversen et al., 2004). The presence of *Cronobacter* species and other Enterobacteriaceae was surveyed in 82 PIF milk and 404 other food products by both the FDA method and DFI chromogenic medium (Iversen and Forsythe, 2004). *Cronobacter* spp. was isolated from 67 samples, whereas only 19 samples were found to be positive for *Cronobacter* spp. by the FDA method. Combine with API-20E test, there was 72.9% false positive presumptive *Cronobacter* spp. by FDA method, compared with 38.5% by DFI medium. Perhaps the combined use of Enterobacteriaceae enrichment (EE) and VRBGA medium could allow the other Enterobacteriaceae strains outgrow *Cronobacter* spp. resulting in false negative result. Meanwhile, the DFI medium detected the organism 2 days before the conventional FDA procedure, since a 2-day incubation period on tryptic soy agar (TSA) is not required prior to confirming identification using API-20E. It was suggested DFI chromogenic media could be used for sensitive and rapid detection of *Cronobacter* from food samples (Iversen and Forsythe, 2004). On one hand, a number of other species such as *Escherichia vulneris*, *Pantoea* spp., and *Citrobacter koseri* were tested positive for α-glucosidase activity, which easily resulted in false positive signals on DFI medium. Additionally, some *Cronobacter* spp. cannot generate blue-green colonies on DFI medium (Iversen and Forsythe, 2007). Therefore, pretreatment of food samples and confirmation of presumptive *Cronobacter* strains are very important for correct detection of *Cronobacter.* A simple and rapid cultural method for detection of *Cronobacter* spp. from environmental samples was developed by Guillaume-Gentis et al. (2005). This assay was performed using modified lauryl sulfate tryptose (mLST) broth as a selective step to enrich *Cronobacter* spp. and suppress non-*Cronobacter* species (Guillaume-Gentis et al., 2005). All of 99 *Cronobacter* spp. could grow in mLST at 45°C, whereas 79.6% (35/39) strains belonging to other Enterobacteriaceae were suppressed. It was concluded that sodium laury sulfate and vancomycin played important roles in selective enrichment step. However, the recent study also found that some *Cronobacter* spp. could not grow in mLST broth at 44°C (Iversen and Forsythe, 2007). A chromogenic agar, R&F *E. sakazakii* chromogenic plating medium (ESPM), was developed for isolating presumptive colonies of *E. sakazakii* from foods and environmental sources (Restaino et al., 2006). On ESPM, 76/79 pure culture strains of *Cronobacter* spp. yielded blue-black colonies after 24 h at 35°C. Other enteric organisms yielded white, yellow, green, or clear colonies. In addition, most false-positive results on ESPM were eliminated by *E. sakazakii* screening medium (ESSM) biplate, which was used to evaluate sucrose fermentation on one side of the plates and acid formation from melibiose on the other side. In an analyzes of 240 samples, the number of samples positive for *Cronobacter* spp. by the ESPM–ESSM method and the FDA were 27 and 16 respectively, with sensitivity and specificity values of 100% and 96.9% versus 59.3% and 43.7%, respectively.

The artificial Neural Networks model was developed for identification of the *Cronobacter* spp. from other related *Enterobacter* species (Iversen et al., 2006). This model was applied to biochemical and 16S rDNA data derived from 282 strains of Enterobacteriaceae, including 189 *Cronobacter* spp. The model resulted in a predictive performance for blind (validation) data of 99.3% correct discrimination between *Cronobacter* spp. and closely related species for phenotypic and genotypic data. Comparison between *Cronobacter* spp. and other strains also constitutively positive for expression of the enzyme α-glucosidase lead to a predictive performance of 98.7% for 16S rDNA sequence and 100% for phenotypic data. The model not only showed a remarkable ability in reducing data dimensionality and complexity to eliminate noise from the system and facilitate the speed and reliability of a potential strain identification system, but also provided valuable information about the population and distribution of individual species for rapid identification of pathogens (Iversen et al., 2006). However, this model has limited values as a method for detection of *Cronobacter* from actual food samples. Recently, to provide sensitive and accurate detection and identification of *Cronobacter* isolates for microbiological safety of food, especially PIF, the comparison of different selective enrichment media for detection of *Cronobacter* spp., was carried out (Iversen and Forsythe, 2007). In addition, the molecular and biochemical methods for detection of *Cronobacter* spp. were also evaluated. Results indicated that some *Cronobacter* strains were unable to grow in selective media (FDA, 2002; Guillaume-Gentis et al., 2006; Druggan and Iversen, 2009). By a comparison of different media for selection, isolation, and confirmation of *Cronobacter* spp. (Cawthorn et al., 2008), identification of *Cronobacter* strains using yellow pigment production was demonstrated to have a low sensitivity, specificity, and accuracy (87%, 71%, and 74%, respectively), while both chromogenic DFI and chromogenic *E. sakazakii* (CES media) were sensitive, specific, and accurate (100%, 98%, and 98% respectively) for the detection of *Cronobacter* spp. (Cawthorn et al., 2008). A new selective, differential, and cost-effective medium (Kim and Rhee, KR-medium) for the isolation of *Cronobacter* spp. was developed. This new medium used salicin as a differential agent, and *Cronobacter* spp. generated typical colonies with characteristic violet-colored centers surrounded by a transparent to opalescent border, while the pure culture from 40 non-*Cronobacter* strains were inhibited or produced visually distinguishable colonies. Using 96 strains including 56 *Cronobacter* and 40 strains of other species to test the specificity and sensitivity of KR medium, the KR medium had a high specificity (98%) and sensitivity (100%), with no false-negative results (Kim and Rhee, 2011).

Capillary gas chromatography with flame ionization detection (GC-FID) was used to determine the cellular fatty acid (CFA) profiles of 134 *Cronobacter* spp., and these were compared to the CFA profiles of other closely related *Enterobacter* and *Citrobacter* species. For GC-FID analyzes, whole cell fatty acid methyl esters (FAMEs) from cells cultured on Brian Heart Infusion (BHI) agar at 37°C for 24 h were obtained by saponification, methylation, and extraction into hexane/methyl tert-butyl ether (Hoffmann et al., 2008). A database for *Cronobacter* spp. was prepared using fatty acid profiles from the 134 strains. Principal component analyzes (PCA) based on CFA profiles for *Cronobacter* strains shows separation of *Cronobacter* spp. subgroups A and B. Likewise, a PCA model based on CFA profiles for *Cronobacter* spp. clearly showed separation of *Cronobacter* spp. from closely related *Enterobacter* and *Citrobacter* species. Analysis of FAMEs from *Cronobacter* grown on BHI agar by a rapid GC-FID method can provide a sensitive procedure for the identification, and this analytical method provides a confirmatory procedure for the differentiation of *Cronobacter* from closely related *Enterobacter* and *Citrobacter* species (Hoffmann et al., 2008).

8.4.3 Genotypic Methods

8.4.3.1 Molecular Detection Methods

Compared with conventional methods, molecular detection methods are sensitive, rapid, and accurate (Wang et al., 2012). Recently, a number of new molecular methods for rapid detection of pathogens have been developed using the 16S rRNA and the 16S-23S rDNA internal transcribed spacer (ITS), *ompA* or other targeting genes as most potential molecular targeted markers, including

PCR, real-time polymerase chain reaction (RT-PCR), DNA hybridization, and probe detection methods as listed in Table 8.2. A PCR-based technique targeting 16S rRNA gene was developed for rapid detection of *Cronobacter* spp. in the granular sludge used to remove the chemical oxygen demand (COD) from winery wastewater (Keyser et al., 2003). Another specific PCR assay was recommended and evaluated based on analyzes of full-length of 16S rRNA (Lehner et al., 2004). Various strains were analyzed for comparison of detection results of this method and the Keyser PCR system (Lehner et al., 2004). They found Keyser system gave negative signals for *Cronobacter muytjensii* ATCC51329 and positive for non-*Cronobacter* species such as *E. cloacae*, *Serratia liquefaciens*, *Serratia ficaria,* and *Salmonella enteritidis.* A set of primers and probe was designed utilizing the *Cronobacter* species partial macromolecular synthesis operon: the *rpsU* gene at 3′ end and the primase (*dnaG*) gene at 5′ end in this detection protocol (Seo and Brackett, 2005). A second RT-PCR assay was developed using TaqMan and SYBR Green to identify *Cronobacter* spp. after enrichment in mLST and BHI. The result showed all (thirty-five) *Cronobacter* spp. was positive, but all *non-E. sakazakii* (*Cronobacter*) species were found to be negative. By inoculation of serial dilution ($1.1–3.9 \times 10^{-1}$ to $1.1–3.9 \times 10^{8}$ CFU) into 100 g PIF, this method detected 1.1–3.9 CFU/100 g PIF after enrichment in mLST-BHI and the definite result was accomplished within 2 working days (Liu et al., 2006a). PCR and oligonucleotide array assays for detection of *Cronobacter* spp. in PIF were developed. All of the *E. sakazakii* (*Cronobacter*) produced positive reaction, and no cross-reaction was observed with non-*E. sakazakii* (*Cronobacter* spp.). Sensitivity of detections was 1.3 CFU/100 g PIF combined with the selective enrichment (Liu et al., 2006b).

The outer membrane protein A, along with its flanking sequence from *Cronobacter muytjensii* ATCC51329, was cloned and sequenced (Mohan Nair and Venkitanarayanan, 2006). Based on the regions of the *ompA* gene unique to *Cronobacter* spp., two primers were designed to develop and optimize a *Cronobacter* genus-specific PCR. The expected fragment, 469 bp, was produced after amplification from all ($n = 17$) *Cronobacter* spp., but not from other non-*Cronobacter.* The PCR detected as few as 10^3 CFU/mL of *Cronobacter* species in the PIF directly and 1.0 mL suspending solutions (10^{-1} CFU/mL) into 9.0 mL sterile PIF after 8 h enrichment step. It was

TABLE 8.2
Molecular Methods for Detection of *Cronobacter* spp.

