Trends in Food Safety and Protection

Edited by

V Ravishankar Rai • Jamuna A Bai

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Preface

Trends in Food Safety and Protection explores the recent developments and ongoing research in the field of food safety and protection. The book covers improvements in the existing techniques and implementation of novel analytical methods for detecting and characterizing foodborne pathogens, the mechanisms of biofilms of food-related bacteria, the techniques used to authenticate foods, and supply chain management in the context of foods. It has topics on the application of next-generation sequencing in food safety, and the design and development of biosensor platforms for food diagnostics. Topics pertaining to the effects of climate change on food safety and security have also been discussed. The book features highly evolving topics in food safety and protection, such as the development and commercialization of nanobiosensors for food application, and the use of nanotechnology for improvements in food production to food consumption. Emerging topics of concern in the food industry such as reconditioning and utilizing waste generated by food processing, wild game meat food safety and security, and regulations and remedies in retail food have also been covered in the book.

The book comprises of chapters on the topics of Next-Generation Sequencing and Its Application in Food Microbiology and One Health; Molecular Methods to Characterize Foodborne Microbial Pathogens; Molecular Recognition: Versatility and Challenges in the Design of Nanobiosensors for Food Security; Nanotechnology in Food: From Farm to Fork; Biofilms: A Community-Based Strategy for Bacterial Persistence and Relevance to Food Safety; Spectroscopic Methods for Fresh Food Authentication: An Overview; Reconditioning of Vegetable Wash Water by Physical Methods; Food Safety and Food Security Implications of Game; Managing Food Supply Chains for Safety and Quality; Retail Food Safety: Concerns, Regulations, Remedies; Sustainability of Food Security in the New Era; and Nanobiosensors and Their Application in Food Safety.

The advances made in the field of food safety and protection from farm to table and the various existing opportunities and challenges have been explored in this book. The contributions on these topics are from international experts in the field. Though food safety comprises a vast field, all the important aspects pertaining to the topic have been covered, with focus on recent advances and a perspective on future trends in the field.

Graduate students and food industry researchers will find the book a valuable tool as it contains the latest developments and advances in the field of food safety and protection. It contains a vast compilation of comprehensive

work and provides innovative technology and solutions for safer foods from farm to table.

I would like to thank all of the eminent scientists for contributing the chapters and sharing their knowledge.

V. Ravishankar Rai
Jamuna A. Bai

Editors

V. Ravishankar Rai earned his MSc and PhD from the University of Mysore, India. Currently, Dr. Rai is working as a professor in the Department of Studies in Microbiology, University of Mysore, India. He was awarded fellowships from the UNESCO Biotechnology Action Council, Paris (1996), the Indo-Israel Cultural Exchange Fellowship (1998), the Biotechnology Overseas Fellowship, Government of India (2008), the Indo-Hungarian Exchange Fellowship (2011), and the Indian National Academy Fellowship (2015). Presently, he is the coordinator for the Department of Science and Technology, Promotion of University Research and Scientific Excellence, and University Grants Commission innovative programs.

Jamuna A. Bai completed her MSc and PhD in microbiology at the University of Mysore, India. She is working as a researcher on a University Grants Commission-Sponsored University with Potential Excellence Project, University of Mysore, India. She has previously worked as an Indian Council of Medical Research senior research fellow on food safety, the role of quorum sensing and biofilms in food-related bacteria, and the development of quorum-sensing inhibitors. Her research interests also include the antimicrobial application of functionalized nanomaterials against foodborne pathogens.

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Next-Generation Sequencing and Its Application in Food Microbiology and One Health

Jorgen Schlundt

CONTENTS

1.1 Introduction

Human and animal populations share many infections, either directly or through food. A number of factors, including poverty, increased food trade, and changes in food production and food preservation methods have over recent decades resulted in an increased foodborne disease burden and significant potential of global spread of pathogens through food. However, a thorough and scientific estimation of this disease burden has until lately been missing, at national as well as international level. On the other hand, it could be argued that even in the case where a specific disease burden cannot be attributed to food, preventive or proactive action could still lead to improved safety of the food we eat. However, one of the great disappointments in the food safety and food control area in most countries has been the lack of efficient collaboration and data sharing between relevant sectors (primarily agriculture and health).

Although the (sometimes deliberate) non-collaboration between sectors has been going on everywhere for many decades, it was primarily the global outbreaks of avian influenza in 2003–2004 that alerted the world, including political leadership, to the need for a cross-sectoral approach, linking animal and human health. From 2004, this was referred to as the "One World, One Health" concept, and later as the "One Health" concept, based on the recognition that human and animal health are inextricably linked (World Bank, 2008).

1.2 Size of the Foodborne Disease Problem

Although the estimated disease burden related to food is very significant in all countries, the major focus on food safety has over recent decades mostly been related to well-publicized outbreaks of foodborne disease or foodborne contamination events. Most consumers all over the world have heard of bovine spongiform encephalopathy (BSE) spreading out from the United Kingdom in 1996, about the spread of highly pathogenic avian influenza (HPAI) from Asia in 2003 and 2004, and about the major outbreak of *Verotoxin*-producing *Escherichia coli* (*VTEC*) in a number of European countries in 2011. Food safety authorities, however, in many countries often like to point out, and are actually correct in pointing out, that these events—highly publicized as they might be—do not really reflect the food safety reality and have in effect caused a limited disease burden even at the global level. The same authorities, however, often use similar statements when more localized outbreaks caused by *Salmonella*, enterohemorrhagic *E. coli*, or *Listeria* occur because of contaminated eggs, contaminated produce, or contaminated ready-to-eat foods. But here the real, total disease burden is actually very significant. The best estimate we have of the comprehensive (sporadic and outbreak) foodborne disease burden comes from the United States where it is estimated that one-third of the U.S. population has a case of microbiological foodborne disease every year (Mead et al., 1999). People confronted with such figures often comment that such disease is just simple diarrhea and will only result in a few days of discomfort. Although this is true in many cases, the same study estimates that every year approximately 5000 U.S. citizens (later down-regulated to 3–4000) die from such disease, and a significantly higher figure is hospitalized, often with long-term effects lasting years. A Dutch study (Havelaar et al., 2012) estimated the burden of disease from 14 food-related pathogens in the Netherlands to be 13,500 Disability Adjusted Life Years (DALY) in 2009, lower than the burden of pneumonia (72,000) but similar to urinary tract infections (15,600). Extrapolating Dutch and American figures to the rest of the world gives us

an astounding disease figure related to the preventable contamination of something we all need on a daily basis: our food.

What about the situation in other parts of the world? Based on national reporting, the World Health Organization (WHO) estimates that around 1.8 million children under 5 years of age die every year from diarrhea caused by contaminated food or water. As usual, the poorest part of the population is at the highest risk: in general, malnutrition can result in a 30-fold increase in the risk for diarrhea-associated death. When considering these estimates it is important to realize that they do not include the very significant burden of microbiological diseases not confined to diarrheal expression, including dangerous blood, renal, and brain infections; Reiters syndrome (reactive arthritis); Guillain-Barré syndrome (autoimmune nerve disease); abortions; and so on.

Foodborne diseases not only significantly affect people's health and well-being but also have economic consequences for individuals, families, communities, businesses, and countries. These diseases impose a substantial burden on healthcare systems and markedly reduce economic productivity. The loss of income due to foodborne disease perpetuates the cycle of poverty. In a study from the United States, Scharff (2012) estimated the aggregated annual cost of foodborne (microbiological) illness to be $78 billion ($29–$145 billion). Estimates of the economic consequences of food contamination events or foodborne disease outbreaks through altered conditions affecting national food export are not forthcoming. However, in several major single incidents, the total health and socioeconomic costs have been measured in hundreds of millions of U.S. dollars. The major *E. coli* outbreak in Europe in 2011 is claimed to have cost almost $3 billion in human damage (health costs, sick leave, and so on), while European farmers estimated their losses to more than $400 million per week (outbreak lasted at least 4 weeks).

Efficient prevention of food safety problems requires full integration of the food production chain: "from farm to fork" or "from boat to throat," recognizing that the critical point for efficient prevention might be at the farm for some problems or at the retail level for others. Most present-day food safety systems are not built according to this important principle. Incoherence of the systems has led to inconsistency and in some cases inefficiency of food safety systems. Although prevention of foodborne disease, of course, has to be based on good general hygienic practices, it is important to focus our efforts toward the real risks in the population. Any system to efficiently prevent foodborne disease problems should be based upon a solid evidence base. Therefore, the data-gathering efforts related to food contamination and foodborne disease need to be coordinated. Likewise, monitoring, surveillance, and control efforts should be geared toward common goals. This coordination is missing in many food safety systems currently, thus leading to a very weak evidence base and, potentially, to uninformed decisions and poor political support.

1.3 The One Health Concept: Linking the Problems between Sectors

The One Health concept is a worldwide strategy for expanding interdisciplinary collaborations and communications in all aspects of healthcare for humans, animals, and the environment. Recognizing that human health, animal health, and ecosystem health are inextricably linked, One Health seeks to promote, improve, and defend the health and well-being of all species by enhancing cooperation and collaboration between physicians, veterinarians, and other scientific health and environmental professionals and by promoting strengths in leadership and management to achieve these goals. One Health seeks to improve communication and encourage collaboration between these professionals to find multidisciplinary solutions to shared challenges, and microbial food safety must certainly be counted amongst the most important of such challenges currently before us.

The One Health concept promotes a holistic approach as the basis for understanding, protecting, and promoting the health of all species. Whether it is emerging infectious diseases, antibiotic resistance, globalization, or natural disasters, human and veterinary medical communities must work together. Food safety is an increasingly important public health issue and a central part of One Health, which is highlighted by the fact that one of the major issues in food safety over the most recent decades has been the lack of cross-sectoral collaboration across the food production chain. The development of some of the major food safety events recently have been impacted by the lack of collaboration between the animal health, food control, and human health sectors (Wielinga and Schlundt, 2013).

One Health formulates clearly both the need for and the benefit of cross-sectoral collaboration in food safety and control. Some foodborne diseases have global epidemic—or pandemic—potential, resulting in dramatic action from international organizations and national agricultural and health authorities in most countries, for instance, as was the case with avian influenza. Other diseases relate to the industrialized food production chain and have been—in some settings—dealt with efficiently through farm-to-fork preventive action in the animal sector, for example, *Salmonella* and antimicrobial resistance in foodborne pathogens (Wielinga et al., 2014). While, in theory, most national regulators in the food and health sectors agree that links between sectors are needed, there is not always agreement between sectors on how such links should be established and, in effect, how a One Health system should be introduced. When national agencies are accustomed to regulating and controlling specific areas, one of the most difficult changes seems to be a change resulting in the responsibility for such areas shifting from one to another ministry. National examples where One Health–like changes took place include Denmark, 1996: the prime minister announced a

new Ministry for Food; the United States, 1997: President Clinton announced a "Food Safety from Farm to Table" plan; the European Union, 2002: the E.U. Parliament and Council adopted a General Food Law following "Farm to Table" principles and created the European Food Safety Authority (EFSA). In all these cases, the highest governmental authority had to oversee change; change did not just come from intersectoral collaboration. It would seem that introduction of regulatory change to introduce One Health principles and frameworks needs a thorough and comprehensive policy reform in order to succeed.

Thus, an important microbiological problem which is clearly One Health in nature is the problem of antimicrobial resistance (AMR). The rapid spread of AMR in microbial populations now challenges sustainable food production and public health in general. A recent U.S. presidential report (Anon., 2015) states that antibiotic resistance is developing at an alarming rate and that efforts must also aim at decreasing current overuse in animals. While recent E.U. regulations have banned the use of antimicrobials for all animal growth promotion, such additional use of antimicrobials continues in the rest of the world. It is estimated that maybe as high as 70% to 85% of all antimicrobials are used in the animal sector. The published estimates of the proportion of antibiotics consumed in animal agriculture—84% (for 36 antibiotics) in China and 70% in the United States—would suggest that global agri- and aquacultural use clearly exceeds human use (noting that usage data from most other parts of the world is missing) (Robinson et al., 2016). A large part of this use is justified and valid on veterinary grounds, but there is significant misuse in the agricultural sector, including both growth-promoter use and excess use for treatment and prevention. With such large consumption levels, it seems likely that agricultural use contributes significantly to AMR. A recent review suggested that misuse of antimicrobials in animal production is a clear and substantial driver of AMR (Holmes et al., 2016).

In order to characterize the nature and extent of the AMR problem we need to document the passage of AMR genetic elements from agri- and aquaculture to humans through food (Figure 1.1), applying the One Health paradigm. Therefore, we need to look at the dynamic interactions that occur at the interfaces between human, animal, and ecosystem sectors (Lammie and Hughes, 2016). Such documentation is now possible through use of the novel technology of next generation DNA sequencing (NGS) to characterize the transmission of AMR determinants from animals to humans and the nature and extent of transfer in real life.

The novel use of NGS to quickly and accurately characterize microorganisms genetically (including AMR genetic elements) represents a new uniform and efficient accounting methodology for the investigation of food contamination issues throughout food production chains and all the way into the human sector. Cross-sectoral use of NGS technology can provide the scientific background for a potentially revolutionizing surveillance

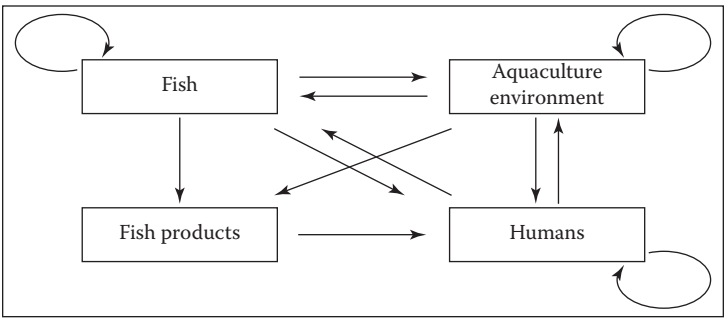

FIGURE 1.1
A One Health description of routes of transmission of AMR between fish, aquaculture environment, fish products and humans due to antimicrobial usage in aquaculture and human medicine. There is a similar interaction in other animal production systems.

system in support of food production and food control. This system will align identification and typing methodology across health and agriculture sectors, something which was never possible with traditional microbiological methodology. This is because the traditional microbiological methodology requires (1) prior knowledge of the identity of tested bacterial species before further characterization, (2) availability of suitable typing systems and reagents for identification and characterization, and (3) multiple different analyses to be done for confirmation of bacterial identity and the genes that are responsible for AMR.

1.4 Surveillance of Foodborne Diseases and the Agents Causing Them

Microbial detection and surveillance form the backbone of all systems currently used to control infectious diseases, including foodborne diseases, worldwide. However, surveillance is still typically targeted at a relatively limited number of specified microorganisms and diseases, and maybe more importantly there is a very significant global disparity in national disease detection systems and methodology. In particular, public health efforts in the food area are hampered by several obstacles:

1. The use of different, specialized, expensive, and difficult-to-compare detection techniques
2. The use of separate (between sectors) characterization systems for relevant pathogens

3. The lack of efficient, reliable typing systems for most pathogens, hampering efficient disease attribution to food sources
4. Poor collaboration between microbiological fields (*Listeria*, *Salmonella*, viruses)
5. (Inter)national politics on the disclosure of surveillance and research data
6. Intellectual property rights
7. Lack of sufficient diagnostic capacities, particularly in developing countries.

A more effective and rational approach is needed and efforts to mitigate the effects of foodborne and other microbial threats, focusing on improved surveillance and diagnostic capabilities, are crucial in relation to bacterial as well as viral infections (Osterhaus and Smits, 2013).

While some national foodborne disease surveillance systems mainly collect information on the number of outbreaks and the number of cases involved in the outbreaks, for most foodborne diseases the majority of cases are sporadic cases. Therefore surveillance systems must include a recording of sporadic cases to enable a realistic estimation of the foodborne disease burden. New, active surveillance systems are likely in the future to blur the difference between what have traditionally been referred to as outbreaks and sporadic cases. The possibility of comparing genetically pathogen isolates from human cases in a broad national system enables the linking of cases, previously considered single, sporadic in nature, as truly part of an outbreak spread over a larger geographical area (Gerner-Smidt et al., 2005). It is thus likely that our understanding of the relative importance of outbreaks and sporadic cases will change in the near future.

As a result of the increased global trade in food it is also likely that outbreaks covering larger areas and affecting several countries will be recognized more in the future. Surveillance of foodborne diseases provides information for action. The use of laboratory data in surveillance enables the identification of pathogens and the potential sources of infection. Integrated surveillance including human data as well as animal- and food-monitoring data will in the future provide the basis for preventive action along the entire food production chain.

With recent technological advances and declining costs in the next-generation sequencing field, these tools will play an increasingly important role in the surveillance and identification of new and previously unrecognized pathogens in both animals and humans. Inherently, an enormous increase in the amount of published whole genome sequences (WGS) is to be expected, providing a wealth of information to aggregate, share, mine, and use to address global public health and clinical challenges.

1.5 Next-Generation Sequencing and Whole Genome Sequencing: A New Potential for Integrated Surveillance and Prevention of Foodborne Diseases

Surveillance is a key component of preparedness for the spread and development of infectious diseases, and is needed at the global level to monitor trends in endemic diseases (e.g., influenza, dengue, salmonellosis), to monitor eradication efforts (polio, measles, brucellosis), or to signal unusual disease activities (including new diseases or outbreak of known diseases). Molecular diagnostic tools based on short pieces of unique genome sequence (e.g., PCR and microarray [biochip] technologies) have been used routinely for surveillance in different areas. However, genomic re-assortment events may easily be missed if surveillance is relying on molecular diagnostic tools that target only small microbial genome fragments. Therefore, whole genome sequencing (WGS) provides a much more interesting, new methodology. WGS is a laboratory process that determines the complete genome sequence of an organism under study. This can have important implications; for instance, during the recent outbreak of MERS (Middle East Respiratory Syndrome) coronavirus in the Middle East, analysis of small genome fragments did not provide sufficient phylogenetic signal for reliable typing and separation of virus variants whereas WGS would enable such separation (Smits et al., 2015). Classically, whole microbial genome sequences were determined by PCR and Sanger sequencing. Nowadays, next-generation sequencing (NGS) techniques are used increasingly in human genomics, and are also widely used to identify and genotype microorganisms in almost any microbial setting (Wielinga and Schlundt, 2014).

NGS advancements and the development of NGS software tools have decreased the cost of WGS much faster than predicted 10 years ago (Figure 1.2). It is likely that the price of WGS analyses will decrease to a point where it can seriously compete with traditional routine diagnostic identification techniques, whereas the speed will seriously out-compete traditional methodology. The potential of WGS in the investigation and surveillance of infectious and foodborne diseases has been demonstrated in many studies, including the tracking and tracing of the cholera outbreak in Haiti in 2010 (Hendriksen et al., 2011), the EHEC (enterohemorrhagic *E. coli*) outbreak starting in Germany in 2011 (Mellmann et al., 2011) and others (Allard et al., 2012). During the EHEC outbreak, scientists from around the globe performed NGS and shared their results for analysis. The collaboration between these researchers allowed for joint and rapid analysis of the genomic sequences, revealing important details about the involved new strain of *E. coli*, including why it demonstrated such high virulence. Similar collaborations have been created globally during emerging viral infections such as MERS coronavirus.

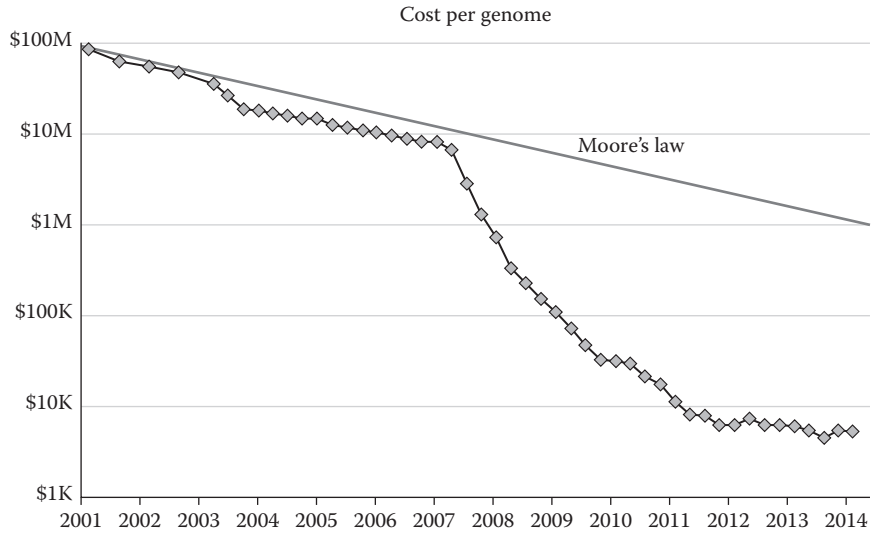

FIGURE 1.2
NGS cost per raw megabase of DNA sequence. (Taken from the National Human Genome Research Institute, http://www.genome.gov/sequencingcosts/)

It is not the intention of this chapter to describe in any detail the different existing sequencing platforms that are used for NGS studies and now also increasingly in routine laboratory. Illumina, Ion Torrent, Pac-Bio, Roche and Nanopore are but a few of the platforms available. For a more thorough description of these platforms and their respective strengths and weaknesses, go to Fournier et al. (2014). In general, a number of advantages can be attributed to these new platforms and to the use of NGS in general in food microbiology. Time and cost are typically mentioned as advantages, because the time to achieve a full bacterial genome is now estimated at 24–48 h, so even if another 24 h is added for the bioinformatics part, we would arrive at a full typing result in 48–72 h. Traditional typing can easily take more than a week with existing microbiological methodologies. Discriminatory power of NGS in separating isolates down to clonal level is also often mentioned as an advantage, and it seems clear that if the sequencing is performed flawlessly NGS performance in this area is clearly ahead of traditional typing methodology and will open new potential both in microbiological characterization and in epidemiological understanding. Unfortunately, sequencers do, however, not yet perform flawlessly and herein lies probably one of the most important present limitations in NGS. Errors in sequencing and in genome assembly will lead to—potentially serious—errors in identification outcome. However, one of the most important advantages of NGS is the fact that these DNA sequences can be collected and collated in big national, regional, or even global databases, so we at some stage will actually have libraries of the

full DNA of all existing microorganisms. This basically means that we will be able to create a global machine for the identification and characterization of all microorganisms (i.e., the basic idea of the Global Microbial Identifier described in this chapter).

Notably, the most dramatic potential change in microbiological identification and characterization methodology is likely to take place in developing countries. Current diagnostic methods, as applied in food control laboratories in most developing countries, are diverse and require specialized training. The introduction of NGS methodology in all relevant labs (food control, clinical, environmental) holds the potential of a simple one-size-fits-all tool for characterization of microorganisms—again relevant for framing food microbiology within a One Health paradigm. Such cross-sectoral introduction of comparable methodology will dramatically improve the capacity and efficiency of food and public health laboratories in developing countries. At a systemic level, the use of NGS will enable uniform laboratory, reporting, and surveillance systems for the identification of microorganisms in all habitats. At the same time, the development of new centralized and decentralized diagnostic systems will be significantly simplified with the potential of real-time characterization of microorganisms in individual laboratories with sequencers and internet link-up. Recent studies have shown that it is possible to determine the species, type, as well as the antimicrobial/antiviral susceptibility of both bacterial and viral pathogens, even when using sequencing directly on clinical samples (Hasman et al., 2014; Prachayangprecha et al., 2014). This would be even more valuable for clinical laboratories in developing countries that do not currently have the same diagnostic capacities as most developed countries.

It should be noted, though, that if partners or sectors involved in One Health programs (typically the health, agricultural, and food sectors) do not embrace—and apply—NGS identification and typing methodology in a coordinated framework there is a real risk that the introduction of NGS methodology in one sector alone will result in significant intersectoral problems. It is thus very important that One Health partners agree on a strategy for the introduction of such technologies in a coordinated fashion.

Although there are stand-alone examples of how NGS has been included in outbreak investigations with great success, a thorough overview of all developments in the area does not exist. While it is not within the scope of this chapter to present such overview, a few key achievements in the area should be considered. During the major outbreak of *E. coli* O104:H4 in Germany and other European countries in 2011, significant parts of the outbreak investigation were performed with the aid of WGS (Ferdous et al., 2015). After the introduction of NGS in routine *Listeria* outbreak investigations in the United States, the Food and Drug Administration (FDA) and Centers for Disease Control and Prevention (CDC) recorded a significant increase in the number of outbreaks detected as well as a significant reduction in the time needed to solve foodborne *Listeria* outbreaks. In general, this experience shows that WGS can

be adopted for prospective *L. monocytogenes* surveillance and investigated for other pathogens relevant to public health (Kwong et al., 2016; Burall et al., 2016).

As NGS technology spreads more globally, there is an obvious potential to develop a global microbial WGS database to aggregate, share, mine, and use microbiological genomic data, and in the not so distant future to use as a diagnostic tool, much like an international database of fingerprints. In the end, all microbial culprits will be in the database, enabling any laboratory to upload its (WGS) sequence and seek the correct answer, meaning species, type, and antimicrobial resistance. It is important to note that such databases will provide the basis for a potential farm-to-table platform for NGS investigations of all microorganisms, including pathogens as well as microorganisms used in food production, such as probiotic and other industrial strains. Such a system should be deployed in a manner that promotes equity in access and use of the current technology worldwide, enabling cost-effective improvements in plant, animal, environmental, food, and human microbiology. If the system is set up in an "open access" format it would likely enable comprehensive utility of NGS in developing countries, since the development of open databases and relevant algorithm platforms at the global level would enable immediate translation of sequence data to microbial identity and antimicrobial resistance pattern. A global system would benefit those tackling individual problems at the frontline (clinicians, veterinarian, etc.) as well as other stakeholders (i.e., policymakers, regulators, industry, etc.). By enabling access to this global resource, a professional response to foodborne contamination and foodborne disease outbreaks will be within reach of all countries with (even relatively simple) basic laboratory infrastructure. The following section will describe briefly a recent global initiative to prepare for the construction of such an open source database of WGS sequences—the GMI (Global Microbial Identifier).

1.6 Global Microbial Identifier (GMI) Initiative

GMI is a global network of scientists and other experts committed to improving microbiology using WGS. A charter has been drawn in which the network partners have agreed on its mission and vision (see www.globalmicrobialidentifier.org). In short, the mission is to build an interactive worldwide network of databases for standardized identification, characterization, and comparison of microorganisms through whole genome sequences of all microorganisms. GMI's vision is a world where high-quality microbiological genomic information from human, food, animal, and plant domains is shared globally to truly enable One Health sharing of such data and thereby arrive at a solid, cross-sectoral understanding of the microorganisms affecting us through foods or other vehicles.

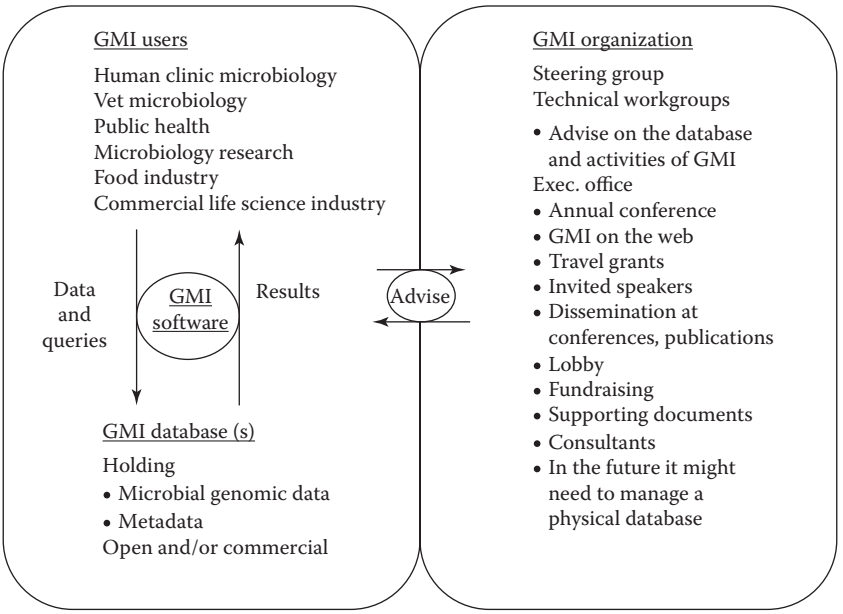

FIGURE 1.3
Schematic outline of GMI. (From Wielinga, PR et al., *Food Control*, 40(2014), 185–192.)

GMI essentially is a global network of stakeholders who take part in shaping how the database and its supporting structures can best be set up and used. Figure 1.3 shows a simplified impression of GMI: the GMI users, the database(s), the GMI software pipelines and other analytical tools, and the GMI organization. The GMI database is defined as all the microbiological WGS data and the linked metadata that can be accessed by GMI software. The GMI organization includes the people creating the database(s), people helping the development of the necessary software, and people active in the GMI working groups and steering committee.

GMI is not the only, or the first, initiative to attempt international sharing of microbiological genomic data. Several internet–based databases/tools could be mentioned: PulseNet compares the PFGE "DNA fingerprints" of bacteria from patients to find clusters of foodborne disease that might be unrecognized outbreaks (http://www.cdc.gov/pulsenet/); MLST-net can be used to compare various bacteria on the basis of multilocus sequence typing (MLST) analysis (http://www.mlst.net/); EuPathDB (http://eupathdb.org/eupathdb/) is a portal for accessing genomic-scale and MLST datasets, related to eukaryotic parasites; and NoroNet is a network of public health institutes and universities sharing virological, epidemiological, and molecular data on norovirus, including a tool for norovirus identification and epidemiology on the basis of sequence comparison (http://www.rivm.nl/en/Topics/N/NoroNet) (Wielinga et al., 2016). While these earlier networks

had to focus their efforts on a single technique and often a limited group of microorganisms, the arrival of cost-effective NGS and WGS means that these different microbiological fields may now work together, which again represents One Health capacity.

There are a number of international issues related to the sharing of microbial DNA sequences over borders. The 1993 Convention on Biological Diversity (CBD) states in Article 3: "States have, in accordance with the Charter of the United Nations and the principles of international law, the sovereign right to exploit their own resources pursuant to their own environmental policies." Microbial genomics are stated as part of these resources. This means that the answer to who owns bacteria and viruses is to be found in domestic laws and regulations and may differ between countries. The Nagoya Protocol (NP) is a supplementary, legally binding agreement under the CBD (1993), intended to create greater legal clarity and help ensure benefit-sharing regarding the utilization of genetic resources. The NP applies to all non-human genetic resources originating from the natural environment, including microbial genetic resources. Whether the utilization of genetic resources will include the digital genetic information of whole genomes is yet unclear. The NP also instructs to pay special legal attention to procedures for rapid diagnostics in health emergency situations, and the NP speaks in regard of emergencies of "present or imminent emergencies that threaten or damage *human, animal or plant health*" "to consider the importance of genetic resources for food and agriculture and their special role for food security."

GMI, in its most recent report (GMI, 2016), mentions a number of relevant issues relative to sharing of microbial WGS data, including sharing samples across borders, dual use regulations, trade/ag regulations, health regulations (including WHO International Health Regulations). Practical issues that are already confronted in real life are, for example: (1) purchase of source materials from a country, (2) patient gets sick abroad, diagnosed after return, and isolate is sequenced at home; (3) isolate sequenced in a home country, sequence transmitted to international repository residing in another country, (4) the existence of a public domain of microbiological genome data and so on. These issues all need clarification under NP as well as under other international instruments before real benefit can be gained from international WGS data sharing.

GMI is an initiative open to anyone interested in and attempting to develop synergistic solutions for the different issues at hand. The roadmap for the development of the database that has been proposed with a vision of constructing an international system by 2020 is as follows:

- Development of pilot systems
- International structural start-up, with the formation of an international core group
- Analysis of the present and future landscape to build the database

- Diplomacy efforts to bring the relevant groups together
- Development of a robust IT backbone for the database
- Development of novel genome analysis algorithms and software
- Construction of a global solution, including the creation of networks and regional hubs

1.7 Future of One Health

While political focus on the problems caused by collaborative failure between agriculture and health sectors clearly was the primary factor in driving the creation of the One Health paradigm (World Bank, 2008), the novel developments in the genomic sequencing area will most likely provide the technological drive toward real One Health change in microbiological identification, typing, and epidemiological characterization. However, the full potential of this new technology will only be realized if agreement is reached between countries as to how WGS data is to be shared. There is a real opportunity for all sectors to agree that genomic data in the microbiological area should be shared across borders just as easily as the original microorganisms are shared across borders (in humans, in food, in animals, and in the environment). If databanks of such genomic data are created, the world community will have a dramatically novel tool to investigate the development and spread of microorganisms (both pathogenic and nonpathogenic), enabling analysis—and thus control—in real time, but also supporting further investigations into historic developments in support of epidemiological understanding of how, when, and where pathogens developed and spread. Such tools would be inherently One Health in nature since all data from all sectors will be available in consistent and comparable format. Our understanding of microbiology in general—and indeed also specifically related to food—will grow manifold and enable future efficient One Health preventive action.

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2

Molecular Methods to Characterize Foodborne Microbial Pathogens

Qiongqiong Yan and Keith A. Lampel

CONTENTS

2.1 Introduction

Food production is a complex process that involves many different components of the "farm to fork" paradigm to market a final product for human and animal consumption. The food industry has grown to become a more impactful influence on the health of the world's population; therefore, the importance of a safe food supply transcends every national border. Food safety has developed into a tripartite partnership with food producers; regulatory entities at the international level, for example, the World Health Organization and the Food and Agriculture Organization, and agencies such as the European Food Safety Authority (EFSA), the U.S. Food and Drug Administration (U.S. FDA), and the U.S. Department of Agriculture's Food Safety Inspection Service (USDA FSIS); and ultimately, the consumer. Although each has a disparate role in the production, preparation, and consumption of foods, the final product, that is, safe foods, is a shared responsibility.

Foodborne illnesses exact a heavy toll around the world on a daily basis, with millions of people adversely affected annually. Many of these infected individuals succumb to the lethal effects of microbial pathogens, toxins, and abiotic agents, that is, chemicals. Therefore, the goals of food safety and risk analysis programs are focused on mechanisms to prevent or reduce the number of foodborne outbreaks.

However, foods present a difficult challenge, from the wide range of ingredients to their complex matrices, to ensure that they are free from hazardous or deleterious materials that pose a serious threat to human health. The complexity of food production reflects the basic challenges to reduce or eliminate foodborne outbreaks and illnesses. To illustrate this point, with regard to foods that are farm grown and are sold directly to the consumer or are used as an ingredient, the issue is whether these foods require further preparation or cooking by the consumer or the retail/restaurant establishment or are consumed directly. In addition, the range of commodity type, that is, matrix composition, spans from the simple to the complex. These physical attributes of foods pose a significant challenge to the food industry, regulatory

agencies, and the consumer with respect to food safety and product integrity. Good manufacturing protocols, as well as integrating food safety programs such as hazard analysis and critical control point (HACCP), are designed to address these concerns. However, this does not ensure against the potential introduction of pathogenic microbial presence in a food manufacturing facility or ensure a safe environment for product processing. Furthermore, there are other points of entry of hazardous biological and abiotic agents along the "farm to fork" paradigm that include the transportation of foods, the food preparer, and perhaps the environment where foods are prepared, for example, retail markets and the home.

With this in mind, the total spectrum of food analysis ranges from sample preparation to final determination of the status of the food product that is, whether it is safe for consumption. As a critical component in any food analytical laboratory, sample preparation covers the range from the direct examination of the food sample, particularly for culture-independent microbes, to more detailed processing, usually incorporating one or several enrichment steps. Therefore, sample preparation is a critical step in food analysis and must fit the downstream process, whether that method is bacteriological, serological, or molecular based.

In the continuing pursuit to develop more efficient means to detect, isolate, and identify microbial pathogens, the methods currently available have transformed the landscape of microbiology. A few decades ago, emphasis was placed on developing liquid and solid media to select for specific pathogens and select against the non-targeted organisms. Of note, these media were designed for culturable microbes and not much was accomplished for many culture-independent pathogens, such as several parasitic protozoans, for example, *Cyclospora*, and viruses, such as noroviruses. In addition to the non-culturable pathogens, another concern was the time necessary to complete the analysis of food samples, from a few days to over a week. Many food products, notably fresh produce, may have a limited shelf life which is a significant factor in marketing these commodities in a timely fashion. Therefore, prolonged analysis time was not in the interest of the food purveyor. Molecular-based methods, primarily targeting DNA molecules, offer a rapid, robust, and accurate means to detect and identify microbial foodborne pathogens compared with phenotypic-based conventional methods. Today, these methods have the advantage of higher discriminatory resolution to characterize isolated strains as well as a more rapid turnaround time of analysis. Figure 2.1 depicts an estimated time flow for analyses of food samples.

Some of the challenges confronted in food analytical laboratories include the difficulty in isolating and detecting pathogenic microbes present in low numbers or in matrices with complex compositions that prevent the release of these agents during sample preparation. To circumvent these problematic areas, many protocols require an enrichment step to increase the number of target pathogens. In addition, there are microbes that, to date, are not

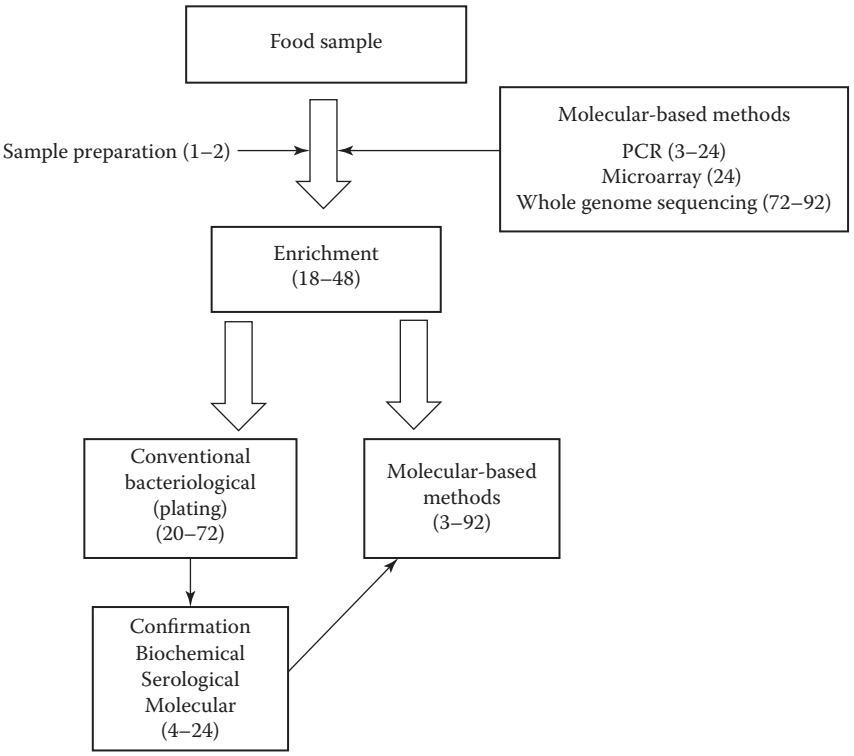

FIGURE 2.1
Estimated time of food analysis using different molecular-based methods. Food samples can be analyzed by several different molecular-based methods, each with a different amount of time for set-up and execution. Estimated times are indicated within parentheses. These estimates do not include time frames for sample preparation since there are many variations to processing innumerable food matrices for analysis. In some instances, such as with PCR, no specific means of amplification/results are designated, but the different PCR platforms, for example, conventional, real-time, LAMP, are covered. Time estimation for WGS begins with an isolated colony and includes library preparation, run-on sequencer, and analysis; it does not include enrichment and plating and incubation on agar medium.

culturable; therefore, their presence in foods is detected through culture-independent diagnostics (CID). As with any method, certain critical metrics must be met to ensure that the method applied will accomplish its intended objective, including detection, isolation, and identification. Therefore, the level of detection, specificity, and sensitivity; the generation of false positive and negative results; and the appropriate use of controls should be provided for each technique used. In addition, other factors, such as ease of use, cost, and degree of difficulty to operate and analyze data, influence the adaptability of any method for routine use in a food analytical laboratory.