Molecular Methods	Primers (5′–3′)	Applicons Sizes	References
PCR on 16S rRNA	F:cccgcatctctgcaggattctc R:ctaataccgcataacgtctacg	832 bp	Keyser et al. (2003)
PCR on 16S rRNA	F:agagtttgatymtggctc R:cakaaaggaggtgatcc	1500 bp	Lehner et al. (2004)
PCR on gluA	F:atgggatcctgaaagcaatcgacaagaag R:ccgaagcttactcattacccctcctgatg	1680 bp	Lehner et al. (2006)
PCR on ITS	F:gggttgtctgcgaaagcgaa R:gtcttcgtgctgcgagtttg	282 bp	Liu et al. (2006b)
PCR on ompA	F:ggatttaaccgtgaacttttttcc R:cgccagcgatgttagaaga	469 bp	Mohan Nair and Venkitanarayanan (2006)
duplex-PCR on ompA-ITS	Same to ITS and ompA above	282 and 469 bp	Zhou et al. (2008)
PCR on unknown gene	F:cccagacgaaactcgccgag R:gggataagcagataatgcgataaa	634 bp	Ye et al. (2008)
Real-time PCR on dnaG	F:gggatattgtcccctgaaacag R:cgagaataagccgcgcatt	78 bp	Wang et al. (2012)
–	Probe:agagtagtagttgtagaggccgtgcttcc gaaa	–	Seo and Bracket (2005)

ITS, internal transcribed spacer.

concluded that this PCR, with enrichment, had potential to be used as a rapid tool for detecting the presence of *Cronobacter* spp. in PIF (Mohan Nair and Venkitanarayanan, 2006). To look for the specific gene and development-specific detection method, the bacterial artificial chromosome (BAC) library of *E. sakazakii* strain 858 DNA was constructed and BAC 10C10 clone from 800 clones of this library were elaborately selected for sequencing and analysis of the insert, the molecular basis of the α-glucosidase activity in *Cronobacter* spp. was characterized (Lehner et al., 2006). Elucidation of the molecular basis would provide novel targets suitable for the development of more specific and direct identification systems for this organism. The different methods including TSA, DFI, Chromocult *E. sakazakii* (CES), and PCR were evaluated for detection of *Cronobacter* spp. in South African infant formula milks and the processing environment using DNA sequences as targeted markers to design a pair of species-specific primer (Druggan and Iversen 2009). The specificity of the PCR amplifications ranged from 8% to 94%, emphasizing the need for rigorous primer testing against closely related species (Druggan and Iversen 2009). Zhou et al. have developed the duplex-PCR for detection of *Cronobacter* spp. on the 16S–23S rDNA ITS and *ompA* gene (Zhou et al., 2008). To further differentiate different species within the genus *Cronobacter*, the six pairs of primers were designed and detection method was developed on *rpoB* gene (Stoop et al., 2009). However, recommended are two simplex PCR steps for detection and for differentiating *C. sakazakii* from *C. malonaticus* since the *rpoB* gene sequences of two species are closely related. A comparative evaluation of three commercial diagnostic systems, namely the BAX® System PCR Assay *E. sakazakii* (DuPont, Qualicon, Wilmington, Delaware), the Assurance GDS™ *E. sakazakii* (BioControl, Bellevue, Washington), and the foodproof® *E. sakazakii* Detection Kit (Biotecon Diagnostics, Potsdam, Germany) for the rapid identification of *Cronobacter* spp. indicated that a specificity of 100% was observed for the Assurance GDS™ *E. sakazakii* and the foodproof® *E. sakazakii* Detection Kit for pure cultures as well as artificially contaminated PIF samples (Fricker-Feer et al., 2011).

8.4.3.2 Molecular Typing Methods

Farmer et al. (1980) described 15 biogroups within *E. sakazakii* (*Cronobacter* spp.) based on the biochemical characterizations of 57 strains. In addition to phenotypic characterizations, advances were made in molecular typing techniques, for example, randomly amplified polymorphic DNA-PCR (RAPD-PCR), pulsed-field gel electrophoresis (PFGE), ribotyping, enterobacterial repetitive intergenic consensus (ERIC)-PCR, restriction fragment length polymorphism (RFLP), multilocus sequence analyzes (MLSA), multilocus sequence typing (MLST), which are listed in Table 8.3 (Nazarowec-White and Farber, 1999; Caubilla-Barron et al., 2007; Drudy et al., 2006; Pei et al., 2008; Ye et al., 2008, 2010; Kuhnert et al., 2009; Strydom et al., 2011; Joseph and Forsythe 2011; Joseph et al., 2012 a,b). Eighteen *E. sakazakii* (*Cronobacter* spp.) strains represented 10 ribotypes by ribotyping with EcoR1 restriction endonuclease (Nazarowec-White and Farber 1999). Clark et al. confirmed ribotyping was more discriminatory than restriction endonuclease analysis (REA) (Clark et al., 1990). Eighteen *Cronobacter* spp. were analyzed by PFGE using the restriction endonuclease Xba1 and each isolate was found to have one unique pattern (Nazarowec-White and Farber 1999). Van Acker et al. (2001) used arbitrarily primed polymerase chain reaction (AP-PCR) to determine the common sources of the outbreaks of diseases on the basis of description of 12 cases of necrotizing enterocolitis in 1998 (Van Acker et al., 2001). Three AP-PCR profiles were obtained for patients and milk isolates with 14 milks isolates matching profiles from three patients. Moreover, the original strain isolated from a gastrostomy tube of a neonate that fed the same type of milk 4 years before had an AP-PCR profile (the profile Ib) highly similar with the AP-PCR profile Ia from the 14 milk and patients isolates, which indicated a persistent contaminated problem in milk samples. They also found RAPD-PCR and PFGE to be the effective typing methods because the food and clinical isolates with same ribotypes had different PFGE profiles and were distinguishable by RAPD-PCR using the primer 5′-CGCGTGCCAG-3′ (Van Acker et al., 2001). According to the analysis of BioNumerics software, 29 strains of *E. sakazakii* (*Cronobacter* spp.) were typed by

TABLE 8.3
Molecular Typing Methods for *Cronobacter* spp.

Molecular Typing Methods	Primers or Genes or Restriction Enzymes	References
RAPD-PCR	cgcgtgccag	Nazarowec-White and Farber (1999)
ERIC-PCR	atgtaagctcctggggattcac, aagtaagtgactggggtgagcg	Ye et al. (2008, 2010)
PFGE	XbaI and SpeI	Pei et al.(2008); Brengi et al. (2012)
MLSA	*recN*, *rpoA*, and *thdF*	Kuhnert et al. (2009)
MLST	*atp*D, *fus*A, *gln*S, *glt*B, *gyr*B, *inf*B, and *pps*	Joseph et al. (2012b); Baldwin et al. (2009)
RFLP	*ropB* based on Csp6I and HinP1I	Strydom et al. (2011)
Multiple types	DNA hybridization, f-AFLP, 16S rRNA analyzes	Iversen et al. (2008)

RAPD-PCR, randomly amplified polymorphic DNA-PCR; ERIC-PCR, enterobacterial repetitive intergenic consensus-PCR; PFGE, pulsed-field gel electrophoresis; MLSA, multilocus sequence analyses; MLST, multilocus sequence typing; RFLP, restriction fragment length polymorphism; f-AFLP, fluorescent-labeled amplified fragment length polymorphism.

PFGE and gave different patterns except two isolates viz. ES004 and ES005 from PIF (Pei et al., 2008). Repetitive intergenic elements as important molecular marks were used to typing study. Among them, ERIC is a group of highly conserved DNA sequence that are about 124–127 bp long located mainly within the intergenic regions of chromosomes of species of Enterobacteriaceae and has ability to form stable stem-loop structure by the inverted repeat sequences (Sharples and Lloyd, 1990). ERIC as an important molecular marker combined with PCR was used to epidemic investigation even as a molecular typing method to trace the contamination and infection of pathogens such as *Escherichia coli, Salmonella* spp., and *Vibrio* spp. (Versalovic et al., 1991). The reproductive and stable ERIC-PCR fingerprinting was optimized and applied by Ye et al. (2008, 2010). They found that ERIC-PCR could be used for typing of *E. sakazakii* (*Cronobacter* spp.) from PIF.

The genome sequence of type strain ATCC BAA-894 was described and comparative genomic hybridization analysis with other *Cronobacter* species was also carried out (Kucerova et al., 2010). MLSA of *recN*, *rpoA,* and *thdF* genes was done on more than 30 species of the family Enterobacteriaceae with a focus on *Cronobacter* and the related genus *Enterobacter.* Phylogenetic analysis showed that the genus *Cronobacter* formed a homogenous cluster related to recently described species of *Enterobacter*, but distant to other species of genus *Enterobacter.* The comparative analysis gave important insights into the phylogeny and genetic relatedness of the family Enterobacteriaceae and would serve as a basis for further studies and clarifications on the taxonomy of this large and heterogeneous family (Kuhnert et al., 2009). The combination of genotypic (PFGE, 16S rRNA gene sequencing, and automated ribotyping) methods with traditional phenotypic biochemical methods was to characterize a collection of *Cronobacter* spp. from various origins. Results obtained here also further verified the high genetic heterogeneity within *Cronobacter* genus (Miled-Bennour et al., 2010). An RFLP based on *rpo*B gene by the combined digestion with restriction endonucleases Csp6I and HinP1I was developed, which could successfully ensure rapid differentiation between the five species of *Cronobacter* (Strydom et al., 2011). A multilocus sequence typing MLST approach *atp*D, *fus*A, *gln*S, *glt*B, *gyr*B, *inf*B, and *pps* was developed for successfully identifying and discriminating between *C. sakazakii* and *C. malonaticus* (Baldwin et al., 2009). Based on the same seven genes, the multilocus sequence typing MLST approach was carried out for investigating the phylogenetic relationship of 325 *Cronobacter* species isolates. Intraspecific variation showed major difference within different species of genus *Cronobacter.* Furthermore, *C. sakazakii* such as ST4 was the predominant species from clinical cases of meningitis (Joseph and Forsythe 2011; Joseph et al., 2012b).

8.5 CONTROL, PREVENTION, AND MANAGEMENT OPTIONS FOR SPOILAGE MICROORGANISMS

To reduce the risk of infant formula causing neonatal *E. sakazakii* infections, interim guidelines were summarized in the United States (Himelright et al., 2002), drawing from recommendations issued by the American Dietetic Association (1991) for proper procedures for preparing, feeding, and storing PIF in health-care facilities. These procedures included the following: (1) Formula products should be selected based on nutritional needs; alternatives to powdered forms should be chosen when possible. (2) Trained personnel should prepare powdered formula using aseptic techniques in a designated preparation room. (3) Manufacturer's instructions should be followed; product should be refrigerated if not fed immediately, and discarded if not used within 24 h after preparation. (4) The administration or hang time for continuous enteral feeding should not exceed 4 h. (5) Written hospital guidelines should be available in the event of a product recall, including notification of health-care providers, a system for reporting, follow-up of specific formula products used, and retention of recall records. However, it must be noted that these guidelines are for health-care facilities and do not apply to home settings (Baker, 2002).

To control and prevent from contamination of *Cronobacter* spp. in powdered milk, the methods presented are as follows: (1) The hazard analysis and critical control points (HACCP) system should be developed during the production of powdered milk and the critical control point should be scientific and reasonable and monitoring measures must be valid. (2) The disinfection and sterilization measures in environmental sanitation is also important for controlling the contamination of *Cronobacter* spp. from environmental samples. (3) The scheduled training and propaganda of scientific knowledge should be in much in need for strengthening awareness of food safety in workers and consumers.

Finally, the potential hazards such as biofilm formation should be emphasized because biofilm on the surfaces of processing environments and equipment is the main source of persistent contamination of foodborne pathogens during the food production. In recent years, the biofilm formation of *Cronobacter* spp. was described (Jung et al., 2013; Lee et al., 2012; Ye et al., 2015b). In addition, inhibitory effects of D-tryptophan (D-Try) on biofilm development by the foodborne *Cronobacter sakazakii* has been studied using crystal violet staining, scanning electron microscopy, and confocal laser scanning microscopy (Li et al., 2015). Results indicated that, compared with biofilm formation of the corresponding control, the reduction of biofilm formation by 10 mM D-Try at 24, 48, and 72 h was 87%, 84%, and 76%, respectively. In addition, a nutrient-rich medium contributes to maintenance of existing biofilm, but favors detachment when exposed to D-Try. Further analyses indicated that inhibitory effects of D-Try on biofilm development might be attributed to changes of initial adhesion between cells and the properties of the extracellular matrix. To reveal the genes related with biofilm formation of *C. sakazakii*, several possible genes such as cellulose biosynthesis and flagella related factors in *C. sakazakii* were revealed to influence the biofilm formation through random transposon mutagenesis (Hartmann et al., 2010). Subsequently, the proteomics was used to determine the factors related with biofilm formation at protein level (Ye et al., 2016). Result showed that compared with proteins in planktonic cells, three proteins (PPlase, Flagellar hook protein FlgE, and DsbC) were expressed only in biofilm cells, which might be the important factors involved in biofilm formation of *C. sakazakii*.

8.6 CONCLUSION AND FUTURE TRENDS

Although PIF was considered to be the main transmission vehicle of *Cronobacter* infections, the true reservoir of the organism is still unknown (Adamson and Rogers, 1981; Muytjens et al., 1983; Arseni et al., 1987; Biering et al., 1989; Gurlter et al., 2005). Consequently, the valid control of contamination sources of *Cronobacter* should be developed in the production of PIF. Several media were used to detect and isolate *Cronobacter*, but current media designed for identification of *Cronobacter* did not support the growth of all *Cronobacter* phenotypes (Iversen and Forsythe 2007; Druggan and Iversen, 2009). Thus, modification or improvement of current methods is also necessary for correct

identification of *Cronobacter* spp. Although several typing methods have been used for the subtyping of *Cronobacter* spp., there is no standard method for tracing origins of *Cronobacter* (Caubilla-Barron et al., 2007; Drudy et al., 2006; Nazarowec-White and Farber 1999; Pei et al., 2008; Ye et al., 2008, 2010). Therefore, to monitor *Cronobacter* isolates from different sources, a combination of several typing methods will be an important trend for correctly tracing the origins of *Cronobacter.*

In addition, pathogenicity difference of *Cronobacter* spp. was observed for the first time (Pagotto et al., 2003). Furthermore, the roles of OmpA or OmpX on virulence potential of *Cronobacter* spp. were described (Singamsetty et al., 2008; Mittal et al., 2009; Mohan Nair et al., 2009; Kim et al., 2010). Some novel factors such as *LuxS*, flagella genes, *ompW*, and *sdiA* were found to relate with the virulence of *C. sakazakii* (Ye et al., 2015a). In the future, detailed functions of these molecular factors related with virulence and their regulation will be of importance for precaution and control of *Cronobacter* spp. infections through powdered milk chain.

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9 Spoilage Microorganisms in Egg Products

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CONTENTS

9.1 INTRODUCTION

An increasing proportion of eggs are processed by the egg-product industry (also known as the egg-breaking industry). Egg products are intended for human consumption, with the removal of eggshells and eggshell membranes, from eggs' inner components, which are difficult to be separated or mixed together. In Europe, the microorganism is responsible for the majority of the cases of foodborne disease due to the consumption of egg or egg products. *Salmonella enteritidis* (90%) was the predominant serotype causing salmonellosis in 2007 (EFSA, 2009). Controlling the microbiological quality of egg products is imperative, especially when they are used as ingredient in raw or lightly cooked food.

In this chapter, an overview is provided for the detection, classification, and characteristics of the spoilage microbial species, current spoilage hazards status, evaluation techniques, management systems, prevention tools, and related mechanisms in egg products. In addition, future trends and further advices are also discussed.

9.2 TAXONOMY OF SPOILAGE MICROORGANISMS IN EGG PRODUCTS

Microbial spoilage may occur in a variety of different egg products such as shell eggs and liquid eggs (LEs). Since the processed eggs (cooked, frozen, dried, and baked) are complex, we just list the most common spoilage microbial species of different egg products. Microbes, causing spoilage of shell eggs and LEs, are Gram-negative microorganisms, but a low number of Gram-negative bacteria can also be found (Baron and Jan, 2011).

9.2.1 Pathogen Populations on Poultry Farms

Two major foodborne pathogens in poultry production, *Salmonella* and *Campylobacter*, are known to be a significant source. Consideration will be given to the prevalence of these organisms in different types of poultry flock and the control measures used. However, the presence of an organism in a particular part of the poultry production pyramid does not necessarily create a significant risk, such as *Campylobacter* in egg production or breeding flocks, noninvasive *Salmonella serovars* in egg production, so the relationship between organisms and production sectors will also be considered. A good understanding of possible source of infection is vital to effective control, so the major source, such as feed, hatchery, vertical transmission, persistent farm infection, and environmental contamination, will be discussed. Once these organisms are introduced onto the farm, it is important to understand their likely behaviors with regard to direct and indirect spread, involving farm pests, contamination of equipment and personnel, airborne spread, and survival in environmental niches. Only then can the organisms be effectively controlled.

9.2.1.1 Foodborne Pathogens in Poultry and Their Significance

Modern food production is so complex that full control of pathogens at every stage is very difficult, since not all components of the supply chain may be under close, central control (Sanders, 1999). Every time there is a change in the way the food chain operates, a new selective pressure may be created for novel or previously insignificant pathogens (Miller et al., 1998). Organisms are responding to environmental challenges the whole time and new forms, such as *Escherichia coli* O157, *S.enteritidis* PT4, PT8, and related egg-invasive strains (WHO, 1989; Haeghebaert et al., 1998; FAO/WHO, 2003), as well as the *Salmonella typhimurium* DT104 complex, are recent examples of this (Miller et al., 1998; Rabsch et al., 2001). There is a large range of potential pathogens that may be present in the commercial poultry and therefore may appear, theoretically, on poultry meat or eggs (EC, 2000). The list also includes commensal organisms with antimicrobial resistance, such as *E. coli* or *Enterococcus faecium* that may, in some circumstances, act as opportunist pathogens or as a potential reservoir of resistance genes, such as *VanA* in *E. faecium* or plasmid-mediated

trimethoprim/sulphonamide resistance in *E. coli*. Environmental spread of pathogens and resistant organisms originating from poultry waste is also important, particularly when fresh salad vegetables or water supplies become contaminated (Meng and Doyle, 2002). Although the range of poultry-associated pathogens is large, the most significant organism in present context is *Salmonella* and *Campylobacter* (Thorns, 2000). They are responsible throughout the world for 90% of identifiable, bacterial zoonoses that are foodborne.

9.2.1.2 *Salmonella*

Salmonella is an important foodborne pathogen worldwide, because of the frequency of its occurrence and its potential pathogenicity (Barrow, 2000; Meng and Doyle, 2002). An important feature of the organism is the epidemic spread of certain clones, for instance, *S. enteritidis* PT4 or *S. typhimurium* DT104 (Threlfall et al., 1993; Low et al., 1997). *Salmonella* survives well in the environment and has been involved in large outbreaks of foodborne illness where vegetables, fruits, and spices have been contaminated by animal or human fecal waste or contaminated water (Bryan and Doyle, 1995; EC, 2000). It can also cause long-term contamination of industrial facilities, such as incubators for hatching eggs, feed mills, and poultry processing plants. A major concern with *Salmonella* is the decrease in susceptibility to newer antimicrobials, such as fluoro-quinolones and extended-spectrum cephalosporins (Rabsch et al., 2001). In recent years, a type of *S. newport* with multiple resistances has emerged and spread widely in the United States, as has *Salmonella paratyphi* B var. Java, which also has multiple resistances, in some European countries. It is important that the international spread of such strains is limited as far as possible.

9.2.1.3 *Campylobacter*

This organism, especially *Campylobacter jejuni*, is the leading cause of foodborne zoonoses in most developed countries (Harris et al., 1986; WHO, 2001). It is widespread in poultry production (Evans, 1992; Bryan and Doyle, 1995), and poultry is often implicated in case-control studies (Adak et al., 1995), but the proportion of human cases caused by contaminated poultry meat is unclear, because there are numerous other potential sources (Siemer et al., 2004), including pets, environmental contamination, contaminated vegetables and bottled water, and foreign travel (Evans et al., 2003; Gillespie et al., 2003; Neimann et al., 2003). Community outbreak is rare, but it is often related to contamination of milk (Gillespie et al., 2003). As well as acute disease, *Campylobacter* infection may be associated with long-term sequelae, such as Guillain–Barré syndrome, reactive arthritis, and immune-proliferative small-intestinal disease (Lecuit et al., 2004). Large volume of poultry meat and eggs are consumed and the potential for cross-contamination of uncooked products, particularly in catering establishments, which makes a high priority of control of *Salmonella* and *Campylobacter* in poultry (Lee, 1974; Bryan and Doyle, 1995; Gillespie et al., 2003).

9.2.2 Prevalence of Salmonella

S. enteritidis is predominant in laying flocks and *S. typhimurium* is relatively uncommon in most countries. There are large variations in the figures, which partly reflect sampling methods: 300 spent hens per flock is likely to give far greater sensitivity than only 20. In general, the level of *Salmonella* in Sweden, Finland, and Norway is very low, because of the long-standing, strict control policies, although there has been an increase in prevalence more recently (EC, 2003). No data are available for Mediterranean countries, but infection rates are thought to be high in some areas and, in particular, eggs imported into the United Kingdom from Spain have been highly contaminated with *S. enteritidis*. Schluter et al. (1994) recorded an individual bird prevalence of 7.2% in pullets, 13.3% in laying birds, and 6.2% in broiler flocks.