Although the transition from a totally bacteriological-based analysis to an all-encompassing molecular biology–based era has not occurred, significant

progress has been accomplished so that a myriad of molecular-based methods is routinely used in an analytical laboratory. As an intermediate in this transition, serological applications not only served as an important tool in the identification of microbial pathogens but are also used in combination today with several different molecular-based methods for analysis.

In the past few decades, analytical laboratories have evolved from the "shake and plate" bacteriological applications to the application of more sophisticated, newer technology. The latter has been greatly influenced by the advancements made in unraveling the mystery of DNA structure and replication, leading to paradigm shifts in science. Examples are the transition from chemically based sequencing (Maxam and Gilbert, 1977) to biologically based (Sanger, 1977); the advent of polymerase chain reaction (PCR) (Saiki et al., 1985); the automation of DNA sequencing, from polyacrylamide gels to capillary electrophoresis; and current instruments for whole genome sequencing (WGS), used to determine the sequence of whole genomes whereas the early technology had its limitations in the number of bases sequenced in one run. As detailed in this chapter, there are other instruments that provide critical data from analysis of foods that are not dependent directly on sequence analysis.

As a major step to the molecular era, the PCR can be considered as one of the more modern technologies that has impact, not only as a stand-alone method but also in the influence it has had on the development of novel technology, from DNA sequencing using dideoxy analogues to today's next-generation sequencing (NGS) era. Refinements made to the original protocol, leading to improvements, greater utility, and accessibility, have meant that PCR has become an integral component of many food, clinical, and research laboratories. The power of sequencing-based technologies can provide critical data on the pathogen's serotype, genotype, or pathotype. This is exemplified as a tool in the traceability of pathogens as etiological agents in foodborne outbreaks as well as a historical "document" in food production/processing environments.

NGS instruments have become an increasingly utilized and integral technology in analytical food laboratories for several reasons. The price of the instruments has decreased significantly; the data generated can reveal much information about strain characteristics, such as strain identity and virulence potential; and access to WGS databases facilitates strain comparisons. However, as noted by Ercolini (2015), the strengths and weaknesses of high-throughput sequencing (HTS; e.g., WGS) for the food industry should be noted and considered before implementation.

This chapter provides an overview of the molecular-based methods used to detect, isolate, and characterize foodborne pathogens that are currently used in food analyses, or have been published in the scientific literature. An excellent review of the current status of molecular-based methods available for food safety and how data generated from this technology can be interpreted is presented by Ceuppens et al. (2014), and for the basis for molecular

methods, see Mangal et al. (2016). Figure 2.1 provides a general flow diagram of food analysis with an estimate of the time to complete each step with the integration of molecular-based methods.

2.2 Blotting

2.2.1 Colony Hybridization

One of the earliest applications of the nucleic acid hybridization technique for the detection of pathogenic foodborne pathogens was the development of colony hybridization by Grünstein and Hogness (1975). Typically, food samples were processed via enrichment in liquid media followed by plating dilutions of the cultures onto agar medium. After overnight growth, colonies were transferred to different types of filter papers (e.g., nitrocellulose, nylon); lysed, typically by alkaline treatment with NaOH or microwaving; air-dried; baked *in vacuo* at 80°C for 2 h; and then hybridized with radioactive (^{32}P) or non-radioactive probes, for example, biotin or digoxigenin-labeled. Probes were double-stranded DNAs that lent themselves to labeling at either 5′ or 3′ ends or synthetic ss-DNA. Filters were hybridized with probes (dsDNA probes are denatured prior to hybridization) under specific time and temperature conditions to increase the specificity of the probe/target interaction. After appropriate washing and drying of the filters, they were subsequently either exposed to x-ray film with radioactively labeled probes, or processed to produce signals directly on the filter. One example was to incubate filters with antidigoxigenin antibody conjugated with alkaline phosphatase, and the final step was to add nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium salt (Riley and Caffery, 1990).

2.2.2 Other Filter-Based Hybridizations

Other types of filter-based hybridization methods that were used to identify foodborne pathogens are Southern (Southern, 1975) and Northern (Alwine et al., 1977) blotting. For Southern blots, DNA is digested, subjected to electrophoresis, transferred and fixed to a solid membrane, and subsequently hybridized to a labeled probe, either radioactive (^{32}P) or chemiluminescent. With radioactive probes, the filters are exposed to x-ray film and developed after an appropriate time. For probes labeled with a chemiluminescent compound, luminescence is captured on film after treatment with an enzyme such as alkaline phosphatase. An early application of Southern blotting examined the efficacy of an oligonucleotide probe to detect *Listeria monocytogenes* (Wesley IV et al., 1990).

Northern hybridizations involved the separation of RNA by electrophoresis and the subsequent hybridization of membrane-bound RNA with a single-stranded, labeled probe. Since RNA is used in Northern blotting, the data generated from this analysis may infer bacterial cell viability, unlike Southern blots. In addition, RNA is more abundant in a bacterial cell than DNA. In one study, Northern blot analysis was used to address the killing effect of UV and heat-killed treatments on Enterotoxigenic *Staphylococcus aureus* and *Escherichia coli* O157:H7 (McKillip et al., 1998). Other types of blotting techniques that have been developed and used for the detection of proteins and nucleic acids include dot blots, Western (protein immunoblots), Southwestern and Northwestern, and Eastern blots.

2.3 Polymerase Chain Reaction

PCR is an *in vitro* means of amplifying targeted sites in either DNA or RNA to generate sufficient and detectable quantities of amplicons (PCR products) for downstream detection and, in some cases, sequence analysis. First described by Saiki et al. (1985), PCR revolutionized the application of molecular methods for the detection and identification of microbial pathogens. Over time, PCR chemistry, instrumentation, and consistency in reaction performance due to high-quality reagents marketed in manufacturers' kits have made this technology a cornerstone in many analytical laboratories. One of the earliest applications of molecular-based methods was the development of PCR assays that targeted foodborne pathogens (Hill, 1996). Over time, PCR emerged as a powerful analytical tool for microbial diagnostics as well as a means to evaluate genomes, which has been well documented.

Briefly, nucleic acid targets are amplified by means of the hybridization (annealing) of a specific sequence of short DNAs, commonly short synthetic molecules (oligonucleotides/primers) that are complementary to targeted regions on the DNA or RNA molecules. After annealing, a DNA polymerase extends double-stranded DNA or DNA/RNA to complete the cycle. The specificity of these reactions is driven by the primer sequence and the annealing temperature. The advantages of PCR stems from its ability to detect specific microbes based on its inherent specificity and to detect non-culturable organisms and identify atypical colony formers, such as sorbitol-fermenting *E. coli* O157:H7. In addition, PCR can be performed rapidly and provides critical information on the serotype, genotype, or pathotype. However, as compared with culture-based methods, PCR, without the addition of specific compounds such as ethidium monoazide (EMA) or propidium monoazide (PMA), cannot distinguish live from dead bacterial cells (Nocker et al., 2006) and, with viruses, infectious versus non-infectious viral particles (Fittipaldi et al., 2010).

PCR has been used to detect pathogenic microbes since the 1990s due to its strengths: specificity, relative ease of use, automation, and acceptance and familiarity worldwide. One application that has gained interest in the area of food fraud has been the development and implementation of DNA barcoding, a powerful tool for food traceability (Bhattacharya et al., 2016). PCR primers are synthesized that target specific sequences in different organisms, such as distinguishing different types of fish, for example, salmon.

Its drawbacks are its provision of limited information compared with WGS data, the presence of PCR inhibitors in foods that negatively impinge on the reaction, the low numbers of targeted pathogens present, and the recommended requirement to physically separate each step of sample prep and PCR to minimize cross-contamination. In addition, PCR data and culture-based results may be incongruent, where an isolated colony is obtained and the PCR reaction is negative. In this case, the level of sensitivity of the PCR assay was not sufficient or the reactions were inhibited due to the presence of chemicals from the food matrix. Obviously, the converse can occur where PCR detects the presence of a pathogen but no isolated colony is obtained. Furthermore, in instances where the etiological agent is not culturable, PCR may be the only alternative means to determine if the food is pathogen-free.

Several platforms have evolved from the original description of PCR. With the use of automated technology, some have extended the utility of PCR to increase the number of samples that can be analyzed within a specific time period, provide a broader range of detection by targeting multiple sites in one reaction, and, in some cases, utilize isothermal reaction conditions to eliminate the need for expensive instruments.

PCR-based assays, on different platforms and formats, have targeted many important foodborne pathogens. A few examples for selected microbes include non-O157:H7 Shiga toxin-producing *E. coli* (STEC), including the "Big 6" serotypes (O16, O45, O103, O111, O121, and O145) identified by USDA FSIS in 2010 (Fratamico et al., 2011; Wasilenko et al., 2012); EHEC (Feng and Monday, 2000; Yoshitomi et al., 2006); *L. monocytogenes* (Rodríguez-Lázaro et al., 2004); *Salmonella* spp. (Rahn et al., 1992); *Shigella* (Deer and Lampel, 2010); *Yersinia enterocolitica* (Ibrahim et al., 1992); *Vibrio* spp. (Brauns et al., 1991; Tada et al., 1992); *Cronobacter sakazakii* (Chen et al., 2010); and *Campylobacter* spp. (Sails et al., 2003). A more in-depth review of PCR-based methods is provided by Umesha and Manukumar (2016).

2.3.1 Conventional PCR

Traditional PCR combines the amplification process with the analysis of amplicons via gel electrophoresis, usually with agarose gels. PCR products are loaded onto gels, separated based primarily on size, and with concurrent running using a known molecular weight standard, a close estimate to the amplicon size can be determined. Confirmation of the final product as to whether it is, or not, the intended targeted amplification sequence can be

accomplished by several techniques: Southern hybridization, DNA sequencing, nested PCR (use of internal primers to amplify a known product). In addition, conventional PCR has the flexibility, depending on the reaction design, to generate short to long fragments.

A major drawback of conventional PCR is that it usually takes a longer period of time to arrive at a final analysis compared with other platforms, such as real-time (RT) PCR. Other concerns include the possibility of cross-contamination by opening/closing the reaction tubes that can lead to questionable conclusions. However, overall, conventional PCR remains a solid and useful tool to analyze samples for the detection and, in some cases, identification of microbial pathogens.

2.3.2 Real-Time PCR

As the name implies, the amplification process can be monitored in real time, that is, as the reaction continues, the increase in the number of amplicons can be followed immediately by recording the fluorescence or the incorporation of DNA dyes and/or fluorescent probes after each cycle. An advantage of RT PCR is that the availability of multiple dyes and probes that fluoresce at different wavelengths allows for the development of multiplex assays using this rapid detection method. In addition, the time of analysis, from 2 to 4 h in conventional PCR with subsequent gel electrophoresis, has been reduced to less than 1 h in a number of different commercially available instruments. During RT PCR reactions, a critical aspect of amplification is determined at a point in which a cycle value (Ct) threshold is obtained. This can be standardized to quantify the original template present, indicate the specificity of the product, and eliminate the false positive reaction due to primer dimer formation. In lieu of using ethidium bromide, the RT reactions are monitored using a fluorescent dye, SYBR-Green, which binds to the amplified double-stranded DNA generated from the targeted genome sequence (Morrison et al., 1998). Over time, the number of amplicons increases and, as indicated earlier, the amount of fluorescence reaches a point where a Ct value can be associated with the reaction. Lastly, the specificity of the reaction can be ascertained by melting curve analysis.

Later modifications to RT PCR utilized the addition of molecular beacons and TaqMan® probes as an alternative to SYBR-Green. Molecular beacons are short length, stem-loop structured DNA that are labeled at separate termini with reporter and quencher molecules. The beacon binds to a location between the two primers used in amplification. If an amplicon is generated, the probe binds and the quencher molecule is too distant to affect the reporter fluorophore and, hence, fluoresces. When no amplification occurs, a complementary DNA of the probe keeps the quencher in close proximity to the reporter molecule and no fluorescence will be generated. TaqMan® probes, designed also with reporter and quencher molecules attached to one end of the oligonucleotide, will bind to the target region between the two primers

used in amplification. If attached at the specific site, the 5′ exonuclease activity of the DNA polymerase will cleave the reporter molecule from the probe. This release allows for fluorescence from the reporter molecule and is detected.

Another use of RT PCR–based assays is as a quantitative PCR (qPCR) technique. In this case, the number of amplicons can be quantified during each PCR cycle. With fluorescent labeled dyes, such as SYBR-Green that intercalate in the newly synthesized double-stranded DNA (amplicons) or with labeled probes, that is, TaqMan®, Molecular Beacons™, the amount of synthesized DNA can be determined during the log (linear) phase of amplification. Usually, a known gene sequence, for example, housekeeping gene, is used to monitor the amplification efficiency for each input target molecule and, from a calibration curve, the quantity of amplified DNA can be determined.

As with any PCR-based method, reactions can be inhibited by the presence of chemicals, for example, from food samples, that interfere with some aspect of amplification. The incorporation of internal amplification control (IAC) has become an essential quality control measure (Hoorfar et al., 2004; Malorny et al., 2003, 2004). Typically, a non-target DNA has been constructed, along with a specific set of primers, and added to the PCR reaction. The IAC will be amplified whether the targeted DNA is present or absent if all the reaction conditions, that is, reagents, are not compromised. If an amplicon is not generated from the IAC, this would indicate that a negative reaction with the targeted DNA should be counted as a false negative result. A positive result should be composed of two amplicons, one from the targeted DNA and the other from the IAC.

The convenience of RT PCR can also be attributed to the minimum number of manipulations, the assay design to include a probe that is used to confirm the specificity of the amplicon, and, in many cases, use of the chemistry on several different platforms. However, one of its drawbacks is the proprietary move by manufacturers to limit the use of the reaction chemistry to only their instrument.

2.3.3 Multiplex PCR

The ability to detect multiple targets in a single PCR set-up has several advantages. The amount of set-up manipulation and the total number of reactions are significantly reduced as well as the overall cost of food analysis by PCR. Whether by conventional, RT PCR, or other platforms, for example, isothermal, targeted sequences can include a range of specified DNAs, from genus or species-specific regions, such as *E. coli* O157:H7, *Bacillus cereus*, *Vibrio parahaemolyticus*, *Salmonella* spp., *L. monocytogenes*, and *S. aureus* in ready-to-eat foods (Lee et al., 2014) and *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* in meats (Suo et al., 2010) or genes that can delineate pathogens from non-pathogens (Feng and Monday, 2000). One challenge in developing a multiplex PCR is to ensure that all targets are equally amplified in order to not generate any false negative data. This would entail that all primers

(or probes for RT PCR) and buffer reagents are maximized to generate amplicons that can be distinguished from each other.

2.3.4 Digital PCR

Digital PCR has been developed to be performed on several different platforms, such as microfluidic or droplet systems. With the latter PCR system, the DNA template molecules are separated on droplets (~20,000 nanoliter-sized) that are formed in a water–oil emulsion. In this manner, digital PCR partitions nucleic acids (template) onto hundreds to thousands of individual droplets; partitioned templates on some droplets will be amplified whereas droplets without template will not. The incorporation of dye-labeled probes into amplified products will generate signals that are subsequently captured and read on the instrument. In other platforms that use tubes or wells, reactions (thermal cycling) will occur similarly, as described earlier, on each droplet but are then analyzed by a flow cytometer. Regardless of the platform-based amplification system, digital PCR can also be used to quantitate the amount of template initially analyzed. Another advantage of this technology is that it is less sensitive to PCR inhibitors that confront classical and other means of RT PCR amplification. In addition, digital PCR can be used for allelic-specific or allelic-discrimination applications, an important tool in use for single nucleotide differences between bacterial strains and viruses. For high-throughput PCR analysis, RT PCR may be suited to this use rather than digital PCR, since the latter requires more hands-on time to set up the assays. A recent publication set some standards for the application of digital PCR to several common uses, notably species identification, genetically engineered events in plants, and food fraud (Lievens et al., 2016).

2.4 Isothermal PCR

One of the drawbacks with conventional and RT PCR is that they usually require instruments, some expensive, that can cycle through different temperatures necessary for amplification reactions. Isothermal reactions, on the other hand, can be performed at a uniform temperature, thereby eliminating the need for thermal cyclers. These types of techniques include loop-mediated isothermal amplification (LAMP) of DNA, transcription-mediated amplification, nucleic acid sequence-based amplification (NASBA), strand displacement amplification, rolling circle amplification, isothermal multiple displacement amplification, and helicase-dependent amplification (HDA) (for reviews, see Gill and Ghaemi, 2008; Law et al., 2015). DNA or RNA can be used as target molecules for amplification. Currently, commercially available isothermal-based kits are available to detect microbial pathogens in foods, including *E. coli* O157:H7, *Salmonella*, and *Listeria* species.

2.4.1 Helicase-Dependent Amplification

HDA uses a DNA helicase to generate single-stranded templates for primer hybridization and subsequent extension by a DNA polymerase with all reactions performed at one temperature. Since the double-stranded DNA unwinds enzymatically by the DNA helicase, the initial steps in conventional and RT PCR, that is, heat denaturation, and subsequent steps are eliminated. Primers anneal to specific regions of the unwound DNA and the DNA polymerase extends the DNA from that point.

2.4.2 Loop-Mediated Isothermal Amplification

LAMP-based assays, first described by Notomi et al. (2000) and later updated by Notomi et al. (2015), use the *Bst* DNA polymerase with four primers, two outer and two inner primer sets that target six regions. In this amplification process, there are repetitions of two elongations that occur from loop regions. From these loop regions, self-elongation occurs at the 3′ end of the loop that is extended by the binding of primers to this region. The specificity of the LAMP assay is driven by the sequence of the inner primers that has sequences complementary to the 3′ terminus of the amplified chain, and the other inner primer is identical to the 5′ end. Further amplification, that is, elongation reactions, is mediated by the DNA polymerase through strand displacement synthesis. Amplified products are either visualized by gel electrophoresis or by turbidity or, more commonly, by fluorescence. In the latter, SYBR-Green dye can be incorporated into the reaction mix and, by simple visualization, a positive reaction can be seen. The LAMP-based assays can be performed in 1 h, are simpler to use, do not require expensive equipment, and can be as sensitive, or more so, than PCR. This format has been developed to detect several microbial pathogens in a range of food matrices such as *Salmonella* Typhimurium in pork (Techathuvanan et al., 2010) and seven serogroups of Shiga toxin-producing *E. coli* in produce (Wang et al., 2012).

2.5 Nucleic Acid Sequence-Based Amplification

Among the advantages of NASBA is that the platform is an isothermal amplification system and its usual target molecule is RNA. RNA, particularly mRNA, can indicate the viability of a bacterial cell and, unlike DNA, has been used to differentiate live from dead cells. As described by Compton (1991), NASBA utilizes three enzymes: avian myeloblastosis virus reverse transcriptase, T7 RNA polymerase, and RNase H. Basically, NASBA uses two primers and begins with the synthesis of cDNA from mRNA by reverse transcriptase from the point where one primer is attached, followed by the degradation of the RNA molecule by RNase H. The other (second) primer

anneals to the cDNA and a complementary strand is produced by reverse transcriptase yielding double-stranded DNA. The T7 RNA polymerase generates a complementary RNA molecule, and the second attaches to the new RNA and reverse transcriptase generates a cDNA that then acts as a template to make more double-stranded DNA. Amplified products can be detected by gel electrophoresis, now transformed into a RT format. As for the latter, molecular beacons using fluorescently labeled probes can be used to detect the amplified RNA amplicons.

Applications of the technology have been cited for *Vibrio cholerae* spiked into environmental water samples (Fykse et al., 2007), *Salmonella* Enteriditis in liquid whole eggs (Cook et al., 2002), *Salmonella* in different food matrices (D'Souza and Jaykus, 2003), and several foodborne pathogens, such as *L. monocytogenes* and *Campylobacter* spp. (Cook, 2003).

2.6 Reverse Transcription PCR

A disadvantage of PCR is its inability to discriminate between live and dead microbial cells (infectious vs. non-infectious viruses). The addition of PMA or EMA in RT PCR can differentiate live cells from dead cells. The dyes bind to double-stranded DNA molecules and, in live cells, this dye does not enter the cell since the cell membrane is intact. However, in dead cells, PMA or EMA can bind to dsDNA since they are able to enter the cells due to leakage in the cell membrane and, therefore, block amplification by PCR.

Reverse transcription PCR targets RNA, usually mRNA, and uses the enzyme reverse transcriptase to generate a complementary DNA (cDNA) molecule. mRNA is usually associated with live cells since the half-life of this molecule is short, and, therefore, amplification from this nucleic acid molecule is indicative of a viable cell. The application of reverse transcription PCRs requires an initial step to copy the mRNA into DNA, followed by amplification by a DNA polymerase. RT, reverse transcription PCR assays can be performed either as a one-step or a two-step process, where in the latter the RT step and PCR amplification are carried out sequentially whereas in the former both reactions are combined.

2.7 Other PCR-Based Methods

A number of PCR-based assays are used in tandem with other methods or as stand-alone protocols, such as for strain genotyping. Amplified fragment length polymorphism (AFLP) is used as a genotyping technique. Bacterial genomic DNA is initially digested with a restriction endonuclease that is

a frequent cutter followed by the ligation of short adapter sequences to the ends of the restriction fragments. PCR primers, with one to three mismatched sequences to the adapter site, are used to amplify products from the generated fragments, initially under stringent conditions to prevent mismatched binding. This reduces the number of PCR products. Amplified DNAs are subjected to electrophoresis, and the pattern generated is used for strain comparisons (Vos et al., 1995). When fluorescent dye-labeled PCR primers are used, automated readout can be accomplished on a DNA sequencer (Tamada et al., 2001).

Another method used for molecular typing is the random amplified polymorphic DNA (RAPD) technique (Swaminathan and Barrett, 1995). In this PCR assay, arbitrary primers are annealed under non-stringent conditions. The generated amplicons are electrophoretically separated and the banding patterns are then used as a fingerprint pattern for each bacterium. These patterns provide a means to evaluate relatedness and differentiate pathogenic from non-pathogenic strains (Williams et al., 1990; Franklin et al., 1999).

Repetitive element PCR (Rep-PCR) targets repeated DNA sequences elements found in the bacterial genome (Versalovic et al., 1991). Primers are specifically designed to the flanking regions to these repeated elements and, after amplification, the PCR products are separated by gel electrophoresis. The resulting banding patterns can be used to assess the genetic relatedness between isolates. Another variation of Rep-PCR focuses on the enterobacterial repetitive intergenic sequences and, hence, is referred to as ERIC PCR (Falcao et al., 2006).

Variable number of tandem repeat (VNTR) and multiple locus VNTR analysis (MLVA) also target repeated sequences resident in bacterial genomes that vary in size from a few nucleotides to over 100 base pairs. For genotyping applications, PCR primers target the flanking regions of repeated sequence motifs of interest. Therefore, the discrimination of one species from another is dependent upon the pattern of the number of repeats in the bacterial genome (Lindstedt et al., 2005). This process can be automated with the use of fluorescently labeled PCR primers and a DNA sequencer. MLVA utilizes amplification with the PCR primers labeled with different fluorophores. In this manner, multiple loci can be analyzed. This method provides a higher level of discrimination and accuracy and has been used to genotype several foodborne pathogens such as *Salmonella* (Lindstedt et al., 2003) and *E. coli* O157:H7 (Keys et al., 2005).

2.8 DNA Fingerprinting Methods

2.8.1 Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) involves the use of restriction enzymes, usually low cutting frequency enzymes such as *Sma*I and *Not*I to digest total genomic DNA; the fragments are then separated based on size through electrophoresis. However, different parameters are used compared

with conventional agarose gel electrophoresis as the movement of DNA fragments by PFGE is dependent on the application of an alternating electrophoretic current, which is "pulsed" in different directions over a gradient of time intervals. This allows a range of DNA molecules, including those of mega-base size, to be resolved through these gels. PFGE is highly discriminatory, successfully differentiating at the strain level; is reproducible; and generates a banding pattern that is easy to interpret. However, there are cases in which a single enzyme digestion is unable to resolve closely related strains and multiple enzymes may have to be used to obtain data to discriminate highly related microbes.

2.8.2 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP), which means to generate DNA patterns from microbial genomes, was accomplished by digesting DNA with restriction endonucleases, with the resulting fragments separated by gel electrophoresis. DNA was transferred to solid membranes and then hybridized with labeled probes to generate a "fingerprint" pattern. A later version bypassed these steps and implemented PCR to target specific regions (genes or loci) on the bacterial chromosomes to generate a unique pattern. PCR-RFLP is a common technique used in the food industry and, in addition, can be used to identify fish and meat species. Further details of the technology are provided in a review by Säde and Björkroth (2014).

2.8.3 Ribotyping

Ribotyping is based on the hybridization of targeted digested chromosomal DNA with specifically labeled 5S, 16S, or 23S rRNA gene probes. Overall, microbial DNAs are isolated, then digested with restriction enzymes, and then separated by agarose gel electrophoresis. Subsequently, the DNA is transferred to a solid membrane, usually nitrocellulose or nylon, followed by hybridization with labeled 16S, 23S, or 5S ribosomal RNA (rRNA) gene probes. The discriminatory power of this technique is based on the hybridization pattern of the probes to the digested bacterial chromosome fixed to either a nitrocellulose or nylon membrane. Since bacterial chromosomes contain multiple copies of the rRNA operons, a distinctive microbial fingerprint can be used as an identification tool. In this manner, species, rather than strain-level identification, can be accomplished. These fingerprint identification patterns have been used in the food industry for starter culture characterization, identification of cross-contamination, and tracing of the source of contamination.

2.8.4 Multilocus Sequence Typing

Another means to characterize microbial pathogens is the use of multilocus sequence typing (MLST). This protocol exploits the sequence differences

(variations) that exist among bacterial pathogens at selected housekeeping genes, usually 7–8 loci. The internal amplified PCR products of these housekeeping genes are sequenced and the alleles defined, based on nucleotide sequence variations at each of these loci, and are representatives of a sequence type (ST). This allelic profile is reflective of and used as an identification marker for that strain type. Therefore, the relatedness of bacterial species can be accomplished by the comparison of STs. This approach has been used for phylogenetic and epidemiological studies, and the constructed dendrogram based on the allelic differences can show relationships between strains.

2.9 Microfluidics/Lab-on-a-Chip

2.9.1 DNA Microarray

A microarray is an orderly arrangement of "spots/probes" that are immobilized on a solid support platform, such as glass, silicon chips, or even microscopic beads, which contain known DNA or RNA sequences. The underlying basis of the array is that the probes on the chip are arranged in specific order so that the hybridization of an unknown sample (DNA or RNA) can reveal several important genetic characteristics to identify the microbe and other details, such as virulence capacity. Based on the probe that hybridizes to its complementary sequence of the unknown, the identity and perhaps the specific characteristics of the tested target can be determined. Since each array can hold more than 10,000 spots, arrays can provide a broad coverage of genomes such that the data generated can be used to identify an unknown microbe (Patel et al., 2016), can provide critical information as to the virulence status of bacteria (Patel et al., 2016), and, as a metagenomics tool, can be applied to samples to determine the microbial population present (Patro et al., 2015). As an example, an array, the FDA-*E. coli* Identification microarray, can discriminate between pathogenic and non-pathogenic *E. coli* due to its ability to molecular serotype and determine virulence profile based on specific genotypic sequences and SNPs embedded in the array (Patel et al., 2016). Although PCR-based assays have been used to determine *E. coli* O- and H-serotypes (Wang et al., 2003; DebRoy et al., 2011), the microarray described by Patel et al. (2016) has shown greater accuracy and utility.

2.10 Biosensors

A means to automate pathogen detection may be accomplished through the application of biosensors, analytical tools that are basically composed of a biologically based recognition system (biorecognition element) coupled

with a transducer (see Ahmed et al., 2014, for review). When the biorecognition element binds to a specific analyte, the signal generated is amplified, processed, and displayed. Typical biological probes include DNA and RNA molecules as well as proteins, such as monoclonal and polyclonal antibodies, and bacteriophages. Biosensors are classified based on the method of signal transduction: optical, electrochemical, piezoelectric, or thermometric. In regard to biosensors as a means to assess food safety, generally two targets are used: (1) whole cells or (2) an internal bacterial molecule, such as DNA, RNA, enzyme or toxins, which then requires an additional step to lyse the bacterial cell. For the former application, bacterial extracellular structures, that is, proteins, glycoproteins, and lipopolysaccharides, can act as antigens/targets for the biorecognition element. Foodborne pathogen detection by biosensors has been used to target *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, *S.* Enteritidis, and *Bacillus cereus* with varying limits of detection ranging from less than 100 CFU/mL (25 CFU/mL for *Salmonella* and *E. coli*) to over 10^6 CFU/mL (see Zhao et al. [2014] for a comprehensive list).

2.10.1 Automated

Several manufacturers of automated detection systems have used molecular-based technology as their underlying science to test for microbial pathogens in foods. Basically, the process involves the capture of targeted microbes with specific antibodies followed by amplification with an isothermal platform, with the final output that detects the presence of specific foodborne pathogens. A common target is rRNA, an abundant molecule in a bacterial cell. Assays for the detection of *Salmonella*, *L. monocytogenes*, and STECs, including *E. coli* O157:H7, are commercially available.

Some systems provide the means to take a food sample from the preparatory step through final pathogen identification. These provide a rapid, high-throughput means, with little hands-on manipulation, to analyze food samples. Other companies provide a detection process that includes sample preparation and automated PCR analysis of the food sample. In these cases, the amplification facet of the protocol is proprietary with little flexibility to modify the system for expanded uses.

2.11 Aptamers

Aptamers are single-stranded DNA or RNA that are designed to specifically bind to target molecules such as proteins. The folding pattern of each aptamer drives the binding specificity in a manner similar to antibody-antigen complexes. These single-stranded nucleic acids are processed through an *in vitro* systematic evolution of ligands by exponential enrichment (SELEX),

a selection method to identify potential aptamers from a random library of synthetic molecules numbering from 10^{13} to 10^{15} (Tuerk and Gold, 1990; Ellington and Szostak, 1990). DNA and RNA aptamers are generated and selected through a similar SELEX process whereas dsDNA library is transcribed into RNA by T7 RNA polymerase. The basic design of an aptamer incorporates a 20–80 randomized sequence of nucleotides that are flanked by a short sequence (18–21 nucleotides), which act as PCR primers. The potential use of aptamers in foodborne pathogen detection spans several modes of methods, including portable, handheld biosensors, that currently employ antibody-based platforms.

2.12 DNA Sequencing and Genomics

2.12.1 DNA Sequencing

The double-helix model of DNA was proposed by Watson and Crick (1953). DNA is composed of two strands of nucleotides coiled around each other and linked together by hydrogen bonds. The canonical structure of DNA has four bases: thymine (T), adenine (A), cytosine (C), and guanine (G), with an A on one strand always paired with a T on the other, and a C always paired with a G. This structure allows each strand to reconstruct each other and pass on hereditary information between generations.

DNA sequencing is the process to determine the physical order of these four bases in a DNA molecule. The first DNA sequences method, which involved a location-specific primer extension strategy, was developed by Wu (1970) and applied to sequence the cohesive ends of bacteriophage λ and 186 DNA. Sanger et al. (1977) adopted this primer extension strategy and developed a more rapid sequencing method using chain-terminating inhibitors. Maxam and Gilbert (1977) also developed a sequencing method based on the specific chemical degradation of the DNA molecule.

The first full genome sequence was completed by Sanger et al. (1977) for the bacteriophage φX174. As DNA sequencers, such as Direct-Blotting-Electrophoresis-System GATC 1500 by GATC Biotech, ABI 370 by Applied Biosystems, and Genesis 2000 by Dupont, became available and affordable, these instruments promoted a surge in genome sequencing efforts. The first whole genome shotgun sequencing was published for the bacterium genome of *Haemophilus influenzae* (Fleischmann et al., 1995). Several new sequencing methods were also developed in the middle to late 1990s and were implemented in commercial DNA sequencers by 2000. Advances in NGS or HTS technologies have led to the reduction in cost of DNA sequencing.

This section of the chapter will describe a list of DNA sequencing technologies for the identification and application of foodborne pathogens in food

safety programs. Different sequencing platforms can afford several outputs for specific applications. Recent efforts to develop CID include the use of metagenomics where either shotgun sequencing or targeted sequencing of the 16S rRNA genes is used to identify the bacterial species or population in samples. In addition, the availability of HTS methods provides millions to billions of sequences of varying lengths (50–500 bases) that can be assembled and subjected to bioinformatics analysis to determine the identity of the bacterium or bacterial populations in the sample.

As the cost of NGS has decreased, the standardization of sequencing protocols and downstream bioinformatics programs remains an issue to be addressed so that it becomes a routine technology for food safety programs. As an example, as indicated in a recent review by Ronholm et al. (2016), the application of NGS can (1) circumvent the identity of non-culturable pathogens, whether by the lack of methods to isolate these microbes or due to their physical state in the food matrix; (2) be used to construct phylogenetic trees to assess the relationship(s) of pathogens of interest; and (3) be used in traceback or surveillance programs to link foods with outbreaks/illnesses.

Of note, the genomes of many foodborne pathogens, including bacterial and viral agents, have been sequenced with this technology. Whole genome sequences have been deposited in publicly accessible databases, such as GenBank at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/), the European Molecular Biology Laboratory (EMBL) (www.embl.org), and the DNA Database of Japan (DDBJ) (http://www.ddbj.nig.ac.jp/).

2.12.2 Maxam–Gilbert Sequencing

Maxam and Gilbert (1977) developed a DNA sequencing technique that determines the nucleotide sequence of a terminally labeled DNA molecule by specific cleavage at thymine (T), adenine (A), cytosine (C), or guanine (G) with chemical agents. The DNA molecule was initially radioactively labeled at either the 5′ or 3′ end, usually with γ-^{32}P. The chemical treatment generates breaks at one or two nucleotide bases in each of four reactions (G, A+G, C, C+T). When the products of those four reactions are resolved by size on a polyacrylamide gel using electrophoresis, the DNA sequence can be read from the pattern of radioactive bands; therefore, the length of the labeled fragments defines the positions of that base. Usually, at least 100 base sequences can be determined from the point of end-labeled DNA. The utility of this method is limited by the resolving power of the polyacrylamide gel and the use of chemical reagents.

2.12.3 Sanger Sequencing

The chain-termination method developed by Sanger et al. (1977) became the method of choice due to its relative ease, reliability, and greater amount

of sequence data that can be read from one run. This method uses DNA polymerase, which makes use of nucleotide inhibitors, specifically dideoxy nucleotides that terminate the newly synthesized chains at specific residues. As an example, the chemistry of the reactions has been designed such that at any one base, such as at a thymidylic acid (dT) position, either a dA or ddA will be incorporated. If the former is incorporated, strand synthesis continues, whereas if the latter is inserted in the growing strand, synthesis ceases. This method uses fewer toxic chemicals and a lower amount of radioactivity than Maxam–Gilbert sequencing. This method is commonly referred to as Sanger sequencing and was widely used from the 1980s until the middle 2000s. Several techniques, such as fluorescent labeling, capillary electrophoresis, and general automation, were introduced to improve the method, which has led to more efficient sequencing and lower cost.

2.12.4 Shotgun Sequencing

Shotgun sequencing can be used for sequencing DNA fragments longer than 1000 base pairs, up to an entire chromosome. The DNA molecule is first randomly fragmented by a prescribed shearing process. Each individual fragment is sequenced from both ends, resulting in paired-end reads that can then be assembled into longer DNA sequence fragments (contigs) based on the overlapping reads (Staden, 1979). The limitation of this method is that its assembly is complex and difficult, in particular, sequence repeats often cause gaps in genome assembly.

2.12.5 Illumina Dye Sequencing

The Illumina dye sequencing method is based on reversible dye-terminators that enable the identification of single bases as they are introduced into DNA strands. This technology is based on three steps: amplification, sequencing by synthesis, and analysis. Purified DNA is fragmented into short segments and ligated with adaptors on both ends. The modified DNA is loaded onto a specialized chip where amplification and sequencing take place. Millions of oligonucleotides that hybridize to the adapters to capture the DNA fragments are anchored on the chip. Once the fragments have attached, the cluster generation phase begins in which approximately 1000 identical copies of each DNA fragment are generated. After the clustering phase, fluorescently labeled dNTPs enter the chip. A single dNTP is added to the template DNA fragments, and the fluorescent dye is imaged and then cleaved enzymatically to allow the next dNTP to incorporate. A computer determines which nucleotide has been added based on the wavelength of the fluorescent tag and records it for every cluster on the chip. This process continues until the specified read length (<300 bp) is sequenced. Sequencing then begins on the other end of the DNA fragments to determine the sequence of the corresponding paired-end read.

2.12.6 Other DNA Sequencing Platforms

Several other sequencing systems are available that can be used for specified and selected purposes. In the broad scheme to identify microbial pathogens from foods or food manufacturing facilities, these methods can be implemented but have been used on a limited basis. Pyrosequencing was originally developed by 454 Life Sciences (Margulies et al., 2005). This method uses 60×60 mm^2 fiberoptic slide containing 1,600,000 individual wells and is able to sequence 25 million bases with over 99% accuracy in a 4 h run. The shotgun sequencing and *de novo* assembling of *Mycoplasma genitalium* genome was utilized to determine its accuracy and robustness. The results showed that the 454 pyrosequencing system has 96% coverage at 99.96% accuracy in one run. Another sequencing platform, Ion Torrent semiconductor sequencing, converts the chemical reaction of base incorporation into a strand of DNA into digital data captured on a semiconductor chip. The polony sequencing method was developed by Shendure et al. (2005). This approach converts an epifluorescence microscope to an automated rapid non-electrophoretic DNA sequencing technology. Shendure et al. (2005) demonstrated the utility of this method by developing a pipeline from library construction, amplification, and sequencing. They demonstrated that this approach had "less than one error per million consensus bases" with a cost of one-ninth as much as conventional sequencing (Shendure et al. 2005).

2.13 Metagenomics

Two major metagenomic approaches, shotgun sequencing and targeted sequencing of specific genes, namely the 16S rRNA genes, are used to generate a profile of the microbial population found in a sample, such as environmental or food. Without any means of culture enrichment, the sequence data generated corresponding to each member of the microbial community will be dependent upon the percent abundance of each microbe present. Thus, detectable levels of a foodborne pathogen will be limited by the depth of sequencing obtained using a particular sequencing protocol and platform. Downstream bioinformatics and computational analysis of 16S rRNA sequence data will allow the identification of members of a microbial community, while shotgun metagenomic sequencing data can be used for assembly, gene prediction, taxonomy identification/phylogenetic trees, and gene function annotation. With such a large volume of data generated via NGS, most laboratories have established bioinformatic analysis pipelines to handle the sequence analyses. An example of the metagenomic analysis of a food product was accomplished by a CID with dietary supplements with intentionally added live microbes (Patro et al., 2016).

2.14 RNA Characterization

Two major approaches have been developed to analyze RNA in cells and viruses. Transcriptomics, as the name implies, is the study of the RNA transcript population in a cell that results from gene expression under specified conditions. A common means to identify transcripts is to capture these molecules on microarrays. The underlying basis of the technology is the hybridization of fluorescently labeled cDNA to known oligonucleotide probes spotted on the microarray.

Another means to analyze the transcriptome of bacterial cells and viruses is RNA sequencing (RNA-Seq) (Wang et al., 2009). This method relies on RNA fragmentation via hydrolysis or nebulization followed by the generation of double-stranded cDNA libraries that are then sequenced using high-throughput NGS technology. It has a significant advantage compared with microarrays in terms of increased sensitivity, a broader dynamic range, and the ability to detect novel transcripts. An exquisite use of RNA-Seq is to investigate the response of the pathogen to environmental conditions. For instance, the genetic determinants that allow a microbe to adapt to a host or persist or survive under specific environmental conditions, for example low moisture foods, can be identified. With a combination of RNA-Seq and quantitative reverse transcriptase PCR, genes were identified from *Salmonella* spp. that were differentially expressed during growth on different vegetables, including sprouts, leafy salad, spinach, and lettuce, compared with cells grown in minimal medium (Brankatschk et al., 2014). This technique has also been used to analyze the transcriptomes of sweet potato (Wang et al., 2010).