There is little reliable prevalence data for turkey, duck, and goose flocks (EC, 2003). The incidence of *Salmonella* in turkeys is thought to be low in Finland, Sweden, Norway, the Netherlands,

and Ireland, but that may not be the case with ducks. *S. typhimurium*, including the DT104 complex, appears to be more common in turkeys than in broilers. In Canada, the high prevalence of 86.7% among 270 flocks was found positive in 1990–1991, which was associated with contaminated feed (Irwin et al., 1994).

9.2.3 *Campylobacter*

Manufactured feed is considered too dry to allow survival of any *Campylobacters* and hatchery infection is not thought to be a significant source of the organisms. Evidence for the entry of *Campylobacter* into hatcheries has been obtained by the polymerase chain reaction (PCR) (Hiett et al., 2002), but not by culture. In some studies, similar genotypes of *C. jejuni* were found in breeding and associated broiler flocks (Cox et al., 1999, 2002), but not in other work (Petersen et al., 2001), where numerous clones occurred in breeding flocks, so apparent associations may reflect sampling variation or a common environmental/fomite source. Although *Campylobacter* may be found on fresh eggs, its survival is poor, unlike *Salmonella* (Sahin et al., 2003). The lower prevalence of *Campylobacter* in winter and the occurrence of mainly *Campylobacter*-free farms within an integrated company would also be incompatible with the hatchery being a significant source (Shane, 2000).

9.3 SPOILAGE MICROORGANISMS CHARACTERISTICS AND POSSIBLE MECHANISMS

Although the contents of fresh eggs are usually sterile, commercially produced egg products (liquid, frozen, and dried) used to be heavily contaminated with bacteria; the contamination of the egg occurs after being laid and the access of microorganisms into the egg is most common through cracks in the shell. 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 (Baron and Jan, 2011).

Epidemiological studies show that poultry meat and eggs are important sources for consumers to expose themselves to zoonotic pathogens, such as *Salmonella* and *Campylobacter* (Svensson et al., 2007). Egg and egg products are the major cause of food poisonings in France; between 2006 and 2008, they were implicated in 15% of the cases of confirmed food poisoning, *Salmonella* being identified as the main causative agent (76%) (Delmas et al., 2010). There are six main types of microorganisms as potential hazards in egg products, as discussed in the following sections.

9.3.1 *Salmonella*

Salmonella is present in the environment and on birds in egg laying barns, and states laying operations. *Salmonellae* are isolated from 30% to 72% of environmental samples, such as water, ventilation fan, egg belt, and egg collectors. Seven of ninety eggshells (7.8%) before washed contain *Salmonella* (Jones et al., 1995). In studies of three united of the foodborne disease outbreaking in nursing homes, *Salmonella* was the most frequently reported pathogen accounting for 52% of outbreaks and 81% of deaths. *S. enteritidis* outbreak accounted for 56% of the *Salmonella*-associated deaths. The implicated food vehicles in *S. enteritidis* outbreak were made from eggs or prepared with equipment contaminated with eggs. Sporadic outbreaks of salmonellosis in the United States has been potentially associated with pasteurized egg products from the consumption of food items, such as crab cake, scrambled eggs, and chile relleno (Gurtler et al., 2015). The U.S. Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) has published a risk assessment model (FSIS, 1995–2013) in which they estimate that there may be approximately 5500 U.S. cases of salmonelloses per year that stem from contaminated pasteurized liquid egg products (Latimer et al., 2008).

9.3.2 *Bacillus cereus*

Bacillus cereus has been found in raw and pasteurized liquid whole egg, and bakery products are made from liquid whole egg in the United Kingdom. *B. cereus* group bacteria can contaminate the processing chains of egg products and survive the low heat treatments because of their ubiquitous and spore-forming characteristics. Their growth is a putative source of both (1) food safety problem, due to their ability to produce enterotoxins and/or the emetic toxin (ceramide), and (2) economic losses for the sector (Svensson et al., 2007).

9.3.3 *Campylobacter*

The organism is frequently carried by poultry, and can reach eggs. As the study shows, *Campylobacter* was isolated from 27.9% of unpasteurized liquid whole egg samples and 36.0% of unpasteurized LE yolk samples. The contamination levels of *Campylobacter* ranged from <3 to 240/100 m (Sato and Sashihara, 2010).

9.3.4 *Listeria* Spp.

Egg contents are less likely to be contaminated; *Listeria monocytogenes* was present in 2 of 42 samples of commercially broken raw LE in the United States. The recent data concerning egg product contamination by *L. monocytogenes* show that *L. monocytogenes* was found in 17.4% of raw whole egg products and 2.1% of pasteurized egg products (Rivoal et al., 2010). The contamination of raw egg products could be the result of the use of eggs with shells that had been contaminated by *L. monocytogenes* in the environment. The contamination of pasteurized egg products is a problem to be concerned; post-pasteurization contamination of the samples cannot be ignored, particularly in view of the ability of *L. monocytogenes* to persist in the environment of food industry plants and to grow at low temperatures (Warriner and Namvar, 2009).

9.3.5 *Staphylococcus aureus*

In the processing of egg products, the origin of *Staphylococcus aureus* was not determined, but it presumably came from the farm. *S. aureus* has caused intoxications in Canada from boiled eggs but these were hardboiled, decorated, and stored at room temperature for more than 1 day (Todd et al., 1991). A similar problem occurred in Sweden when 46 cases (four fatalities) resulted from the consumption of improperly stored hardboiled eggs. The study of the degree of contamination of table eggs indicated a relatively high degree of contamination of table eggs by *Staphylococcus* bacteria. In 1125 bacteriological tests conducted on whites, yolks, and shells of eggs, *Staphylococci* were found in 514 cases (Stepien-Pysniak et al., 2009).

9.3.6 *Yersinia enterocolitica*

Experiments have shown that *Yersinia enterocolitica* can penetrate shell and membrane over a period of time, which is similar to *Salmonella*, and there is a possible risk of penetration and proliferation for this microorganism into eggs during storage. *Y. enterocolitica* has been isolated from liquid whole eggs, the surface of eggshells (Favier et al., 2005), and unwashed commercial shell eggs in different countries (Musgrove et al., 2004).

9.3.7 Persistent Farm Contamination

A major source of *Salmonella* infection in poultry is due to the contamination of the farm environment, particularly that remaining inside houses and on equipment after cleaning and disinfection (Rose et al., 1999, 2000). This situation is common with less invasive serovars, particularly

those originating from feed (Limawongpranee et al., 1999), which may become environmentally adapted. Invasive serovars, especially *S. typhimurium*, are less likely to persist on farms or in hatcheries and feed mills (Chriêl et al., 1999), and infected breeding flocks or other animal sources are more important. *Salmonella* originating from the flock may also persist outside poultry houses in anterooms or in dust and litter from the house. Wildlife with access to such materials may then become infected (Davies et al., 2001). In the United States, routine cleaning and disinfection of broiler houses are not carried out after every crop but, despite this, it is not inevitable that new birds placed in a contaminated house will become infected. Poor cleaning and, in particular, insufficient disinfection may increase the risk of infection by distributing the organisms and leaving a damp environment.

The presence of breeding colonies of mice within poultry houses is a major factor that contributes to the chances of infection (Henzler and Opitz, 1992; Garber et al., 2003; Liebana et al., 2003). This is not a common problem in modern broiler or breeder/layer rearing houses, as there are few harborages for, or easy access to, feed. On broiler-breeder farms, on the other hand, there may be considerable harborages in mini-pits and easy access to feed in slave hoppers. There is a similar risk in barn or free-range egg production, but it is in cage houses that mice are particularly persistent, because they can breed in droppings pits or house insulation and readily gain access to feeders, without interference from the birds.

Persistence of *S. enteritidis* and, to a less extent, other serovars on laying farms is a considerable problem because of multiage sites. Unlike breeding, rearing, and broiler farms, these farms are occupied continuously, so there is always a source of feed, contaminated dust, and other materials, as well as operatives that may rapidly infect newly housed flocks. The risk is greatest when houses are linked by common egg belts and dung belts/channels (Davies and Breslin, 2003a). Typically, normal cleaning and disinfection of caged-layer houses and pest control are insufficient to eliminate *Salmonella*, and much reliance is placed on vaccination against *S. enteritidis* to suppress infection (Berghold et al., 2003; Davies and Breslin, 2003b). In Denmark, prolonged heating of cage houses, using steam containing formaldehyde, has been used successfully to eliminate infection on problem farms (Gradel et al., 2004).

Persistence of contamination inside conventional broiler houses is not thought to be a major problem with *Campylobacter*, but farms with nonwaterproof electrical fittings or roof-mounted extractor fans that are not easily cleaned can be at increasing risk, suggesting that there may be some 114 food safety control persistence in the poultry industry (Shane, 2000). Organic flocks may become infected at a young age during the housed brooding period, because powerful disinfectants are rarely used (Dr Vivian Allen, personal communication). Occasionally, the organism may be found in poorly cleaned broiler houses (Stern et al., 2001; Davies R.H., unpublished), but it is not always associated with an infection. *Campylobacter* can persist in biofilms in water distribution lines, but is not found in houses holding negative flocks and, in infected flocks, the genotypes in the house are often different from those in birds (Zimmer et al., 2003). Because *Campylobacter* survives poorly in dry environments and is sensitive to oxygen and disinfectants, it is likely that significant survival occurs only in poorly cleaned and dry houses, where contaminated wash-water is allowed to collect on surface or in equipment (Shane, 2000).

9.4 DETECTION AND EVALUATION METHODS FOR SPOILAGE MICROORGANISMS

9.4.1 Detection of Microorganisms

Most microorganisms associated with egg spoilage can be detected and isolated by routine and standard methodology. In recent years, more and more studies on rapid detection method of pathogen are going on. The rapid detection of *Salmonella* in eggs and its products is of great significance in preventing foodborne salmonellosis. Gold standard culture methods (e.g., International Organization

for Standardization Method 6579 [ISO], U.S. Food and Drug Administration's Bacteriological Analytical Manual Chapter 5: *Salmonella*), which require up to 5 days (including biochemical and serological confirmations) (11, 15), do not suffice in routine and rapid monitoring of these samples. In recent years, novel real-time PCR assays were developed for the rapid detection of *Salmonella,* particularly from eggs. Rapid detection of an average of 5.9 stressed *Salmonella* cells in 25 g of food product using immune magnetic separation (IMS) and PCR is described in 1999 (Rijpens et al., 1999).