2.15 Protein-Based Methods

In some instances, the target for detection is not a viable microorganism but products made by microbes, such as toxins. Assays have been developed that utilize primarily antibodies directed at specific epitopes on the toxins and, in some instances, the microbial organism. Several platforms are available to conduct tests for the presence of bacterial and fungal toxins as well as foodborne pathogens such as *Salmonella*.

2.15.1 Enzyme-Linked Immunosorbent Assay

The underlying principle for the immunological-based method, enzyme-linked immunosorbent assay (ELISA), is the attachment of a capture antibody to the intended antigen or analyte followed by the addition of a detection antibody that is enzyme conjugated. After appropriate washing, a substrate

is added and a color formation is produced from the enzymatic reaction. Two common enzymes used in these reactions are horseradish peroxidase and alkaline phosphatase. In this sandwich format, the antigen is bound (sandwiched) between the two antibodies. As a means to improve the efficacy of immuno-based assays, streptavidin/avidin-biotin is often used.

The detection of pathogenic foodborne bacteria by ELISA has been developed for *V. parahaemolyticus* in seafood (Kumar et al., 2011), targeting the thermostable direct hemolysin (TDH)-related hemolysin. A common application of ELISA is testing foods for the presence of staphylococcal enterotoxins A, B, C, and E; *Clostridium perfringens* α, β, and ε toxin (Aschfalk and Müller, 2002); *Clostridium botulinum* toxins (Hansbauer et al., 2016); *B. cereus* toxins (Tallent et al., 2015); and *E. coli* Shiga toxins (Gehring et al., 2014). Commercially available kits target *Salmonella*, *L. monocytogenes*, *B. cereus* emetic toxin-cereulide (peptide), and *E. coli* O157:H7 in different food matrices, such as pork, fruits, vegetables, cheese, fish, and beef.

An alternative form of antibody-antigen agglutination is reverse passive latex agglutination (RPLA) in which antibodies are attached to latex particles and react to antigens present in the food sample. Commercial kits are available to test foods for the presence of staphylococcal enterotoxins A, B, C, and D using this format. The sensitivities of these assays run from 10^3 to 10^5 CFU/g without any enrichment of the food sample to less than 2 CFU/g and for proteins or toxin targets, a few nanograms per milliliter^{-1}.

2.15.2 Lateral Flow Immunoassays

Another antibody-based detection system for foodborne pathogens is the lateral flow immunoassays with two used platforms: dipstick and immunochromatographic strips. The basic design of a lateral flow device is composed of a sample pad where the sample fluid is applied. As the liquid moves through the nitrocellulose membranes of the device via capillary action, eventually the analyte interacts with either an antibody or an antigen (conjugate) that is labeled with a colored reagent. This interaction occurs at the test lines in the nitrocellulose membrane and if an antigen–antibody complex does occur, this would indicate that the tested analyte is present. Commercial immunochromatographic test strips are available for the detection of foodborne bacterial pathogens, such as *Listeria*, *Salmonella*, and *E. coli* O157. Although these lateral flow immunoassays are simple, easy to use, and rapid, they are not feasible for high-throughput analysis.

2.15.3 Immunomagnetic Separation

Immunomagnetic separation (IMS) is the application of antibodies to a specific bacterial or viral pathogen that is used as a means to concentrate the target microbe from an enriched food sample. This approach affords the analytical laboratory a higher probability of detecting the pathogen in a food

sample. Typically, immunomagnetic beads are coated with antibodies and mixed with the targeted pathogen, and this complex is concentrated by a magnetic force. Thereafter, the immunoprecipitated microbe can be analyzed by conventional bacteriological means (plating) or a variety of nucleic acid–based methods, such as PCR.

2.15.4 Fungal Targets

Mycotoxins that are a concern for food safety primarily are products of three fungal genera: *Aspergillus*, *Fusarium*, and *Penicillium*. Aflatoxins are secondary metabolites from *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* that are toxic and carcinogenic compounds. There are approximately 20 aflatoxins found in foods, such as peanuts and maize. *Fusarium graminearum* produce trichothecene mycotoxins that are quite destructive to plants; they are present on the crop plant before or just after harvesting. The mycotoxins produced by *Aspergillus* and *Penicillium* species are generally found in food products during drying and storage. Another fungal toxin of concern is ochratoxin A, produced by *Penicillium* and *Aspergillus* species. This mycotoxin is a class B carcinogen in humans and is known to be nephrotoxic, immunotoxic, and teratogenic (Pfohl-Leszkowicz and Manderville, 2012).

The detection of aflatoxins in foods uses an immunological-based method, ELISA, and immunochromatographic test strips. The advantages of using ELISA protocols are that they are easy to use, inexpensive, rapid, and sensitive. Either polyclonal or monoclonal antibodies can be used to detect the toxins. The former are relatively inexpensive to produce, whereas monoclonals are more specific and can provide more consistent results. However, other chromatography-based methods, for example, high performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), offer the same level of sensitivity as ELISA, but the latter lacks the ability to quantify the amount of toxin present (Iqbal et al., 2014). Alternative nucleic acid detection methods to protein-based technology have also been published that incorporate LAMP (Luo et al., 2014) and RT and reverse transcription PCR (Mahmoud, 2015). However, the ability to discern the production of the toxin, rather than just the presence of the aflatoxin genes, was more reliant on reverse transcription PCR than the other methods (Mahmoud, 2015).

2.16 Summary

The rapid, sensitive, and accurate detection and identification of foodborne pathogens remain a critical objective to ensure a safe food supply. Although culture-based isolation methods still prevail and are considered the gold

standard for food analysis, CID testing has made significant inroads into the analytical laboratories. For the latter, these techniques provide depth of information suitable to accurately identify the microbial pathogen. Furthermore, its use circumvents the issue of detecting and identifying those microbes that are not currently culturable in the laboratory. Norovirus, human parasitic protozoa such as *Cyclospora*, and microbes that have been physiologically impaired are examples in which there presently no means to either propagate or resuscitate these organisms from food samples.

Advancement in molecular-based technology since the days of DNA/colony hybridization has certainly impacted the pace and accuracy of detecting and identifying microbial pathogens in foods.

Although PCR, and now the NGS era, have marked significant progress in detection technology, most of these methods require expensive equipment, reagents, and, with respect to NGS, a high level of computation and bioinformatic expertise. However, molecular technology lends itself to miniaturization, and, with current efforts, the development and implementation of handheld devices could be the next wave of tools for pathogen detection (Nugen and Baeumner, 2008). The adaptation of molecular-based protocols should address the needs of the end user, such as the food industry (is the final food product safe to consume?), regulatory agencies (safe foods, source traceback in outbreaks, and surveillance programs), and the consumer, who expects that foods marketed are safe to eat, nutritious, and wholesome. Other applications for molecular-based tests can be in identification strategies to discriminate one genus from another and strain-specific identification. In this manner, they can serve as an important tool for differentiating and identifying strains used in food production and in foods that have the intentional addition of live microbes. Table 2.1 provides a quick reference to the commonly used molecular-based methods, with some advantages and disadvantages associated with each.

The application of high-throughput "omics," that is, genomics and transcriptomics (RNA-Seq), has impacted the analysis of foods on several levels. Culture-independent protocols can provide rapid analysis of foods, and the resulting *in silico* data of pathogenic and non-pathogenic microbes can provide deep phylogenetic analyses of microbial populations present. These types of data can elucidate the means by which pathogens emerge and adapt to their environment or host, as well as providing some insight into their route and history of transmission. Of note, the cost and improvements made in sequencing platforms have certainly made NGS an attractive means to analyze foods, perform comparative genomics for epidemiological studies, and undertake traceback analysis in foodborne outbreaks. However, currently, there is a range of challenges confronting this technology. These include processing ability and time to accomplish analysis with large data sets, storage, and standards to define acceptable levels of base-calling accuracy, coverage, and quality of reads. In addition, not every bioinformatic algorithm used for sequence analysis, whether commercially available or developed in-house, will generate the same output data in regard to sensitivity and accuracy.

TABLE 2.1

Overview of Molecular Methods Used to Detect and Identify Foodborne Pathogens

Molecular Method	Description	Advantages	Limitations
Nucleic Acid–Based			
Colony hybridization	Hybridize labeled (radioactive or non-radioactive) probe to lysed bacterial cells	Isolated colony needed (live cells)	Not rapid; much hands-on needed
PCR	*In vitro* amplification system with different platforms; conventional, real-time, reverse transcription, isothermal, digital, loop mediated	Can be very specific for pathogen detection and identification; high sensitivity; real-time PCR can be rapid; mainstay in most analytical laboratories; can be automated, multiplexed; some formats (isothermal) use inexpensive equipment	Does not distinguish between live or dead cells unless PMA or EMA is added; can be inhibited by food components; real-time PCR requires expensive instrument; easy to cross-contaminate
DNA fingerprinting	PFGE was the gold standard for pathogen identification and relatedness; other protocols include MLST and ribotyping	Standard PFGE protocols enabled analytical laboratories worldwide to compare profiles and track foodborne pathogens/outbreaks; MSLT applicable for phylogenetic and epidemiological analyses	PFGE requires 4–5 days for completion; poor resolution with some pathogens
Microarray	Oligonucleotides can be generated to detect specific regions in pathogen genome; allows highly specific hybridization with targeted microbe	Has high specificity; multiple pathogen detection possible; serotype identification; not labor intensive; some platforms can be high-throughput	Expensive equipment and reagents
Aptamers	Single-stranded synthetic oligonucleotides that when folded (3-D structure) bind to target molecules	High affinity and specificity to target molecules; are used to detect toxins, pathogens	Can be a protracted process (SELEX) to produce aptamers of choice

(*Continued*)

TABLE 2.1 (CONTINUED)

Overview of Molecular Methods Used to Detect and Identify Foodborne Pathogens

Molecular Method	Description	Advantages	Limitations
Sequencing	From Maxim–Gilbert to Sanger dideoxy protocols to current platforms that yield whole bacterial genome sequences	Relatively short time period (4–5 days) to achieve the sequence of the entire bacterial genome: Bacteria identity, serotype, virulence potential, resistance profile, protein analysis	Time-consuming for rapid diagnostics; expensive; technically challenging
Metagenomics	A broad stroke sequence analysis of the entire bacterial community present in analyzed sample	Approach can identify most, if not all, bacteria present in food samples	If below the threshold for identification, possibility to yield a false negative result for pathogen identification
RNA-based methods	Target macromolecule is RNA; PCR-based assays use reverse transcriptase to generate cDNA	Has been used to determine viability of bacterial cells; used to detect and identify foodborne viruses (e.g., norovirus)	RNA is more unstable than DNA
Protein-based enzyme-linked immunosorbent assay (ELISA)	Primarily based on antibody–antigen interaction	Used to detect non-culturable microbes, e.g., viruses, and toxins such as staphylococcal enterotoxins; easy to use; has been automated	Not as sensitive as some nucleic acid technology; preenrichment may be necessary; specialized equipment is needed; may require enrichment to achieve detectable levels; cross-reactivity between closely related microbes possible
Lateral flow immunoassays	Same principle as ELISA but in different format	Easy to use; no expensive equipment required; colorometric end result provides easy read; relatively inexpensive; rapid	Lack of sensitivity; may require enrichment to achieve detectable levels
Immunomagnetic separation	Typically magnetic beads coated with specific antibodies to capture targeted cells as a means of concentrating bacteria from a liquid culture or diluted food matrix	With appropriate antibodies, can be used to concentrate target microbes, thereby reducing time of analysis and increasing chance of detecting pathogen	Requires antibodies to each specific target pathogen; may require expensive instrument

The impact of CID methods on food safety is reflected in a recent publication by the United States Centers for Disease Control and Prevention (Huang et al., 2016). In the United States, FoodNet, the Foodborne Diseases Active Surveillance Network, is an active surveillance mechanism to monitor laboratory-confirmed foodborne infections caused by nine pathogens. According to this publication, there is a shift from the paradigm to isolate a pathogen via culture-based methods toward CID tests. As noted by these authors, the transition to CID methods provides some advantages, such as faster diagnosis and, perhaps, strains characteristics that will not only impact food safety but also enable medical personnel to administer the appropriate treatment for infection. Lastly, the impact of public health policies and practices has been noted, particularly in regard to the ability to identify the pathogen more quickly and ascertain its pathogenicity potential, such as with STECs, and the microbes' antibiotic resistance profile.

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Molecular Recognition: Versatility and Challenges in the Design of Nanobiosensors for Food Security

Mihaela Puiu and Camelia Bala

CONTENTS

3.1 Biosensing for Food Control: What's Next?

Food safety is a global issue and a goal. Since agricultural crops, raw materials, and foodstuffs are unceasingly prone to contamination by various pathogens and toxins, fast and accurate methods are required along the food chain for the detection and monitoring of contaminants (Narsaiah et al. 2012). Even if most foodborne contamination is from bacteria, toxins also play a major role. Toxins usually occur naturally in the food supply, yet the contamination may occur before harvest, during harvesting and drying, and in storage. Furthermore, some toxins can persist even after rigorous food processing (Puiu et al. 2014, Taitt et al. 2008). It is generally accepted that food poisonings are caused mainly by protein toxins such as Staphylococcal enterotoxin B (SEB) and botulinum toxins (BoNTs) that have been secreted into contaminated foodstuffs by bacterial growth, while several fungi-produced mycotoxins are the most powerful natural carcinogens (Zheng et al. 2006). A major worldwide concern is raised by the group of molds and mycotoxins of *Aspergillus* spp. (producing aflatoxins B1, B2, G1, G2, M1, ochratoxin A (OTA), and patulin), *Fusarium* spp. (producing T-2 toxin, deoxynivalenol [DON], zearalenone [ZEN], and fumonisin B1 [FB1]), and *Penicillium* spp. (producing OTA) (Chauhan et al. 2016). The amount of toxin required to cause harm varies from toxin to toxin, for example, an OTA fatal ingested dose for humans is about 1 ng/kg (Puiu and Bala 2016a), while a similar dose of SEB would cause minor problems (Taitt et al. 2008).

A biosensor represents an integrated device consisting of a biological recognition species in direct contact with a transduction element (Figure 3.1). A transducer is a detector device that converts a biological response signal (change) resulting from interaction with the target analyte into a quantifiable electrical signal. The biological recognition element responds to the measured analyte, and the transducer converts this observed change into a measurable signal that is proportional to the concentration of the analyte (Ansari et al. 2010). Therefore, biosensors can be categorized according to the biological recognition element (immuno, enzymatic, nucleic acids, and whole-cell biosensors) or the signal transduction method (electrochemical, optical, mass-based, and thermal biosensors) (Wanekaya et al. 2008, Turner 2013).

Today, great importance is paid to "nanosized" biosensors, called "nanobiosensors," which may have a huge impact on quality control, food safety, and traceability (Pérez-López and Merkoçi 2011). Thereby, the newest multidisciplinary approaches involve nano-inspired sensing/biosensing devices, amenable to miniaturized instruments with rapid response and minimal sample preparation.

The latest advances in transducer technology at the micro/nanoscale corroborated with the integration of nano-biorecognition materials have resulted in more powerful tools for multiplex analysis and nanotracking

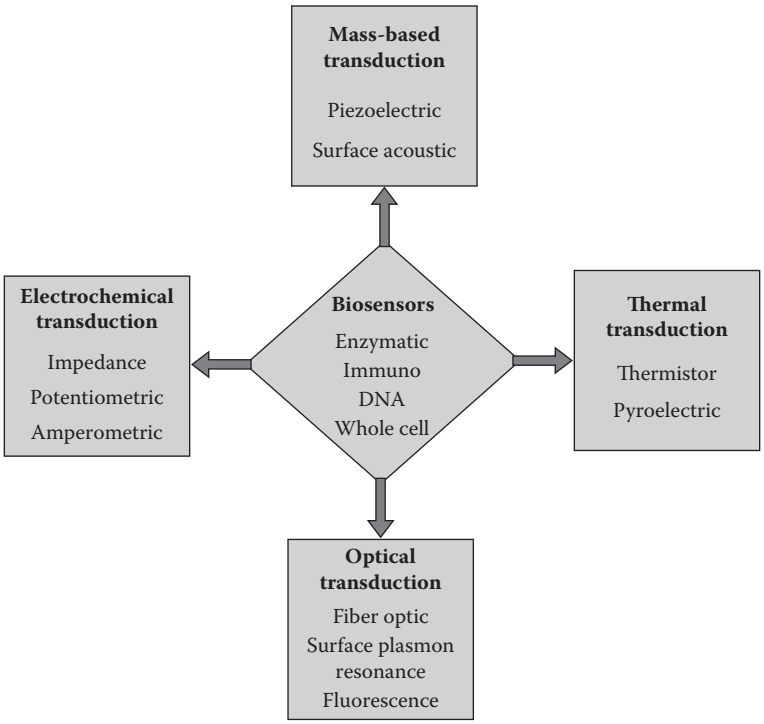

FIGURE 3.1
General classification of biosensors. (Reproduced from Wanekaya, A. K. et al., *J Environ Monitor*, 10 (6), 703–712, 2008 with permission of RSC Publishing.)

systems able to fulfill the rapid monitoring and control need of the food chain (Tothill 2011, Wang and Dai 2015). Nevertheless, the technology is still under development and it may take time before real commercial products are available on the market. Nanotechnology lies at the boundary of biology, chemistry material sciences, physics, and engineering. Here, the high precision provided by nanostructured materials and molecular manipulation are connected. The unprecedented properties of nanomaterials, which are highly different from the bulk (controllable size, shape, surface charge, and physiochemical characteristics), enhance the biosensor performances by increasing sensitivity and lowering detection limit with several orders of magnitude (Chen and Chatterjee 2013, Holzinger et al. 2014). Moreover, since they display high specific surface, nanomaterials can be easily functionalized with increased amounts of bioreceptor elements in order to achieve better biocompatibility and specific targeting (Hayat et al. 2014, Puiu and Bala 2016b). These features have made it possible to incorporate nanomaterials in lab-on-a-chip devices for real-time pathogen and toxin detection. Lab-on-a-chip (LOC) is a device that integrates several laboratory functions on one small platform, typically only millimeters or centimeters in size (Figure 3.2)

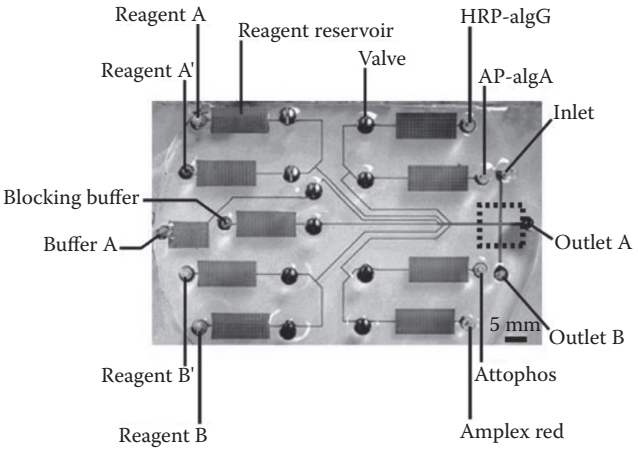

FIGURE 3.2
A lab-on-a-chip contains a network of channels and wells (Attophos is a highly sensitive fluorimetric substrate for the detection of alkaline phosphatase, and Amplex red is a fluorogenic substrate for peroxidase). (Reproduced with permission from Yoon, J-Y. and Kim, B., *Sensors*, 12 (8), 10713, 2012, licensed under CC by 4.0.)

(Yoon and Kim 2012). A LOC device usually involves the handling of very small fluid volumes; this introduces the area of "microfluidics" dealing with the control and manipulation of fluids constrained to a small, sub-millimeter scale.

Lab-on-a-chip enables sample handling, mixing, and detection on a single integrated system (Estevez et al. 2012). These devices can be cost-effective and highly beneficial for the food industry in ensuring high safety and quality of food. The sensing capacity of the detection systems is being improved by using nanomaterials and nanostructures such as magnetic nanoparticles (MNPs), carbon nanotubes (CNTs), nanorods (NRs), dendrimers, quantum dots (QDs), nanowires (NWs), nanochannels (NCs), noble metals nanoparticles (AuNP, AgNP), and nanoshells (Pérez-López and Merkoçi 2011); therefore, the area of biosensing applications for food contaminant detection is expanding rapidly.

Overall, the integration of nanobiosensors with the microfluidic systems creates a powerful analytical tool that will be an advanced step toward the high-throughput screening of toxins in the field, which will benefit both developing and developed countries (Luka et al. 2015). With the advent of nanobiosensor integration in microfluidic systems for *multiplexed* detection of toxins, several important aspects need to be considered, such as type of transduction, signal increase, noise decrease, fluidics design (sample injection and drainage, reduction of sample consumption, etc.), surface immobilization chemistry (analyte capture efficiency, elimination of non-specific

binding, etc.), detection format (direct binding, sandwich-type binding, competitive binding, etc.), and processing (information regarding analyte concentration, affinity, binding kinetics, etc.) (Narsaiah et al. 2012). First, the bioreceptor molecule is immobilized in a suitable matrix to form a biorecognition layer, which is then placed in the immediate vicinity of a transducer. Then, the reactive surface of biorecognition elements generates a measured response upon binding with an analyte; the transducer itself then converts the recognition event into a measurable optical or electrical signal (Hunt and Armani 2010, Rackus et al. 2015). In this case, the recognition element is responsible for the transducer's ability to selectively detect the target analyte (Cella et al. 2010). The immobilization of the receptor molecule on the sensor surface is a key point for the performance characteristics of the biosensor (Putzbach and Ronkainen 2013, Villaverde 2003). These characteristics include the limit of detection (LOD), the full-scale output (FSO), linearity, and hysteresis. The LOD represents the minimum detectable concentration of an analyte (Chambers et al. 2008), which is dependent also on the noise of the system. The FSO is the difference in output between the highest and lowest values. Linearity describes closeness of the sensor's calibration curve to a straight line and is expressed as a percentage of the FSO. Hysteresis is the maximum difference in the forward and reverse response of the sensor. Additional parameters include saturation and selectivity. Saturation is the point at which there is no further output signal, even if more analyte reaches the biosensor's surface. Selectivity describes the suppression of incorrect signals, such as binding of incorrect biological molecules or environmental interference (Hunt and Armani 2010). The repeatability/reproducibility of the biosensor describes how the measured signal changes if the same measurement is performed under the same conditions. Finally, the performances of the latest developed biosensors were increasingly improved due to the progress of surface chemistry and physics; the design of more efficient and highly selective surfaces at the nanometer scale resulted in miniaturized sensing devices with high sensitivity and selectivity (Kim and Kang 2008).

3.2 Molecular Recognition and Bioassay Formats

The biorecognition element dictates the specificity and selectivity that ultimately allows the biosensor to respond to a specific target or group of analytes, minimizing interferences with non-target compounds (Luka et al. 2015). As previously mentioned, the functionalization of the sensing surface (transducers or label nanoparticles) can be achieved through the attachment of organic molecules via physical adsorption, covalent bonding, or biochemical interaction (Kim and Kang 2008). The choice of

the functionalization format depends on the type of transduction of the output signal (i.e., electrochemical, optical absorption, fluorescence, surface plasmon resonance [SPR], piezoelectric, or magnetic) (Puiu et al. 2014). Both functionalization and signal transduction can be improved through the use of nanomaterials—carbon nanotubes (CNTs) and graphene in electrochemical systems and nanoplasmonic structures in optical systems (Liu et al. 2012). The goal of employing nanomaterials for immobilization on a transducer surface is to reduce diffusion limitations and maximize the surface area to increase biomolecule loading (Zhu et al. 2015). Nanomaterials have a strong tendency to adsorb biomolecules and, therefore, are helpful in the immobilization of biomolecules onto the transducer's surface. The adsorption of biomolecules directly onto bulk materials may result in denaturation and loss of bioactivity, while nanosized materials preserve the bioactivity of biomolecules. It is of great importance that several nanoparticles carry charges providing electrostatic surface to attach the biomolecules with different charges (Hayat et al. 2014). According to the type of recognition elements immobilized onto the transducer's surface, biosensors are classified as

- Catalytic biosensors that utilize enzymes, cells, tissues/organelles, and microorganisms as the recognition element (Pearson et al. 2000)
- Affinity biosensors that utilize entire antibodies or antibody fragments, nucleic acids/aptamers, lectins, molecularly imprinted polymers, and novel engineered, scaffold-derived binding proteins as the recognition agent (Lee et al. 2008, Yun et al. 2009)

The selection of the biological recognition element depends on the target analyte (e.g., antibodies and aptamers are more suitable for the detection of bacteria or pathogens, whereas enzymes are more suitable for catalytic reactions) (Luka et al. 2015).

The affinity biosensors are designed to maximize association and minimize dissociation of target analytes; often, these sensors become saturated and may not provide kinetic information about fluctuations in the level of the analyte over time. On the other hand, enzyme-based biosensors are based on enzymatic turnover and, therefore, can be used to record changes in analyte concentration over time (Liu et al. 2012). Enzymes are optimal biorecognition molecules for electrochemical sensors, because the enzyme layer catalyzes the production or depletion of an electro-active species; when a voltage is applied to the electrode, for example, in amperometric sensors, a redox reaction of the electro-active species is induced, generating a measurable signal (Ronkainen et al. 2010) (Figure 3.3). This electrical signal is correlated with the concentration of analyte in the sample. A variation in electrode potential can also be used as the measurable transducer response in potentiometric sensors. Finally, a signal processor connected

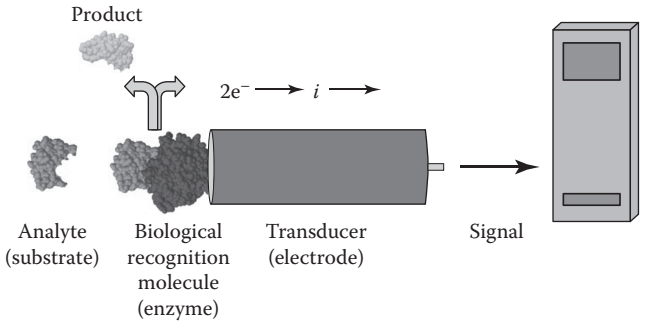

FIGURE 3.3
Schematic representation of an enzyme biosensor. (Reproduced with permission from Putzbach, W. and Ronkainen, N., *Sensors*, 13 (4), 4811, 2013, licensed under CC by 4.0.)

to a transducer collects, amplifies, and displays the signal. Electrochemical detection also offers additional selectivity as different electroactive molecules can be oxidized/reduced at different potentials (Putzbach and Ronkainen 2013). The main challenge in developing enzyme-based biosensors is to overcome the often inefficient electron transfer between the enzyme and the electrode surface (Marcus and Sutin 1985). This is generally due to the redox active site being embedded deep within the different regions of the enzyme and the inability of the enzyme to orient itself favorably with respect to the electrode surface for fast and efficient electron transfer (Putzbach and Ronkainen 2013).

Enzyme-based assays are typically achieved as direct or indirect format. In the direct format, the analyte increases the catalytic activity of an enzyme (either acting as a co-factor for the enzyme or in tandem with an affinity binding event to confine the enzyme near the analyte), in a reaction where a measurable product is formed (the analyte concentration is proportional to the signal) (Rackus et al. 2015). In the indirect format, the analyte acts as an enzyme inhibitor, decreasing the rate of the measurable product formation; here, the analyte concentration is inversely proportional to signal (Newman and Setford 2006).

Immunosensors are affinity-based biosensors relying on the antibody/antigen specific interaction. Immunoassays are implemented in several formats, including

- Direct format, following the binding of an unlabeled antigen to an unlabeled antibody
- Competitive format, following the competition for binding of an unlabeled (target) antigen to a labeled antigen to an antibody (Rackus et al. 2015, Mitchell 2010)

- "Sandwich" format featuring an antigen with two epitopes (antibody-recognition sites) that binds to an immobilized primary antibody and also to a labeled- or enzyme-modified secondary antibody (when the secondary antibody is enzyme-modified, the technique is known as an "enzyme-linked immunosorbent assay" or ELISA) (Darwish 2006)
- Inhibition format featuring competition between an analyte and a primary antibody for binding to a labeled (or enzyme-modified) secondary antibody (Figure 3.4) (Sapsford et al. 2002)

Nucleic acid–based biosensors are affinity sensors that exploit the sequence-specific Watson–Crick base pairing between nucleic acids and their complements (Rackus et al. 2015). The most common forms of nucleic acid sensors are formed from a single-stranded DNA (ss-DNA) probe that is immobilized onto the surface of a transducer (Wang 2002). Upon recognition of its complementary ss-DNA or RNA analyte (or target) by hybridization, transduction is facilitated by electrochemical, optical, or mass-sensitive

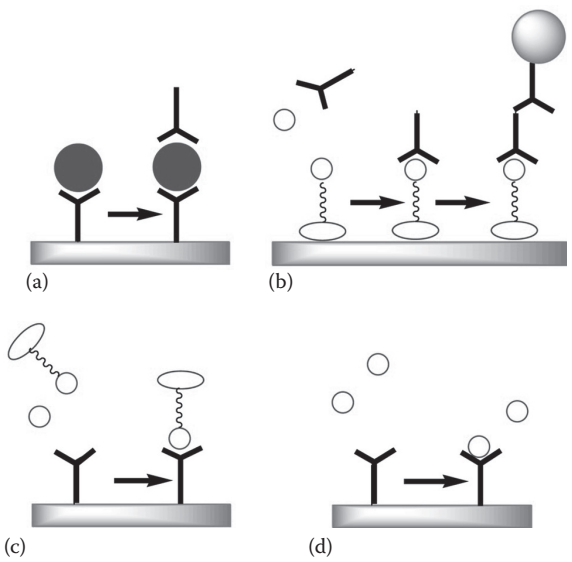

FIGURE 3.4
Immunoassay formats according to the analyte type: (a) sandwich immunoassay for large molecules, (b) protein conjugate immobilized indirect inhibition immunoassay with optional secondary antibody-gold nanoparticle labeling in a second step, (c) protein-labeled inhibition immunoassay, (d) direct small molecule immunoassay. (Reproduced with permission from Mitchell, J., *Sensors*, 10 (8), 7323, 2010, licensed under CC by 4.0.)

techniques. The DNA probe–DNA target hybridization is exploited through the following formats:

- One assay uses peptide nucleic acid (PNA) probes in which the negatively charged sugar-phosphate backbone of DNA is replaced by a neutral pseudopeptide chain. PNA probes have higher binding affinities (relative to their analogous ss-DNA probes) for ss-DNA targets, and the reduced charges on these probes confer advantages for some forms of electroanalytical techniques.
- "Sandwich" assay, when an immobilized probe binds a specific region of an analyte, and a second, labeled probe binds a different region of the analyte.
- "Molecular beacon" assay featuring probe-sequences that form stem-and-loop or hairpin structures through self-binding (Figure 3.5). Complementary targets compete for binding with such structures (requiring the probe to undergo a change in conformation), which can enable very sensitive detection of small numbers of targets. The most useful nucleic acid biosensors allow for differentiation between the binding of a target that is perfectly complementary to the probe and a target that has a one base-pair mismatch with the probe (Rackus et al. 2015).

Aptamer-based biosensors feature an alternative form of affinity biorecognition relying on synthetic oligonucleotide (single-stranded DNA or RNA molecules) probes; in contrast to conventional nucleic acid sensors (which bind only their complements), aptamers can be designed to bind any type of target. Aptamers are prepared by a combinatorial approach called systematic evolution of ligands by exponential enrichment (SELEX) (Blind and Blank 2015). SELEX is an iterative process in which a library of oligonucleotides with varying sequences is generated, and the population that binds best to

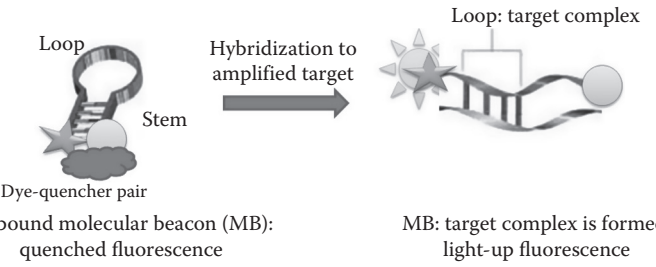

FIGURE 3.5
Detection of amplified DNA by molecular beacon (MB). (Reproduced with permission from Astakhova, K., *Chemosensors*, 2 (3), 193, 2014, licensed under CC by 4.0.)

a specific target is selected and isolated. The best binding sequence(s) then serve(s) as the basis to generate new sequences, to generate a product with high affinity binding to the target of interest. In most assays, the aptamers are modified with electroactive indicators, fluorescent tags, nanoparticles, and enzymes (Rackus et al. 2015).

3.2.1 Affinity Analysis

Affinity biosensors are based on a probe binding a target analyte and can thus be treated in terms of receptor-ligand binding theory (Leech 1994). The affinity of the analyte for the immobilized probe (or biorecognition element) can be estimated using the Langmuir adsorption isotherm. The Langmuir isotherm describes surface binding for identical non-interacting binding sites (Daniels and Pourmand 2007). For a simple bimolecular interaction with molecules A (analyte) and B (biorecognition element or probe) forming the complex AB, the equilibrium association constant (or affinity constant) K_a and dissociation constant K_d are given by Equations 3.1 and 3.2 (Puiu et al. 2012).

$$K_a = \frac{[\mathrm{AB}]_{eq}}{[\mathrm{A}]_{eq}[\mathrm{B}]_{eq}}\left(\mathrm{M}^{-1}\right) \tag{3.1}$$

$$K_d = \frac{1}{K_a}(\mathrm{M}) \tag{3.2}$$

The fraction of probe bound at equilibrium (θ) is determined by the relative values of the dissociation constant K_d and analyte concentration [A].

$$\theta = \frac{[AB]}{[B]+[AB]} = \frac{[A]}{K_d+[A]} \tag{3.3}$$

where [AB] and [B] represent the surface densities of the biorecognition element/analyte complex and the unbound biorecognition element. Since the sensor's response is directly proportional to the amount of the bound analyte, the normalized response of the sensor R_{norm} (calculate as the ratio of the equilibrium response of a certain analyte concentration R and the sensor's response when all the binding sites are saturated) is also equal to θ. Therefore, the concentration of the analyte can be calculated from the linear part of the Langmuir plot R norm versus concentration of the analyte (Puiu et al. 2012). Increasing the surface density [B] leaves θ unchanged but allows the measured surface property change—typically related to the actual density of target molecules bound [AB]—to increase. However, higher probe densities may inhibit the analyte binding due to steric hindrance or mass transport limitations (Daniels and Pourmand 2007). Despite the ability to

monitor real-time biointeractions, the majority of affinity assays make measurements only after equilibrium has been reached. However, kinetic considerations are particularly important at low concentrations, where detection limits are usually determined (Kusnezow, Syagailo, Goychuk et al. 2006) and when analyte diffusion to the probe surface takes longer than the binding interaction (Daniels and Pourmand 2007, Kusnezow, Syagailo, Rüffer et al. 2006).

3.3 Immobilization of Bioreceptors: Design of Tunable Sensing Surfaces

The main questions for both catalytic and affinity biosensors are how recognition moieties are immobilized on specific transducers and how the nanomaterials are incorporated into the sensor devices. Prior to immobilization of bioreceptors onto the transducer's surface (or onto nanostructures), one needs to create an organic layer able to capture the biorecognition element and to bind to the surface. The progress of surface chemistry has led to the design of more efficient and selective surfaces, ultimately accounting for the particular features of the transducers and nanostructures. In the last decade, continuous emphasis was put on enzyme and immunosensors and also on the emergence of new recognition approaches, particularly those employing aptamers and phages. Currently, research into enzyme-based biosensing systems is focused on the areas of (1) enzyme immobilization; (2) integration of enzymes with specific nanostructures such as AuNPs, CNTs, and graphene to enhance electron transfer properties (Chen and Chatterjee 2013); and (3) enzyme engineering to improve selectivity, stability, and immobilization (Pérez-López and Merkoçi 2011, Holzinger et al. 2014). For affinity biosensors, beside antibody-based approaches, attention is paid to (1) aptamer-based biosensors where the most activity is in designing various aptamer structures and conjugation of aptamers with different physiochemical transducers (Cella et al. 2010, Labuda et al. 2010), and (2) phages as a specific biorecognition structure that improves the specificity and the stability of the biosensor (Liu et al. 2012). According to the chemical composition, almost all nanomaterials can be equipped with suitable functions through direct functionalization (sometimes already during synthesis), or via coating with functional polymers without affecting their specific properties (Holzinger et al. 2014). Figure 3.6 depicts the main pathways to functionalize nanomaterials with biological elements.

Efficient methods for the bio-functionalization of nanomaterials are depicted in several excellent works (Hunt and Armani 2010, Hayat et al. 2014, Kim and Kang 2008, Putzbach and Ronkainen 2013). Briefly, non-covalent approaches are represented by electrostatic interactions, π–π stacking,

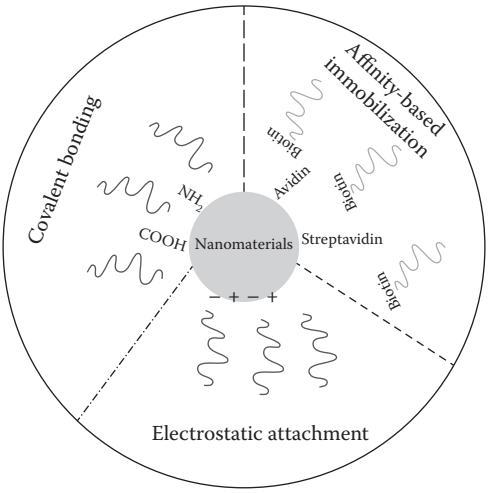

FIGURE 3.6
Nanomaterials-based immobilization strategies. (Reproduced with permission from Hayat, A. et al., *Sensors*, 14 (12), 23439, 2014, licensed under CC by 4.0.)

entrapment in polymers, or van der Waals forces between the nanomaterial and the biological element. Covalent binding or the strategy to covalently anchor biomolecules to nanomaterials has an advantage in terms of stability and reproducibility of the surface functionalization and lowers unspecific physical absorption. Covalent links can be formed, for example, by classic amide coupling reactions, cross-linking, or "click-chemistry." One drawback is the uncontrolled anchoring of the biomolecule that can affect the domain that is responsible for the recognition event. Other immobilization strategies that raised great interest include the immobilization via supramolecular or coordinative interactions—the biotin/avidin (or streptavidin) pair (Wilchek and Bayer 1988). Biotinylated biomolecules can be attached to biotinylated substrates via avidin (or streptavidin) bridges (Figure 3.7). Other reported affinity systems were nitrilotriacetic acid (NTA)/Cu^{2+}/histidine complex (Holzinger et al. 2014).

3.3.1 Integration of Biorecognition Elements with Gold Nanoparticles and Gold Supports

One of the most studied nanomaterials in biosensor approaches is colloidal gold. It is manufactured from small octahedral units called primary units. The AuNPs' size and morphology depend on the employed method of synthesis (Putzbach and Ronkainen 2013, Holzinger et al. 2014). The gold nanoparticles are usually stored in an aqueous solution. An interesting optical property of AuNPs is that their optical absorption and scattering peaks

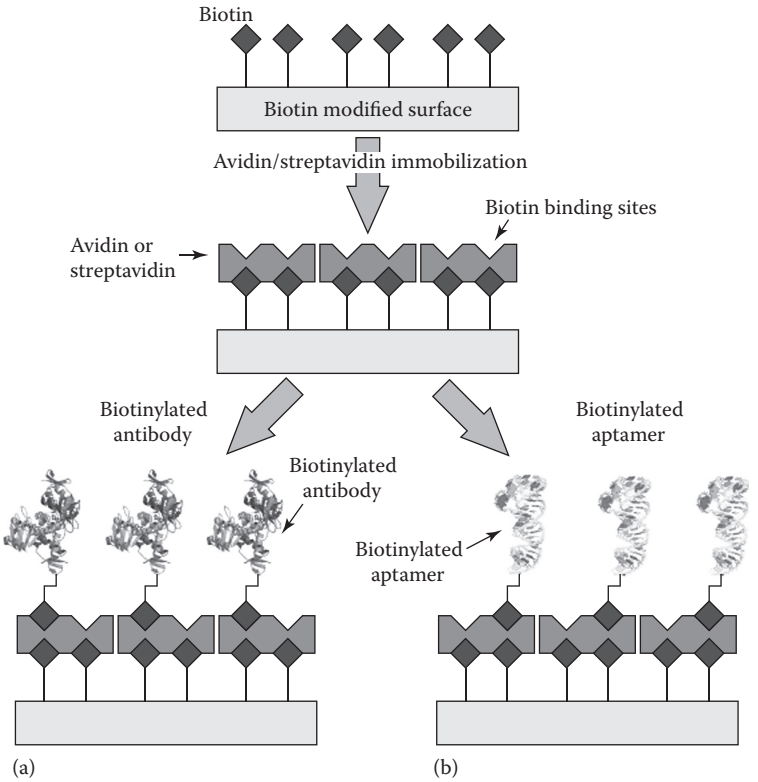

FIGURE 3.7
Receptor immobilization via biotin-avidin/streptavidin strategy. Initially, biotin is physically or covalently bound on a solid support and modified further with multivalent avidin or streptavidin. A biotin labeled antibody (a) or a biotin labeled aptamer (b) is then attached. (Reproduced with permission from Santos, A. et al., *J Anal Bioanal Tech*, S7, 016, 2014, licensed under CC by 4.0.)

can be tuned by varying their size (at around 20 nm range) and shape; in particular, these peaks can be put in the near-infrared (NIR) optical window (800–1300 nm) (Das et al. 2013). They are capable as well of efficiently transferring electrons between different electro-active species and electrodes. In addition, the light-scattering properties and extremely large enhancement ability of the local electromagnetic field enable AuNPs to be used as signal amplification tags. Modification with AuNPs allows the attached enzyme or antibody more freedom of orientation, weakening the protein layers that are covering the active/binding site (Justino et al. 2015). AuNPs have also demonstrated their advantages in the biosensing applications with SPR transduction. This method is usually based on the change of the dielectric constant of propagating surface plasmons' environment of gold films

where the detection of the analyte can be recorded in different ways like the changes of the angle, intensity, or phase of the reflected light (Puiu and Bala 2016b, Guo 2012). AuNPs possess the ability to form a transduction platform for single molecule detection by refractive index sensing of localized surface plasmon resonance (LSPR) coupled with enzyme-linked immunosorbent assay (ELISA) using isolated gold nanoparticles of 60 nm sizes. The model enzyme horseradish peroxidase (HRP) was immobilized onto AuNPs via biotin streptavidin linkage (Chen et al. 2011). Although gold is an inert metal, it was found that the high surface-volume ratio and surface properties (Benetti et al. 2013) and quantum-scale dimensions (Alivisatos 1996) provide colloidal gold with enhanced catalytic activity (Haruta 2004). This virtually eliminates the need for over-potentials.

3.3.1.1 Physical Adsorption onto AuNPs

Physical adsorption is a simple and fast method for developing enzyme biosensors. It involves reducing the AuNPs with a negatively charged ligand such as citrate. The reduced AuNPs are then allowed to associate with the ligand, insulating the GNPs from electrostatic repulsion and offering it stability. The resulting citrate layer imparts a negative charge onto the colloidal particle surface. Positively charged amino acid residues allow enzymes or antibodies in solution to be electrostatically adsorbed on the surface by solely dipping the modified electrode into the solution.

However, physical adsorption can result in monolayers of randomly oriented recognition elements, leading to a reduction in functionality (Hunt and Armani 2010). Moreover, this technique creates a weak attachment of the recognition element to the surface, which could be disadvantageous for biosensors that are part of a flow-through cell (Luppa et al. 2001).

3.3.1.2 Chemisorption and Covalent Attachment onto Self-Assembled Monolayers (SAMs)

Chemical adsorption involves direct covalent binding between the enzyme and the electrode or the AuNP surface. Chemisorption is achieved through the covalent interaction between the –SH groups of cysteine residues and Au on the AuNP surface (Holzinger et al. 2014). On the other hand, SAMs of amphiphilic molecules such as long-chain n-alkylthiols are highly ordered 2-dimensional structures that act as an interface between the surface and the recognition elements (Wink et al. 1997, Chaki and Vijayamohanan 2002). In this technique, the hydrophilic "head group" has a special affinity for the surface (such as thiols for gold), while the hydrophobic "tail group" can be attached to the recognition elements. Due to the nature of the self-assembly and the chemisorption, this technique can create stable and uniform surface coverages with oriented recognition elements that are generally more stable than physical absorbed films (Figure 3.8).

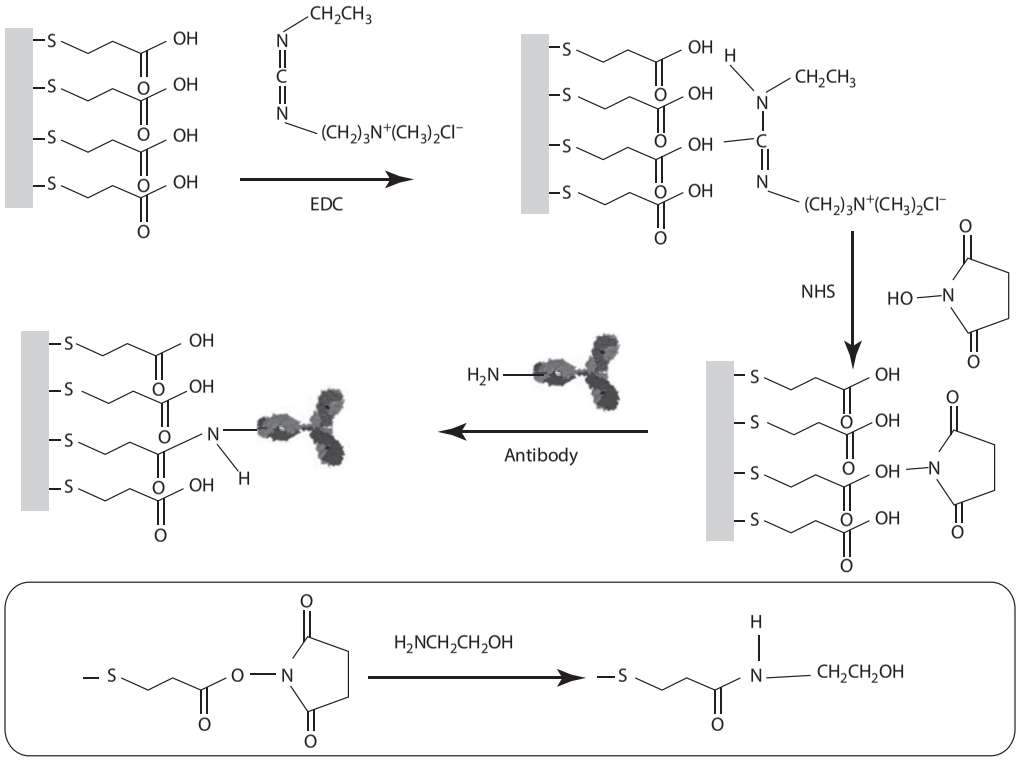

FIGURE 3.8
Immobilization of the anti-*Staphylococcus aureus* antibodies onto 3-Mercaptopropionic acid (MPA) SAM on gold through covalent binding via N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC)/N-hydroxysuccinimide (NHS) esters. After antibody attachment, the remaining activated carboxylic groups were blocked with ethanolamine. (Reproduced with permission from Braiek, M. et al., *Biosensors*, 2 (4), 417, 2012, licensed under CC by 4.0.)

Monolayers of this type can also be formed from heterobifunctional polymers, such as polyethylene glycol (PEG) subunits with thiol terminations (Gobi et al. 2007) as well as dendrimer-terminated n-alkylthiols (Hunt and Armani 2010).

3.3.2 Integration of Biomolecules with Carbon Nanotubes

3.3.2.1 Characteristics of Carbon Nanotubes

Carbon nanotubes (CNTs) are fullerene-related molecules, composed of graphene sheets that are wrapped into a cylindrical shape (Battigelli et al. 2013). They may be closed at either end with caps containing pentagonal rings, or they may be left open. Multi-wall carbon nanotubes (MWCNTs) follow the same layout as single-walled CNTs (SWCNTs), except that there are multiple layers of CNTs, each enclosing each other (Putzbach and Ronkainen 2013). Electrodes incorporating single- or multi-walled CNTs displayed faster electron transfer rates compared with that of traditional catalytic electrochemical biosensors. Because of their structural heterogeneity, the walls of the tubes have distinct properties from the ends of the tubes. Thus, the rate of electron transfer may be significantly affected by changing the orientation and arrangement of CNTs on the electrode surface (Dumitrescu et al. 2009). MWCNTs usually have 2–100 nm diameter with an internal diameter of 2–10 nm, while SWCNTs usually have about 0.2–2 nm diameter (Mehra et al. 2014). CNTs have a high surface area to weight ratio of 300 m^2/g, and most of this surface area is accessible to both electrochemistry and immobilization of biomolecules. CNTs themselves do not provide any measurable signals for sensing biomolecules. Thus, the development of functionalization methods that can endow the CNTs with both a molecular recognition and a signal transduction function is crucially required (Yoon and Kim 2012).

3.3.2.2 Non-Covalent Integration of Biorecognition Elements of CNTs

The non-covalent functionalization of CNTs is generally based on hydrophobic, π–π stacking and Van der Waals interactions among CNTs and recognition elements. Enzymes and antibodies exhibit mostly hydrophobic interactions, biopolymers and lipids show Van der Waals interaction; finally nucleic acids display π–π stacking (Mehra et al. 2014).

3.3.2.3 Covalent Immobilization of Biorecognition Elements onto Chemically Modified CNTs

The modification of CNTs usually involves the ends, side-walls, or defects that result from the oxidative acid pre-treatment of CNTs and are rich in

CNT-bound carboxylic groups. Thus, the treatment of pristine (raw synthesized) CNTs with strong oxidative agents (concentrated H_2SO_4:HNO_3 3:1) or hydrogen peroxide is the most extensively used approach and involves, as a primary step, the generation of oxygen containing functional groups like carboxylic, ketone, phenolic, and ester at "ends and defects" sites of CNTs (Karousis et al. 2010). This treatment not only generates functional groups on CNTs, but also cut and shortens the CNTs into the smaller units. Side-wall functionalization introduces a higher concentration of covalently attached functional groups on the CNTs' surface but also induces a significant perturbation in the electronic structure (Jain et al. 2007). Side-wall functionalization is achieved through gas-phase oxidation, involving treatment with nitric acid vapors (Mehra et al. 2014). Due to their large surface area, CNTs may easily be conjugated with biomolecules of interest. The linkages between the functional groups of the biomolecules and CNTs, usually involving coupling agents, are typically based on the formation of amide and ester bonds. For example, amine-terminated DNA strand was conjugated onto the open ends and defect site of oxidative functionalized SWCNTs (Villa et al. 2008) (Figure 3.9). One crucial step in the adsorption of CNTs on a transducer substrate (gold, platinum, glassy carbon, carbon fiber, and glass) is the prevention of CNTs' coagulation when they are placed in aqueous media.

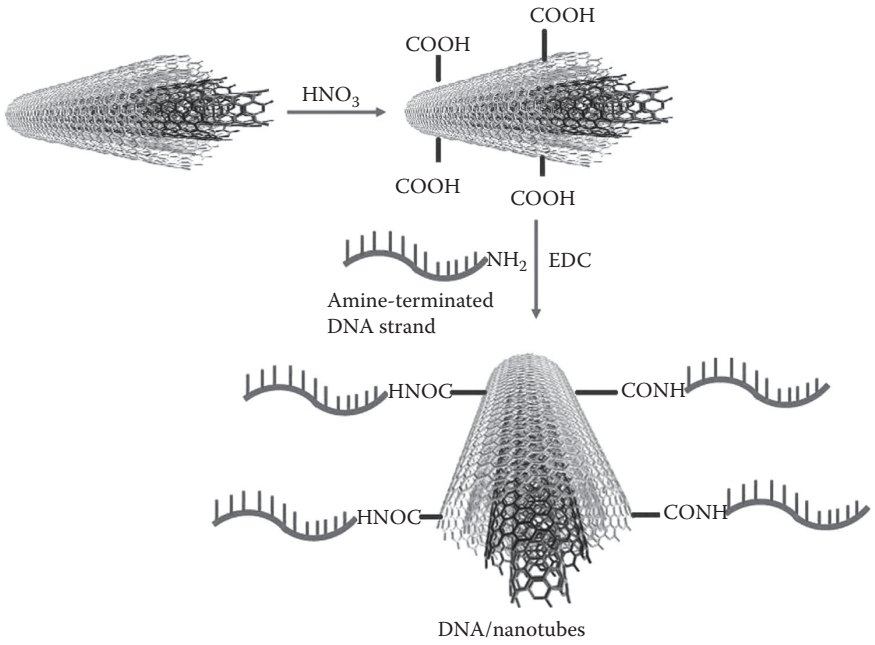

FIGURE 3.9
Modified SWCNTs bearing single stranded oligonucleotide. (Reproduced from Mehra, N. K. et al., *Biomaterials*, 35 (4), 1267–1283, Copyright (2014), with permission of Elsevier.)

Homogeneous CNT dispersions were obtained by dissolving CNTs in non-polar organic solvents (N,N-dimethylformamide [DMF] or chloroform) followed by sonication (Baj-Rossi et al. 2012). The CNT dispersions were used further to spin coat transducer surfaces. A porous, 3-D structure of CNTs on the electrode surface was obtained after the evaporation of the solvent. The major limitation of adsorption immobilization is the resulting random distribution of nanomaterials that is not reproducible on the transducer surface (Putzbach and Ronkainen 2013).

3.3.3 Integration of Biomolecules with Quantum Dots (QD)

Quantum dots are semiconductor structures below 10 nm, fabricated from materials taken from elements of groups II-VI and III-V (Warriner et al. 2014). The QD emission wavelength is strongly dependent on the size and shape of the individual crystal. The QDs-aptamer/antibody/protein-conjugates are usually obtained through the covalent binding of biomolecules to chemically modified QDs, via EDC/NHS chemistry. Biosensors using QD are based on the changes of emission spectra due to fluorescence resonance energy transfer (FRET) or photo-induced electron transfer. QDs are often used as FRET donors and their emission can be size-tuned to optimize spectral overlap with acceptor dyes. Different-colored QDs can be excited by a single light source, yet produce specific, narrow, and symmetric emissions of different colors, thus opening the way for multiplex analysis (Hayat et al. 2013).

3.4 Nano-Inspired Biosensors for Toxin Detection in Food

In addressing the requirements for toxin detection in various food samples, biosensors need to meet the following expectations. (1) Minimal if any sample preparation is to be used. Solid food samples should be homogenized and either filtered or centrifuged. Fluid samples may be diluted with a buffer to control pH or to reduce viscosity, but pre-concentration or fractionation steps should be avoided. (2) The assay time should be adapted for the application. In many cases, rapid responses are more important than maximum sensitivity, and 10–15 min assays are the norm (Taitt et al. 2008). For assays where sensitivity is more important than assay speed, 30–60 min assays are conducted. (3) The specificity of the assays should be adapted to user needs. For a high degree of selectivity, immunoassays are the method of choice. Several recent excellent reviews and works have already discussed the state-of-the-art of actual high-sensitive methods for small toxins (mycotoxins) and protein toxins analysis (Narsaiah et al. 2012, Raeisossadati et al. 2016, Pérez-López and Merkoçi 2011, Tothill 2011, Chauhan et al. 2016, Pauly et al. 2009). Therefore, we will briefly discuss the latest label and label-free

approaches using miniaturized devices, emphasizing the role of nanoparticles in the improvement of sensitivity, selectivity, and stability of biosensing surfaces. In this context, a short classification of biosensors for toxin detection according to transducer type will follow.

3.4.1 Electrochemical Detection

Electrochemical biosensors have superior properties over other existing measurement systems, because they can provide rapid, simple, and low-cost on-field detection. Electrochemical measurement protocols are also suitable for mass fabrication of miniaturized devices (Vestergaard et al. 2007). At minimum, two electrodes are needed in electrochemical measurements, but usually three electrodes are used. The current is measured at the working electrode (WE) and is functionalized with the probe (biorecognition element). In order to establish a desired voltage between the WE and solution, electrical contact is made with the solution using a reference electrode (RE) and/or counter electrode (CE). The RE maintains a fixed, reproducible electrical potential between the metal contact and the solution, allowing a known voltage to be applied. The CE supplies current to the solution to maintain the desired electrode-solution voltage, usually in electronic feedback with the RE monitoring the solution voltage (Daniels and Pourmand 2007). Voltammetry and amperometry involve measuring the current at an electrode as a function of applied electrode-solution voltage; these approaches are DC or pseudo-DC and intentionally change the electrode conditions. In contrast, electrochemical impedance spectroscopy (EIS) biosensors measure the electrical impedance of an interface in AC steady state with constant DC bias. Voltammetric methods for toxin detection also include linear sweep voltammetry (LSV), differential pulse voltammetry (DPV), square-wave voltammetry (SWV), and alternating current voltammetry (ACV) (Rackus et al. 2015).

3.4.1.1 Amperometric and Voltammetric Approaches

Few studies were dedicated to toxin detection, particularly mycotoxins, with enzyme biosensors due to difficulty of finding the appropriate enzyme inhibition assay. Several studies revealed that the enzyme acetylcholinesterase (AChE) can be inhibited by aflatoxins (Puiu et al. 2012). Based on this principle, an enzymatic biosensor for the assessment of aflatoxin B1 (AFB1) in olive oil was developed (Ben Rejeb et al. 2009). The reported LOD was 0.10 ng/mL. Another amperometric AFB1 biosensor was based on aflatoxin-oxidase (AFO), embedded in sol-gel, linked to MWCNTs-modified Pt electrode (Li et al. 2011). AFO, a recombinant expressed enzyme, has shown selective oxidative activity towards AFB1. Its immobilization on MWCNT allowed the biosensor to attain a 1.6 nmol/L (0.5 ng/mL) LOD with a linear range within 3.2 nmol/L to 721 nmol/L (Puiu et al. 2014).

Amperometric immunosensors use antibodies or antigens as biorecognition elements immobilized onto an electrode surface, but also require enzyme-labeling. To date, the most successfully used enzyme labels have been horseradish peroxidase (HRP) and alkaline phosphatase (ALP). Several amperometric biosensors were developed for OTA in wheat (Alarcón et al. 2006) and wine (Prieto-Simón et al. 2008) and for AFM1 in milk (Micheli et al. 2005). The detection of AFM1 was based on either direct or indirect competitive immunoassays onto screen-printed electrodes coated with a monoclonal antibody. A LOD of 25 pg/mL was achieved with a working range between 30 and 160 pg/mL. AuNPs coupled to OTA-bovine serum albumin (BSA) antigen were immobilized onto a screen-printed electrode in an indirect immunoassay for ochratoxin A detection. A LOD equal to 0.86 ng/mL was achieved (de la Escosura-Muñiz et al. 2010). An immunosensor based on Protein G functionalized magnetic beads (MBs) as solid phase for affinity reaction between OTA and OTA monoclonal antibody (mAbOTA) was recently reported. This immunosensor was based on a direct competitive assay between OTA in wine samples and OTA labeled with HRP (OTA–HRP) (Perrotta et al. 2012). The HRP, in the presence of hydrogen peroxide, catalyzed the oxidation of pyrocatechol to benzoquinone, whose back electrochemical reduction was detected on a carbon screen printed electrode by SWV. The achieved LOD was 0.08 ng/mL. An amperometric sensor for BoNT/A using gold nanoparticles as electronic bridges and signal amplifiers was recently reported (Liu et al. 2014). Here, AuNPs were first tethered with mixed PEG-aryl-diazonium salts and attached to the glassy carbon (GC) electrode by forming Au–C bonding, which displayed greater stability compared with Au and NH–Au-S bonds. Then, the tethered AuNPs were functionalized with the capture antibody. The tethered AuNPs were functionalized with HRP-labeled anti-BoNT/A antibody for the sandwich assay in a similar manner. Hydroquinone was used as a mediator for HRP electron transfer. The linear range was 4–35 pg/ml, and the detectable concentration of BoNT/A was 5 ng/ml in spiked milk samples. A similar sandwich ELISA format was developed recently for BoNT serotype E. This method relied on graphene nanosheets—aryldiazonium salt modified glassy carbon electrodes (GCE) as sensing platform and enzyme-induced silver nanoparticles (AgNPs) deposited on AuNPs as signal amplifier (Narayanan et al. 2015). This immunosensor could detect BoNT/E with linear range from 10 pg/ml to 10 ng/ml with LOD of 5 pg/ml in orange juice and milk. The deposited AgNPs on electrode surface were determined by LSV.

In comparison to antibodies, aptamers have several advantages such as ease of synthesis and modification with a variety of functional groups, stability, and cost-effectiveness (Chauhan et al. 2016). An electrochemical competitive aptamer-based biosensor for OTA was recently developed. Paramagnetic microparticle beads (MBs) were functionalized with an aptamer specific to OTA and were allowed to compete with a solution of the

mycotoxin conjugated to the enzyme horseradish peroxidase (OTA–HRP) and free OTA. The modified MBs were confined on disposable screen-printed carbon electrodes under a magnetic field, and the product of the enzymatic reaction with the substrate was detected with DPV. The magnetic aptasensor showed a linear response to OTA in the range 0.78–8.74 ng/mL and a LOD of 0.07 ng/mL and was applied to extracts of certified and spiked wheat samples. (Bonel et al. 2011). The selectivity of the amperometric biosensors is governed by the redox potential of all the electro-active species in solution, and, consequently, the measured current may include the contribution of several chemical species (Ansari et al. 2010).

3.4.1.2 Impedimetric Approaches

Compared with the amperometric immunosensors, EIS immunosensors have been found to be more sensitive and reliable. There are two classes of impedance sensors:

- Capacitive, where the electrode's surface is completely covered by a dielectric layer. The entire electrode assembly behaves as an insulator. No redox probe is present in the solution, and the capacitive current is measured under small amplitude sinusoidal voltage signal, at low excitation frequencies (typically 10–1000 Hz). The binding of the analyte (small toxin or protein) is expected to cause a decrease of the measuring capacitance, since less polar molecules replace water molecules from the electrode surface (K'Owino and Sadik 2005).
- Faradaic, where the electrode's surface (partially or entirely covered by a non-insulating layer, or partially covered by an insulating layer) is able to catalyze a redox probe, which exists in the measuring solution. The measured parameter is the charge transfer resistance (the real component of impedance at low-frequency values, typically 0.1–1.0 Hz); antibody/antigen interactions are expected to cause an increase of the charge transfer resistance, as the faradaic reaction becomes increasingly hindered (Daniels and Pourmand 2007). Regarding mycotoxin detection, most studies were pitched on the signal amplification. Thus, amplification has been required for the development of an impedimetric immunosensor for AFM1 detection in milk. Colloidal gold has been, as a label, necessary for the amplification procedure based on silver electrodeposition. The linear range of the described AFM1 immunosensor ranged between 15 and 1000 ng/L with a LOD of 15 ng/L (Vig et al. 2009). Another work reporting a label-free impedimetric-based immunosensor for OTA used antibodies attached onto 4-carboxyphenyl modified gold surface (Radi et al. 2009), providing a linear range of 1.20 ng/mL with LOD of 0.5 ng/mL, but lacked implementation in food samples.

3.4.2 Optical Detection

Optical biosensors are particularly appealing in food security since they may detect analytes in complex matrices with minimal sample treatment. In optical biosensors, the immunoaffinity element is immobilized onto the transducer and responds to the interaction with the target either by generating an optical signal (fluorescence, phosphorescence), or by undergoing changes in optical properties, such as absorption, emission, reflectance, and refractive index (Warriner et al. 2014). A vast number of optical techniques (based on optical fiber and planar waveguide transducers) are used to create optical changes, for example, fluorescence, total internal reflection fluorescence (TIRF), surface plasmon resonance (SPR), localized surface plasmon resonance (LSPR), and surface-enhanced Raman spectroscopy (SERS) (Puiu and Bala 2016a).

3.4.2.1 Fluorescence–Based Immunoassays

In fluorimetric sensors, immune-reagents are immobilized on various segments of an optical fiber, planar waveguides, or microgranules. Fluorimetric sensors provide broad opportunities for multianalysis. An indirect competitive fluid array fluoroimmunoassay quantified the mycotoxins FB1 and OTA, with both toxins immobilized to the surface of microspheres using the binding of biotinylated "tracer" antibody tracers (determined through flow cytometry using streptavidin-phycoerythrin conjugates) and the Luminex100 flow cytometer (Anderson et al. 2010). The binding of biotinylated monoclonal antibodies to FB1 and OTA was competitively inhibited by different concentrations of those toxins in solution. Concentrations of FB1 giving 50% inhibition were 300 pg/mL in buffer, 100 ng/g in spiked oats, and 1 μg/g in spiked cornmeal; analogous concentrations for OTA were 30 ng/mL in buffer, 30 ng/g in spiked oats, and 10 ng/g in spiked corn.

The simultaneous detection of BoNT/A/B, ricin, abrin, and staphylococcal enterotoxin B in milk, yoghurt, iced coffee, and carrot juice using a suspension-bead-array–based optical (fluorescence) sensor (Pauly et al. 2009). Toxin-specific antibodies were immobilized to magnetic beads that were differentially fluorescently tagged. After mixing with homogenized samples, the beads containing toxin bound to the capture antibody were magnetically isolated, concentrated, and washed before a secondary fluorescently labeled antibody was introduced. BoNT serotypes A and B were detected in food and animal sera using a "sandwich" ELISA with electrochemiluminescence (ECL) immunoassay platform developed by Meso Scale Discovery (Cheng and Stanker 2013). The assay achieved a LOD of 2 ng/mL with a 2–200 ng/mL linear range.

3.4.2.2 Surface Plasmon Resonance (SPR) Detection

SPR biosensors are optical sensors exploiting special electromagnetic waves due to fluctuations in the electron density at the boundary of two materials. Surface plasmons are the collective vibrations of an electron gas or plasma

surrounding the atomic lattice sites of a metal (Narsaiah et al. 2012). When plasmons are coupled with a photon, the resulting particle propagates along the surface of the metal until it decays, either by absorption or by radiative transition into a photon. A change in mass due to the immobilization of a bioreceptor and further interactions that take place when analytes are passed over the sensor surface cause a shift in the resonance to a longer wavelength and, hence, introduce a refractive index change easily detected by the SPR instruments (Puiu et al. 2014). These changes are related to the molecular weight of the antigen as well as the conformational change of the antibody/antigen complex on binding. Most commercial SPR devices are based on a prism-coupled; this SPR configuration is simple, robust, and sensitive but not amenable to miniaturization. Over the last decade, optical fibers and waveguide structures have been proposed as an alternative to prism-transmitting surface plasmon excitation (Hoa et al. 2007). There are few papers dedicated to mycotoxin detection with miniaturized optical fiber devices but are to be mentioned: the simultaneous detection of AFB1, OTA, DON, FB1, and ZEN (Byrne et al. 2009) and detection of OTA reporting a LOD of 0.1 μg/mL (Yu and Lai 2004).

3.4.3 Nanomechanical Detection Using Microcantilevers

Microcantilevers are devices acting as micromechanical beams that are anchored at one end, detecting changes in cantilever bending or vibrational frequency; they can be readily fabricated on silicon, polymers, and other materials. Microcantilever-based sensors operate in two modes: (1) static mode, where the cantilever bends due to an attached mass or force acting upon it, and (2) dynamic mode, where the shifting of the resonant frequency due to the mass getting bound to the structure is monitored. The difference in resonance is correlated to the amount of attached mass or any other type of force acting upon it (Chaudhary and Gupta 2009). In order to obtain a functionalized surface able to bind recognition elements, a metal layer, usually gold, is often evaporated onto the cantilever's surface. A thin gold film (i.e., 20 nm) can be used as a reflecting layer if the bending of the cantilever is monitored through an optical beam deflection method. Cantilevers are miniaturized sensors and several parallel cantilevers can be manufactured at low cost in large amounts. This enables multiplexed analysis of several targets in one sample by functionalizing individual cantilevers by different antibodies (Skottrup et al. 2008).

3.5 Advantages and Limitations of Nanobiosensors for "On-Site" Detection

Current sensing approaches such as SPR, amperometric/voltammetric techniques, and cantilevers will most likely play major roles in "on-site" toxin

detection from food matrices. Still, there are some issues inherent in these technologies to be addressed, before commercially nanobiosensors can be fully developed. Special attention should be paid to biosensor design and the recognition elements for different types of toxins. Electrochemical biosensors are generally very sensitive, have a rapid response, and are designed to be selective toward the target of interest. Easily miniaturized and incorporated into microfluidic systems, they can often offer a less expensive readout than optical systems (Lafleur et al. 2016). Amperometric and voltammetric biosensors are time-consuming and sensitive to fluctuations in the surrounding environment. Additionally, they require a redox system for signal amplification (Luka et al. 2015). Impedimetric biosensors have the advantages of simplicity and performing real-time measurements, but they require bulky devices and theoretical stimulation for data analysis. SPR biosensors are more amenable to real-time and label-free detection and display high sensitivity, but the surface modification involves laborious and time-consuming steps (Skottrup et al. 2008, Guo 2012). Microcantilever-based biosensors are characterized by simplicity and are implementable in miniaturized devices; they can perform real-time and label-free detection. As disadvantages, one can mention the sensitivity to temperature and stress (Luka et al. 2015). Regarding the recognition elements, enzymes are highly selective to their targets and are suitable for redox reactions; however, they are prone to loss of activity after immobilization onto a transducer and are restricted to small analytes detection such as urea, glucose, and lactate from food samples. Antibodies are preferred for affinity biosensors, being suitable for small analytes such as mycotoxins or for large targets such as proteins and bacteria; still, they require additional labeling steps for the indirect detection of small targets, thus increasing the cost and time needed for analysis. They may also display short-term stability. Aptamers are highly selective and sensitive, inexpensive, and stable and display flexibility to labeling without losing their performance or binding properties. Compared with antibodies, they display weaker binding to analytes.

A brief overview of the latest achievements on bioassays formats involving nanostructures for several food toxins are summarized in Table 3.1.

In the food industry, biosensors mainly focus on analysis of food security (detection of contaminants, toxins, pathogens, additives, allergens, etc.), analysis of food composition, and online process control (Bahadır and Sezgintürk 2015). Commercially available electrochemical platforms mainly use disposable SPEs from Dropsens (Spain), Kaniki (UK), and Zensor (Taiwan) (Kadara et al. 2009). SPE-based sensors provide excellent platforms for modification with a variety of nanoparticles and nanomaterials, requiring no pre-treatment (electrode polishing or electrochemical pre-treatment via electrodeposition), as is common with other conventional electrodes. SPEs not only address the issue of cost-effectiveness but also satisfy criteria of reproducibility and sensitivity toward target, while maintaining low-cost production through scales of economy (Metters et al. 2011). On the other hand, optical

TABLE 3.1

Recent Works on Food Toxin Detection Using Nanobiosensors

Target	Detection Method	Type of Nanoparticles Or Nanomaterials/Role in the Assay Format	Assay	LOD (pg/mL)	Ref.
AFB1	Electrochemical (SWV)	Quantum dots/signal amplification	Competitive immunoassay	18	(Zeng et al. 2015
	Optical	Gold nanorods/sensing platform		160	(Xu et al. 2013
	(UV-Vis absorption)	Gold thin film/biomolecule immobilization	Competitive immunoassay	30	(Zhou et al. 2016)
	Nanomechanical (microcantilever)		Direct immunoassay		
OTA	Electrochemical (EIS)	Iridium oxide NPs/surface modification	Direct aptamer assay	5.65	(Rivas et al. 2015)
	Optical (fluorescence)	Silver nanoclusters/ surface modification	Direct aptamer assay	2	(Chen et al. 2014)
DON	Electrochemical (EIS)	Fullerene/surface modification	Direct immunoassay	0.3	(Zhilei et al. 2011)
	Electrochemical (DPV)	CNTs/surface modification	ELISA (indirect competitive)	5	(Qing et al. 2016)
BoNT/A	Electrochemical (amperometric)	Gold NPs/Surface modification and antibody labels	ELISA (sandwich)	1	(Liu et al. 2014)
BoNT/E	Electrochemical (LSV)	Silver NPs/signal amplifier Gold NPs/catalyst Graphene nanosheets/surface modification	ELISA (sandwich)	5	(Narayanan et al. 2015)
SEB	Optical (fluorescence)	Fluorescent Tris (2,20 -bipyridyl) dichlororuthenium(II) NPs/label	ELISA (sandwich)	10	(Lian et al. 2010)
	Optical (chemiluminescence)	CNTs/ Surface modification	ELISA (sandwich)	100	(Yang et al. 2010)

detection has evolved on the market through SPR instruments. The demand for high-throughput analysis led to the development of the multichannel SPR sensor that recently evolved into SPR imaging sensing platforms. The most notable SPR instruments available on the market for food toxin detection are Biacore (GE Life Sciences), SR7500DC (Reichert), and BI-SPR 1000 (Biosensing Instrument) for laboratory-based applications; SPREETA (Texas Instruments), SPIRIT (Seattle Sensing Systems), and SPR micro (KMAC), which are compact and portable devices; and SPR imaging instruments such as Flexichip (Biacore), IBIS-MX96 (IBIS Technologies), and ProteOn XPR 36 (Bio Rad Laboratories) (Couture et al. 2013).

3.6 Conclusions and Perspectives

The past decades have witnessed the advance of novel functional nanomaterials and engineered biorecognition elements in the biosensor field for food security. The development of transduction methods and surface functionalization techniques can provide insight into and understanding of biological processes. The synergistic action of multifunctional materials, recognition elements, and electrochemical or optical transducers is improving the performance of biosensors in terms of selectivity, stability, and reproducibility. Compared with catalytic biosensors, antibody-based affinity sensors are not suitable for long-term monitoring due to their loss of activity during the regeneration steps. From this perspective, aptamer-based sensors are more amenable to continuous monitoring since aptamers are chemically stable and can be easily regenerated by denaturation of the target analyte.

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4

Nanotechnology in Food: From Farm to Fork

Joseph C. Hannon and Enda Cummins

CONTENTS

4.1 Introduction

The current world food supply, according to the Food and Agriculture Organization (FAO) (2009), will not be sufficient to feed the world's population by 2050, even with proposed increases in food production. This highlights the need for some form of intervention, whether it is investment in agricultural production or implementation of more sustainable policies. Technological innovations in the area of agricultural production, food processing, and food safety play a major role in meeting the world's current and projected food needs. Nanotechnology is placed among eight sustainable innovations to be the focus of research and development investment to improve the security of the world food supply chain (STOA Project 2013). In addition, nanotechnology can benefit many other associated technologies such as sensor technology, smart packaging, and more energy-efficient processing technologies, for example, anti-fouling coatings on piping for food processing lines, reducing the amount of energy and water required to clean (Barish and Goddard 2013).

Although nano-scale materials have, unwittingly, been used for hundreds of years, while also existing in nature, it is only in the last few decades that

attempts have been made to understand and define these nano-scale substances and structures—particularly as engineered nanomaterials (ENMs) are currently being developed for a myriad of applications. The term "engineered" is used to describe manmade nanoparticles and has been used in association with nanomaterials being developed in the food industry. In addition, materials at the nano-scale that have always been present in the food industry (e.g., certain additives and the formation of certain nanoparticles due to cooking [Sk et al. 2012]), whether it be unintentional or incidental of manufacturing processes, also fall into this category. Alternatively, nanomaterials (NMs) present in nature could be termed naturally occurring NMs, for example, casein micelles in milk. Common trends exist between the definitions used in different regions. In Europe, the European Commission definition makes reference to the source of the NM, whether it is natural, incidental, or manufactured. It also makes reference to the aggregate state (unbound or aggregate) and minimum percentage (50%) of the particle size distribution that contains at least one dimension in the size range 1–100 nm (European Commission 2011). The absence of a clear and common definition for nanotechnology is just one of many uncertainties, along with ENM potential toxicity and bioaccumulation, which have contributed to the cautious uptake of ENMs. Consequently, assessments must be performed to evaluate the human and environmental safety of ENMs and gaps in knowledge must be filled to safeguard human and environmental health and foster consumer trust.

This chapter looks at the current application of ENMs in the food industry and takes a look at safety aspects along the farm-to-fork continuum, with particular emphasis on risk assessment strategies.

4.2 Application of ENMs in the Food Industry

The improved properties of ENMs, due to their nano-scale dimensions, have formed a firm basis for the use of ENMs in the food industry. Although nanomaterials have gained more momentum in certain regions, primarily due to more lenient regulation and favorable public perception, substantial research and development in the area points to the use of nanotechnology in a substantial number of products and processes in the food industry in the future (Figure 4.1).

In recent years, analytical techniques have allowed for the detection of nanoparticle fractions in food additives such as silica (SiO_2 or E551) (Dekkers et al. 2011) used as an anti-caking agent, silver (E174) (Verleysen et al. 2015) used as a coloring, titanium dioxide (TiO_2 or E171) (Weir et al. 2012) used as a coloring, and zinc oxide (ZnO) (Wang et al. 2014) used as an antimicrobial. It could be argued that these nano-fractions are present incidentally

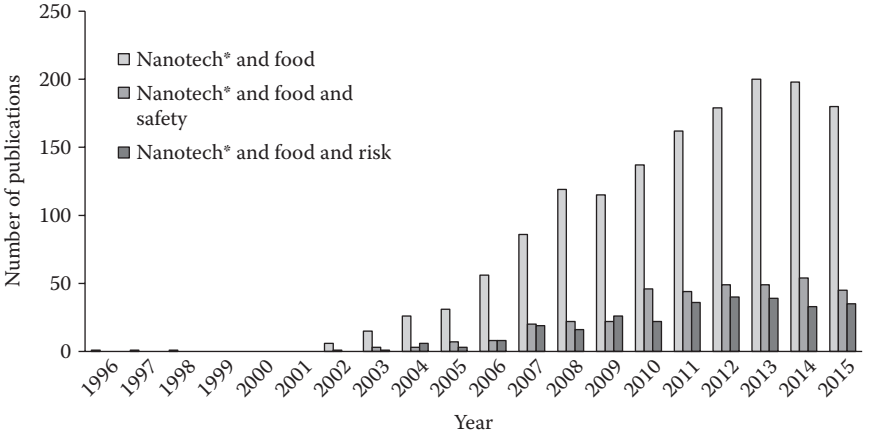

FIGURE 4.1
Trends in research and development related to nanotechnology in the food industry (SCOPUS search on 10th March 2016 using terms: nanotech*, food, safety, and risk).

as a result of the additive manufacturing process. Raw milk, however, has been found to contain nano-fractions of casein micelles that are naturally occurring nanoparticles and not incidental of processing (Martin et al. 2007). Some components of food such as food proteins fall within the nano-size range, with many lipids and polysaccharides having 2 nm thickness (Rossi et al. 2014). Other sources of ENMs exist within the food industry (Maynard and Michelson 2016); however, a large portion of the products feature unsupported claims on the use of nanotechnology (Bouwmeester et al. 2014). This adds uncertainty when attempting to measure current and future use. Furthermore, this issue is exacerbated by a lack of regulation governing the mandatory labeling of nanomaterials in the food industry (Amenta et al. 2015). Peer-reviewed journal papers provide detailed information on the use of ENMs in the food industry. A non-exhaustive list of peer review publications focusing on the development of nanotechnologies within the food industry is presented in Table 4.1.