9.4.2 Evaluation of Freshness

9.4.2.1 Sensory Evaluation

Sensory tests can be divided into three parts: discriminative test, descriptive test, and affective test (G, 1991). Descriptive and discriminative tests are objective analytical tests in which a trained panel is used. Affective test is a subjective consumer test that is based on a measure of preference or acceptance. The choice of method depends on the purpose of the application of the sensory evaluation and whether it is used in product development, quality control, consumer studies, or physicochemical research methods.

The characteristic of fresh eggs will change during aging, being influenced by both storage temperature and environmental conditions (concentrations of O_2 and CO_2, relative humidity). The albumen has the major influence on overall interior egg quality. Thinning of the albumen is a sign of quality loss. The most widely used method for the determination of albumen quality is the Haugh units, which is based on determining both the weight on the intact weight and the albumen height of a broken egg. Another index used to evaluate egg freshness is air-cell height, which is affected by egg weight and relative storage humidity. And pH has also been used to determine the egg freshness.

9.4.2.2 Freshness Determination of Nondestructive Eggs

Near infrared (NIR) spectroscopy is widely used in the determination of organic constituents in feeds, foods, pharmaceutical products, and related materials. In the sector of egg, the application of NIR to the determination of egg freshness is rather limited. Only a few studies have been published on the potential of this technique in determining the freshness of eggs. The spectral region of wavelengths below 1100 nm is often called near-NIR. Schmilovitch et al. have used near-NIR to determine the freshness of eggs. The results obtained from the partial least square (PLS) showed that the variables, days after hatching, air chamber size, weight loss, and pH could be predicted by near-NIR with correlation coefficients varying from 0.9 to 0.92. Many overlapping signals are the NIR, which give rise to spectrums with broad peaks, making it difficult to interpret compared to the conventional mid infrared (MIR) spectrum. In addition to the above, the front-face fluorescence spectroscopy has been used extensively in the field of dairy products. However, in the literature, only preliminary studies explored the application of front-face fluorescence to the determination of egg freshness. This may be explained by the fact that eggs are complex products containing numerous fluorescent compounds, which makes it difficult to derive molecular information from their spectra.

9.4.3 Implications for Detecting *S. enteritidis* in Eggs

The nature of *S. enteritidis* deposition in eggs has a profound effect on the methods that have evolved for detecting contamination. Because *S. enteritidis* deposition is evidently a highly infrequent event and, since contaminated eggs have usually been found to contain very low concentrations of *S. enteritidis* cells, large numbers of eggs must be sampled to ensure that the pathogen is detected with adequate sensitivity (Gast, 1993). This imposes several significant constraints on practical and dependable methods for detecting these organisms in eggs that were taken from commercial laying flocks. To sample large numbers of eggs without overwhelming available laboratory resources, the contents of up to 20 eggs are often pooled together. However, pooling eggs

introduces a dilution of the already small numbers of *S. enteritidis* cells. Accordingly, incubation of egg pools, before applying subsequent enrichment culture steps, is essential to permit the multiplication of *S. enteritidis* more consistently in detectable levels (Gast, 1993; Gast and Holt, 2003). Supplementing these pools with concentrated sources of iron and other nutrients can improve the growth rate of *S. enteritidis* in incubating egg content pools (Gast and Holt, 1998; Chen et al., 2001). Innovative rapid technologies for *S. enteritidis* detection can be applied to eggs to replace traditional culture methods, but they still are dependent on a preliminary egg-pool incubation step to achieve satisfactory detection sensitivity (Gast and Holt, 2003).

9.4.4 Sampling Techniques

Effective sampling and handling of samples is the most important aspect of *Salmonella* monitoring. Sampling must be planned carefully according to the objective of the test and the likely variation in infected birds (Andersen-Sprecher et al., 1994); in practice, infection may be clustered, especially in laying houses (Riemann et al., 1998; Hayes et al., 2000; Mallinson et al., 2000). Although feces or tissues taken from individual birds at post-mortem may be preferable for comparative purposes (Altekruse et al., 2003), these are normally insufficiently sensitive and too costly for routine use (Davison et al., 1995), and it is considered that environmental monitoring, especially at key points of contamination, is superior (Kradel and Miller, 1991). In most types of poultry house, dust is the best sample to take, since competitor organisms do not survive as well as *Salmonella* (Davies et al., 1998, 2003; Davies and Breslin, 2001). In most types of house, accumulation of dust is commonly greatest on extraction vents but, where these are very high or sunk in a pit, dust on ledges close to the birds may be preferable. In houses with automated egg collection, the most productive dust is often the accumulated material from egg elevators or brushes/spillage trays after belts run. In cage houses, dust underneath the cage is an excellent sample and, for houses where pens of breeding birds are divided by solid walkways, sweepings from these will often identify adjacent infected pens (Davies et al., 1998). Where air filters are used, dust can also be gathered from these, particularly in hatcheries and feed mills (Kwon et al., 2000).

In cage houses, where droppings belts or scrapers are present, the best site to detect fecal contamination is the scraper, after belts run or droppings boards scrape. When birds are on litter, representative fecal sampling is appropriate, but more laborious. At the Veterinary Laboratories Agency (VLA), gauze swabs (Kleenex Readiwipes) are used (Davies and Wray, 1996). These are autoclaved in 225 mL amounts of buffered peptone water (BPW) in plastic jars and allowed sampling of a large area of the litter surface or of pooled feces on ramps, pop-holes, and weighers. Some workers claim that cellulose sponge swabs, presoaked in maximum recovery diluent, are superior (osterblad et al., 2003). If the samples are not to be cultured on the same day, then maximum recovery diluent should be used and the swabs kept cool until cultured. Many poultry companies take samples of pooled litter or feces, but it is essential that these are collected in a representative manner and mixed well before subsampling (Cannon and Nicholls, 2002). One approach is to create a slurry, using an equal volume of BPW, mix well then add 50 mL of the slurry to 200 mL of BPW for subsequent culture. It is much better to mix pooled samples manually, with care, rather than blend or "stomach" them, as this process can release inhibitory factors and directly damage low numbers of organisms. The same consideration is being applied to egg contents (Seo et al., 2003). For best detection of *Salmonella* in feces, it may be beneficial to culture an additional 1:10 dilution of the initial sample, since, at higher concentrations, particulates, and other competitive factors may influence the test result. With other samples, such as dust, feed, or carcasses, (Simmons et al., 2003) direct selective enrichment of feces is used and indicates that the larger volume of the material, the more sensitive of the test (Funk et al., 2000).

Indirect methods of sampling feces have been devised. These usually take the form of drag swabs or boot swabs/socks. Originally, drag swabs consisted of three large, moistened gauze pads, pulled behind a stick, along the length of a poultry house or droppings pit (Mallinson et al., 1989).

Such swabs are an effective means of sampling (Kingston, 1981), but the sampling assembly is cumbersome in occupied houses, and good technique and subsequent handling of samples are crucial (Opengart et al., 1991; Opara et al., 1992). Commercial attempts to scale down the drag swab do not produce equivalent results, since swabbing is not sufficient to identify an infected flock in all cases, and the identification of positive flocks increases in proportion to the number of assemblies used (Caldwell et al., 1994). It has been claimed that drag swabs are more sensitive than boot swabs (Caldwell et al., 1998), but this depends on the situation being sampled, the type and method of drag swabbing, and the type and others work well (Aho, 1992; Heyndrickx et al., 2002). In Denmark, a tube of gauze bandage has been used as a boot or "sock" swab and two to five pairs per house have given reasonable sensitivity, compared with testing large numbers of individual feces in pools (Skov et al., 1999; Gradel et al., 2002). For monitoring noncaged flocks, a combination of boot swabs and dust is the most practical and sensitive method, and initial sampling is best carried out about 3 weeks after housing to correspond with the accumulation of *Salmonellas* from the early peak of infection (Gradel et al., 2002; Heyndrickx et al., 2002). At the end of the crop, wash-water from the house provides a sensitive pooled sample (Fu et al., 2001) and it is essential to sample effectively after cleaning and disinfection of a contaminated house (Davies and Wray, 1996).

Sampling in the hatchery is important, and culture of only dead-in-shell or cull chicks will significantly underestimate contamination problems (Hafez and Jodas, 1992). Many poultry companies also culture hatcher fluff samples, but hatcher and delivery-box liners and macerated waste are the better sources of contamination. At the best, it will be necessary to increase flock-specific or incubator-specific sampling to identify the source of the infection. Sampling in feed mills and abattoirs also needs to be upgraded, as these can act as sensitive sentinels for the appearance of new *Salmonella* serovars within a population. Thus, dust accumulating in bins and auger systems, around equipment and in coolers in different parts of feed mills can reflect contamination of the ingredient or finished-product area, while unloading and waste areas in poultry processing plants can yield *Salmonella* serovars originating from processed flocks.

Sampling for *Campylobacter* has been less well researched than that for *Salmonella* (Reilly and Gilliland, 2003), since the high individual-bird prevalence and large numbers of organisms excreted by infected birds means that detection is usually straightforward (Newell and Fearnley, 2003). However, low-prevalence infection can occur after thinning, before there has been a chance for widespread infection to occur. Cloacal swabs or caecal contents are normally used for monitoring by poultry companies, but fresh droppings, particularly caecal droppings, may give better results and boot swabs are increasingly used in research studies (Stern and Robach, 1995; Eifert et al., 2003; Dr Vivian Allen, personal communication). It is essential that samples are maintained in a moist condition away from the air, thus, cloacal swabs in charcoal transport medium, collected directly into culture media, or full, tightly closed feces sample pots, with no air space, are required. *Campylobacter* in water supply is supposed to be detected by culture or PCR tests carried out on filters which are used to process large volumes of water.

9.5 CONTROL, PREVENTION, AND MANAGEMENT OPTIONS FOR SPOILAGE MICROORGANISMS

9.5.1 Management Options for Spoilage Microorganisms

9.5.1.1 Implications for Effective Control

The procedures described in this chapter will provide a framework for evaluating the safety of chemical residues that occur in edible poultry tissues. However, ensuring the safety of food supply depends on proper enforcement of key principles. Without a strong surveillance and compliance effort, intentional, illegal, or inadvertent exposure to chemicals may produce harmful residues in the food supply. Many countries have active regulatory supervision to ensure compliance and to detect and eliminate harmful residues (Botsoglou and Fletouris, 2001). For example, in the United

States, the U.S. Food and Drug Administration (US-FDA), the USDA-FSIS, and the Environmental Protection Agency (EPA) all collaborate actively in an effort to prevent the occurrence of harmful residues. The USDA-FSIS has a national residue-monitoring program that targets specific residues in edible animal tissues, including those of poultry (Paige et al., 1999). This program monitors not only foods in the United States, but also those imported from elsewhere.