Despite the current uncertainties regarding the usage of engineered nanomaterials in the food industry, there is no doubt that nanotechnology will greatly impact the lifecycle of many products and processes in the food industry (Figure 4.2).

During the creation of a food product and at the beginning of the farm-to-fork lifecycle, the improved properties of ENMs will allow for less raw material to be used to provide similar function, for example, less fillers required in clay nanocomposites demonstrating superior exfoliation to traditional composites (Silvestre et al. 2011). This will reduce costs while reducing the product's environmental impact. Throughout transport and storage, nano packaging containing ENMs improving barrier properties (i.e., clay

TABLE 4.1

Food Industry Applications for Nanoparticles

Stage of Lifecycle	Engineered Nanomaterial	Nanoparticle Function			Reference
Primary agricultural activities	Fe_3O_4	Efficient irrigation improving the absorption of water			(Liu and Lal 2015)
	CaNPs	Targeted and efficient pesticides and fertilizer			(Liu and Lal 2015)
	Polyethersulfone	Nanofiltration that reduces bio-fouling and improves removal of contaminants			(Shanmuganathan et al. 2015)
	Liposome	Bioavailable veterinary medicines			(Underwood and van Eps 2012)
	CuO and Al_2O_3	Nanofuels and oils providing superior thermal properties			(Gumus et al. 2016)
	SeNPs	Animal feed improving bioavailability			(Shi et al. 2011)
Processing	Solid lipid NPs	Low energy processing			(Finke et al. 2014)
	Ni-P-PTFE NPs	Coatings for improved chemical resistance and anti-fouling			(Barish and Goddard 2013)
Transport and storage	AgNPs	Food contact materials	• Active	• Antimicroabial	(Azlin-Hasim et al. 2015)
	CNTs and silicon NPs		• Intelligent	• Nanosensor	(Vanderroost et al. 2014)
	Carbon ENMs			• Time tamper sensors	(Vanderroost et al. 2014)
	Carbon ENMs			• Nanobarcodes	(Chen et al. 2013)
	MMT		• Improved	• Strength and barrier properties	(Rostamzad et al. 2016)
Product	Nanoemulsion	Reduced fat, cholesterol and allergen dairy products			(Ghazaei et al. 2015)
	Lipid Nanoencapsulation	Delivery of ingredients, flavours, and supplements through encapsulation			(Fathi et al. 2012)
	lycopene	Food supplement improving bioavailability			(Boon et al. 2008)
End of life	dendrimers	Re-use			(Yen et al. 2017)
	MMT	Biodegradability			(Avella et al. 2005)
	Fe_3O_4, SiO_2, etc.	Waste remediation			(Sánchez et al. 2011)

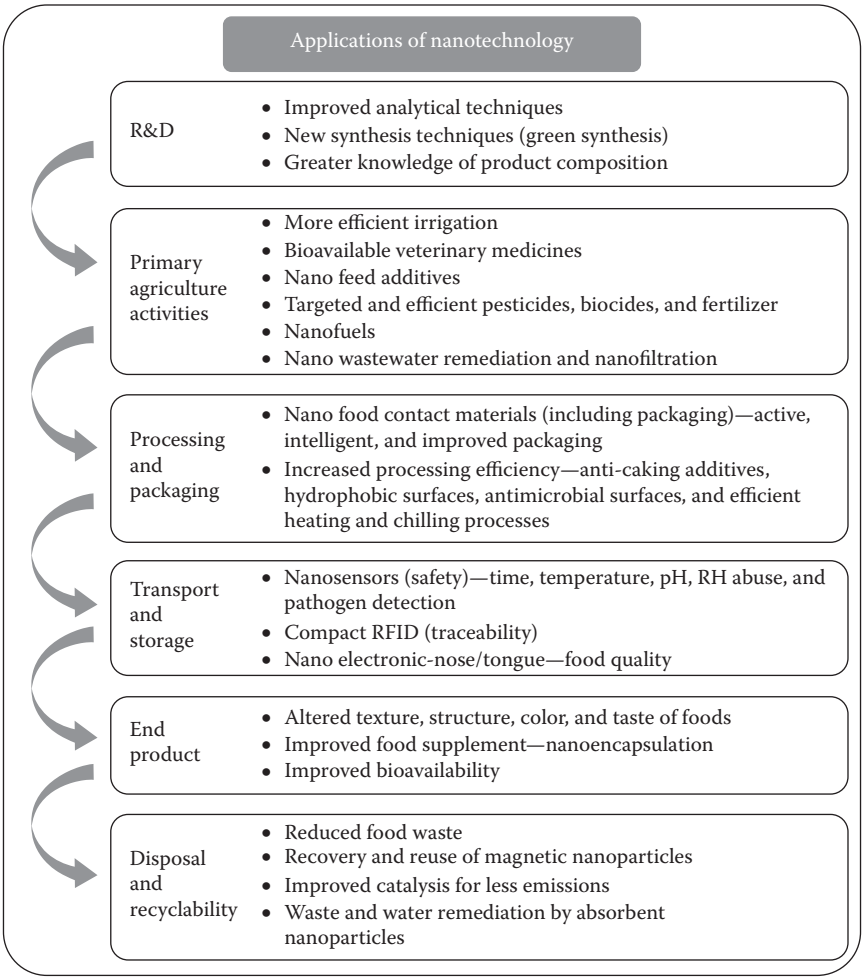

FIGURE 4.2
Potential impact of nanotechnology on the food industry along the farm-to-fork continuum.

nanoparticles) will control the release of gases in and out of the packaging. Active antimicrobial and oxygen scavenging nanoparticles in the packaging will prevent food spoilage while maintaining food safety (Hannon et al. 2015). Intelligent nanosensors incorporated in the packaging will monitor the gas levels, microbial counts, and temperature conditions affecting the contained food. Printable nano electronic labels on the face of the packaging will warn the consumer of potentially harmful foodborne bacteria or food spoilage through visual changes of the label. Compact nano RFID tags on the packaging will give information related to the origin of the product and its journey from the farm to store and on to the consumer. In addition, these tags will give the industry and regulatory authorities the ability to track the

product's journey through the supply chain in order to monitor sales but also determine its final destination in the event of a recall (Vanderroost et al. 2014). Once the food has been removed from the packaging, the improved composition of the nano packaging would allow it to biodegrade (Avella et al. 2005) or be recycled and reused. The aforementioned ENM products cover a very broad range of applications within the food industry, including food production, processing, transport, storage, and disposal. There are concerns over the potential human exposure to ENMs present in food originating from different products and processes in the farm-to-fork lifecycle.

4.3 Engineered Nanomaterial Release, Transport, and Toxicity

To date, few studies have investigated the kinetic behavior of ENMs in food and beverages, instead placing a focus on the unintentional release of ENMs into food from food contact materials (Hannon et al. 2015) and consumer products (Tulve et al. 2015, Quadros et al. 2013, Mackevica et al. 2016). The heightened mobility of ENMs would suggest that ENMs have improved diffusion characteristics influencing their distribution in food or beverages and subsequently the amount to which humans are exposed. If ENMs accumulate at the surface of food or beverage containers, the likelihood of consumption may be reduced depending on how much of the foodstuff is consumed and how much remains in the container after consumption. Food crops is a food category that has been investigated in terms of ENM mobility. In a review by Rico et al. (2011), it was noted that nanomaterials accumulated in food crops impacting on human exposure levels, that is, consumer preference to different parts of the food crop, that is, root, stem, and leaf. Many of the studies reviewed placed a considerable emphasis on the toxicity of ENMs toward different plant species and a lesser focus on the accumulation of ENMs within the plant. A recent study by Ebbs et al. (2016) investigated the accumulation of multiple metal oxide ENMs and ionic species released from soil into carrots' peel, flesh, and root. The authors observed higher uptake and accumulation of ionic species in the carrots when compared to ENMs, establishing a need for further research focused on the potential human exposure and not just plant eco-toxicity. Further research is needed in the area of ENM release, transport, and accumulation in order to identify other potential sources of ENM human exposure, whether it be through direct inclusion in the food chain or unintentional contamination of food from ENMs that have been released in the environment, for instance, ENM uptake by carp fish from the environment (Sun et al. 2006). It is evident from the literature that fewer studies have investigated the behavior of ENMs in food and the environment when compared with studies focusing on the fate and toxicity of ENMs in the human body.

The novel behavior of ENMs, lent by their nano-scale size, has caused concern over potential negative health effects influencing their behavior and fate within the human body once consumed. An example of where ENMs' novel behavior can produce both positive and negative effects is the improved antimicrobial ability of silver nanoparticles that consequently also raises a concern regarding toxicity against mammalian cells (Arora et al. 2008). Other potential adverse effects that may arise from exposure to nanomaterials would include allergenic effects. In the human body, it is probable that immune system cells are susceptible to interactions with ENMs due to their role in eliminating pathogens and contaminants from the body (Shannahan and Brown 2014). Size-dependent allergenic responses were witnessed in mice injected with amorphous silica nanoparticles (Hirai et al. 2012). In the literature, there are limited studies that investigate the potential allergenic responses in the human body from exposure to ENMs used in the food industry. Given the broad range of applications of ENMs across all industries, ENMs have the potential to enter and interact with the human body through many of the possible routes of exposure, such as oral, dermal, respiratory, ocular, and cervical routes (Hannon et al. 2015). In the food industry, the most likely exposure route is through the oral route from consumption of a foodstuff.

There is great uncertainty surrounding ENMs' fate in the human gastrointestinal tract (GIT). One concern is the potential penetration of natural mucus in the GIT by ENMs, aided by their smaller size, surface coating, and surface charge (Bouwmeester et al. 2009), challenging the applicability of existing bulk or ionic material adsorption, distribution, metabolism, and excretion (ADME) data. In addition, ENMs' improved mobility in the GIT promotes their potential transfer to organs within the body, followed by possible accumulation and toxicity (Cockburn et al. 2012). For ENMs that possess a heightened ability to encapsulate and absorb contaminants, it is also likely that ENMs may cause a "Trojan Horse" effect, carrying harmful contaminants to otherwise inaccessible parts of the body (Lomer et al. 2002). Alternatively, it is argued that the human digestive system can process food components at the nano-scale (Chaudhry et al. 2008). In the literature, studies that investigate the fate of ENMs in the GIT have been identified as an area that requires further research to improve the understanding of the ADME of ENMs in the GIT (Dekkers et al. 2013).

4.4 Risk Assessment

Risk assessment is a systematic approach that has been outlined as a crucial tool in the acceptance and uptake of nanotechnology in the food industry. This approach is used to quantify the level of risk posed to humans and

the environment using four steps: hazard identification, hazard characterization, exposure assessment, and risk characterization (FAO 2006). The risk characterization, which is the product of the hazard and exposure (Warheit et al. 2007), is used to estimate the probability of a potentially adverse effect occurring in a given population. Numerous studies have identified a lack of risk assessments specific to the use of ENMs in the food industry (Coles and Frewer 2013, Handford et al. 2014). A non-exhaustive list of risk assessments for the use of ENMs in the food industry can be seen in Table 4.2.

In 2009, scientific opinion by the European Food Safety Authority (2011) proposed that the chemical risk assessment paradigm is valid for ENMs, provided that ENMs be assessed on a case-by-case basis. In the United States, the Food and Drug Administration (FDA) holds a similar stance on the case-by-case testing of ENMs but places more of an emphasis on the safety of the finished product in its given application (Amenta et al. 2015). In the literature, chemical risk assessment has been identified as a method to evaluate the human health risk from exposure to nanomaterials in food (Hristozov and Malsch 2009). However, a number of issues have been identified with the application of traditional chemical risk assessment to ENMs (EFSA 2011). These issues are spawned from a lack of suitable data and gaps in current knowledge and require attention before chemical risk assessments can be applied to ENMs.

4.5 Issues Identified with Traditional Risk Assessment of ENMs

4.5.1 Physicochemical Properties

One of the most significant issues associated with the application of traditional risk assessment to ENMs is the lack of data related to their unique physicochemical properties and how these affect their bioavailability, ability to accumulate, potentially heightened exposure, and toxicity (Rossi et al. 2014, Jacobs et al. 2015). In traditional risk assessment, the physicochemical properties of a bulk material have limited bearing on their risk. Therefore, there is not a significant emphasis placed on the characterization of a material's physicochemical properties, such as particle size, shape, surface area, surface charge, crystal structure, surface chemistry, and level of agglomeration, when compared with the nano-scale material (Warheit et al. 2007). As a result, traditional methodologies may require alterations to fully detect unique behavior related to physicochemical properties that would otherwise go unnoticed (Rossi et al. 2014), that is, increased dissolution rate of ZnO nanoparticles into Zn^{2+} aided by vitamin C that was linked to increased cytotoxicity (Wang et al. 2014). The Organisation for Economic Co-operation

TABLE 4.2

Risk Assessments for the Use of Nanoparticle in the Food Industry

Model	Approach	Stochastic/ Deterministic	Application	NP Type	Size	Surface Area	Predicted Exposure	Results	Recommendations	Reference
Hazard identification	1. Nanomaterial characterization, 2. Acute oral toxicity studies, 3. Screening genotoxicity and aquatic toxicity studies	N/A	Not stated	TiO_2	136–149.4 nm	18.2–38.5 m^2/g		Very low oral toxicity, negative for bacterial reverse mutation, negative for chromosome aberration	Bridge' robust hazard data with material with few specific hazard data	(Warheit et al. 2007)
Exposure assessment	1. Release studies: Real food matrix, 2. Exposure = (migration x consumption)/ body weight	Stochastic	Food packaging	Ag	50 and 10 nm (packaging precursor)	57.2 and 11.5 m^2/g		Consumption of Ag not likely to be above provisional ingestion limit	More toxicity studies needed	(Cushen et al. 2013)
Exposure assessment	1. Nanomaterial characterization, 2. Child non-dietary exposure assessment	Deterministic	Child sippy cup, bandage, fabric, wipes, breast milk storage bags, kitchen scrubber, plus toy and tooth brush	Ag			1.53 μg Ag/kg	Ag exposure was 60% of EPA national secondary drinking water standard	More studies needed to detect more commercial nanomaterials	(Tulve et al. 2015)
Hazard and exposure assessment	1. Nanomaterial characterization, 2. Nano fraction quantification, 3. Human exposure assessment	Deterministic	E551 as anti-caking and thickener in food products	SiO_2	12 and 7 nm (Aerosil 200F® and Aerosil 380F®)	199.1 and 200 m^2/g	9.4 mg/kg bw/day	Scenario 1:MOS = 66 (not likely to be safety concern), Scenario 2: too many uncertainties to conclude	Prioritize research on how nanosilica is absorbed in the gastrointestinal tract	(Dekkers et al. 2011)
		Deterministic	E551 as anti-caking and thickener in food products	SiO_2						(Dekkers et al. 2013)

(*Continued*)

TABLE 4.2 (CONTINUED)

Risk Assessments for the Use of Nanoparticle in the Food Industry

Model	Approach	Stochastic/ Deterministic	Application	NP Type	Size	Surface Area	Predicted Exposure	Results	Recommendations	Reference
Hazard and exposure assessment	1. Human exposure assessment	Stochastic	E551 as anti-caking and thickener in food products	SiO_2	From (Dekkers et al. 2011)	From (Dekkers et al. 2011)	From (Dekkers et al. 2011)	95% confidence 99% of Dutch population would experience no risk	Probabilistic methods are preferable over deterministic methods	(Jacobs et al. 2015)
Exposure assessment	1. Nanomaterial characterization, 2. Nano fraction quantification, 3. Human exposure assessment	Stochastic	E171 as food coloring	TiO_2	110 nm (range of 30–400 nm)		1–2 mg/kg bw/day (<10 years age), 0.2–0.7 mg/kg bw/day (>10 years age)	Exposure higher for children <10 years		(Weir et al. 2012)
Hazard characterization	1. Release studies: Food simulants, 2. *In vitro* cytotoxicity and mutagenicity study	N/A	Improved properties of packaging	Nano clay	Not stated	Not stated	N/A	No cytotoxic or genotoxic effects from migrant extracts	Further toxicological studies are necessary	(Maisanaba et al. 2014)
Exposure assessment	1. Collection of toxicokinetic data from the literature, 2. Physiologically based pharmacokinetic model		Dietary intake and three consumer products	Ag	15–150 nm	Not stated	Not stated	Silver levels below levels that would cause adverse effects	More toxicological studies required for fate in GIT	(Bachler et al., 2013)

and Development (OECD 2012) has emphasized the lack of unified hazard identification and characterization approaches for ENMs that would enable the distinction between toxic ENMs and non-toxic ENMs. A suite of analytical techniques have been proposed for the identification and characterization of ENMs in consumer products, food, and biological media to combat this shortfall (Tulve et al. 2015). The substantial number of specialized techniques, in combination with significant resources necessary to carry out such analysis, extenuates the difficulties of properly characterizing and quantifying ENMs in food industry applications. In addition, poorly characterized ENMs in food and biological media have been identified as a weakness in many toxicity studies, negatively impacting their usefulness within risk assessments (Magnuson et al. 2011). Failure to fully identify the driving physicochemical properties of an ENM's toxicity would risk overlooking the unique behavior exhibited by ENMs, for example, the potential differences in toxicity between synthetic amorphous silica (E551) and nanosilica (Dekkers et al. 2013). The inability of studies to establish a relationship between physicochemical properties and the driving mechanisms of toxicity supports the EFSA's (2011) use of a case-by-case approach for the risk assessment of ENMs.

4.5.2 Animal Testing

The proposed EFSA case-by-case strategy presents its own issues for the successful risk assessment of ENMs. Given the potential future growth of nanotechnologies in the food industry, there could be significant costs associated with the toxicity testing of ENMs. This is especially true considering the substantial range of ENM types and sizes for which toxicity studies are lacking (Bouwmeester et al. 2009). Currently, a battery of *in vitro* toxicity assays and *in silico* modeling techniques are proposed for the toxicity assessment of ENMs in food, to prioritize and reduce the number of costly *in vivo* toxicity studies (Warheit et al. 2007). In a EFSA (2011) guidance document for the risk assessment of ENMs, a number of toxicity tests are proposed that include *in vitro* genotoxicity studies, ADME studies, repeated-dose 90-day oral toxicity rodent studies, *in vitro* digestion studies, reproductive studies, developmental toxicity studies, chronic toxicity studies, carcinogenicity studies, and other specific toxicity tests. The number of toxicity tests performed is dependent on the persistence of the ENM throughout the lifecycle of the food product before and after consumption. An issue that can be identified with these toxicity testing strategies is that a reliance on *in vitro* studies may not fully identify the long-term behavior of ENMs in the human body, particularly insoluble ENMs that could accumulate in secondary target organs (Silvestre et al. 2011). Therefore, *in vitro* studies provide only an indication of ENM toxicity and would require additional supporting information on the long-term behavior, toxicity, genotoxicity, and carcinogenicity of ENMs. Other aspects of these testing strategies that require attention to improve

their effectiveness would include investigations into the influence of food components on the physicochemical properties and toxicity of ENMs. This is especially relevant for *in vitro* studies where ideal biological testing media are used for toxicity testing.

4.5.3 Dose Metrics

A commonly raised issue with regard to ENM risk assessment is the metrics used to quantify ENM exposure. It has been argued that in ENM exposure assessment, a mass-based metric, as used in chemical risk assessment (i.e., mg/m^3), would exclude the number of particles and reactive surface area present in the system (Warheit and Donner 2015). Therefore, a human exposure assessment using a mass-based concentration of ENMs in food could fail to capture the physicochemical properties of an ENM that enable it to pass natural barriers, bio-accumulate, or cause toxicity. An example where mass-based metrics are used for release studies would include the European Commission (2011) Regulation on food contact materials No. 10/2011. It proposes that unauthorized substances in food contact materials do not migrate into food in doses greater than 0.01 mg/kg of food. This is a common trend among most regulatory bodies who are cautious to enforce nano-specific provisions, as it may affect products already on the market with a long history of safe use, for example, food additives. In the European Union, great efforts have been made in the past decade to relate toxicity to other dose metrics and reevaluate the safety of a long list of food additives, such as food colors, preservatives, emulsifiers, stabilizers, and gelling agents (European Commission 2010). Similar to the exposure assessment of ENMs in food, uncertainties exist around the correct dose metrics to use for toxicity testing. Unlike traditional dose response studies that rely heavily on the mass-based dose administered orally, for ENMs there is a substantial number of additional factors that require consideration, such as particle concentration, size, shape, aspect ratio, level of agglomeration/aggregation, zeta potential, and coating (Kong et al. 2011). These additional factors would lead to an exponential growth in the number and complexity of toxicity studies required to evaluate the broad range of ENMs used in food applications. Human exposure assessments entailing detailed information on ENM metrics could be used to prioritize the toxicity testing of ENMs effectively by providing clarity during the experimental design stage regarding the ENM characteristics and concentration that should be investigated. The absence of regulatory labeling and a comprehensive inventory on the use of ENMs has contributed to difficulties in modeling human and environmental exposure to ENMs used in the food industry. These shortfalls, similar to ENM hazard identification and characterization, are largely attributed to deficiencies in analytical techniques to characterize and screen for ENMs, and consequently slow development of ENM regulation (Warheit et al. 2007).

Techniques for the characterization and quantification of ENMs in food, food simulants, and biological media are well reviewed by the scientific community (Laborda et al. 2016, Bandyopadhyay et al. 2013). Commonly used techniques for the identification and characterization of ENMs would include scanning and transmission electron microscopy, atomic force microscopy, UV-vis spectroscopy, and light scattering techniques such as multiple angle light scattering, nanoparticle tracking analysis, and dynamic light scattering. For the quantification of nanoparticles in food and biological media, frequently used techniques include inductively coupled plasma—atomic emission spectrometry, optical emission spectrometry and mass spectroscopy, atomic absorption spectroscopy, and high performance liquid chromatography. A pitfall of these techniques, with the exception of single particle inductively coupled plasma mass spectrometry (ICP-MS), is their inability to distinguish between nanoparticles and dissolved species of the same element. The aforementioned techniques for characterization and quantification of nanoparticles each presents its own limitations in terms of limit of detection, detection range, and ability to differentiate particles based on chemical composition. There is also an issue related to the separation of nanoparticles from food and biological media, as these techniques have limited ability to analyze nanoparticles in complex samples. Often, a pre-treatment is necessary that includes dilution, digestion, solvent extraction, centrifugation, filtration, dialysis, and separation using a complementary technique, that is, asymmetric flow field-flow fractionation. These pre-treatments present their own problems regarding the influence they have on the ENM's structure and state, sample dilution, and bias toward specific size ranges. To aid the safe development of ENMs in the future, these issues related to analytical techniques for the characterization and quantification of ENMs in complex samples must be overcome.

4.6 Risk Assessment Frameworks

The development of risk assessment frameworks for the use of ENMs in the food industry is still very much in its infancy. Frameworks have been proposed for the use of ENMs in occupational and environmental scenarios (Jacobs et al. 2015); however, a significant shift in research interest has occurred toward human and environmental health impacts arising after the ENM production stage of the farm-to-fork lifecycle. This trend may be in response to the growing number of consumer products that involve direct contact with consumers and the environment, unlike products already on the market that contain immobilized and inert ENMs, that is, textiles and electronics. For these products, the most probable exposure group are workers developing ENMs and also the environment that is exposed to discarded

products and production waste, which would explain the preference given to these risk assessment frameworks over food-bound ENM risk assessments. Given the growing number of food and beverage products being reported worldwide (Maynard and Michelson 2016), there has never been a greater need for risk assessment frameworks to provide a strategic approach toward the safety assessment of a wide range of ENMs in the food industry. The risk assessments present in the scientific literature (Table 4.2) give an indication of approaches that can be used to evaluate the safety of ENMs; however, their applicability to other ENMs must be confirmed before they are embraced by industry and regulators. The issue around the applicability of traditional risk assessment to ENM risk assessment may partly contribute to the lack of risk assessment frameworks in the literature for ENMs used in the food industry. Additionally, the tremendous logistical challenge associated with the number of ENMs that require consideration when formulating such frameworks hinders progress. To the best of the authors' knowledge, the European Union (E.U.) and United States have provided the only approaches for the risk assessment of ENMs in the food industry in the form of non-binding guidance documents (Table 4.3).

Outside the E.U. and Switzerland, there have been limited alterations to existing legislation to incorporate nano-specific provisions. In the U.S., New Zealand, Canada, and Australia, the use of ENMs in the food industry has been regulated under existing regulation, regarding existing regulatory frameworks capable of adapting to the use of ENMs (Amenta et al. 2015). In other countries such as Thailand, Taiwan, and Iran, labeling and tracking systems for ENMs have been introduced. In many Asian countries, certification and standards have been provided to industry to foster the development of ENMs. Currently, the only nano-specific provisions in legislation for the use of ENMs in the food industry have been made by the E.U. and Switzerland, with other countries providing guidance for industry.

In the E.U. and U.S., non-binding guidance documents support a tiered approach for the risk assessment of ENMs. In 2014, the U.S. FDA (2014) published a complete guidance document with the purpose of identifying products and processes that fall under the category of an ENM, so that a decision can be made at an early stage of product development on whether a risk assessment requires further provisions to account for nano-specific behavior. Following the guidance document outlined by the FDA (2015), if an ENM is present in a product or process, a food additive petition submitted for premarket evaluation must include identification and characterization of the ENM's physicochemical properties, details of the manufacturing processes used and potential by-products or impurities that may contribute to the product's toxicity, details on the ENM's stability under the conditions of use, details surrounding potential use and exposure scenarios, and sufficient human toxicity and eco-toxicity assessment to confirm the safety of the product in its proposed application. The document gives general guidance

TABLE 4.3

Non-Binding Regulatory Guidance Documents for the Use of ENMs in the Food Industry

Organization	Year	Document	Link
EFSA	2011	Guidance on the Risk Assessment of the Application of Nanoscience and Nanotechnologies in the Food and Feed Chain	http://www.efsa.europa.eu/en/efsajournal/pub/2140
USFDA	2014	Guidance for Industry: Assessing the Effects of Significant Manufacturing Process Changes, Including Emerging Technologies, on the Safety and Regulatory Status of Food Ingredients and Food Contact Substances, Including Food Ingredients that Are Color Additives	http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ucm300661.htm
USFDA	2015	Guidance for Industry Use of Nanomaterials in Food for Animals	http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM401508.pdf
ECHA	2012	Guidance on Information Requirements and Chemical Safety Assessment	http://echa.europa.eu/web/guest/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment
ILSI Europe	2011	Practical Guidance for the Safety Assessment of Nanomaterials in Food	http://www.ilsi.org/Europe/Documents/ILSI-12-005%20Rep_food_02.pdf
LIFE REACHnano	2015	Guidance on Available Methods for Risk Assessment of Nanomaterials	http://www.lifereachnano.eu/index.php/dissemination/documents?download=31:guidance-on-available-methods-for-risk-assessment-of-nanomaterials

that recommends consultations at an early stage of development to give more specific guidance for the ENM product being assessed.

In the EFSA's (2011) non-binding guidance document, more detail is given, following the traditional risk assessment paradigm with a tiered approach to limit the number of nano-specific provisions. Similar to the FDA approach, the first tier is used to identify the presence of ENMs by characterizing physicochemical properties. The second tier involves a traditional risk assessment approach with a preliminary step aimed at identifying exposure scenarios where additional toxicity testing could be waived in the event that it can be proven that there is no exposure to ENMs in food, that is, complete transformation of ENMs in food prior to consumption or no release of ENMs from food contact materials (zero exposure scenario). This step, which precedes toxicity testing, is the reason that a small group of food contact materials containing ENMs have been accepted for use in the E.U. (European Commission 2011), due to evidence proving that no ENMs are released from the materials.

Given the general and non-binding nature of these guidance documents, it is evident that more work is necessary to support the development of ENMs and build consumer confidence. In the literature, these frameworks have been supplemented by a number of studies presenting their own frameworks that have been applied successfully to ENMs in food. For example, under the DF4nanoGrouping project, Arts et al. (2015) emphasize the use, release, transport, and exposure routes as "qualifiers" in a grouping framework, similar to the exposure scenarios outlined by the EFSA (2011). The authors propose a three-tiered approach to group nanomaterials into four main categories (soluble ENMs, bio-persistent high aspect ratio ENMs, passive ENMs, and active ENMs), with the aim to reduce unnecessary studies by prioritizing testing for ENMs that are most likely to cause adverse effects. If a material is not found to release nanomaterials, then it would potentially lead to the waiving of further unnecessary testing. This is one potential approach that could ease the burden associated with the case-by-case risk assessment of ENMs proposed by EFSA, which has been identified as an inefficient approach in the long term (Amenta et al. 2015). In the literature, it is apparent that few studies use this approach when assessing the safety of nanomaterials, opting for unrealistic high-dose toxicity studies using ideal test media and pristine nanoparticles (NPs).

Assuming that the ENMs are released and remain persistent in food up to consumption, prompting further investigation according to the framework by Arts et al. (2015), there are additional quantitative analyses that can be conducted to reduce unnecessary testing. A risk assessment framework proposed by Lai (2015), which uses quantitative structure activity relationship (QSAR) analysis to assess potential human hazards from respiratory exposure to nanoparticles, contains elements that are also applicable to the oral route of exposure. The model ranks the inhalation hazard concern level from high to low using specific nanoparticle physicochemical characteristic threshold values that were found to cause concern within *in vivo* and

in vitro studies. Although this model was generated for respiratory exposure, a model with a similar structure would be invaluable for oral exposure scenarios. The proposed model has advantages over a fully tiered risk assessment approach, as it requires less data to populate the risk ranking model. It also has the ability to reduce the number of animal tests required to provide toxicity endpoints and can, therefore, lead to cost and time saving. With this said, the framework has its limitations, providing only an initial indication of adverse health effects. There is the possibility that the unique behavior of ENMs in the gastrointestinal tract may be missed in the assessment, hiding potential detrimental consequences (Bergin and Witzmann 2013). Furthermore, if the assessment shows indications that there are potential health effects from exposure to the ENMs in question, a full quantitative assessment, including a series of toxicity studies, may be necessary.

A similar approach proposed in a study by Cockburn et al. (2012) established a two-tiered approach for the safety assessment of ENMs in food. The first tier involves an initial screening of the material using *in vitro* and *in silico* modeling to determine its behavior in food and the human GIT compared with its bulk counterpart. Where differences are identified, *in vivo* studies are carried out to gain a greater understanding of toxicity, which can provide a basis for tier 2 if the ENM is found to behave in a fashion meriting further investigations. In tier 2, the authors suggest that toxicological differences between ENMs and their bulk counterparts be established using sub-chronic 90-day *in vivo* studies. Where differences are observed, further testing must be conducted covering more stringent study parameters (i.e., reproductive or mechanistic studies if indicated). The flexibility incorporated into the model by the two-tiered system allows for dramatic cost and time savings in scenarios where the second tier can be excluded due to insignificant differences observed between toxicological profiles for ENMs and bulk materials. This view is supported by the World Health Organization (WHO)/FAO in a recent report on the risk assessment and management of nanotechnologies in the food and agriculture industry (WHO/FAO 2013) that used an example of nano-salts to illustrate the potential for *in vivo* testing to be waived following a first-tier assessment.

Other ENM risk assessment frameworks are present in the literature, although not dedicated solely to the oral route of exposure, such as the risk assessment for TiO_2 NPs applied to a workplace exposure scenario (including oral exposure) performed under the Nano Risk Framework (nanoriskframework.org 2007) (Warheit and Donner 2015). Using this framework, the authors found negligible uptake of TiO_2 NPs in the bloodstream from orally administered NPs that were excreted from the gastrointestinal tract. In addition, following sub-chronic oral toxicity studies in rats, a very low NOAEL of >1000 mg/kg_{bw}/day was observed. It is important to note that the results for the evaluated occupational exposure scenario would have limited relevance to alternative scenarios, where the predominant route of exposure is through consumption of food containing ENMs. The differences between

consumer. In this case, the main differences between consumer and occupational exposure scenarios includes the dose and frequency to which humans are exposed to ENMs via the oral route. In the light of this statement, the hazard data presented is relevant to scenarios where ENMs are present in food. Due to a lack of food industry–specific ENM risk assessment frameworks, it is important to consider well established frameworks generated for alternative industries and other routes of exposure to form a foundation for frameworks dealing with ENMs in the food industry.

4.7 Conclusions and Recommendations

In response to the projected growth of ENM use in the food industry, there is an urgent need to provide robust ENM risk assessments to ensure consumer confidence and safeguard human and environmental health. There is consensus that the traditional risk assessment paradigm is applicable for ENM risk assessment. However, it is apparent from the literature that gaps in knowledge related to each aspect of the traditional risk assessment paradigm, when applied to ENMs, are hindering progress. Considering hazard identification and hazard characterization, there is uncertainty related to the fate of ENMs in the human GIT (i.e., ADME). Furthermore, standard toxicity testing (*in vivo* and in vitro) requires attention to determine additional criteria that should be implemented to identify ENMs' unique characteristics that may cause adverse effects. In particular, physicochemical properties such as size, shape, solubility, stability, and surface area should be determined to identify the driving mechanisms behind ENM toxicity. A deficiency in physicochemical property reporting in toxicity studies has been emphasized as a factor prohibiting the use of such studies in ENM risk assessments. Furthermore, the issues surrounding the reporting of physicochemical properties are exacerbated by uncertainties related to additional dose metrics (e.g., surface area) that should be used alongside mass-based dose metrics as used in traditional toxicity studies.

Developments in analytical techniques for the identification and characterization of ENMs in food and biological media would benefit both toxicity and exposure assessment approaches, improving the reporting of physicochemical properties in toxicity studies and identifying potential sources of ENM human exposure throughout the farm-to-fork lifecycle. Due to the limited knowledge on the number of food products containing ENMs currently in use, it is difficult to quantify current human exposure; however, human exposure assessments with a forward-looking approach should be conducted to determine predicted exposure levels.

In regions (e.g., U.S. and E.U.) that have set out a strategy to deal with the risk assessment of ENMs in the food industry, a case-by-case approach has

been chosen to deal with the unique behavior witnessed between different ENMs. It has been argued that this is not the most sustainable method for ENM risk assessment in the long term; however, until gaps in knowledge are filled, it is the most appropriate approach to ensure safe development of ENMs for use in the food industry. In light of the additional studies required to cater for case-by-case risk assessments of ENMs, approaches have been suggested that aim to reduce the number of studies necessary by undertaking a tiered hazard-based approach that identifies ENMs that are potentially harmful and others that are toxicologically comparable with well documented non-nanoform materials. These approaches have been favored by regulatory bodies who have included decision-making frameworks in guidance documents that are designed to screen for potentially harmful ENMs.

Research attention should be focused on providing comprehensive risk assessments of a variety of ENMs in wide-ranging food applications to support the formation of integrated risk assessment frameworks for the use of ENMs in the food industry at an international level.

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5

Biofilms: A Community-Based Strategy for Bacterial Persistence and Relevance to Food Safety

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CONTENTS

5.1 Introduction

Biofilms are a microbial strategy to persist in challenging or changing environments. Fossilized records indicate putative microcolonies of biofilms that are over three billion years old (Hall-Stoodley et al., 2004). Formation of biofilms was also evidenced in the most ancient lineages of archaea and bacteria, indicating that the ability to assemble into multicellular aggregates is a trait that evolved early as a strategy for attachment and persistence (Hall-Stoodley et al., 2004). Biofilms can be found in soil, sediment, and

water ecosystems and might be one of the most common modes of bacterial existence in nature, contributing significantly to ecological processes such as carbon and nitrogen cycling (Høiby, 2014; Hall-Stoodley et al., 2004).

One of the first observations of biofilms was made by Anthony van Leeuwenhoek while observing scrapings of the tongue. He described his observation of microbial cells as "animalcules" after studying a dental biofilm commonly known as plaque. While dental biofilms have been associated with caries and periodontitis, the spoilage of food and the persistent occurrence of foodborne pathogens such as *Listeria monocytogenes* and *Salmonella enterica* in food processing plants could also result from biofilm formation. Louis Pasteur depicted biofilms in his sketches while describing the process of vinegar formation. Biofilms forming on beech wood chips used in vinegar production were described as "*la matiere visqueuse*" or the viscous material. The viscosity of biofilms is attributed to the formation of an extracellular matrix that encompasses a population of homogenous or a community of heterogeneous bacterial species. Studies on bacterial biofilms in association with pipes and ship hulls and as biofouling agents have indicated that bacteria are capable of attachment to abiotic surfaces such as glass and stainless steel. As a result, opportunistic and pathogenic bacteria are capable of biofilm formation on medical devices and food contact surfaces, resulting in a source of persistent nosocomial infection or food contamination, respectively. Biofilms on biological tissue of animals and plants form an important feature of microbiomes, resulting in a beneficial relationship with the host. Biofilm formation with opportunistic pathogens, on the other hand, leads to infections or detrimental effects to the host, depending on the type of bacteria encompassing the biofilms. Notably, 90% of a biofilm biomass consists of exopolymeric substances, with only 10% comprised of bacteria. The formation of the exopolymeric substance is the main feature that distinguishes sessile and adhering cells from a biofilm (Høiby, 2014; Hall-Stoodley et al., 2004).

5.2 A House with Many Building Materials: The Architecture of Biofilms

The architecture of biofilms is complex and varies depending on the sugar-based polysaccharide, proteins, and lipids produced by the bacterium in the extracellular polymeric substance (EPS); substratum for attachment; competing bacterial species; and biofilm age. The bacterial cells that are enclosed in a biofilm matrix are shielded by many macromolecules. The polymeric matrix that sheaths bacterial cells in a biofilm was formerly thought to consist mostly of extracellular polysaccharides or exopolysaccharides (EPS). Deeper analysis of the biofilm matrix has revealed a more complex consortium of proteins, glycoproteins, glycolipids, and extracellular DNA. Hence,

EPS now refers to the extracellular polymeric substance, to encompass all these components. The EPS forms a medium that aids attachment to surfaces, retains water, and allows dissolution of nutrients as it interacts with the environment.

5.2.1 Role of DNA

The EPS matrix of certain bacteria could incorporate a large amount of extracellular DNA (eDNA). Experiments using spectroscopy of the EPS of *Pseudomonas* revealed peaks at 260 nm and susceptibility to DNAse I, an enzyme that specifically cleaves DNA fragments but not RNA (Whitchurch et al., 2002). The source of eDNA is likely lysed bacterial cells (Allesen-Holm et al., 2006). The high DNA concentration within a fluid matrix supporting high cell densities could provide an ideal environment for horizontal gene transfer amongst its constituent microorganisms. High levels of conjugation have been observed in bacterial biofilms (Hausner and Wuertz, 1999), while evidence also supports the idea that cell competence is enhanced in biofilms, allowing for higher rates of non-conjugative transformation (Molin and Tolker-Nielsen, 2003; Maeda et al., 2006). The role of eDNA in a biofilm could be multifarious, serving not only as a source of DNA horizontal gene transfer but also for organizational integrity. The high molecular weight and viscosity of double-stranded eDNA provide a scaffold and give the matrix hydrophobicity in which bacterial microcolonies can develop. Treating 24 h biofilms of various bacterial species with DNAses had marked dose-dependent reductions in biomass and microcolony numbers following treatment, with cell-free patches forming within the biofilm (Tetz et al., 2009). Further evidence that eDNA plays a role in maintaining the structural integrity of a biofilm came from a study utilizing a biofilm of *Escherichia coli* BW25113; DNAse treatment for 8 h reduced the biofilm, while supplementing the system with exogenous DNA augmented the biofilm (Zhao et al., 2013). In *Neisseria meningitidis*, eDNA might also help in the establishment of microcolonies of bacteria by providing resistance against shear force and contributing toward host interaction of the pathogen (Lappann et al., 2010). The eDNA matrix forming the scaffold for the biofilm matrix can also contain enzyme complexes from lysed cells, hence acquiring enzymatic capabilities that are advantageous for the population. Such benefits may include the ability to inactivate antimicrobials, as well as releasing cells back into the environment upon detection of suitable conditions (Flemming et al., 2007).

Pseudomonas aeruginosa and *E. coli* have served as models for a wealth of biofilm research, leading to important findings regarding biofilms of enteric pathogens relevant to food safety. For instance, eDNA is an important component of *Listeria monocytogenes* biofilms and might play an important role in both initial attachment to substrates and early biofilm formation (Harmsen et al., 2010). On the other hand, extrapolating knowledge from model systems to enteric pathogens relevant to food safety research is not always

straightforward. Contrary to previous findings, a study on the role of eDNA in *Salmonella* biofilm formation on abiotic surfaces indicated that the presence of DNase I resulted in more biofilm being formed and better attachment to abiotic surfaces. The addition of extracellular DNA, on the other hand, resulted in inhibition of biofilm formation (Wang et al., 2014). Hence, to fully understand the role of biofilms in the attachment and persistence of foodborne organisms on abiotic surfaces, studies with foodborne pathogens of interest are needed.

5.2.2 Curli: Sticky Protein Scaffolding

Curli are defined as amyloid fimbriae, non-branching fibers that constitute the protein fraction of certain biofilms. In organisms such as *E. coli*, *Salmonella*, and *Staphylococcus*, curli play an important role in adhesion and biofilm formation. Curli-expressing *E. coli* O157:H7 strains formed stronger associations with lettuce and cabbage surfaces compared with weak curli-expressing strains (Patel et al., 2011). In *E. coli*, curli formation is controlled by the csgBA and csgDEFG operons. CsgA is the major curli subunit that polymerizes into β-sheet-rich amyloid fibers by the CsgB nucleator. CsgD is the outer membrane protein of the curli fiber, while CsgE and CsgF are responsible for the stability of the subunit and the nucleator proteins and their transport to the cell surface (Reichhardt et al., 2015). In *Salmonella*, curli biogenesis is similar to that in *E. coli* and the biogenesis of curli is mediated by the expression of the CsgD regulator of the csgBAC operon (Jonas et al., 2007). A study assessing biofilm formation among 15 clinical, 31 meat-associated, and 25 produce-associated *Salmonella* isolates revealed that all isolates were capable of developing biofilms. Of these, all the clinical and meat-associated isolates and 20 of the produce-related isolates were determined to be curli-producing strains (Solomon et al., 2005).

The formation of curli is assayed by the Congo Red plate assay, where the organism is streaked on a low osmolarity medium containing the dye. Congo Red agar plates were first introduced by Freeman et al. (1989). Biofilm forming *Salmonella* or *E. coli* have been known to produce an "rdar" morphotype expressing aggregative fimbriae that tightly bind cells (Römling et al., 1998). The "rdar" morphotype stands for red, dry, and rough and describes the colony structure formed on Congo Red agar due to binding of the hydrophobic dye with the β-strand structure of the curli subunits, contributing to a rugose appearance, together with cellulose production. Deficiency of curli causes the formation of smooth, pink colonies, while deficiency of cellulose causes brown, dry, and rough colonies (bdar). Deficiency of both curli and cellulose results in the formation of smooth and white colonies (saw). Hence, this medium has proven useful in the assessment of mutants used to study biofilms.

Outside the animal host, the presence of curli can result in increased fitness as the pathogen transitions to another animal host. Curli synthesis

mediates better attachment of bacteria to abiotic surfaces and to plant surfaces. The ability to produce thin aggregative fimbriae by *Salmonella enterica* and simultaneous cellulose synthesis enhanced biofilm capabilities of the tested strains on abiotic surfaces (Jain and Chen, 2007). This advantage has been demonstrated also for biotic surfaces. Mutant strains of curli-deficient *E. coli* O157:H7 were impaired in their ability to attach to spinach leaf surfaces (Macarisin et al., 2012). Variability in leaf roughness among various spinach cultivars also benefitted attachment in curli-expressing strains of this pathogen (Macarisin et al., 2013). Curli expression, however, conferred no advantage during internalization of *E. coli* O157:H7 via spinach root uptake (Macarisin et al., 2014). There are several benefits to having curli-reinforced biofilm scaffolds. Curli bolster the stability of the matrix and augment resistance to proteases and detergents. Curli expression is highly stratified even within a single colony. When the rdar morphotype is formed, the bacteria on the rugose portion of the colony that are exposed to oxygen express curli while the cells within the colony do not. The bimodal colonies are triggered by oxidative stress, and rdar morphotypes are resistant to many oxidizing sanitizers (Evans and Chapman, 2014). In addition to curli, other putative fimbrial proteins, including type 1 fimbriae, type 3 fimbriae, and BAP-like adhesins, might play important roles in the adhesion of foodborne pathogens to surfaces such as host cells (Römling et al., 2014; Steenackers et al., 2012).

5.2.3 Polysaccharides in Biofilms

Polysaccharides serve as major constituents of a biofilm structure existing as fine strands attached to cell surfaces, forming complex networks. The formation of polysaccharides is important in biofilm formation, and bacterial species that do not produce bacterial polysaccharides often exist with those that do in mixed species biofilms. Many bacteria produce complex heteropolysaccharides in the exopolymeric matrix, and the sugars often contain charged or neutral residues (Flemming and Wingender, 2010).

E. coli and *Salmonella enterica* biofilms may contain cellulose, but also capsular polysaccharides and lipopolysaccharides. Solomon et al. (2005) identified cellulose production in 73%, 84%, and 52% of the clinical, meat-associated, and produce-associated *Salmonella* isolates tested. The bcsABZC and bcsEFG operons are needed for cellulose synthesis in *Salmonella* Typhimurium and *Salmonella* Enteritidis (Solano et al., 2002). The emerging foodborne pathogen *Cronobacter* spp. also carries the bcsABZC operon. The genes bcsABC are needed for cellulose synthesis and were detected in most of the 231 clinical, food, environmental, and other isolates tested (Hu et al., 2015). Specifically, this study found that bcsA and bcsB were necessary not only for cellulose production but also for biofilm formation and cell aggregation. By contrast, cellulose formation did not appear indispensable for biofilm formation of *E. coli* O157:H7 strain EDL933 on spinach leaves (Macarisin et al.,

2012). Regardless of whether bacteria themselves are able to synthesize specific carbohydrates, plant cell wall–derived carbohydrates released during processing of fresh produce can influence attachment. The matrix formed between cellulose fibrils and pectin in a plant cell wall model demonstrated that the structure itself and not the specific components aided retention of *Salmonella* cells within the matrix (Tan et al., 2016).

Biofilms formed by mucoid stains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients contain alginate, while *E. coli* biofilms also contain a polyanionic polysaccharide known as colanic acid. Colanic acid production in *E. coli* is responsible for complex three-dimensional structure formation of biofilms but not for initial attachment (Danese et al., 2000). A gene cluster similar to that of *E. coli* for the production of colanic acid has also been identified in *Salmonella* (Stevenson et al., 2000).

Capsular polysaccharides might play an important role in adhering to surfaces, which is the first step required for biofilm formation. Capsular polysaccharides might help in repelling van der Waals forces, improving adhesion, and can survive in the biofilm matrix in excess of a decade. A fluorophore-assisted carbohydrate electrophoresis (FACE) analysis of the capsular polysaccharide of *Salmonella* Typhimurium DT104 indicated that the main sugars were glucose, mannose, and galactose (de Rezende et al., 2005). In *Listeria monocytogenes*, the presence of *N*-acetylglucosamine along with eDNA resulted in increased adhesion to abiotic surfaces, and the complexity and size of these intracellular molecules might play an important role in cell adhesion and biofilm formation (Harmsen et al., 2010).

5.3 Biofilm Formation on Food Contact Surfaces and Food

Biofilms form in a process of bacterial succession initiated by cells that attach to a surface, followed by accretion of the biofilm and recruitment of other species to continue to develop a multispecies biofilm dictated by physicochemical conditions and intricate and complex trophic interactions. The steps involved in biofilm formation include: (1) adhesion to the substratum and overcoming forces that might repel the microorganism, (2) microcolony development followed by formation of exopolymeric substances, (3) maturing/development of the biofilm through the formation of columns and channels, and (4) release of bacterial biofilm residents into the environment.

5.3.1 Food Contact Surfaces

Foodborne pathogens of concern in the modern food processing environment such as *Salmonella enterica*, pathogenic *E. coli*, and *Listeria monocytogenes* have

been shown to form biofilms on abiotic surfaces (Table 5.1). In a study evaluating the ability of *Listeria monocytogenes* to form biofilms on different surfaces in a food processing plant, the pathogen was capable of biofilm formation on Teflon, stainless steel, nylon, and the polyester floor sealant (Blackman and Frank, 1996). In the presence of a nutritionally weak medium, the pathogen did not produce biofilm on the polyester floor sealant and at a temperature of 10°C, while it produced weaker biofilms on Teflon and stainless steel (Blackman and Frank, 1996). A comparison of 122 *Salmonella enterica* serotypes isolated from food, animals, and humans for their biofilm production capabilities revealed that all serotypes produced biofilm in plastic microtiter plates (Stepanović et al., 2004). *Salmonella enterica* produced more biofilm in comparison to *Listeria monocytogenes.* Interestingly, *Salmonella enterica* biofilm formation in a nutrient-poor medium was superior in comparison to *Listeria monocytogenes* (Stepanović et al., 2004). On surfaces commonly encountered in food processing plants, such as cement, high-density polyethylene, and stainless steel, it was observed that *Salmonella* Weltevreden formed most biofilm on plastic and the least on stainless steel. The cell density of the organism was also found to be reduced on stainless steel (Joseph et al., 2001).

Stainless steel is less hydrophobic than rubber, and it is known that bacterial cells attach better to more hydrophobic surfaces. Stainless steel is, hence, a commonly used material in food processing industries. The surface finish of stainless steel could also play an important role in biofilm formation. When stainless steel surfaces with different finishes were evaluated for their ability to support *Salmonella* attachment and biofilm formation, it was observed that the presence of cracks or abrasions resulted in improved attachment

TABLE 5.1

Biofilm Formation by Foodborne Pathogens on Produce Surfaces and Equipment

Organism	Food/Food Contact Surface on which Biofilm is Formed	References
Salmonella enterica	Plastic, cement, stainless steel, parsley, melons	Joseph et al., 2001 Lapidot et al., 2006 Annous et al., 2005
Listeria monocytogenes	Plastics, cement, stainless steel, condensate forming surfaces, lettuce leaf	Hassan et al., 2004 Joseph et al., 2001 Ölmez and Temur, 2010
E. coli O157:H7	Stainless steel (type 304, no. 2b finish) and HDPE (smooth finish) surfaces used in beef processing facilities Romaine lettuce and spinach leaf surfaces.	Dourou et al., 2011 Niemira and Cooke, 2010
Enterobacter sakazakii	Stainless steel and enteral feeding tubes	Kim and Beuchat, 2006
Campylobacter jejuni	Plastic, possibly chicken house nipple drinkers	Trachoo and Frank, 2002
Bacillus cereus	Lettuce and cabbage surfaces	Elhariry, 2011

of *Salmonella enterica* to the surfaces. On rough surfaces, the thickness of *Salmonella* biofilm after 5 days was four times higher than on the first day, with a 77% increase in biofilm formation (Schlisselberg and Yaron, 2013). In comparison to *Listeria monocytogenes*, biofilms were comparatively shallower than the *Salmonella enterica* biofilm on the first and fifth days. The electropolished surface was the smoothest amongst the stainless steel finishes tested and also had the least number of attached cells. Both electropolished and machine-sanded stainless steel surfaces had the lowest amount of biofilm formation over the 5 day duration (Schlisselberg and Sima, 2013). The presence of biofilms of increased thickness can result in cross-contamination to other surfaces due to the dispersal of microcolonies, as well as enhanced resistance to disinfection.

In *E. coli* O157:H7, the presence of curli played a significant role in the formation of biofilm on stainless steel coupons (Ryu et al., 2004). The type of food contact surface and the medium present in the environment could play an important role in foodborne pathogen biofilm formation. *E. coli* O157:H7 formed increased biofilms on stainless steel and high-density polyethylene when ground beef was present. Attachment of *E. coli* O157:H7 to the surfaces was also observed at 4°C.

5.3.2 Biofilm Formation on Produce

Different types of foods provide ideal surfaces for biofilm formation by way of uneven surfaces and nutrients for attachment and bacterial utilization (Table 5.1). Foodborne pathogens isolated from poultry, meat and produce such as cabbage and spinach are capable of forming biofilms on contact surfaces (Wang et al., 2013; Igbinosa, 2015). Tomato outbreak–associated *Salmonella* serotypes were capable of producing biofilm on quartz dust particles. The amount of EPS produced by the serotypes tested changed over time with the highest amount of EPS being measured around day 6 followed by reduction of EPS at days 10 and 14. The results from the study indicate that sediments could harbor *Salmonella* due to biofilm formation by the pathogen and could serve as a source of contamination in water bodies such as irrigation ponds, ditches, and canals (Dev Kumar et al., 2013). Specific ecological niches may select for stronger biofilm capabilities. Produce-recovered isolates of *Salmonella enterica* were better adapted at forming biofilms on abiotic surfaces and spinach leaf surfaces compared with poultry-recovered isolates (Patel et al., 2013).

The role of enteric pathogens in plant-associated biofilms is not known. *E. coli* phylogroups vary by ecological fitness and their ability to form biofilms on plant tissues. Recent outbreaks associated with *E. coli* O157:H7 and *E. coli* O104:H4 along with the innocuous occurrence of non-pathogenic *E. coli* from produce indicated adaptation to epiphytic existence. *E. coli* isolates from plants produced more biofilm than other mammalian and human isolates (Méric et al., 2013).

Biofilms are frequently multispecies, and cooperation among constituents may be one advantage to this lifestyle. Natural multispecies biofilms on sprouts of various seeds have been visualized with scanning electron microscopy, showing biofilms on root, hypocotyl, and cotyledon structures (Fett, 2000). Enteric pathogens could be recruited to multispecies biofilms in the phyllosphere, although whether the nature of their interaction with neighboring taxa is competitive or cooperative remains to be investigated. The presence of certain taxa may favor the establishment of new species based on positive microbe–microbe interactions. This synergistic effect on biofilm formation may occur between plant native microbiota and foodborne pathogens. It was observed that *Burkholderia caryophylli* isolated from a fresh-cut produce facility increased the survival of *E. coli* O157:H7 by 180% while simultaneously growing its own population (Liu et al., 2014). Similarly, curli-negative variants of *E. coli* O157:H7 were capable of synergistic biofilm formation with native microbiota present in produce wash water (Dev Kumar and Ravishankar, 2014).

Biofilm formation by foodborne pathogens could be an important strategy to persist on plant and animal tissues, which in turn may have important implications for food safety. The presence of an EPS matrix on plant surfaces might result in decreased efficacy of sanitizer treatment. Biofilms of *E. coli* O157:H7 were able to form on Romaine lettuce and spinach, conferring enhanced tolerance to chlorine washes and even irradiation (Niemira and Cooke, 2010). Location of the biofilm, as opposed to exopolymeric substances, however, has been described as an important factor in conferring this protection from antimicrobial treatments, as was demonstrated for *E. coli* O157:H7 biofilms on spinach leaf surfaces versus spinach leaf edges (Lee et al., 2016). The observation of *Salmonella*-inoculated rinds of cantaloupe with scanning electron microscopy indicated biofilm occurrence 24 h after inoculation by the pathogen (Annous et al., 2005). Biofilms formed on plant surfaces also provide the contaminating microorganism with protection against the plant defense system. It was observed that *Pseudomonas aeruginosa* was resistant to the rosamarinic acid produced by sweet basil when the cells were in biofilms but not when the cells were in planktonic state (Walker et al., 2004).

Cellulose could play an important role in *Salmonella* Typhimurium biofilm formation on tomato surfaces, as cellulose-deficient mutants were significantly impaired in biofilm formation compared with the wildtype (Fatica, 2013). The use of episcopic differential interference contrast microscopy to observe *Salmonella enterica* interaction with native microbiota and biofilm formation on spinach leaves indicated that plant leaves can harbor 5 Log CFU bacterial cells per mm of leaf surface and that biofilm material is present on leaves. *Salmonella enterica* was able to attach to cell margins and around stomata where biofilm microcolonies were present (Warner et al., 2008). As mentioned earlier, plant-derived carbohydrates released during cutting and chopping of fresh produce may also play a role in building a network in which *Salmonella* cells can become lodged.

Aside from pathogen risk, the presence of biofilms on food may impart undesirable sensory factors and impact marketability. Biofilms could impart a slimy texture to a food surface, and consumers may gauge the quality or "freshness" of foods by feel, coloration, and odor, which may be directly impacted by the presence of biofilms.

5.3.3 Dispersal of Bacteria from Biofilms

While the formation of biofilms on food surfaces results in contamination and spoilage of food, the release of bacteria from biofilms might result in contamination of contact surfaces and equipment and even in dispersal of the organism to non-contaminated stocks. The dispersal of cells from a biofilm consists of three steps: (1) detachment of cells from a biofilm, (2) transfer of cells to a new location, and (3) attachment of cells to a new substratum.

Dispersal of cells from a biofilm can be achieved though both active and passive processes. Active dispersion results from internal mechanisms within the biofilm, while passive dispersal occurs as a result of physical forces such as shearing. Dispersal of biofilms can be categorized as erosion, sloughing, and seeding based on the amount of cells and rapidity by which cells are being disseminated. Dispersal of cells can be facilitated by enzymatic activity such as by DNases, proteases, and glycosidases that break down the structural components of the biofilm architecture. Release of cells from biofilms can also occur following treatment with detergents such as rhamnolipids or dispersins that affect fimbrial adhesion. Cells might also be shed from the biofilm matrix by cell division where the progeny are not bound by the adhesive forces of the biofilm.

The presence of quorum-sensing molecules can regulate many processes in the biofilm life cycle from the aggregation and attachment of cells to channel formation and cell dispersal (Kaplan, 2010). In *Xanthomonas campestris*, the diffusible signaling factor (endo-β-1,4-mannanase) can cause the dispersal of the organism from a biofilm (Solano et al., 2014). Other biomolecules capable of promoting dispersal of bacteria from biofilms include fatty acids and nitric oxide. The fatty acid, *cis*-2-decenoic acid, is a diffusable signaling factor that can result in the release of *Pseudomonas aeruginosa* cells from its biofilm (Davies and Marques, 2009). Nitric oxide is a key signaling molecule involved with plant defense against pathogens. Nitric oxide donors resulted in the dislodging of native, sessile *Salmonella enterica* and *E. coli* O157:H7 from plastic and stainless steel surfaces, indicating that the dynamic changes in plant tissue after a contamination event might result in bacterial dispersal from biofilms (Marvasi et al., 2014). Nitric oxide formation was also capable of inducing the release of *Salmonella enterica* from biofilms at 4°C. The potential of fatty acids, nitric oxide, and other molecules to cause shedding of foodborne pathogens from produce-associated biofilms requires further exploration as these molecules are frequently associated with plant surfaces.

5.3.4 Recalcitrance of Microbial Biofilms

The architecture of a mature biofilm contributes to its recalcitrance. Biofilms often have a high population of bacterial cells stacked in columns while encased in the polymeric sheath. When challenged with an antimicrobial or a sanitizer, the physical barriers presented by the biofilm directly result in reduced efficacy. *Salmonella* in a biofilm matrix can resist chlorine treatments as high as 500 mg/L while planktonic cells are susceptible to 10% of the same concentration. A study of chlorine treatments of parsley contaminated with EPS-producing *Salmonella* revealed that chlorination was ineffective in inactivating the pathogen even when high concentrations of the sanitizer were used (Lapidot et al., 2006). When assessing the efficacy of the disinfectants sodium hydroxide, sodium hypochlorite, and benzalkonium chloride, only the former was capable of completely eradicating a 2-day-old *Salmonella* Agona biofilm, and this ability was lost when treating more mature biofilms (Corcoran et al., 2014). Factors that can result in reduced efficacy of sanitizers on biofilms include reduced depth of penetration and inactivation of the sanitizer by neutralizing enzymes. When hydrogen peroxide was used to treat biofilms formed by *Klebsiella pneumoniae,* catalase-based inactivation of the sanitizer was observed (Anderl et al., 2003).

These responses often overlap with strategies employed by bacterial biofilms to resist antibiotics. The possible mechanisms of antibiotic resistance are numerous and can include environmental and genetic factors. Horizontal transfer of genetic materials such as plasmids or bacterial conjugation can result in the exchange of antibiotic resistance genes between bacterial species (Annous et al., 2006; Høiby et al., 2010). Antibiotic resistance genes common among human microbiota, food animal microbiota, and *Salmonella enterica* indicate that a broader linkage of resistance genes exists in the farm-to-food cycle (Marshall and Levy, 2011; Zhao et al., 2003). Food processing facilities such as harvesting facilities and produce packaging houses often result in an interactive diaspora that promotes genetic exchange.

Biofilms pose a sanitation challenge to the food industry because they provide foodborne pathogens with protection from physical injury, promote horizontal gene transfer, and enhance communication between cells that could allow metabolic interactions (Sauer, 2007). These factors could provide increased protection from antimicrobial agents and aid in the acquisition of new genetic traits and survival in challenging environments (Speranza et al., 2011). *Salmonella enterica* is capable of attaching to equipment surfaces and persisting in processing environments by forming biofilms. Biofilms can develop on solid surfaces and water interphases such as floors, cutting and deboning equipment, and produce processing and washing equipment (Annous et al., 2006). Bacteria in a biofilm can be dispersed into the environment under certain conditions, such as abrasions, changes in the environment, and pH and chemical oxidation (Sauer, 2007). When released back into the environment as planktonic cells, these cells could carry antibiotic resistance

genes, traits acquired during their stint within biofilms. The increase in the spread of antibiotic resistance among foodborne pathogens clearly suggests this. A 1998 *Salmonella* Typhimurium outbreak in Denmark resulted from strains that were nalidixic acid–resistant and displayed reduced fluoroquinolone susceptibility (Mølbak et al., 1999). The strain was isolated from patients, pork samples, herds, and the harvesting facility, indicating various routes of spread (Mølbak et al., 1999). Bacterial cells can undergo phase variations and contain antibiotic resistance genes for enzymes that break down antibiotics or for efflux pumps through genetic exchange and adaptations in biofilms (Høiby et al., 2010). These genetic and physiological adaptations of biofilm existence could also result in cross-protection to sanitizers that are commonly used in food processing, resulting in survival of the pathogen after processing plant sanitization (Alexander et al., 2010).

A previous study on 26 strains of *Staphylococcus* aureus isolated from fisheries indicated that the biofilms formed by the microorganisms made them more resistant to the sanitizers than their planktonic counterparts (Vázquez-Sánchez et al., 2014). This indicates the importance of finding alternate sanitizers that have the capability to prevent biofilm formation and exhibit permeability through biofilm matrices. This last point is significant, since individual constituents of bacterial biofilms often do not retain their resistance to antimicrobials and sanitizers during a planktonic stage. Effective penetration of an antimicrobial, therefore, may boost its activity against biofilms.

5.3.5 Control of Biofilms

Biofilms can be controlled through physical, chemical, and biological mechanisms. Physical mechanisms involve producing enough shear force and abrasion to disrupt the structure of the biofilm, while chemical treatments can destabilize the architecture of the biofilm. Biological methods involve the use of microbial competition to reduce resources for the target organism. One of the possible caveats to keep in mind while using these methods to control biofilms is to ensure that the method does not result in further dispersal of the component organisms in a biofilm as it is being degraded by the control treatment. An example of this was reported in a study assessing the efficacy of flushing an irrigation water distribution system in a crop field with chlorine, with the intention of controlling biofilms in the drip tape and ensuring water quality. Instead, water at the end of the line following chlorine flushing had higher bacterial loads, indicating sloughing off of cells from the biofilm, and, hence, degrading the quality of the water delivered to the fields (Callahan et al., 2016).

5.3.6 Biochemical Sanitizers

Chemical treatments involving disinfectants such as chlorine and antibiotics have been notoriously ineffective against biofilms. Recently, some strategies

have been demonstrating more promising results. A combination of steam for 20 s and iodophore at a concentration of 20 ppm resulted in the reduction of viable cell counts in a mixed pathogen biofilm consisting of *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on stainless steel coupons (Ban and Kang, 2016). In general, however, chemical treatment alone is insufficient to control biofilms. For this reason, several other strategies involving plant-derived active compounds or enzymes are being explored. Essential oils have potent antimicrobial activity as they can disrupt cell membranes of bacteria and bring about ATP leakage. The potential antimicrobial activity of carvacrol, an essential oil derived from oregano, thyme, or marjoram, was tested against dual species *Salmonella* and *S.* aureus biofilms. The continuous use of a 5 mM emulsion of carvacrol resulted in no viable *Salmonella* and complete inhibition of *S.* aureus biofilm formation (Knowles et al., 2005). Hence, the use of essential oil emulsions in wash waters and flume tanks during washing of fresh produce could potentially prevent biofilm formation by pathogenic organisms on produce washing equipment. Extracts of *Punica granatum* L. (pomegranate) and *Rhus coriaria* L. (Sicilian sumac) with high polyphenol content and antioxidant activity were effective against biofilms of bacterial species relevant to food safety, *Listeria monocytogenes* and *E. coli* (Nostro et al., 2016).

Enzyme treatments such as ones targeting the constituents of the EPS matrix could prove effective in the reduction of biofilm biofouling. Options for reducing biofilms include the use of anti-quorum-sensing enzymes such as *N*-acyl homoserine lactonases and acylases, proteases, polysaccharide-degrading enzymes, and phages that can produce polysaccharide depolymerases capable of degrading the EPS. The combination of a physical disruption method, such as sonication and ultrasound, and a chemical sanitizer has often proved to be more effective than a single treatment (Meireles et al., 2016).

5.3.7 Biological Control

Considering the problematic recalcitrance of biofilms to chemical disinfection, and the tendency for biofilms to recur, the prospect of completely eliminating biofilms with other biological agents holds much promise. The released exopolymeric material produced by *Lactobacillus acidophilus*, a lactic acid bacterium that plays an important role as a probiotic and as a starter for many fermented foods, was able to impair biofilm formation by *E. coli* O157:H7 and also contributed to decreased adherence to intestinal cells by the pathogen (Kim and Kim, 2009). Anti-biofilm activity of released exopolymeric material produced by *Lactobacillus acidophilus* was also observed against *Salmonella enterica*, *Yersinia enterocolitica*, and *Listeria monocytogenes*, although the reduction in biofilm formation by *E. coli* O157:H7 may be due to the suppression of genes required for curli formation and chemotaxis in this pathogen (Kim and Kim, 2009). Lectins are non-catalytic sugar binding

molecules produced by a vast range of microorganisms with important functions associated with cell signaling and interactions. Lectins have been proposed as potential antimicrobial agents, and the anti-biofilm potential of *Lactobacillus rhamnosus* was explored. The lectin molecules, LLP2 in particular, reduced biofilm formation by *Salmonella*. Observation of biofilms indicated damage to the structure of the biofilms. No such effects were observed when plant-derived lectins were used (Petrova et al., 2016). *E. coli* O157:H7 population in mixed biofilm communities from spinach lysates decreased significantly after 48 h due to competition with native microbiota for nutrients (Carter et al., 2012). The reduction in *E. coli* O157:H7 population was supported through metagenomics analysis that indicated a shift in gene composition during biofilm maturation. The epiphytes appeared to be more successful in assimilating available nutrients than *E. coli* O157:H7, and thus out-competed the pathogen (Carter et al., 2012).

A different strategy to control biofilms could come from lytic viruses of bacteria—bacteriophages and their enzymes. The bacteriophage-encoded enzymes that may present a successful approach could include enzymes that degrade the EPS, dissolving the biofilm matrix, or enzymes that break down bacterial cell walls, neutralizing cells directly (Chan and Abedon, 2015). Control of foodborne enteric pathogens by bacteriophages has been evaluated for certain pathogen-commodity pairs. Spraying or directly applying *Listeria monocytogenes*-specific phages to fresh-cut honeydew melons resulted in remarkable reductions in the pathogen populations. In combination with a bacteriocin from lactic acid bacteria, nisin, phage treatment was enhanced and effective also on apple slices (Leverentz et al., 2003). The efficacy of lytic phage specific to *E. coli* O157:H7 EDL933 was also evaluated on pepper and spinach surfaces, with successful log reductions of the bacterial pathogen over 72 h (Snyder et al., 2016). Our work has also demonstrated log reductions of *Salmonella enterica* Newport on the surface of cucumbers in a temperature-dependent manner, with activity at 10°C but not 22°C (Sharma et al., *submitted*). The effectiveness of lytic bacteriophage treatment of biofilms containing enteric pathogens or post-harvest pathogens requires further research, but this treatment holds promise as a way to control contamination on produce and possibly extend shelf life.

Quorum-sensing molecules play an important role in cell to cell communication and for cells in a biofilm to behave in unison. Quorum-sensing molecules can influence attachment, formation of cell aggregates, filamentation, and swimming motility, factors important for biofilm formation and maturity (Kalia, 2013). *Salmonella* Typhimurium mutants that were deficient in quorum-sensing molecules were less capable of forming biofilms on gallstones (Prouty et al., 2002). Quorum-sensing molecule disruptors are being employed to disrupt biofilms as antibiotics are often ineffective. The plant essential oil carvacrol demonstrated anti-biofilm activity against *Salmonella* but not *Pseudomonas*. Concentrations of carvacrol that were sub-lethal to planktonic cells were effective against biofilm formation but ineffective against mature

biofilms. The effectiveness of carvacrol against quorum-sensing molecules produced by *Chromobacterium violaceum* indicates that its mode of action may result from the disruption of quorum-sensing signaling (Ojo-Fakunle et al., 2013). The fatty acid, *cis*-2-decenoic acid, which resulted in the release of bacteria such as *E. coli* and *Klebsiella* from biofilms, could be a component of a diffusable signaling molecule produced by *Pseudomonas aeruginosa* (Davies and Marques, 2009). Signaling interference using quorum-sensing quenchers and disruptors of signaling molecules and their analogs has potential as a biofilm control strategy.

5.3.8 Physical Methods

Physical methods to control biofilms include developing materials that reduce adherence of pathogenic organisms and consequentially preventing the establishment of biofilms. Hydrophilic polymers such as polyethylene glycol have been incorporated on surfaces, resulting in decreased bacterial adhesion. Materials containing antimicrobials or antimicrobials that can be activated are also new options being explored. The incorporation of titanium dioxide nanoparticles on glass and stainless steel surfaces was explored for its antimicrobial potential against *Listeria monocytogenes* due to free hydroxyl radical generation by activated titanium dioxide particles. After a 180 minute-long treatment of stainless steel surfaces, *Listeria monocytogenes* cells were not detected but the presence of biofilm was still observed (Chorianopoulos et al., 2011).

While biofilms are recalcitrant to many chemical sanitizers, they can be disrupted by physical mechanisms. Sonication results in cavitation bubbles that can result in the dislodging of microorganisms. The combined use of ozone at a concentration of 0.5 ppm in combination with sonication (20 kHz and at 100% amplitude) resulted in a greater decrease of the pathogen in biofilm than either of the treatments alone. The combined use of these treatments resulted in excess of 4.5 Log CFU/ml of *Listeria monocytogenes* cells embedded in a biofilm matrix. One of the disadvantages of the treatments is that complete submersion of the item to be sanitized is required (Baumann et al., 2009).

5.3.9 Methods to Detect Biofilms

Bacterial biofilms are a dynamic environment and are often difficult to analyze. Chemical analysis of bacterial biofilm constituents is often challenging. The simplest method for quantifying bacterial exopolymeric substance is staining and quantifying the attached or bound stain. Biofilm formation can be measured by culture-based methods where sessile cells are dislodged by vortexing or sonication. Alternative techniques have been developed for quantitation of biofilms such as crystal violet (CV) staining and Syto9 staining (Coenye and Nelis, 2010). Stains like CV can be used to quantitate cells

in biofilms. CV staining was first described by Christensen et al. (1985) and has since been modified for increased accuracy and to allow biofilm biomass quantification. CV binds to negatively charged surface molecules and polysaccharides in the extracellular matrix (Li et al., 2003; Peeters et al., 2008). Dye extraction was performed by Prouty et al. to determine the amount of dye retained by bacterial cells attached to glass slides (Prouty et al., 2002).

The Calgary Biofilm Device (CBD) consists of 96 pegs suspended from the lid into wells similar to a 96-well microtiter plate. The advantage of this device is that biofilms formed from bacteria placed in the wells are of the same thickness and architecture. Hence, the effect of different chemicals, antimicrobials, and biocontrol agents can be tested (Ceri et al., 1999). The biofilms develop on the pegs, and the pegs can be detached and studied using staining methods such as viability dyes, nucleic acid stains such as acridine orange, and leptin-based dyes that stain the exopolymeric substances (Harrison et al., 2006). The advantage of the method is consistency and reproducibility of results. Cells of the biofilm can also be further analyzed for population enumeration and gene expression by dislodging them using sonication. The pegs of the CBD are made of polystyrene and have a neutral charge (Harrison et al., 2006). The tip of the peg is rounded and, when immersed into the wells, air surface interface pellicle formation can also be studied at the neck region (Ceri et al., 1999). This device eliminates the need for tubes, decreasing the chances of cross-contamination. Single- and multispecies biofilm architecture can be studied using this device, making it a versatile tool.

Advanced imaging methods such as laser scanning confocal microscopy, scanning electron microscopy, and fluorescent microscopy have been employed. Confocal microscopy can be used to study the depth of cell stacking usually observed in biofilms. Handheld scanners using optical coherence tomography can be used to detect biofilms in real time and detect them on surfaces. Non-invasive methods used to study biofilms include spectroscopy techniques such as Raman spectroscopy and Fourier transform infrared spectroscopy that can obtain the infrared spectrum of the biofilm. Hyperspectral imaging is a novel method to detect and study biofilms in real time. Impedance-based sensors that detect differences in surface charge, resonance, or conductivity might aid in the early detection of biofilms (Tan et al., 2014).

Biofilm architecture is complex, consisting of channels, enzyme reservoirs, and variations in EPS thickness. While atomic force microscopy, confocal microscopy, and scanning electron microscopy have been used to study the presence of organisms in a biofilm matrix, many new, innovative approaches are being explored (Harrison et al., 2006). Harrison et al. studied biofilm architecture by using confocal laser scanning microscopy and creating Z stacks to develop an architectural map of biofilms formed on the pegs of a CBD (Harrison et al., 2006). They processed the images generated by confocal laser scanning microscopy of stained *Candida* biofilms and used 3D

visualization software to create images and study the architecture and the presence of live and dead cells. Much open source and commercial software exists for these purposes (Harrison et al., 2006).

Electron microscopy is often used to study morphological changes in cells and cell structure, providing the advantage of magnification and clarity. Four methods of electron microscopy are conventional scanning electron microscopy (SEM), cryo-scanning electron microscopy (CRYO-SEM), environmental scanning electron microscopy (ESEM), and focused ion beam scanning electron microscopy (FIB SEM). The techniques were compared since biofilms consist of hydrated matrices that become dehydrated following sample preparation for conventional SEM. In CRYO-SEM, the samples were immersed in a slush of liquid nitrogen at –210°C and fixed under a vacuum, while ESEM was carried out in a humidified environment with samples imaged using a gaseous secondary electron detector. In FIB SEM, the biofilm segments were imaged using a high-resolution SEM and 3D images were created of segments to visualize 3D architecture. While SEM provided clear images with high magnification, the structure was altered because of dehydration. CRYO-SEM yielded images of lower resolution than conventional SEM because of lower conductance, while ESEM was inadequate at magnifications above 10,000 X, due to electron beam damage. FIB SEM resulted in a high-resolution image comparable to SEM but provided the added advantage of developing a 3D model of biofilm structure similar to confocal laser scanning microscopy, with the added advantage of higher magnifications (Alhede et al., 2012).

Growth conditions of biofilms play an important role in their study. Biofilms can contain numerous species of bacteria and can develop in dynamic environments where shear force is constant or can change with time. These conditions can be simulated in microfluidic chambers. The use of -omic techniques such as metagenomics, metabolomics, and metatranscriptomics can reveal a better understanding of the complexity in the biofilm's interactions between its constituent species and their biotic surfaces.

5.4 Conclusion

- While biofilms can result in persistence of foodborne pathogens and spoilage organisms, they also have many uses such as fermentation and outcompeting pathogenic bacteria.
- Biofilms establish themselves on a substratum by attachment, microcolony formation, and exopolymeric substance generation. Bacterial cells can be released from a biofilm into the surrounding environment.

- The constituent building blocks of a biofilm can vary depending on the species of bacteria. They can include polysaccharides, extracellular DNA, and fimbriae.
- Horizontal genetic transfer occurs frequently in bacterial biofilms, and genes acquired can also include pathogenic and antibiotic resistance genes. Cells released from a biofilm might also be slow growing and have resistance to certain biocides.
- The biofilm environment is complex and can contain over 500 different species of microorganisms. The relationship between these species can range from competition to synergism based on the presence of nutrient availability and the nutrient assimilation profiles of the resident microbiota.
- Biofilm production can protect microorganisms from hostile conditions such as nutrition depletion and result in enhanced survival in challenging environments.
- The presence of biofilms protects their bacterial inhabitants from antimicrobial agents such as antibiotics and disinfectants at concentrations that are lethal to planktonic cells.
- Sanitation protocols used to treat biofilms require a mix of physical and chemical treatments to reduce attachment to the substrata and to facilitate contact with the sanitizer.
- When bacteria are released from a biofilm, they usually lose their resistance to sanitizers.
- During sanitation treatment of biofilms, it is imperative not to spread fragments of the biofilm due to physical shear as this can result in spread of the biofilm in the food processing environment.

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6

Spectroscopic Methods for Fresh Food Authentication: An Overview

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CONTENTS

6.1 Introduction

Consumers recognize the value of the information supplied on food labels, including the description of the ingredients and information about the production processes applied to the final product. The food consumer's choice often reflects lifestyle, religion, awareness of the nutritional properties of food and health concerns. In fact, the identification and authentication of food play an important role in a healthy diet. The verification and reporting of food product components is therefore needed to prevent the practice of adulteration.

Consumers have, in particular, become more demanding in their meat and fish consumption, in terms of quality, safety, and the origin of the products they consume. Recent reports into the occurrence of food fraud suggest that an effective identification of the species as part of food authentication is required (Andrée et al. 2010, Ballin 2010, Standal et al. 2010, Lin et al. 2014).

Animal products, particularly meat and fish, can be targets of adulteration, such as the substitution or removal of ingredients, addition of other proteins from various origins, and the addition of food additives and genetically modified organisms (GMO) not described on the label, often contributing to increased financial profits. The authentication and determination of quality meat and fish is of great importance in preventing fraud which negatively impacts the food industry and causes health problems for the consumer (Meza-Márquez et al. 2010, Cawthorn et al. 2013). For example, the substitution of fresh meat and fish for frozen-then-thawed products is a typical commercial fraud which may cause economic loss and food safety and quality issues for consumers. These products are characterized by an increased susceptibility to microbial grow and color changes. Temperature fluctuations can result in the formation of ice crystals (Cozzolino and Murray 2004, Ballin and Lametsch 2008, Fajardo et al. 2010, Standal et al. 2010, Alamprese et al. 2013, Ottavian et al. 2013, Lin et al. 2014) due to the migration of water vapor from the product to the surface, resulting in poor quality food products. This defect is recurrently found in frozen foods which have been inadequately controlled.

Food authentication depends on the establishment of databases that contain information about the origin of food including the biological and geographic origin, species, production methods, and other critical information. However, there is a need for reliable analytical methods that can verify the geographic origin of food apart from their biological origin. Un-targeted spectroscopy approaches combined with chemometric analysis were investigated for their potential to classify the geographical origin of meat and predict its value (Sun et al. 2012b). An overview of analytical methods for determining the geographical origin of food products can be found in Luykx and van Ruth's (2008) paper.