In the past, there was no deliberate intention to produce foods containing harmful residues. Even the illegal application of chemicals in poultry production was done for economic benefits and not with malicious intention. Recent bioterrorist activities may change this situation. Both preharvest and postharvest food monitoring will need to be implemented to counteract this type of threat. Fortunately, because of the wide distribution of food production and processing operations in most countries, it would be difficult to contaminate a significant amount of food supply at any one time. Furthermore, many chemicals that may be used deliberately to contaminate growing poultry would also make birds ill and thus serve as an early warning signal. The unintentional contamination of poultry with dioxin in Belgium was discovered because birds became ill (Hume, 2002). However, although the number of affected poultry was relatively small, the product recalls and international ban on selected Belgian animal products had a significant economic impact. Therefore, it is necessary to develop and maintain a strong government regulatory monitoring system to detect and limit food safety problems and subsequent economic impact.

9.5.1.2 Control of *Salmonella*

9.5.1.2.1 Statutory Controls

The European Union (EU) Zoonoses Directive currently specifies monitoring and control measures that are then enacted by specific legislation in Member States. The legislation in place in the United Kingdom is presented previously, which currently applies mainly to breeding chickens (Stewart, 1999), but which will be extended in future to commercial laying flocks, broilers, and turkeys. Statutory controls are normally supplemented by government and industry codes of practice (Anon., 2002).

Some of the Scandinavian countries, such as Sweden, Norway, and Finland, have a zero-tolerance policy for *Salmonella* in poultry production (Wierup et al., 1995) and infected flocks and their products will be heat treated or destroyed, if any *Salmonella* serovar is found. This pathogen-free approach is not possible in most countries, but, in Denmark, monitoring and control have become much stricter in all poultry sectors (Bisgaard, 1992; Wegener et al., 2003) and some poultry meat is sold as "*Salmonella*-free." The basis of S*almonella* control is biosecurity to stop *Salmonella* entering the farm and to eliminate it quickly if it should enter the holding (Humphrey, 1989). This approach should apply throughout the production chain so as to avoid dissemination of the organisms (McIlroy et al., 1989; Anon., 1991; Forsythe, 1996; Noordhuizen and Welpelo, 1996; Johnston, 2000).

Biosecurity is defined as a health plan for measures designed to protect an animal population from transmissible infectious agents (Anon., 1999). This embodies all measures which can or should be taken to prevent viruses, bacteria, fungi, protozoa, parasites, disease carriers (rodents, insects, wild birds, people, equipment, etc.) from entering and endangering the health status of the population.

In poultry industry, biosecurity measures are used to minimize the risk of *Salmonella* entering poultry farms and associated enterprises, such as feed mills and hatcheries. Comprehensive biosecurity measures are costly in terms of capital equipment, use of disinfectants and other antibacterial, testing, and labor. The maximum level of biosecurity is only possible where there is a high-value product and the consequences of *Salmonella* being transmitted to customers are severe. Such measures are normally applied in full only for primary breeding and grandparent flocks, and include heat treatment of feed at higher temperatures and for longer periods than those used in commercial sector, combined with the addition of effective organic acid products at suitable concentrations. Feed is often tested for *Salmonella*, using rapid methods, before delivery to farms, and at feed mills there is monitoring of process and production environment, as well as testing of ingredients

and finished products. There is extremely frequent and comprehensive monitoring for *Salmonella* on farms and in hatcheries, and staff is also monitored. They are not allowed to contact the birds while excreting *Salmonella*. Visitors may also be asked to provide a negative fecal test result before being allowed on the premises. Entry to the premises is via a hygiene barrier, where showering in and out and use of disposable or site-dedicated protective clothing is required. Equipment used by contractors is a high risk (Heyndrickx et al., 2002; Pattison, 2003) and is either supplied by the company or fumigated on entry to the farm. Other farm inputs, such as litter, are carefully sourced to minimize risk, tested, and usually treated with antibacterial substances, such as organic acids or formaldehyde/acid combinations.

One of the basic principles of effective biosecurity in the commercial sector, all-in/all-out production on a whole-farm basis, is often not possible on primary breeding farms, because of the need to maintain and evaluate small groups of birds of high genetic potential. This presents a severe risk from possible persistence of *Salmonella* and hence the high level of biosecurity. This strict biosecurity applies in broiler, primary, and grandparent breeding in most North European countries, but measures may be less strict in grandparent flocks of some layer breeders, turkeys, and ducks (Davies et al., 1998, 2003), where there may be farms or hatcheries that are not completely dedicated to grandparent production.

At parent level, in conventional, but not organic production, all-in/all-out production is normal. Many of the biosecurity principles described earlier are applied, but at a lower intensity, because of cost. Although chicks originating from grandparent flocks are free from *Salmonella*, they may become infected during the rearing or laying stages through any of the routes described earlier, particularly via contaminated feed, except possibly in the case of *S. typhimurium* and *S. enteritidis*, which are rarely found in feed, but can be found regularly in the grain-storage areas of feed mills (Davies and Wray, 1997). Currently, the predominant cause of parent-flock infection with *S. enteritidis* and *S. typhimurium* is unknown, but there are significant levels of both organisms in the human and animal populations, and high levels of *S.enteritidis* can still be found on some commercial laying farms in most countries, and in broiler production in some EU Member States.

All-in/all-out production should ensure that *Salmonella* does not persist for more than one flock cycle, since it is possible to depopulate farms completely, remove all contaminated material, wash, disinfect, and test, to ensure that decontamination has been successful, before restocking the houses. In practice, there is sometimes insufficient time to complete the whole process effectively, before restocking. In particular, carriage of *S. enteritidis* and, to a lesser extent, *S. typhimurium* and other serovars in breeding mouse populations, has resulted in a high level of persistent infection. The mice are harbored in mini pits, storage areas, and wall and roof insulation within the house. In the United Kingdom, the introduction of vaccination in broiler-breeder sector, combined with improved hygiene and biosecurity, was fundamental in breaking the cycle of persistent farm infection, hatchery contamination, and dissemination of *S. enteritidis*. In the parent layer-breeder sector, vaccination is not used regularly, since widespread and persistent infection of flocks has not been such a problem.

For commercial broiler production in the United Kingdom, improvements in the *Salmonella* status of breeding flocks and feed, and improved cleaning and disinfection of houses have reduced *Salmonella* to a low level. At this time, there is considerable interest in further improving on-farm biosecurity to reduce the prevalence of *Campylobacter* and possible introduction of viral diseases, such as avian influenza. These measures include dedicated boots (and, in some cases, protective over suits) for each house, improved facilities and protocols for hand hygiene, step-over barriers between a "clean" and "dirty" part of the service area, or ante-room and improved tidiness outside the house. Such improvements also include in-filling of areas where water can pool and better drainage. Biosecurity in large-scale turkey production is of a similar standard, but there are considerable problems in applying biosecurity on commercial duck farms and laying farms (especially multiage, caged laying flocks). On caged layer farms, movement of mice and other rodents, flies, egg belts, and personnel can spread *S. enteritidis* between houses, despite vaccination (Davies and Breslin,

2003b). Mice and poor cleaning and disinfection are also responsible for persistence of *Salmonellas* on the farm (Davies and Breslin, 2003a). All biosecurity programs should be supplemented by appropriate monitoring to confirm their effectiveness.

Despite these difficulties in maintaining biosecurity, there seems to be little *Salmonella* infection on free-range layer and broiler farms (Davies and Breslin, 2003b; McDowell, 2004), so high levels of biosecurity do not appear to be necessary to limit *Salmonella* infection in less intensive systems.

9.5.2 Prevention Tools and Related Mechanisms

9.5.2.1 Implications for Refrigeration or Pasteurization of Eggs

The nature of *S. enteritidis* deposition in eggs also has significant consequences for the application of refrigeration or pasteurization as measures to protect consumers from egg-transmitted illness. Refrigeration of eggs at 7°C during storage and transportation has been recommended repeatedly for preventing the multiplication of small initial numbers of *S. enteritidis* cells to more dangerous levels (U.S. Department of Agriculture, 1998; U.S. Food and Drug Administration, 2004). However, refrigeration of eggs using conventional technologies may require several days before temperatures within the eggs are reduced sufficiently to prevent further microbial growth (Curtis et al., 1995; Thompson et al., 2000). If SE is deposited in the albumen, where bacterial multiplication is very slow, even at warm temperatures, the extended interval necessary to achieve growth restricting temperatures inside eggs will have little adverse effect. However, if the initial site of *S. enteritidis* contamination is in or on the nutrient-rich yolk, rapid multiplication could produce high levels of the pathogen during the early stages of refrigerated storage, while internal egg temperatures are still in the process of being reduced. Determining how often *S. enteritidis* is in fact deposited in association with the yolk, and whether (and how quickly) it can penetrate through the vitelline membrane into the yolk, is, accordingly, very important in defining the necessary parameters for thoroughly protective application of egg refrigeration. Many of these same considerations affect the ultimate efficacy of egg pasteurization, as the number of bacteria that will survive destruction by heat (either inside intact shell eggs or in LE products) under any specific combination of time and temperature will depend on the number of cells that were present initially (Hou et al., 1996; Brackett et al., 2001). Therefore, the effect of the location of deposition on subsequent growth to high numbers before pasteurization becomes a pivotal consideration in this context as well.

9.5.2.2 Decontamination of Contaminated Premises

9.5.2.2.1 Feed Mill

Persistent contamination of oilseed plants, feed mills, hatcheries, and poultry farms is an important aspect of the epidemiology of *Salmonella*. The main focus of long-term contamination of feed mills is normally the coolers (Davies and Hinton, 2000), from whence recycling can occur via contaminated dust, reworked material, and residual contamination in bins and auger systems. Cooler contamination appears to be particularly problematic in integrated poultry companies, because there is insufficient time to close the mill and complete effective decontamination by cleaning, fumigation, and multiple passage of formaldehyde or acid-treated material, such as wheat feed, while maintaining a feed supply for company flocks. Where cooler contamination does exist, *Salmonellas* present are usually the predominant noninvasive serovars found in live poultry and at slaughter houses. Waves of broiler-flock infection can also occur when there is more transient contamination of mills.