Modern food inspection is under an ever-increasing demand for efficiency in the use of resources, either human or material, and for achievement of purpose through optimal inspection planning and the use of new methodologies. Spectroscopy, based on analytical technology tools, in combination with dynamic predictive models may bring these goals closer to reality (Thygesen 2012). Dynamic chemometric methods have been used in food inspection for quality monitoring in food processing industries (Singh and Jayas 2013). Singh and Jayas (2013) present a discussion on three broad categories of optical sensing techniques, namely, spectroscopic, fiber optic, and imaging. In their work, they describe the working principles, instrumentation, advantages, disadvantages, and the limitations of these techniques. For instance, an ultra-low field magnetic resonance imaging (MRI) system using a high-temperature superconducting quantum interference device (HTS-SQUID) for food inspection was reported in Kawagoe et al.(2016).

There are several methods for the detection of low levels of adulteration (Ballin and Lametsch 2008). Replacement products are often similar to the main material from a biochemical point of view and therefore, adulterant

identification can be particularly difficult (Ghovvati et al. 2009). Recently, researchers have applied various analytical techniques in the detection of food industry fraud. The protein-based methods (Al Ebrahim et al. 2013, Mamani-Linares et al. 2012), the deoxyribonucleic acid (DNA) based methods (Ali et al. 2012, Cammà et al. 2012, Mamani-Linares et al. 2012, Sakaridis et al. 2013, Zhang 2013, Karabasanavar et al. 2014, Lin et al. 2014), the real-time polymerase chain reaction (PCR) techniques and analysis of triacylglycerol (Kesmen et al. 2009, Fajardo et al. 2010, Soares et al. 2010, Druml et al. 2015) and methods based on fat (Abbas et al. 2009, Rohman et al. 2011), have become increasingly important. However, these methods are laborious, technically demanding, slow, invasive, expensive, destructive, and require sophisticated laboratory procedures and highly qualified employees. Moreover, they are not suitable for real-time applications (Damez and Clerjon 2008).

The various multidimensional analytical approaches that permit authentication of food can be divided into targeted and un-targeted methods. The classical authenticity assessment of food is usually based on the analysis of specific marker compounds, which are indicative for a certain property of the product (Herrero et al. 2012). Given that most adulterants are unknown, they are difficult to recognize using the targeted screening approaches typically used in food laboratories. The industry needs non-targeted methods to analyze samples for adulterants to provide proof of origin or to prevent deliberate or accidental undeclared admixture of food samples (García-Cañas et al. 2012). Food fingerprinting approaches are based on a high-throughput screening of samples with the purpose of differentiation or classification of samples. The investigation of food fingerprints provides high potential with regard to the characterization and verification of food identity. These approaches are usually based on spectroscopic and spectrometric data, providing the ability for a comprehensive characterization of the investigated matrices. The aim is to differentiate various food fingerprints in terms of, for instance, possible adulterations or their botanical or geographical origin (Esslinger et al. 2014).

There is a growing interest in methods based on spectroscopic techniques because they offer several advantages. Emerging non-destructive mapping technologies for authentication and traceability include nuclear magnetic resonance (NMR) imaging, fluorescence (FS), visible (VIS), near infrared (NIR), mid-infrared (MIR), and Raman (RS) spectroscopy, sometimes coupled with Fourier transform technique, and multispectral (MSI) and hyperspectral (HIS) imaging. These are simple, non-destructive, non-invasive, low cost, and allow real-time analysis. All spectroscopic techniques require small samples and no further preparation is necessary. They are powerful tools for conducting adulteration tests (Mamani-Linares et al. 2012). The methods presented in this work might be used as a complement or even constitute an alternative to PCR based DNA (Schmutzler et al. 2015).

The techniques NIR and MIR combined with Fourier transform (the latter so called FTIR—Fourier transform infrared) (Cozzolino and Murray 2004,

Ortiz-Somovilla et al. 2005, Rodriguez-Saona and Allendorf 2011, Mamani-Linares et al. 2012, Alamprese et al. 2013, Morsy and Sun 2013b, Rohman et al. 2011, Meza-Márquez et al. 2010), RS (Abbas et al. 2009, Boyaci et al. 2014, Zając et al. 2014, Zhao et al. 2015) and NMR (Rezzi et al. 2007, Aursand et al. 2009, Standal et al. 2010) combined with multivariate statistical methods were largely applied in the authentication of foodstuffs.

In addition, ultraviolet (UV) based spectroscopic methods were used in meat and fish adulteration studies (Alamprese et al. 2013). In recent years, these techniques have received much attention for safety inspection and the quality of food and meat and related products (Kamruzzaman et al. 2013, Barbin et al. 2013, Kamruzzaman et al. 2016, Kamruzzaman et al. 2012, Ma et al. 2015).

This work is an up-to-date literature revision applied to the detection of fresh meat and fish adulteration using spectroscopic methods which could be important for future research and in the development of equipment and methods for commercial markets, allowing detection of adulteration analysis very quickly.

6.2 Methods of Vibrational Spectroscopy

6.2.1 Visible and Near Infrared Spectroscopy

VIS and NIR spectroscopies offer a number of important advantages when compared to traditional chemical methods. These methods deal with the VIS and the NIR region of the electromagnetic spectrum, from about 750 to 2500 nm, corresponding to a photon of energy from 4000 to 13000 cm^{-1} (Huck 2015). When using the NIR region, the spectra can be recorded in reflection or transmission. The interaction of the radiation with matter provides information about the presence of functional groups (Lohumi et al. 2015, Huck 2015, Porep et al. 2015).

In comparison with other vibrational spectroscopic methods, NIR is considered a time-consuming procedure and the detector is often a source of noise. However, the use of an interferometer reduces the time of analysis through the single output signal (spectrum) which has all the infrared frequencies encoded therein. A Fourier transform is also necessary to extract the information from the spectrum. The interferometer coupled to Fourier transform has started to receive great attention for its use in the quantitative analysis of edible fats and oils (Gouvinhas et al. 2015). The attenuated total reflectance (ATR), diffuse reflectance, high-yield transmission and diffuse transmission cells are measuring methods used together with Fourier transform (Lohumi et al. 2015).

Diffuse reflectance or trans-reflectance spectroscopy has also gained attention in the control of fraud in the food industry. However, adequate overall

reflectance is the most widely adopted method for analyzing the quality and authenticity of the final food product (Porep et al. 2015). Reflectance infrared spectroscopy has allowed discrimination between fresh meat and fish products from frozen-then-thawed and mixtures of species that are not permitted in the final product (Mamani-Linares et al. 2012).

NIR spectroscopy has been applied in industrial online setups using a fiber optic probe and in the laboratory to detect different veal meat adulteration with pork parts (Schmutzler et al. 2015). Samples of veal meat with different percentages of pork parts were analyzed using chemometric methods. Control samples without adulteration (100% veal) and samples with increasing levels of adulteration were prepared in 10% steps from the original until a composition of 50% veal and 50% pork parts was obtained. Figure 6.1 shows the second derivative of spectra from 6200 to 5480 cm^{-1} measured with the laboratory setup as a function of the adulteration of the veal meat with pork. Close connection between the signal intensity and the level of adulteration from genuine (no adulteration) samples up to 50% adulteration were found at 5940 cm^{-1} (1683 nm), 5908 cm^{-1} (1693 nm), 5892 cm^{-1} (1697 nm), 5868 cm^{-1} (1704 nm), 5776 cm^{-1} (1731 nm), 5756 cm^{-1} (1737 nm), 5668 cm^{-1} (1764 nm), 5648 cm^{-1} (1770 nm) and 5492 cm^{-1} (1821 nm). Principal component analysis (PCA) was used to obtain a tridimensional projection of samples and to observe the relation between the genuine product and samples with adulteration. It was possible to notice an absence of associations from 20% to 50% of adulteration, with one and two principal components (PC), for laboratory

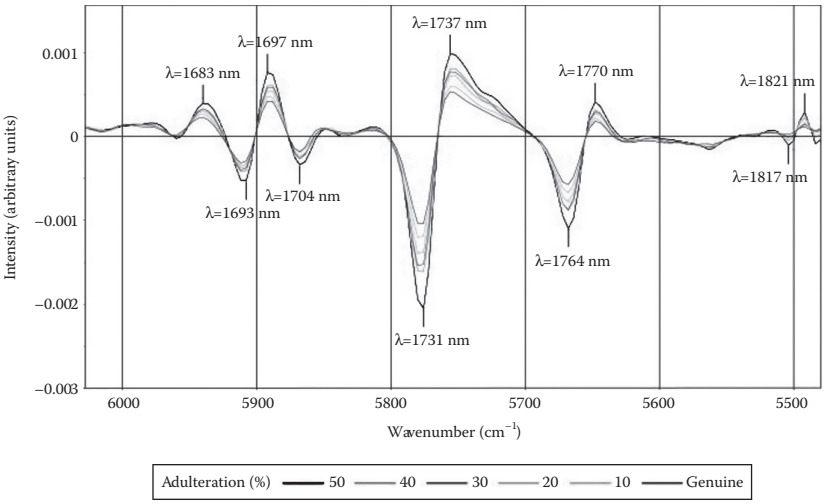

FIGURE 6.1
Second derivative of spectra (from 6200 to 5480 cm^{-1}) measured with the laboratory setup as a function of the adulteration of the veal meat with pork. Adulteration levels from genuine (no adulteration) up to 50%, in 10% steps. (Reprinted with permission from Schmutzler M. et al., *Food Control*, 57, 258–267, 2015.)

and the industrial setups, respectively. However, three PCs were necessary for models applied to the on-site setup. Data from PCA was used as input for classification and validation using support vector machines (SVM). The SVM allowed correct calibration values of discrimination of 94.4% for the laboratory, 91.7% for the industrial and 77.8% for the on-site analyses to be achieved (Schmutzler et al. 2015).

In a study conducted by Mamani-Linares et al. (2012), VIS and NIR reflectance spectroscopy or trans-reflectance methods were used to discriminate meat and meat juices from three livestock species. Meat samples from beef, llamas, and horses were purchased from different butcher shops and supermarkets. 79 samples of *Longissimus lumborum*, 500 g each, were used: 31 of beef, 21 from llama and 27 from horse were thawed at 4°C for 24 h and stored for 4–6 h before measuring the spectra. Another 58 samples of the same muscle (20 of beef, 19 from llama, and 19 from horse) were used to obtain the meat juice. They concluded that the VIS-NIR spectroscopy coupled to PCA, and with partial least squares regression (PLS-R), is a useful tool to discriminate between different species. In addition, it is useful to discriminate the geographical origin and the production system (Mamani-Linares et al. 2012).

The potential of UV-VIS, NIR, and MIR spectroscopies coupled with the chemometric techniques PCA, PLS-R, and linear discriminant analysis (LDA) enabled the detection of minced beef adulteration with turkey meat (Alamprese et al. 2013). Each batch was separately minced and then used to prepare (in duplicate) seven mixtures of bovine meat added with different percentages of turkey meat: 5%–50%, in 5% steps. With NIR, two PCs explained 98% of the total variance and for MIR the first two PCs explained 82.3%. LDA correctly classified 78.6% in the UV-VIS, 88.3% in the NIR and 84.8% in the MIR. PLS-R allowed construction of models with the root mean square error of cross-validation (RMSECV) and the root mean square error of prediction (RMSEP) slightly smaller than for NIR (Alamprese et al. 2013).

NIR has the potential to detect and quantify different adulterants in fresh and frozen-then-thawed minced beef. In addition to the pure beef and pork, fat trimming and offal samples, a series of mixed samples in the range of 10%–90% (w/w) from pork ($n = 144$) and 10%–80% of fat trimming ($n = 112$) was prepared (Morsy and Sun 2013a). The mixtures of samples adulterated with offal were prepared in the range of 2.5%–30%. The PLS-R had determination coefficients (R^2) of 0.96, 0.94, and 0.95 with standard error of prediction (SEP) of 5.4%, 5.1%, and 2.1%. Models based on PLS-R/DA and LDA distinguished between the unadulterated and adulterated classes with a classification of 100% (Morsy and Sun 2013b).

The NIR combined with chemometric analysis was used for data analysis to classify the geographical origins of lamb meat (Sun et al. 2012a). The δ13°C and δ15°N values of defatted lamb meat (Alxa League [37°53′N, 105°23′E, $n = 20$], XilinGol League [42°21′N,115°08′E, $n = 19$] Chongqing City [30°50′N, 108°24′E, $n = 20$] and Heze City [34°48′N, 116°04′E, $n = 20$] and Hulunbuir City [49°06′N, 119°40′E, $n = 20$]) were determined by isotopic ratio mass

spectrometry (IRMS).The analytical precision was lower than 0.2‰ for both. FDA/PLS-R and LDA gave a total correct classification of 88.9% and 75% to the five individual region samples, respectively. The PLS-R/DA and LDA correctly classified 100% of the samples from pastoral and agricultural regions. For PLS-R calibration models, the obtained R^2 value was 0.76 and 0.87 for predicting δ13°C and δ15°N, respectively. The first three PCs explained 98% of the total variance.

The adulteration of beef with pork and chicken was studied by Bilge et al. (2016). The beef samples were adulterated with pork and chicken (concentrations between 10% and 50%). The PLS-R method was used for evaluating laser-induced breakdown spectroscopy (LIBS) spectral data and RMSEC and R^2 values of 2.67 and 0.994 were obtained for beef adulterated with pork. A 83.4% correct discrimination rate between beef, pork, and chicken was achieved by PCA (Bilge et al. 2016).

The samples (43 adulterated and 12 controls), originating from dismantled criminal networks by the Brazilian Police, were analyzed using chemical parameters and ATR in conjunction with FTIR spectroscopy (Nunes et al. 2016). This fraud consisted of injecting aqueous solutions of non-meat ingredients (NaCl, phosphates, carrageenan, maltodextrin, collagen) into bovine meat. The PCA model of ATR-FTIR spectroscopy data was obtained with 4 latent variables (LV), accounting for 95.7% and 26.7% of variance in X and Y blocks, respectively. The PLS-R/DA model correctly detected 91.0% of the adulterated samples (Nunes et al. 2016).

Several strategies have been proposed for determining the substitution of fresh fish with frozen-then-thawed fish (Ottavian et al. 2013). One of the first strategies consists of using the PLS-R/DA method to classify the fresh and frozen-then-thawed status of each sample considering the species altogether. In another approach, a two-level cascade arrangement of PLS-R/DA was developed. In the first level, a PLS-R/DA was used to classify the samples according to their species and in the second level, a different PLS-R/DA discriminated between fresh and frozen-then-thawed samples. In a third strategy, an orthogonal PLS-R/DA was used to remove the information from the spectral data which is not related to the fresh and frozen-then-thawed status of the samples. Depending on the strategy, the overall obtained calibration accuracies ranged between 80% and 91%. The PCA explained 97% of the total variability (Ottavian et al. 2013).

NIR and VIS-NIR spectroscopy has also been used to distinguish fresh from frozen-then-thawed swordfish cutlets (Fasolato et al. 2012). Thirty specimens of swordfish were caught using traditional hooks and fishing. The relevant data was recorded to maintain sample traceability. The samples were vacuum-packed in polyethylene bags and three of them were refrigerated at 2°C. The remaining samples were frozen and stored: 30 samples at −18°C and the remaining 30 samples at −10°C for 30 days. Before analysis, the frozen samples were thawed overnight in the lab at 2°C. The first three PCs of the PCA explained 87.2% of the variability and with milling treatment the

first three PCs explained a higher value (94.8%). The samples were classified using VIS-NIR spectroscopy with a correct classification of 96.7%, whereas this value for NIR was higher than 90.0%.

In another study to distinguish fresh from frozen-then-thawed fish, *Pagrus major* fish were divided into two equal groups and used for further evaluation (Uddin et al. 2005). For fresh or frozen-then-thawed fish, 54 samples were used soon after being killed, whereas the second lot of 54 fish was kept at −40°C. After 30 days, fish were removed and thawed then evaluated as frozen-then-thawed samples. The fresh or frozen-then-thawed status was investigated and discrimination was carried out by soft independent modeling of class analogy (SIMCA), LDA based on PCA. The investigators obtained a classification of 100%. However, the high percentage of water in the fish is a major limitation for the analysis of samples with this application.

Real-time measurement and noise reduction using NIR spectroscopy requires Fourier transform. While this methodology allows detection of small molecules, water interference is a major drawback. However, this method does allow reading through glass or polypropylene containers.

6.2.2 Mid-Infrared Spectroscopy

MIR spectroscopy is fast, non-destructive and does not involve laborious sample preparation. It is an attractive option to identify and quantify adulteration and chemical composition of samples (Rohman and Man 2011). The absorption bands in the MIR region are characteristic of functional groups of molecules (Meza-Márquez et al. 2010, Zhao et al. 2014). The MIR region can be divided in the functional group region, from 4000 to 1500 cm^{-1}, and the fingerprint region, from 1500 to 500 cm^{-1}(Lohumi et al. 2015). MIR spectroscopy associated with FTIR spectroscopy and multivariate analysis require low sample volume and are environmentally friendly. Furthermore, FTIR spectroscopy in combination with PLS-R regression technique and PCA are powerful tools for quantification and classification of adulterants (Rahmania et al. 2015).

Overall, these methods are fast and effective in the detection of contaminants and adulterants (Meza-Márquez et al. 2010, Rodriguez-Saona and Allendorf 2011). Some countries have regulations to determine whether foods are safe, authentic and protect consumers requiring Halal and as such, investigators have conducted studies into the detection of adulterants in this type of food (Kurniawati et al. 2014).

In research investigating the adulteration of deer meat (*Dama dama*) with different percentages of goat meat (0%, 25%, 50%, 75% and 100%), samples were stored at 3°C for periods of time between 12–432 h. The methods used were microbiological analysis, measurement of color, lipid oxidation based on the thiobarbituric acid reactive substances method (TBARS), FTIR, sensory analysis, and statistical methods of multivariate analysis. In Figure 6.2, the average FTIR spectrum of different mixture proportions stored at 3°C

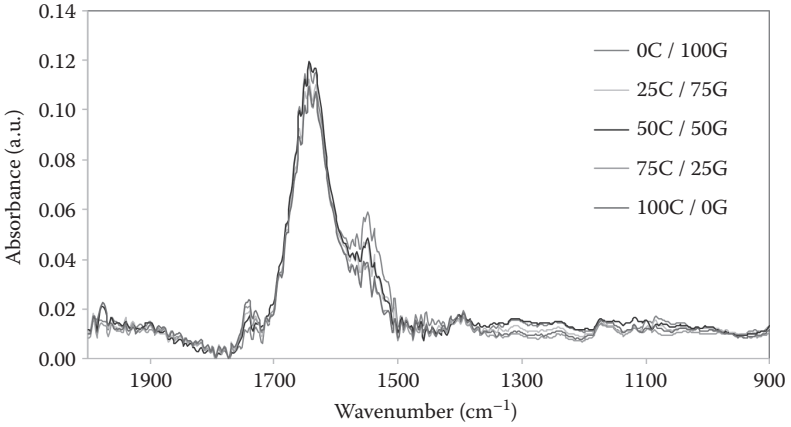

FIGURE 6.2
FTIR spectra of hamburgers containing different percentages of Deer (G) and Goat (C) from 2000 to 900 cm^{-1}. (Reprinted from Silva, A.C.C. da, Study of adulteration of fresh meat using spectroscopic, microbiological, chemical, physical and sensory methods. B.Sc., School of Agrarian and Veterinary Sciences, University of Trás-os-Montes e Alto Douro, Portugal, 2014.)

for 0 h between 2000 and 900 cm^{-1} can be observed. A peak at approximately 1639 cm^{-1} due to the presence of water (O-H stretch) with simultaneous contribution of amide I (C=O) is also visible. A second peak at 1550 cm^{-1} can be associated with the amide II (N-H, C-N). The peak at approximately 1460 cm^{-1} can be assigned to fat (ester CO). The absorptions in the region of 950–1200 cm^{-1} may reflect the content of carbohydrates, especially muscle glycogen. The amide content III can be viewed at about 1300 cm^{-1} and amino acid side chains between 1480 and 1800 cm^{-1}. Figure 6.3 shows the graph of observations of a LDA, where the discriminant factors F1 and F2 explain 96.63% of the total variance. With this analysis, the authors obtained a clear distinction between each blending percentage. The accuracy and performance of the model that relates the current and estimated values obtained from FTIR spectra is shown in Figure 6.4 and at $t = 27$ and $t = 0$ h 432 h, respectively. The PLS-R was used in order to validate and calibrate the model used. The PLS-R model was conducted to determine the relationship between the predicted values and the measured values of the mixtures. The R^2 coefficient shows high values and the RMSEC and RMSECV show low values which demonstrates that the PLS-R model has good predictive accuracy and performance.

In separate research, the adulteration of beef meatballs with the meat of rat (*Rattus argentiventer*) was studied by Rahmania et al. (2015). Rat meat was obtained from farmers while beef was purchased from several local markets. During the preparation of calibration samples, rat meat and beef was prepared by mixing rat meat at concentrations of 0%, 10%, 20%, 35%, 50%, 65%, 80%, and 100% in beef. The FTIR spectroscopy in combination with PLS-R

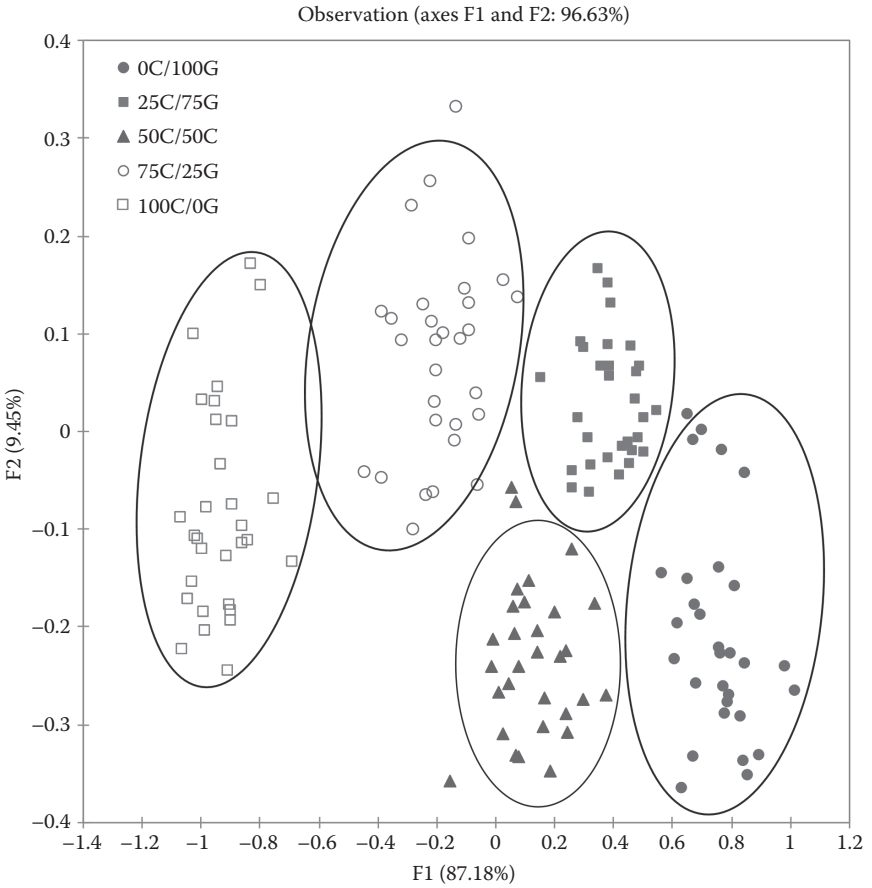

FIGURE 6.3
Projection of the samples according to the storage time (t0 to t14) and the type of meat with two batches where G samples correspond to Deer, and C samples correspond to goat. (Reprinted from Silva, A.C.C. da, Study of adulteration of fresh meat using spectroscopic, microbiological, chemical, physical and sensory methods. B.Sc., School of Agrarian and Veterinary Sciences, University of Trás-os-Montes e Alto Douro, Portugal, 2014.)

and PCA multivariate calibrations were used for the differentiation between rat meat and beef meatballs. The frequency region from 750 to 1000 cm^{-1} was selected during PLS-R and a R^2 value of 0.993 and root mean square error of calibration (RMSEC) of 1.79% was obtained. The PCA modeling method correctly classified the meatball sample with 100% rat meat and 100% beef.

In a similar study, the investigators prepared oils of pork (lard), lamb, beef, and chicken. FTIR and GC analyses were performed. For calibration, a training set of 30 samples consisting of lard, body fats of beef, chicken, and mutton with certain concentrations were prepared. Each sample was subjected to FTIR analysis and gas chromatography (GC). PCA showed that PC1

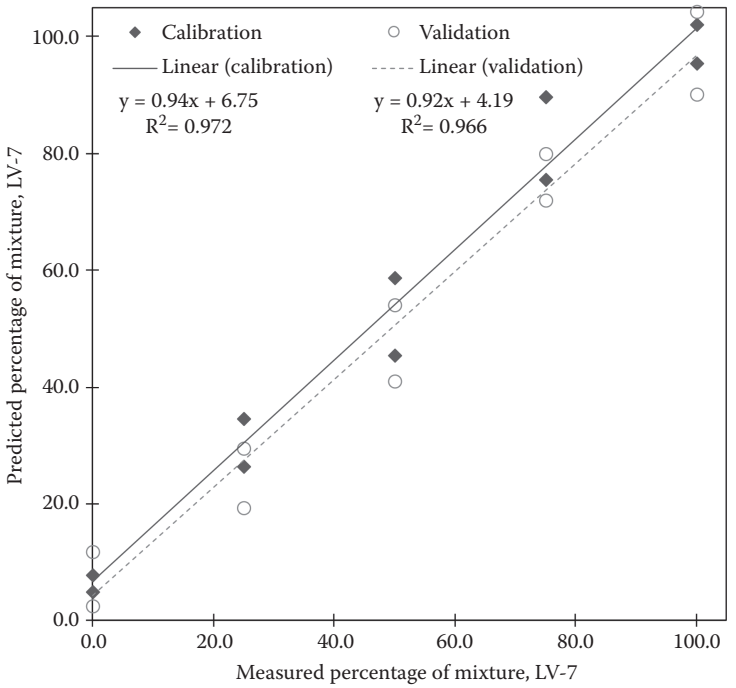

FIGURE 6.4
Illustration of the quality of the PLS-R model conducted to determine the relationship between the predicted values and the measured values of the mixtures. (Reprinted from Silva, A.C.C. da, Study of adulteration of fresh meat using spectroscopic, microbiological, chemical, physical and sensory methods. B.Sc., School of Agrarian and Veterinary Sciences, University of Trás-os-Montes e Alto Douro, Portugal, 2014.)

accounted for 57% of the variation, while PC2 explained 25% of the variation, and PC3 contributed to 13% of the variation. These three first PCs can describe more than 95% of the overall variation (Rohman et al. 2012).

A separate research study by Rohman et al (2011) investigated the adulteration of beef meatballs with pork. The calibration sets were prepared by spiking pork to beef meatball in the concentrations of 1.0%, 3.0%, 5.0%, 10.0% and 25.0%. Samples containing 100% beef and 100% pork were also made to observe the spectral differentiation. The adulteration was detected using FTIR spectroscopy and PLS-R. This regression method was used to develop a calibration model and a R^2 value of 0.999 was obtained.

In another similar research study, pork fat (lard) and beef fat were obtained through a rendering process of the corresponding animal (Kurniawati et al. 2014). The fatty acid composition of lard and beef fat was carried out using GC with a flame ionization detector (GC-FID). A set of standards consisting of lard in beef fat was prepared by mixing both types of fat in the concentration range of 0%–100%. FTIR spectroscopy in combination with PLS-R

and PCA was used for the detection of the substitution of beef fat with lard. PLS-R was characterized by a high R^2 value (0.998). while PCA was used successfully in the region from 1200 to 1000 cm^{-1}.

The adulteration of high quality beef steak with horse meat, beef fat trimming and soybean protein was studied by Meza-Márquez et al. (2010). The beef steak samples were minced using a food processor. Horse meat samples and beef fat trimming were minced separately in the same way as lean beef. Textured soybean protein was rehydrated according to instructions on the packet label. Samples of each type of adulterated mixture (minced lean beef-horse meat, minced lean beef-textured soy bean, and minced lean beef fat trimmings) ranging from 2% to 90% w/w adulterant concentration were prepared in increments of 2%. A methodology using MIR spectroscopy in tandem with chemometrics was developed to discriminate between pure minced meat and adulterated samples. The results of the developed PLS-R models showed, in the region 1800–900 cm^{-1}, values of R^2 greater than 0.99. The SIMCA model showed 100% correct classification for minced beef and for beef adulterated with horse meat, beef fat trimmings or soy protein.

A common adulteration process is the substitution of fresh food by frozen-then-thawed food. Fresh and frozen-then-thawed samples of offal-adulterated beef burgers were analyzed using ATR-FTIR technique and chemometrics methods (Zhao et al. 2014). The authentic beef burgers were produced in two groups, called lean and fat, which correspond to higher (lean) and lower (fat) quality levels. The beef burgers in each of the two groups were made on separate occasions beginning with the highest lean meat content and moving to the lowest. A total of 82 fresh beef burger samples (36 authentic + 46 adulterated) and 82 frozen-then-thawed beef burger samples (36 authentic + 46 adulterated) were prepared. The first three PCA components accounted for 72.9%, 11.3%, and 8.4% of the variability. From the PLS-R models, 100% were accurately classified in calibration and in validation. The SIMCA efficiency values varied from 0.57 to 0.87 for fresh and from 0.62 to 0.91 for frozen-then-thawed beef burgers.

MIR spectroscopy requires the preparation and dilution of samples. In this methodology the interference of water contained in the food may occur. However, MIR with chemometric methods is a promising technique that allows detection of larger functional groups.

6.2.3 Fluorescence Spectroscopy (FS)

Fluorescence is a physical process associated with the emission of photons upon molecular transition from the electronic excited state to the ground state. The emission of photons occurs at a higher wavelength than the wavelength of the incident excitation source (Bridier et al. 2015). FS involves the application of a light beam in the sample, causing excitation of electrons in molecules of certain compounds and the emission of low energy light. It's a fast, sensitive, and non-destructive technique (Karoui et al. 2006, Damez

and Clerjon 2008). A stable fluorescent label is of crucial importance for the sensitivity of quantitative and qualitative detection as well as for the contrast of fluorescent microscopic imaging. Covalently bound fluorescent labels are a promising tool for obtaining highly stable fluorescent labeled particles for a considerable period of time. However, negligible leakage and low signal intensity have also been reported (Weiss et al. 2006).

This method, combined with multivariate statistical analysis, is an effective tool for the discrimination of different beef muscles in relation to the age of the animal, while chemical and mechanical properties make it possible to evaluate the quality and adulteration of the food (Sahar et al. 2016). There are different applications of FS: heterocyclic particular aromatic amines (HAA), tryptophan fluorescence, and nicotinamide adenine dinucleotide phosphate oxidase (NADPH) (Karoui et al. 2006). The NADPH fluorescence spectrum enables differentiation of fresh from frozen-then-thawed fish and the simplicity of this method also allows the extension of the VIS spectroscopy characterization efficiency of the fish (Karoui et al. 2006).

FS also enables aromatic acids of the amino acids to be observed. When using excitation at 250 nm the emission will be at 305–400 nm. For observation of proteins folding, tryptophan fluorescence can use excitation at 290 nm and emission at 305–400 nm (Albani 2012).

This technique can therefore use the presence of fluorescent molecules such as tyrosine, phenylalanine and tryptophan in proteins to detect the environmental and biological origin of samples (Karoui et al. 2006). Adipose tissue contains fluorescent molecules that are specific for fat. Few studies have been conducted with this method in food. However, NADH/FS and tryptophan fluorescence in combination with chemometric methods enables identification of both fresh and frozen-then-thawed fish fillets (Karoui and Blecker 2011, Karoui et al. 2006). Regarding the PCA of tryptophan fluorescence spectra, the first two PCs explained 55.9%and 36.9% of the total variance. On the other hand, the PCA of NADH fluorescence spectra led to 84.9% and 12.1% variance for the first two PCs. Then, PCA applied to the factorial discriminant analysis (FDA) method obtained a 100% accuracy when using the calibration set (Karoui et al. 2006).

The conventional and the synchronous fluorescence spectroscopic method of excitation-emission in combination with chemometric methods, namely predictive and descriptive methods, determines the changes in foodstuffs during technological process and storage. Front-face FS has the potential to reduce the analysis time and costs compared to the enzymatic and biochemical methods (Karoui and Blecker 2011).

6.2.4 Raman Spectroscopy

Raman spectroscopy can be used to observe vibrational, rotational, and other low-frequency modes in a molecule and/or a system. The vibrational modes provide a major contribution to knowledge of the chemical constitution of a

specific analyte. Raman spectroscopy depends upon the inelastic scattering of monochromatic light, usually from a laser in the ultraviolet, visible or near infrared wavelengths (Li-Chan 1996).

Raman spectroscopy was used to determine authentication and quality of foodstuffs (Lohumi et al. 2015). This technique provides specific information about and allows the determination of lipids, proteins, and carbohydrates, and it can be employed to classify microorganisms (Argyri et al. 2013). It has the capacity to provide information on the chemical structure of molecules without causing changes in the samples (Boyacı et al. 2014). It is a very promising method and has high potential for evaluating the quality of foodstuffs during handling, processing, and storage (Boyacı et al. 2014, Zając et al. 2014).

There are techniques that can be used to improve the Raman signal, in particular scattering Raman spectroscopy (SRS) (Tipping et al. 2016), coherent anti-Stokes Raman (CARS) (Roy et al. 2010), resonance Raman spectroscopy (RRS) (Wächtler et al. 2012), shifted excitation Raman difference spectroscopy (SERDS) (Sowoidnich and Kronfeldt 2012), and surface-enhanced Raman scattering (SERS) (Hakonen et al. 2015). Reducing the Raleigh scattering allows high-quality spectra extension to be obtained. SERS is a powerful tool for characterizing a wide range of analytes when combined with biologically relevant nanostructures (Shrestha et al. 2014). The combination of RS with Fourier transform provides high spectral resolution with effective wavelength accuracy and allows the degree of opening of fatty acids in foods to be estimated (Lohumi et al. 2015).

The RS coupled with PCA was developed for the rapid determination of beef adulteration with different concentrations of horse meat. The beef samples were provided from local supermarkets while horse meat samples were bought from local markets. In the scope of study, beef samples containing 0%, 25%, 50%, 75%, and 100% by weight of horse meat were investigated (Boyaci et al. 2014). The PCA exhibited a first PC explaining 96.3% and a second PC explaining 3.2% of the total variance. The developed model system was good enough to differentiate adulterated samples. This method has shown good results because of the short time analysis and simple preparation of the sample (Boyaci et al. 2014). Figure 6.5 illustrates the Raman spectra of meat samples collected between 200 and 2000 cm^{-1}. Raman bands at 555, 678, 815, 1032, 1265, 1392, 1611, and 1706 cm^{-1} were observed in the spectra of both horse and beef samples. The spectral difference between the samples arose from the unique bands of horse fat that were positioned at 919, 974 and 1215 cm^{-1}.

In another study, fresh meat species (cattle, sheep, goat, buffalo, pork, fish, chicken, and turkey) were purchased from the local markets and slaughterhouses and kept in refrigerated conditions (Boyaci et al. 2014). These were utilized in the preparation of salami products and fat was extracted from each meat sample. Raman spectroscopy coupled with PCA differentiated the origin of the meat and meat products. Principal components PC1 and PC2 explained 85.1% and 6.4% of the variance, respectively. After the third

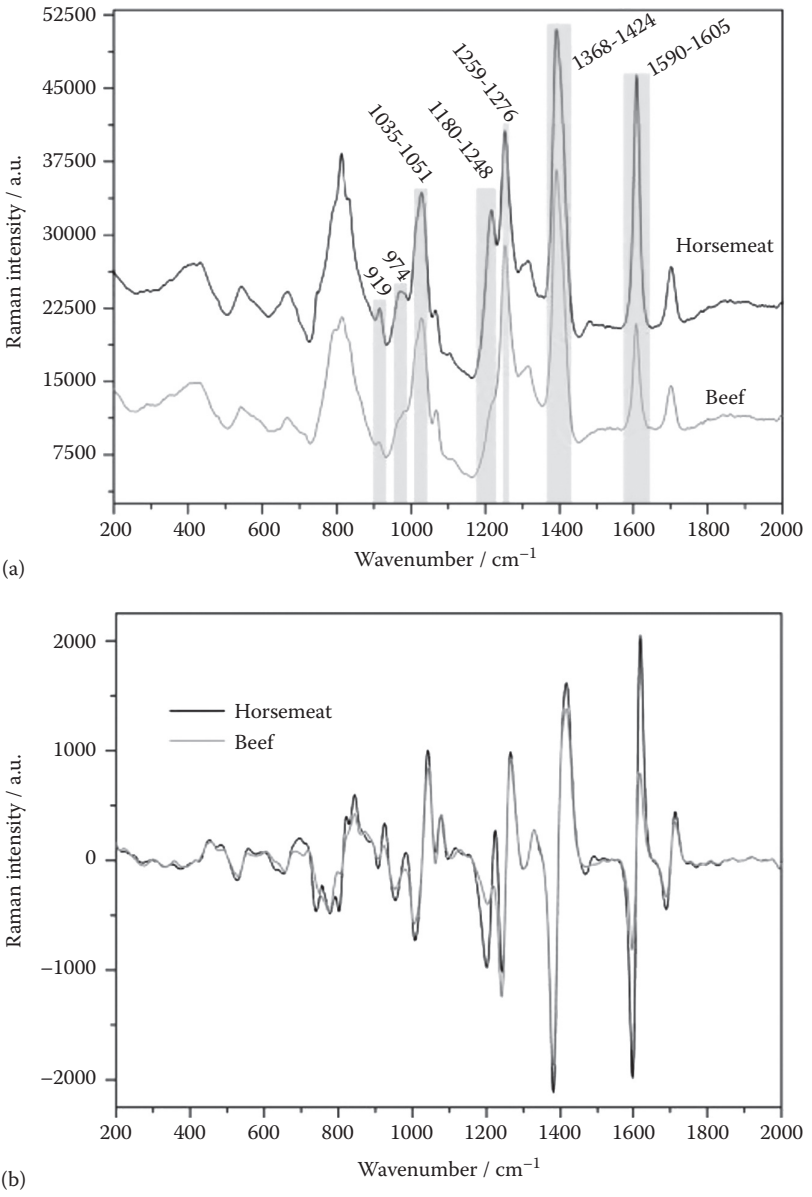

FIGURE 6.5
Original (a) and first derivative (b) Raman spectra of horse meat and beef samples. (Reprinted with permission from Boyaci, I.H. et al., *Food Chemistry*, 148, 37–41, 2014.)

derivative was applied to the spectra, PC1 and PC2 explained the variance of 96.3% and 2.2%, respectively (Boyaci et al. 2014).

In a similar study, fresh samples of horse back muscles were purchased from a local butcher (Zając et al. 2014). The meat mixture was prepared from horse meat and beef in a composition of 1:4, 1:2, 3:4, respectively. The content of horse meat in the samples with beef was detected using the Raman bands at 937, 879, 856, 829, and 480 cm^{-1} (Zając et al. 2014).

Al Ebrahim et al. (2013) applied a 671 nm (50 mW) microsystem diode laser to study the applicability of the RS in the distinction of beef and horse. The fresh muscles of beef and horse were purchased from local butcher shops. The muscles were cut into 2 cm thick slices and packed separately. All slices were stored at 5°C for a period of 12 days in a laboratory refrigerator. The PCA method was applied for data evaluation and presented the PC1 and the PC2 explaining 79% and 18% of the total variance, respectively. Raman spectroscopy showed changes in the spectra for proteins, lipids, and water muscle meat.