9.5.2.2.2 Hatchery

A similar situation exists in hatcheries, where resident *Salmonella* serovars may become established, particularly in hatcher incubators, but, also, occasionally, in setters and tray-wash machines. Again, time constraints are the major problem, as setters may be occupied continuously and hatchers, the main site of contamination, is usually only empty for a few hours before restocking. This

allows only limited time for effective disinfection and, if *Salmonella* is present, to eliminate it. In practice, it is necessary to carry out a very thorough clean, using noxious chemical at high concentrations (Mitchell and Waltman, 2003) to overcome the problem. Disinfectants, such as formaldehyde-glutaraldehyde-quaternary ammonium compound (QAC) combinations or more refined noncoal-tar phenols, are best for disinfection of surfaces and ducting. When *Salmonella* is present, these should be used at Department for Environment, Food and Rural Affairs (DEFRA) General Orders concentrations rather than general application rates, and should be applied to surfaces that have dried after cleaning and power washing with a QAC disinfectant solution. Trays of undiluted formalin should be used throughout the hatch, so that an inhibitory atmosphere is maintained for the whole of the time. This can be handled safely if fans are used to remove the vapor at takeoff. Following successful completion of this program, confirmed by testing, it will be possible to revert to less intensive disinfection, but care must be taken to avoid recontamination from other parts of the building, such as waste-handling areas, and from infected breeding flocks. If infection in a breeding flock does occur, then attempts should be made to channel eggs through a limited range of incubators and handle eggs and chicks separately from those of other flocks. It is also necessary to ensure that there is minimal opportunity for cross contamination via air, equipment, or staff movements (Cox et al., 1990; Davies and Wray, 1994; Davies et al., 2001).

9.5.2.2.3 Poultry Farms

Cleaning and disinfection of premises for primary breeding and grandparent flocks is normally extremely good and well monitored to ensure that *Salmonella* does not gain access on the rare occasions when biosecurity breaks down. Persistent infection of parent chicken flocks used to be a significant problem in the United Kingdom, but improvements in cleaning program and rodent control, plus the use of vaccination for *S. enteritidis*, have changed the situation completely, so persistence of infection is now extremely rare (Davies et al., 2003). The situation in parent turkey and duck flocks is less clear, since there is no statutory monitoring, but results of voluntary monitoring suggest that persistence of *Salmonella* is not a major problem in these sectors.

Persistent infection of commercial broiler, turkey, and duck farms is still a problem in some companies (Crilly et al., 2001). But, overall, the standard of cleaning and disinfection has improved, so this is no longer so important. There are, however, problem farms in many companies and these are often large, modern farms with metal houses that should be relatively easy to clean. Persistent contamination often involves serovars that have originated from persistently contaminated feed mills, so these strains may have become environmentally adapted. There is no evidence of disinfectant resistance among such serovars, but, where they are present, it is necessary to allow more time between flocks for a thorough clean out of all houses, before disinfection is begun. Attention to detail is vital to ensure that there are no residual foci of contamination (Engvall, 1993; Meroz and Samberg, 1995) and this can be done systematically to predict likely disinfection failures (Cruickshank, 2003; Rose et al., 2003). Physical auditing, rather than checking procedures on paper, is essential to ensure that control measures and hazard analysis and critical control point (HACCP) principles are applied properly (Noordhuizen and Frankena, 1999). As with the hatchery, it is necessary to upgrade disinfection until the persistent infection has been overcome, and similar disinfectants should be used, with the longest possible drying period between washing and disinfection and between disinfection and fogging with neat formalin solution. In large, modern houses, the high number of individual pan feeders that accumulate wash-water is a particular problem, made worse by operator fatigue. Before restocking, feed and water lines should be completely emptied. Water lines should be flushed with an acidifier and, if feed lines are the problem, then acid or formaldehyde/acid-treated rations should be used at the end of the old flock and start of the new, to reduce *Salmonella* in the system.

Persistent contamination is more of a problem on commercial laying farms, particularly caged layer units, where *S. enteritidis* may occur, despite vaccination (Davies and Breslin, 2003b). The cause of this problem is multifactorial, but, in general, it is difficult to clean and disinfect cages and droppings pits effectively and mice, rats, and flies are a hazard (Davies and Breslin, 2003a).

However, resistance of *Salmonella* to disinfectants is not involved (Davison et al., 2003). The main weaknesses are the linkage of houses by egg and droppings belt and the close proximity of houses, which may allow dust to transfer *Salmonella* between flocks. In Denmark, where vaccination is not allowed, a strict monitoring and control policy, including heat treatment of eggs from infected flocks, has forced producers to improve procedures. Disinfection of persistently contaminated houses with 30 mg/L formaldehyde in steam at 60°C, 100% relative humidity (RH) has been introduced to overcome previous failures of disinfection (Gradel et al., 2003, 2004). For many other countries, monitoring is insufficient to detect a large proportion of infected flocks, and this makes effective control very difficult (Muller and Korber, 1992). It has been shown experimentally that serial passage of *S. enteritidis* through birds increases the rate of internal infection of eggs (Gast et al., 2003), and there is a concern that persistent infection of farms may lead to the selection of more invasive strains and organisms more likely to evade the immune response provided by vaccination. Ideally, total depopulation of persistently infected farms and an all-in/all-out production system should be used. Although this would be possible theoretically within a large company, since different sites could supply different grades of eggs, the downtime involved in the transition period would present severe economic difficulties (Bender and Ebel, 1992). Control of mice is also difficult once large breeding populations have become established, but, without the intensive baiting, trapping, and tidiness necessary to achieve control, cleaning and disinfection of the premises is wasted. In the United States, Denmark, and the United Kingdom, improved control of egg contamination has led to a significant reduction in human cases of *S. enteritidis* (Patrick et al., 2004).

9.5.3 Vaccination

The cost of attempting *Salmonella*-free production has prompted the development of biological options, but rarely is these fully effective on their own (Barrow et al., 2003). Use of vaccination for *Salmonella* control varies in the different production systems and among EU Member States. In Germany, it is compulsory to vaccinate laying flocks against *S. enteritidis*, but this may be done with a *S. typhimurium* vaccine that has limited efficacy against *S. enteritidis*. In the Nordic countries, no vaccination is permitted at any level of production. Vaccination against *S. enteritidis* is not used at the level of elite and grandparent flocks in the United Kingdom, but almost universally used routinely in broiler parent flocks. Here, vaccination involves a killed *S. enteritidis*/*S. typhimurium* vaccine, a live *S. enteritidis* oral vaccine or an oral and injectable *Salmonella gallinarum* 9R vaccine. In layer, turkey, and duck parent flocks, targeted vaccination may be used to the following identification of a specific infection problem, providing extra protection for birds placed in previously contaminated houses.

No vaccination is used for commercial meat birds, but it is thought that the stimulation of maternal immunity by vaccination of broiler breeders, using an injectable vaccine, may have contributed to the current rarity of *S. enteritidis* in the UK broilers. Certainly, maternal immunity can interfere with the colonization of birds by a live *S. enteritidis* vaccine strain, so the vaccine response will be less effective (Methner et al., 2002). Vaccination against *S. enteritidis* is a requirement for membership of the main quality assurance scheme used by the egg industry in the United Kingdom. A live and oral vaccine has been used most commonly since 2001. Vaccination alone is not successful in caged laying flocks and must be combined with improved cleaning, disinfection, and pest control (Davies and Breslin, 2003b, 2004). There are constant debates about the relative efficacy of killed and live vaccines and, generally, live vaccines are considered to be superior (Zhang-Barber et al., 1999), but fears about the safety and environmental persistence of the vaccine strains (Selbitz, 2001) may lead to the development of commercial vaccines that are considerably attenuated and therefore less robust than necessary for a high level of colonization and stimulation of immunity. Also, there have been difficulties, in some cases, with consistent delivery of vaccine through long water lines and residual antibacterial substances in the water, eggs, or chicks, following a previous flock treatment that may affect the viability of the vaccine. More work is needed to develop and properly

define the immune response to rationally derived live vaccines (Mastroeni et al., 2000) and application methods. For example, when tested under experimental conditions, competitive exclusion treatment and vaccination can give an additive response (Methner et al., 2001) but, in practice, the competitive exclusion product may be given too long after exposure to *Salmonella* to be of value, especially in laying flocks.

In the future, improved vaccine delivery systems that are specific to poultry flocks are required (Tacket and Mason, 1999; Liu et al., 2001; Ryan et al., 2001; Oshop et al., 2002; Mastroeni and Ménager, 2003; Singh and O'Hagan, 2003), as well as the ability to deliver protection against a wider range of *Salmonella* serovars.

9.5.4 Competitive Exclusion

In some countries, the application of competitive exclusion products, which are undefined or partially defined cultures derived from the intestinal microflora of poultry, has been widely used as a part of general *Salmonella* control programs. Elsewhere, they may be used only within certain companies or for problem farms (Mead, 2000; Ricke, 2003). Currently, there are problems with the use of undefined competitive exclusion cultures in some Member States, because of variations in the authorization procedures. The European Commission plans to consider this topic further, since such products can be valuable aids in the control of *Salmonella* and other pathogens.

Many different treatment products are available commercially and these appear to have different levels of efficacy (Nakamura et al., 2002; Ferreira et al., 2003). The efficacy is also related to the level of challenge, but, even when this is high, there is still, usually, some reduction in the prevalence of infection in individual birds and in the numbers of *Salmonella* organisms being excreted. The effect can be used to sequentially reduce the level of excretion and environmental challenge in consecutive flocks, to the point where total elimination is more likely (Mead, 2000). Wider studies are needed to fully define this phenomenon and further developments are in progress (Andreatti et al., 2003). To be maximally effective, competitive exclusion treatment should be administered shortly before exposure to *Salmonella*, so administration by spraying chicks at the hatchery is generally superior to dosing on the farm via drinking water (Cox et al., 1992; Mead, 2000; Patterson and Burkholder, 2003).

9.5.5 Feed and Water Treatments

A wide range of feed and water additives for the control of *Salmonella* are described (Ricke, 2003), but most require large-scale field evaluation (van Immerseel et al., 2002). In-feed preparations of organic acids can reduce the chances of flock infection, both from contaminated feed and environmental challenge (Humphrey and Lanning, 1988; De Oliveira et al., 2000), but the efficacy of different products varies (Hume et al., 1993) and those containing the highest levels of free formic acid in a liquid application appear to perform best.

A combination of formaldehyde and propionic acid appears to be helpful in reducing the shedding of *Salmonella* and environmental contamination in infected laying flocks, where the level of environmental challenge is not overwhelming (Davies, R.H., unpublished data; Anderson et al., 2001, 2002).

Water can be a means of introducing *Salmonella*, so only mains water or water from other sources that has been appropriately treated and tested should be used. Treatment of water supplies with acidic oxidizing agents, such as hydrogen peroxide/peracetic acid or lactic acid (Byrd et al., 2001) or sodium chlorate, and sodium nitrate (Byrd et al., 2003; Jung et al., 2003) appears to have a beneficial effect on broiler contamination at slaughter, and it could be investigated in a wider range of situations. It is possible to use continuous water and air disinfection during the life of a flock by means of organic acid or chlorine-based products (Tablante et al., 2002; Bragg and Plumstead, 2003). Lime can also be used to achieve a high pH and reduce pathogens in litter and around entrances to houses (Bennett et al., 2003; van Immerseel et al., 2004).

9.5.6 Antimicrobials

Antimicrobials are primarily used in poultry for prevention or treatment of disease (ACMSF, 1999), but they may also be used to restrict the spread of *Salmonella*, reduce the chance of flock infection where there is an environmental challenge, or, in some countries, reduce the prevalence of contamination prior to slaughter. Treatment with fluoroquinolones, followed by competitive exclusion treatment, has been used in the control of *S. enteritidis* in broiler-breeder flocks (Goren, 1993; Reynolds et al., 1997) and layer rearing flocks, but it is seldom completely effective (Humbert et al., 1997; Davies et al., 2003). Treatment of eggs and chicks with antimicrobials, such as fluoro-quinolones, cephalosporins, or gentamicin, has also been used to limit vertical transmission (Hafez et al., 1995), but it may interfere with other control measures, such as use of live vaccines or competitive exclusion cultures (McReynolds et al., 2000). Prophylactic or therapeutic use of antimicrobials, such as ampicillin, tetra-cyclines, or spectinomycin, may also suppress *Salmonella*, but, in the case of strains with multiple resistances, such as *S. typhimurium* DT104, use of these compounds may increase the multiplication and spread of the organisms. In recent years, there has been concern about treatment failures involving *Salmonella* and *E. coli* with reduced susceptibility to ciprofloxacin. It is unclear exactly what proportion of the overall problem has been generated by veterinary usage, but there is a temporal association between increasing usage in poultry and reduction of susceptibility in human isolates (Bager and Helmuth, 2001; Malorny et al., 2003). Reduced susceptibility to fluoroquinolones appears to have been a particular feature of *Salmonella* isolates from turkeys (Davies et al., 1999), but following the introduction of prudent-use initiatives, the prevalence of such strains in UK poultry production has fallen (VLA, 2003). Despite the reduction in susceptibility to fluoroquinolones, high-level resistance (i.e., MICs >1.0 mg/L) is rarely seen. This may be due to reduced survival and dissemination capabilities ("fitness") in the strains (Giraud et al., 2003). There is also concern about increasing multiple resistance in *Salmonella* (Jones et al., 2002), and strains such as *S. Paratyphi* B var. Java have spread widely in some countries (van Duijkeren et al., 2003). There is further concern about the possible spread of plasmid-mediated resistance to third-generation cephalosporins, like ceftriaxone (Liebana et al., 2004), among *Salmonella* and *E. coli* via international trade. The reduction in use of antimicrobial growth promoters, that had either no effect or some suppressive effect on *Salmonella* (Cox et al., 2003), and consequent increase in the use of therapeutic antimicrobials, may increase the potential for selection and spread of resistant organisms.

9.5.7 Control of *Campylobacter*

9.5.7.1 General Considerations

Options for control of *Campylobacter* are less well defined than those for *Salmonella*. Feed and vertical/hatchery transmission do not have a major role, if any, in dissemination of infection. Persistence of the organism within depopulated poultry houses is occasionally possible (Petersen and Wedderkopp, 2001), especially when short turnaround times allows pooling of wash-water or when water systems are subject to be contaminated, but it is unlikely that cleaning and disinfection programs that are sufficient to control *Salmonella* would fail to eliminate *Campylobacter*. Prolonged survival in dry materials in inaccessible areas of the house, which is a feature of *Salmonella* contamination, does not occur regularly (Berndtson et al., 1996b), but it may be underestimated in the short term, because of the limitations of present culture methods (Cox et al., 2001). *Campylobacter* is widespread in domestic animals, wildlife, and aquatic environments (Newell, 1999) and, in many ways, acts as a commensal or opportunist organism (Humphrey, 1989). In infected flocks, the organism achieves high levels in intestinal contents, so personnel and equipment that have been in contact with the birds will be highly contaminated in the short term. Viable *Campylobacter* can also be found in dust emanating from houses that hold infected flocks, although the infectivity of the organism in this dry material is unknown. Because of the large potential challenges, it is common for all

houses on a multihouse site to become infected. However, spread can be limited by good biosecurity, suggesting that airborne infection is not a major route.

Having eliminated a number of potential sources from consideration, as discussed earlier, the environment immediately surrounding the house is left as the predominant source of infection. This apparently simple situation is made complex through the variety of potential routes by which contaminated material can be brought into the house (White et al., 1997; Sahin et al., 2002). Despite the apparently straightforward concept of preventing organisms entering the house, hygiene barriers that have been effective for *Salmonella* have had only limited success with *Campylobacter* (Kapperud et al., 1993; Humphrey et al., 1993; Berndtson et al., 1996a; Reiersen et al., 2001). This is probably because of the relatively high numbers of organisms involved and the low infectious dose. Intervention studies have shown a reduction in *Campylobacter* or a delay in establishing infection. Gibbens et al. (2001) showed a 50% reduction in the risk of flock infection and identified lack of water oxidizing on as the most significant risk factor. In Norway, only 6.3% of 3267 flocks were found to be infected, and this favorable situation is thought to be related to a stricter attitude to the maintenance of hygiene barriers (Hofshagen and Kruse, 2003). In Norway, however, farms are smaller, often with only one house on the site; they are more likely to be run by the owner and the climate is colder. A substantial reduction in flock prevalence has been achieved in Iceland, and the beneficial effect on human cases has been further enhanced by freezing broiler carcas (Stern et al., 2003). Observations by the author on UK broiler farms suggest that, despite apparently good company protocols, biosecurity precautions on entry to the house are often not properly observed and the hygiene barrier area becomes contaminated with fecal material, so cannot operate as intended. This is especially problematic on farms where disinfectant foot-dips are no longer used. In addition, proper hand oxidizing is not carried out regularly and poorly dried hands that are not disinfected may present an increasing risk. Furthermore, moveable equipment may not always be properly disinfected between houses or even between sites. In summer, when there may be more organisms around the house as a result of wild-bird and insect movements, and contaminated water supplies, standards tend to fall further, as farm staff do not always use waterproof boots or wear other protective clothing. A study has shown that a high risk associated with children entering poultry houses was reduced when they used house-dedicated clothing (Bouwknegt et al., 2004). It is apparent that attention to detail is needed at all times (Pattison, 2001) and, in broiler-parent rearing flocks, this can keep birds *Campylobacter*-free until they are moved to laying farms, when control becomes impossible (K. Gooderham, personal communication). Rigorous attention to detail is laborious and therefore costly. Thus, it is necessary to provide sufficient incentive to stimulate compliance, and a premium of one penny per bird for flocks that were negative at slaughter would encourage better hygiene and an improved approach to flock thinning. It is also desirable to develop boots and other protective clothing that are light, cool, and pleasant to wear, and can be changed easily and rapidly by farm staff and thinning teams. Thinning would be less of a risk, if washed modules and crates were immersed briefly in a 2%–3% solution of a compound oxidizing disinfectant, but there is still a chance that contaminants from vehicles, catching gangs, and the exterior of the house will be brought in during thinning. It would be desirable, therefore, to devise a barrier system that would allow the remaining birds to be held back from the area where thinned birds had been removed until the following day, when any *Campylobacter* cells introduced would have died off. Even if *Campylobacter* is introduced at thinning, the numbers carried into the abattoir will typically be lower than for flocks infected at an earlier stage and this will reduce the number of human infectious doses per flock (Hartnett et al., 2001).

Since there is a clear relationship between *Campylobacter* and water, it makes sense to ensure that the water supply is as safe as possible. Many farms use bore-hole water, which may be subject to contamination after heavy rain. Even if chlorinated mains water is used, the level of chlorine at the end of a long water line may have dropped significantly. Therefore, it makes sense to use water sanitizers, at least intermittently and strategically, during periods of stress, such as chick placement and dietary changes, at the end of the "lag phase" and during thinning. Water acidifiers often improve

the performance of flocks (Tablante et al., 2002), although there may be differences between products and possible damaging effects on water lines. Silver-stabilized acid products appear to be superior in this respect and other substances, such as lime, aluminum sulphate, or sodium bisulphite, that may be useful to reduce numbers of organisms in litter, possibly after thinning of a partitioned area of the house (Line, 2002).

Since *Campylobacter* does not colonize abattoir equipment or multiply in the environment or on carcasses, testing and logistic slaughter of flocks would have only a minor effect on the total burden of contamination entering the food chain (Rosenquist et al., 2003). However, it would be desirable for flocks that are inevitably contaminated, like organic flocks (Heuer et al., 2001), to be slaughtered only at the end of the day. If a two-log reduction in the number of organisms per carcass could be achieved, this would have a substantial effect on the level of human disease (Stern and Robach, 2003). Methods of achieving a reduction in numbers in infected flocks prior to slaughter should be evaluated. These could include the use of water or feed acidifiers, other harmless inhibitors, bacteriocins, and bacteriophage, possibly combined with competitive exclusion products (Stern, 1994).

9.5.7.2 Antimicrobial Resistance in *Campylobacter*

Although antimicrobials are never used to control *Campylobacter* in poultry flocks, the high prevalence of infection means that these organisms will often be exposed to whatever treatment is given for clinical disease or prophylaxis. *Campylobacter* responds very quickly to the use of antimicrobials by developing resistance (Avrain et al., 2003), especially to fluoroquinolones (Ge et al., 2003; van Boven et al., 2003). Resistance is more frequent in situations where there is high use of fluoroquinolones, such as in developing countries or in Turkey (Ge et al., 2003; Padungton and Kaneene, 2003), but, unlike *Salmonella*, there appears to be little spread of specific, resistant clones (McDermott et al., 2002).

9.6 CONCLUSION AND FUTURE TRENDS

With the growing importance of food safety, there is an increasing interest of food safety management in food production, trade, and services companies. For these purposes, safe egg products may become a major trend in the future, higher animal welfare strategy may be put into effect, and more and more research on the spoilage microorganism shall be taken out.

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