In another study, SERDS was applied for separation of the meat samples into distinct groups (Sowoidnich and Kronfeldt 2012). For each animal species, beef (rump steak), pork (loin chops), chicken (breast), and turkey (breast), 12 randomly chosen slices of fresh meat were bought in a local supermarket and measured at the day of purchase for separation of the meat species into four distinct groups with the PCA. The SERDS method showed enormous potential and demonstrated a quick breakdown for classification of different species of meat.

In a study conducted by Ellis et al. (2005), RS was applied to the identification of meat and poultry based products and showed potential for the rapid assessment of adulteration of food. Samples of pre-packed meat (lamb, beef, pork) and poultry (chicken [skinless breast fillets] and turkey [skinless breast fillets]) were acquired, and for the subsequent experiments, chicken (skinless breast fillets and legs with skin) and turkey (skinless breast fillets and legs with skin) were purchased from retail outlets and stored at 4°C. Raman spectra were collected using an infrared diode laser at 785 nm, using a Renishaw 2000 Raman probe system together with the Renishaw WiRE Grams software package and a CCD detector. Spectra were collected for 10 s and 1 accumulation over the wave number range 100 cm^{-1} to 3000 cm^{-1}. PCA and genetic algorithms multiple linear regression (GA-MLR) and discriminant multiple linear regression (D-MLR) were used.

The Fourier transform Raman spectroscopy (FT-RS) was chosen for the discrimination of animal fat (Abbas et al. 2009). To assess the technique, four mixtures were analyzed: mixture 1 contained 50% bovine, 50% ovine–pork; mixture 2 contained 80% bovine, 20% pork; mixture 3 contained 50% bovine, 50% ovine–pork–avian–former foodstuffs; and mixture 4 contained 55% bovine, 15% ovine, 30% pork, and traces of avian fat. PCA was applied and the first PC represented 67% of the variance while the second one explained 24% of the variance. PLS-R/DA model allowed discrimination between

poultry samples and other components (pork, bovine, ovine fats, and fish oils) obtained a sensitivity and specificity of 0.917 and 1.000, respectively

In a study by Beattie et al. (2007), RS was used to classify adipose tissue from four different species (chicken, beef, lamb, and pork). The samples used in this investigation were from beef, lamb, pork, and from the breast of chicken. In order to obtain a wide range of variation within each species, the samples were obtained from a number of commercial outlets and encompassed a wide variety of breeds and feeding regimes. Complementary fatty acid composition was determined by GC. PCA data reduction on the adipose Raman spectral data set was followed by LDA and allowed 97.6% correct classification of the samples, while using the PLS-R/DA method further improved the correct classification rate to 99.6%

Beef offal adulteration of beef burgers was studied using dispersive Raman spectroscopy and multivariate data analysis to explore the potential of these analytical tools for detection of adulterations in comminuted meat products with complex formulations (Zhao et al. 2015). Fresh beef (brisket), beef offal (kidney, liver, lungs, and heart) and beef fat were purchased from local stores and stored overnight at 4°C. Authentic beef burgers comprised two groups, higher quality burgers contained only lean beef and beef fat; lean meat content varied between 80% and 100% of the burger in 2.5% increments, with fat accounting for the remainder and lower quality burgers contained rusk (5%) and water (20%) in addition to lean beef (45%–65% in 2.5% increments) and beef fat (30%–10% in 2.5% increments). Adulterated beef burgers were formulated with lean beef, beef fat, water, rusk, and offal (liver, lung, kidney, and heart). Multivariate data analysis methods of the DRS spectra comprised PLS-R/DA, SIMCA, and PCA. In relation to the PCA, the first three PCs described 61%, 34%, and 3% of variance, respectively, in the frozen-then-thawed beef burger spectral data set. PLS-DA models correctly classified 89%–100% of authentic and 90%–100% of adulterated samples. The SIMCA has a specificity of 0.64–0.89 and a sensitivity of 0.95–1. In comparison with other studies by these authors (Zhao et al. 2014), using the model of PLS-R/DA, adulterated samples obtained a 74%–91% value for NIR, 73%–100% for Fourier transform-NIR, and 81%–100% for RS. The SIMCA efficiency was 0.62–0.91 for NIR, 0.81–0.94 for Fourier transform-NIR and 0.88–0.97 for DRS.

Raman spectroscopy provides a high rating of detection of adulteration compared to other spectroscopic methods. Water interference doesn't occur when using the RS technique, providing specific information on the matrices of the samples. Samples can be read through glass or polymer packaging. However, the heating from the laser radiation can destroy the samples or hide the Raman spectrum. This process requires only a small sample. It has the ability to supply information about the chemical structure of molecules without causing any alterations. This technique is a new approach to the determination of meat adulteration and showed reasonable results for the determination of fraud meat mixtures.

6.2.5 Nuclear Magnetic Resonance Spectroscopy

NMR is based on the emission and absorption of energy in the radio frequency range of the electromagnetic spectrum. The most commonly measured nuclei are ^{1}H and ^{13}C, the first for proteins because they are rich in hydrogen and the second for larger proteins and lipids (triglycerides) (Aursand et al. 2009, Jakes et al. 2015). The shielding effect of electrons, which decreases resonance frequencies of nuclei, varies with the chemical environment and is, therefore, characteristic of specific structural fragments of organic compounds (e.g., methyl, methylene, or methine^{1}H nuclei) and their substituents (e.g., OH, NH_2, NH, COOH, CONH) (Mlynárik 2016).

This technique presents advantages compared with other spectroscopic methods for foods with a high water percentage because the protons are easily detected. However, it is expensive and time-consuming (Aursand et al. 2009, Jakes et al. 2015, Santos et al. 2014). Low-resolution NMR or time-domain ^{1}H nuclear magnetic resonance (TD-NMR) is an excellent alternative to traditional methods because it is rapid, simple, and has the potential for online and in situ measurements and using permanent magnet technologies, significantly reduces the overall system and running costs (Santos et al. 2014). These benefits make NMR particularly interesting for analyzing the safety of food and consequently, there has been an increase in its use in the food industry (Damez and Clerjon 2013). For example, NMR has shown high sensitivity in differentiating between the structure of muscles in wildlife and farmed animals (Standal et al. 2010). The ^{1}H NMR is also an effective technology for analysis and quantification of triglyceride samples and the use of high resolution (HR) ^{13}C NMR in the analysis of lipids is increasing with lipid analysis being a potential tool for authentication of fish and marine oils (Standal et al. 2010). However, there are few studies with NMR for authentication of meat or fish products.

In a study by Santos et al. (2014), TD-NMR spectroscopy, when combined with univariate and multivariate analysis, provided a valuable tool for tracing the sex and bull race of beef samples. It has been demonstrated that NMR is a fast and accurate method for measuring conjugated linoleic acid (CLA) content in beef samples (Manzano Maria et al. 2010). The beef samples were collected from calves (43 heifers and 56 steer) from different bull race (Angus, Bonsmara, and Canchim) and cows (Simmental-Nellore and Angus-Nellore for cows). The calves were designated according to the bull race and sex, resulting in 14 Angus heifers, 21 Angus steer, 17 Bonsmara heifers, 19 Bonsmara steer, 12 Canchim heifers, and 16 Canchim steer. Carr-Purcell-Meiboom-Gill (CPMG) and Continuous Wave Free Precision (CWFP) pulse sequences were used to obtain time-domain ^{1}H NMR. The PLS-R/DA showed a correct classification higher than 79% either for CPMG or CWFP decays (validation set). The k-nearest neighbor (KNN) showed a correct classification of 75% and 76%, while SIMCA showed a correct classification 66% and 78%, respectively, for the CWFP and CPMG dataset. The SIMCA method obtained a best predictability for the CWFP dataset with correct classification between 85% and 89% for beef samples. ^{1}H NMR coupled CPMG

CWFP and with univariate and multivariate methods obtained a correct classification of more than 80%. The ^{1}H TD-NMR method allowed for authentication and traceability when applied to meat samples.

The 60 MHz ^{1}H NMR method was used to differentiate samples of fresh beef and horse. Peak integration was sufficient to differentiate samples of fresh beef (76 extractions) and horse (62 extractions) using Naïve Bayes classification. Fresh meat samples were purchased from a variety of outlets. It was possible to obtain 100% correct classification of the different samples of beef and horse, exploiting the differences in triglyceride compositions. In relation to the PCA, principal components 1 and 2 described 83% and 12% for lab 1 and 81% and 13% for lab 2, respectively, of variance in fresh beef and horse spectral data set (Jakes et al. 2015). The first two PC scores are plotted against one another in Figure 6.6(a) and (b), with symbols coded according to species. In both cases, the first dimension contains most of the relevant information relating to the difference between the two species. Furthermore, regions of the loading corresponding to the olefinic and *bis*-allylic peaks are positively associated with horse samples in Figure 6.6 (c) and (d).

The differences in quality and price between different species of fish are reasons for falsification and therefore, it is necessary that methods are able to verify the traceability of the correct information to protect consumer rights (Aursand et al. 2009).

The following species and stocks of lean fish were caught outside the coast of Vikna, Nord-Trondelag: Norway north-east arctic cod, Norwegian coastal cod (*G. morhua L.*), haddock (*M. aeglefinus*), saithe (*P. virens*), and pollack (*P. pollachius*). Approximately 90 mg of the oil sample was transferred to 5 mm NMR tubes and diluted with 0.6 mL deuterated chloroform ($CDCl_3$, 99.8% purity, Isotec Inc., Matheson). Lipid was extracted from white fish muscle under the back dorsal fin according the Bligh and Dyer method (Bligh and Dyer 1959). Before analyzing the lipid extract by NMR, parts of the chloroform phase were removed by evaporation. ^{13}C NMR spectroscopy coupled with chemometric methods PCA, LDA and Bayesian belief networks (BBN) authenticated five different gadoid fish species. With PCA, groupings were obtained and the first two principal components accounted for 36% and 9% of the variance. PCA was used as input variables in the LDA. LDA with the three PCs obtained 21/27 correct classifications (78% correctly classified) and the Bayesian belief networks (BBN) showed 100% correct classifications (Standal et al. 2010).

^{13}C NMR spectroscopy in tandem with chemometrics methods classified the Atlantic salmon in relation to its geographical origin and to its identity as wild or farmed. The probabilistic neural networks (PNN) and support vector machines (SVM) showed an excellent breakdown of 98.5% and 100%, to wild and farmed salmon. The geographical origin obtained correct classification of 82.2% to 99.3% for PNN and SVM, respectively (Aursand et al. 2009).

A similar study with NMR and chemometrics methods tried to classify *Gilthead pargo* in accordance with wild or farmed and geographical origin (Rezzi et al. 2007). The LDA and PCA made a clear distinction between wild

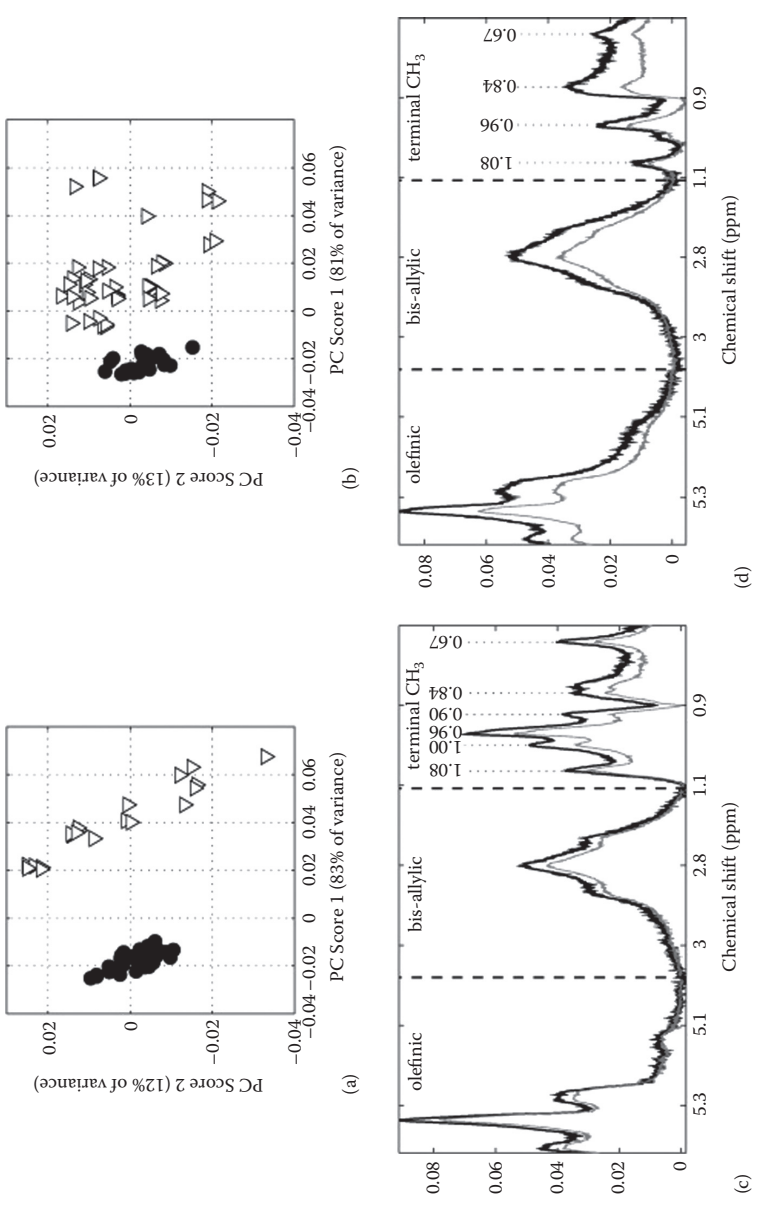

FIGURE 6.6
First versus second principal component scores plots for (a) Lab 1 Training Set data, and (b) Lab 2 Training Set data (black disks = beef, open triangles = horse). (c) and (d) Corresponding loadings plots (black trace), together with the covariance of each dataset with the group membership (grey trace) and peaks picked from the loadings in the CH_3 region. (Reprinted with permission from Jakes, W. et al., *Food Chemistry*, 175, 1–9, 2015.)

and farmed samples. This method showed a rating of 100% of the samples in accordance with wild or farmed and 85%–97% for the geographical origin.

Fourier transform spectroscopy and micro ^{1}H NMR LF are used in studies of changes in the structure of proteins and secondary water distribution (Rezzi et al. 2007, Damez and Clerjon 2013). The NMR technique permits an easy reading of the characteristics of foods with large amounts of water but is highly sensitive, expensive and time-consuming. It is a method that can cause spectra with many peaks. However, TD-NMR is fast, simple and has the potential for online measurement.

6.2.6 Multispectral and Hyperspectral Imaging

Currently, many researchers are using hyperspectral imaging (HIS) methods because they are powerful techniques which can provide spectral data of an object with certain chemical characteristics in a spatially resolved manner (Pu et al. 2015). HIS methods, with the aid of image processing techniques that allow visualization, allow detection of attributes by spectral analysis of the samples. This method is a non-conventional analytical technique, non-destructive, using few reactants and it is fast.

This method allows simultaneous analysis of several samples and was introduced to integrate images and spectroscopy in a system for providing spectral and spatial information of an object (Ma et al. 2015, Lohumi et al. 2015). The images originate three-dimensional data sets that can be analyzed to characterize the object in greater detail than the imaging or spectroscopy techniques (Kamruzzaman et al. 2016). HIS is composed of hundreds of discrete spectral bands for each spatial position for the object (Kamruzzaman et al. 2012). The spectroscopy is used to detect or quantify the biological, chemical and physical properties of samples based on their spectral signature images and transform chemical information steps to viewing space (Kamruzzaman et al. 2013). HIS can be used to ascertain the amount of certain attributes and where they are located in the sample. Spectra can be used to characterize, identify and discriminate classes and types of materials in the image. The most commonly used spectral bands are in NIR, VIS-NIR and VIS (Lohumi et al. 2015). NIR HIS is involved in acquiring a spectrum for each image pixel in micro- and macroscopic scale (Kamruzzaman et al. 2016).

In a study by Kamruzzaman et al. (2013), the identification of the adulteration was conducted using pure minced lamb meat and lamb meat mixed with potential adulterants including pork, heart, kidney, and lungs in 20% proportions. The lamb samples were adulterated by mixing pork in the range of 2%–40%. Both minced lamb (28% fat) and pork (15% fat) were acquired from a local supermarket and transported to the laboratory. NIR HIS detected the level of adulteration of minced lamb using a PLS-R method. PCA was used to interpret and visualize the spectral data to highlight their properties. The first PC represents 87.5% of the variance while the second one explains 8% of the variance. (The two first PCs explained 95.7% variation).With PLS-R

prediction results, it was possible to detect adulteration in minced lamb with high performance in both calibration and cross-validation conditions using five latent variables (LV). The coefficient of determination in calibration (R^2_c) of 0.99, RMSEC of 1.08%, coefficient of determination in cross-validation (R^2_{cv}) of 0.99, and RMSECV of 1.37% were obtained for PLS-R. The calibration model was also evaluated based on the ratio of percentage deviation (RPD) and this value for adulterate detection was 8.51. The multiple linear regression (MLR) model was then built using the reduced spectral data and the results of MLR for predicting adulteration are R^2_c of 0.99, RMSEC of 1.25%, R^2_{cv} of 0.98, RMSECV of 1.45%, and RPD of 8.04. The prediction ability of PLS-R with selected wavelengths was equivalent to the PLS-R with full spectra, with R^2_{cv} (0.99 vs. 0.99), RMSECV (1.42% vs. 1.37%), and RPD (8.51 vs. 8.21).

In a different study, meat samples originating from *Longissimus dorsi* muscles of pork, beef, and lamb were analyzed by Kamruzzaman et al. (2012). The muscles were dissected and then sliced by a mechanical slicer. The slices were labeled and vacuum-packed and transported under refrigerated conditions to the laboratory. HIS with PCA and PLS-R/DA was used for identification and authentication of different red meat species. The first three PCs resulting from PCA explained 99.7% of the variation among samples. The PC1 and PC2 were particularly representative and accounted for 98.9% of the total variance (PC1 – 88.9% and PC2 – 10.1%). The PLS-R/DA showed a classification accuracy of 93.3%, 98.7%, and 97.3% for pork, beef, and lamb, respectively.

NIR HIS coupled with PLS-R/DA was used to distinguish between fresh and frozen-then-thawed samples (Barbin et al. 2013). Fresh samples of pork from the loin muscle were obtained from a commercial food retailer and transported to the laboratory for storage at 4°C. After 24 h, each fresh sample was removed from cold storage and scanned in the NIR hyperspectral system. Pork samples were then vacuum-packed and frozen at –18°C. PLS-R/DA with full cross-validation had coefficients of prediction of 0.97 and 0.89 for R^2_C and R^2_{CV}, respectively, with standard error of calibration (SEC) of 0.23 and standard error of cross-validation (SECV) of 0.46. To verify the potential information carried by the selected wavelengths, frozen-then-thawed samples were correctly identified (sensitivity = 1.00), and no fresh sample was misclassified as frozen-then-thawed (specificity = 1.00). The overall correct classification for this method was 100% to discriminate fresh from frozen-then-thawed samples. The classification of pork samples according to freezing treatment are: fresh samples (85.4%), frozen once (77.9%), frozen twice (60%), frozen three times (70%) and frozen four times (90%). The fresh pork meat and frozen-then-thawed meat was detected with PLS-R/DA and obtained 97.9% accuracy, and with colorimeter method achieved 75% accuracy. The discriminant model PLS-R/DA obtained a variance to LV1 of 58% and LV2 of 39% to identify the fresh and frozen-then-thawed samples. This method can be applied for the benefit of the retail sector and the consumer. Figure 6.7 shows the main configuration of the push room NIR hyperspectral imaging system, reprinted from Barbin et al. (2013).

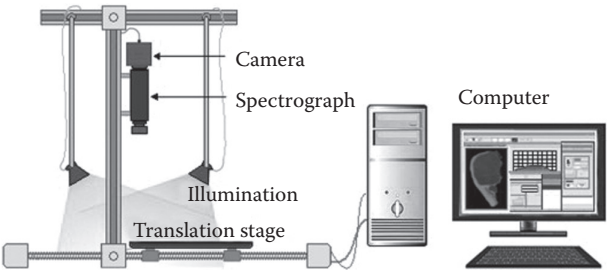

FIGURE 6.7
Hyperspectral imaging system setup. (Reprinted with permission from Barbin, D.F. et al., *Innovative Food Science and Emerging Technologies*, 18, 226–236, 2013.)

In other research, the potential of VIS and NIR HIS with PNN was used for classification of fresh and frozen-then-thawed pork muscles (Pu et al. 2015). Animals with similar conditions (age, weight, feeding environment from the same farm) were obtained for the experiment. The pork samples were divided into three groups: the first group without any freezing treatment was designated as fresh pork meat, the second group was frozen-then-thawed-once, and the third group was frozen-then-thawed-twice. The PC images from HIS were obtained using histogram statistics (HS), gray level co-occurrence matrix (GLCM) and gray level-gradient co-occurrence matrix (GLGCM). For fresh, frozen-then-thawed once and frozen-then-thawed twice meats, PNN showed a correct classification rate of 100% and 97.73% for calibration and validation sets, respectively. The successive projections algorithm (SPA) showed a correct classification rate of approximately 100% for calibration and validation sets. The correct classification rate was reduced to 86.36% and 86.36% for calibration and validation sets, when six optimum wavelengths were used alone. The average classification accuracy of PNN using optimum wavelengths (OW)-GLGCM was the highest (92.0%), followed by OW-GLCM (91.3%), OW-HS (91.3%) and OW (86.4%).

The multispectral imaging (MSI) coupled with PLS-R/DA and LDA was used for the detection of minced beef fraudulently substituted with pork (Ropodi et al. 2015). Different levels of adulteration of minced beef and pork were prepared; fillets were cut into smaller pieces and grinded separately one at a time, using a domestic meat-mincing machine. To achieve different levels of adulteration, ranging from 10% to 90% with a 10% increment, the appropriate amount of each type of meat was used and mixed in conditions that simulate industrial processing. The class of adulteration obtained an overall correct classification, mean per-class recall and precision of 83.3%, 83.3%, and 84.5%, respectively. The classification error for 98.48% of the samples was, at most, 10% for LDA. The overall correct classification, mean per-class recall and precision of pure pork, adulterated and pure beef was over 94% (mean recall, precision and overall correct classification was

94.4%, 99.4%, and 98.5%, respectively). The PLS-R/DA showed a correct classification of 98.5% using 12 PLS-R components after cross-validation.

In a study by Ma et al. (2015), VIS-NIR HIS was used to classify the fresh and frozen-then-thawed pork meats. The pork *Longissimus dorsi* muscles were obtained from a local market. The first group of fresh samples were divided without any freezing treatment, the second group of meat samples were frozen at −18°C for 24 h and then thawed at 20°C for 2 h, and the third group were frozen and then thawed twice. The correct classification rate was applied to assess the performance of the PLS-R/DA classifier for model establishment. The correct classification rate of 97.7% was achieved, confirming the high potential of textures for fresh and frozen-then-thawed meat discrimination. The PCA with three components explained 99.9% of variance and the first three PC images (the optimal GLGCM images) explained 98.1%, 1.3%, and 0.4%, respectively. This method is a powerful tool and allows the analysis of the quality of food and its authenticity.

In a 2016 study, chicken adulteration in minced beef was detected with VIS-NIR HIS (400–1000 nm) and HIS was acquired in the reflectance mode (RM) (Kamruzzaman et al. 2016). The pure minced beef and minced chicken were collected from a local supermarket. The minced beef samples were adulterated by mixing minced chicken in the range of 0% at 50%. Hyperspectral images were transformed into absorbance (A) and used the Kubelka-Munk (KM) function (Nobbs 1985). The performance of PLS-R developed using raw and pre-treatment spectra (1st derivative, 2nd derivative, MSC, and SNV). The percentage of chicken adulteration in minced beef was predicted with R^2_c of 0.97, 0.97, and 0.95 with the corresponding RMSEC values of 2.5%, 2.6%, and 3.3% for RM-PLS-R, A-PLS-R, and KM-PLS-R, respectively. When applied to an independent validation set, they were capable of predicting adulteration with R^2_p of 0.97, 0.97, and 0.96 and the corresponding RMSEP of 2.67%, 2.45%, and 3.18%, for RM-PLS-R, A-PLS-R, and KM-PLS-R, respectively. The ratio of percentage deviation values obtained were 5.84, 6.24, and 4.81 for RM-PLS-R, A-PLS-R, and KM-PLS-R, respectively.

Multispectral and hyperspectral imaging are quick techniques that allow a large number of samples to be analyzed at the same time and provide spectral data on the chemical, biological, and physical characteristics of samples. However, the instrumentation is costly and data processing can limit the use of this method in real time.

6.3 Spectral Data Processing

Spectroscopic methods associated with chemometric methods are tools for the identification of species and foodstuffs that are not on the label. However, identifying regions of interest and features in the spectrum, sometimes

called regions of interest, of the tested substances is important for the optimization of the methodologies (De Jong 1990).

Univariate methods that can be used, namely, the averages and standard deviations, descriptive statistics, box plots, analysis of variance (ANOVA), pair-wise comparisons of mean values with Fisher's LSD test, and t–test. These methods were applied, for example, by Pillonel et al. (2005) in the study of geographic origin of European Emmental cheese. A comparison between univariate and multivariate methods was performed by Moustafa et al. (2015) for evaluating the efficiency of spectral resolution when manipulating ratio spectra applied to ternary mixtures in common cold preparations. Multivariate regression techniques have been widely used to study food authentication. A comprehensive introduction and review on multivariate regression procedures can be found in Higgins (2005), van den Hout et al. (2007), and Cruyff et al. (2016) and references therein.

The application pre-treatment may remove the effects of unsystematic spectral data and eliminate variations, light scattering, random noise, external factors, and base line changes (Rinnan et al. 2009). The most common pre-treatment methods are the standard normal variate (SNV) (Barnes et al. 1989), the multiplicative scatter correction (MSC) (Dhanoa et al. 1994), the Norris-Williams derivation (Rinnan et al. 2009), and the Savitzky-Golay 1st and 2nd derivatives (Savitzky and Golay 1964).

The SNV is an accurate and reliable method for ranking in the spectroscopic field. The SNV is also an ideal technique for classification and validation of the results of PCA (Alamprese et al. 2013, Schmutzler et al. 2015, Ropodi et al. 2015). The MSC method is a simple processing step that attempts to account for scaling effects and offset (baseline) effects. This correction is achieved by regressing a measured spectrum against a reference spectrum and then correcting the measured spectrum using the slope (and possibly intercept) of this fit (De Jong 1990). The Norris-Williams derivation is a basic method developed to avoid the noise inflation in finite differences. This technique was elaborated on by Norris and Williams in 1984 as a way to calculate the derivative of NIR spectra. The NW derivation includes two steps, smoothing of the spectra and first-order derivation (Norris and Williams 1984). The Savitzky-Golay method reveals a larger structure of spectral data resulting in an easy interpretation of the chemical basis of the observed signals. The derivatives can also be used in conjunction with SNV (Press and Teukolsky 1990).

After pre-treatment of the spectral data a few simple statistic methods allow extraction of information from the spectral data.

The PCA method is applied to spectral data to reduce the dimensionality, to classify samples, and to identify outliers. The original variables are transformed into new uncorrelated variables called PC that are a linear combination of the original variables. The main components are linearly independent and represent variations in the dataset in descending order with PC1 describing the largest variance, PC2 the second largest variance, and so on.

The LDA method is a probabilistic classification technique that allows for maximum separation of samples between categories. The number of samples must be greater than the number of variables. This method allows the recognition of supervised patterns where the number of categories and the samples belonging to each category defined above is based on the assumption that samples of the same group are more similar than samples belonging to different groups. This method also allows a linear transformation maximizing the variance between classes and minimizing the variance within the class (Morsy and Sun 2013b, Uddin et al. 2005, Alamprese et al. 2013).

The PLS method permits an associate set of independent variables (predictors, X) to response variables (observations, Y) by reducing the original number of descriptors to a new set of data based on a reduced number of orthogonal factors called latent variables. The PLS-DA method accounts for the maximum separation between the classes in the data where the variable is dependent and categorized (Morsy and Sun 2013b). PLS-R is used to reduce the original predictors to a new variable which has better predictive power (Sun et al. 2012a, Morsy and Sun 2013b).

The SIMCA method provides a useful classification of high dimensional variations and incorporates PCA to reduce the dimensions of spectral information. The computing speed of SIMCA with PCA can be increased by calculating the covariance matrices and the indices. The MIR ATR spectroscopy with SIMCA makes it possible to successfully detect and quantify adulterants(Meza-Márquez et al. 2010, Zhao et al. 2015). The mean difference, standard deviation of difference, RMSECV, and R^2 are used for validation. Generally, a good model should have high R^2 and RPD and low RMSEC and RMSEP.

The PNN method consists of establishing decision limits in feature space with distinct patterns belonging to different classes. This method improves the standards of classification and enables faster speed training (Cheng et al. 2015). The PNN method showed potential for the analysis of the NMR data technique. PNN can be used as a classifier and to find variables with the highest impact in classification (Standal et al. 2010). For the MLR, it is necessary to establish the wavelength that can relate two or more explanatory variables and the response variable (Kamruzzaman et al. 2013). The GLCM method is an image processing method for resource collection textural analyzing the relationship of levels and slope between 2 pixels (Karoui et al. 2006).

6.4 Discussion and Conclusions

Authentication of foodstuffs is crucial due to design food with adulteration by substitution of species, geographical origin, or freshness. NIR spectroscopy detects the number of bands of smaller molecules OH, CH, and NH compared to mid-infrared spectroscopy which detects a greater number of

the molecules in the food matrix in more detail. This involves stretching, bending, and shaking movements of functional groups, such as CC, CH, OH, C = O and NH (Mamani-Linares et al. 2012, Alamprese et al. 2013, Zhao et al. 2014).

MIR spectroscopy in conjunction with Fourier transform and chemometric methods proves to be a promising technique for the analytical determination of adulteration of Halal food (Rahmania et al. 2015). However, the results of the ATR technique are affected by water content contained in food producing noise (signal). The method is fast, non-destructive and does not involve a lot of sample preparation, giving sufficiently reliable results. MIR spectroscopy requires dilution of samples unlike NIR, however this technique has difficulty in reading samples with large amounts of water, such as fish (Uddin et al. 2005, Zhao et al. 2014). NIR spectroscopy is a powerful technique for rapid analysis in line applied to inspections of foodstuffs and discrimination of linear and nonlinear allowing adulteration to be detected with ease (Morsy and Sun 2013b). FS reduces the time and cost of the measurements and analysis of enzymatic bio-analytic chemistry. This method can identify fish and detect fresh from frozen-then-thawed samples (Karoui et al. 2006). In a study by Jakes et al. (2015), investigators used a simple, quick, and inexpensive basic extraction with chloroform to obtain triglycerides in NMR spectra and found that 60 MHz ^{1}H NMR is a viable approach in high yield for the determination of adulteration in meat.

To obtain vibrational spectroscopic results, it is necessary to use chemometric models. The PCA, LDA, SIMCA, and PLS-R/DA methods demonstrated that, combined with spectroscopy methods, these techniques are useful tools for authentication and detection of adulteration in food (Kamruzzaman et al. 2013, Meza-Márquez et al. 2010, Zhao et al. 2014). RS is a promising technique in providing specific information on the identification of sample matrices based compounds (lipids, proteins, carbohydrates), it is sensitive to smaller components such as microorganisms responsible for spoilage and it provides detailed information on molecular vibrations and the chemical structure of molecules without causing damage to the small sample required for analysis (Boyaci et al. 2014, Argyri et al. 2013, Al Ebrahim et al. 2013).

Water interference does not occur with RS and results in water samples can be analyzed by glass or polymer packaging. However, analyzing samples using FS can hide the impurities and heat from the intense laser radiation can destroy the sample or hide the spectrum. To alter this effect, the use of a NIR laser reduces or prevents the fluorescence of the samples. It is a method with a high potential for identification purposes (Lohumi et al. 2015). Although NMR is a technique that allows the detection and analysis of different types of fat, it is an expensive technique, can yield spectra with too many peaks, and requires very concentrated solutions. While NIR, MIR and RS are well-established techniques, they are based on a sample point, a relatively small area of a species, which is not capable of providing the composition gradients yielding mean results compared with the multispectral

TABLE 6.1

Detection Techniques of Different Types of Meat and Fish Species

Type of Adulteration	Food Products	Detection Method	Chemometric Method	References
Substitution or removal of ingredients	Halal and non-Halal Chinese Ham sausages	FTIR	PLS-R/DA and PCA	(Xu et al. 2012)
	Veal	Fourier transform -NIR	PCA	(Schmutzler et al. 2015)
	Lamb	NIR hyperspectral imaging	PCA and PLS-R	(Kamruzzaman et al. 2013)
	Iberian pork sausages	NIR	PCA and MLSD	(Ortiz-Somovilla et al. 2005)
	Beef or bovine meat	UV–VIS, NIR and MIR	PCA, LDA and PLS-R	(Alamprese et al. 2013)
		Raman	PCA	(Boyaci et al. 2014)
		Raman	PCA and PLS-R	(Ebrahim et al. 2013)
		60 MHz 1H NMR	Naïve Bayes classification model, PCA	(Jakes et al. 2015)
		FTIR	PLS-R and PCA	(Kurniawati et al. 2014)
		Mid-infrared	PLS-R and SIMCA	(Meza-Márquez et al. 2010)
		ATR-FTIR	PLS-R/DA	(Nunes et al. 2016)
		FTIR	PLS-R and PCA	(Rahmania et al. 2015)
		FTIR	PLS-R	(Rohman et al. 2011)
		Multispectral imaging	LDA and PLS-R/DA	(Ropodi et al. 2015)
		TD-NMR	SIMCA, KNN and PLS-R/DA	(Santos et al. 2014)
		Fourier transform-Raman		(Zając et al. 2014)
		Mid-infrared ATR	SIMCA, PCA and PLS-R/DA	
	Carp fish fillets	Hyperspectral imaging	SIMCA and PNN	(Cheng et al. 2015)
	Chicken	VIS NIR hyperspectral imaging	PLS-R	(Kamruzzaman et al. 2016)

(*Continued*)

TABLE 6.1 (CONTINUED)

Detection Techniques of Different Types of Meat and Fish Species

Type of Adulteration	Food Products	Detection Method	Chemometric Method	References
Fresh vs. thawed meat	Pork	NIR hyperspectral imaging	PLS-R/DA	(Barbin et al. 2013)
		VIS–NIR hyperspectral imaging	PCA	(Ma et al. 2015)
		Hyperspectral imaging		(Pu et al. 2015)
		VIS and NIR hyperspectral imaging	PNN	(Pu et al. 2015)
	Beef	NIR spectroscopy	LDA and PLS-R/DA	(Morsy and Sun 2013a)
		Mid-infrared ATR spectroscopy	PCA and LDA	(Zhao et al. 2014)
	Fish	Front-face fluorescence	PCA and FDA	(Karoui et al. 2006)
		NIR	PCA and PLS-R/DA	(Ottavian et al. 2013)
Classification of species or origin	Gadoid fish species	13C NMR	PCA and BBN	(Standal et al. 2010)
	Lamb	NIR	LDA, PCA and PLS-R/DA	(Jakes et al. 2015)
	Different meat species	Raman spectroscopy	PCA	(Boyaci et al. 2014)
		VIS and NIR	PLS and PCA	(Cozzolino and Murray 2004)
		Hyperspectral imaging	PCA and PLS-R/DA	(Kamruzzaman et al. 2012)
		VIS and NIR spectroscopy	PCA and PLS-R	(Mamani-Linares et al. 2012)
	Deer	Shifted excitation Raman difference spectroscopy	PCA	(Sowoidnich and Kronfeldt 2012)

image. HIS and MS are important methods for food inspection as analysis is more convenient, fast and they analyze a larger number of samples simultaneously. However, the high initial costs and difficulties in data acquisition have limited the use of this real-time technology (Kamruzzaman et al. 2012) (Table 6.1).

Within this research area of the food industry, the future perspective is the application of multispectral imaging to many foodstuff samples to determine

the quantity of contaminants and the application of spectroscopy techniques to determine adulteration of food (Raman).

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7

Reconditioning of Vegetable Wash Water by Physical Methods

Vicente M. Gómez-López and Parag R. Gogate

CONTENTS

7.1 Introduction

One of the main process steps in the vegetable industry is water washing. Vegetable washing intends to remove soil, debris, and exudates and to reduce microbial load. This process consumes huge amounts of water. Wash water is usually added with disinfectants. The goal for adding disinfectants has been changing in the last year. They were initially used to kill foodborne pathogenic microorganisms that may be present in vegetables; however, recent evidence shows that this effect is negligible. Nevertheless, disinfectants still have a role nowadays in avoiding cross-contamination in the washing tank (Gil et al., 2009). Vegetable wash water is characterized by a high organic load that interferes with the effectiveness of sanitizers by reacting with them. It is a potential vehicle for bacterial pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella*. Ecological reasons and local water scarcity have prompted the search for strategies to reduce the use of water and disinfectants for vegetable washing, among which one can find water recycling and the partial replacing of disinfectants by physical methods. Water disinfection occurs in the washing tank; however, if water is collected at the end of the process for recycling, it must be subjected to cleaning and disinfection treatments outside the washing tank before using it for washing again, a process that has been called water reconditioning (Figure 7.1) (Gil et al., 2015; Van Haute et al., 2015). This process has been classified in four steps: preliminary treatments (separation of large particles), primary treatments (using physical methods of separation such as filtration or sedimentation), secondary treatments (aimed to remove organic matter by biological means), and tertiary treatments (elimination of chemical contaminants and disinfection [Ölmez, 2013]). The Codex Alimentarius states that if water is recirculated for reuse in a process in

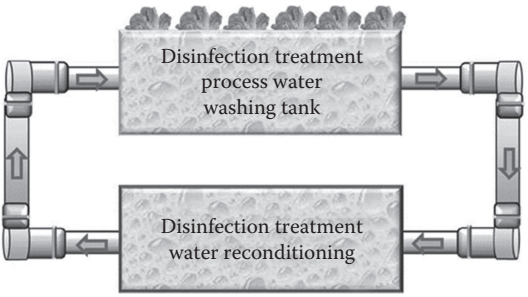

FIGURE 7.1
Difference between water disinfection in the washing tank and water reconditioning. (Reprinted with permission from Springer, *Food and Bioprocess Technology*, Potential of electrolyzed water as an alternative disinfectant agent in the fresh-cut industry, 8, 2015, 1336–1348, Gil, M.I. et al.)

contact with food it should be treated in such a way that it does not pose a risk to the safety and suitability of food (FAO, 2003). Physical methods for process wash water sanitation, disinfection, or reconditioning in the context of this chapter are those methods that do not introduce chemical substances to the water even though they can produce a chemical change. They include different mechanical separation methods, ultrasonication, and UV light treatment. Mechanical separation methods are used to remove substances, mainly solids. Ultrasonication and UV light treatments are focused on microbial inactivation. While these methods are unlikely to fully replace chemicals in the washing tank, they can be used for water recycling. Brief descriptions of these methods are given in this chapter.

7.2 Mechanical Separation

The type of wash water generated during industrial washing steps can widely differ depending on the kind of washed items; in general, it is formed by high amounts of suspended solids and dissolved molecules together with pieces of food. Separation of water contaminants by mechanical means allows a better action of physical disinfection methods such as UV light by removing screeners of its action. The following are methods that can be applied for effective solid removal upon selection of appropriate combinations and sequences depending on the characteristics of the water.

7.2.1 Screening

Coarse solids can be easily removed by using rakes or screens that can be located in the first place in the sequence of methods for wash water treatment.

7.2.2 Coagulation and Flocculation

Coagulation and flocculation are terms that are sometimes used interchangeably but are indeed two different processes that are used sequentially. Wastewater contains compounds in stable suspension and solution. Coagulation is the process that overcomes those factors that make them stable, while flocculation is the process whereby the clots are induced to come together in order to form larger agglomerates (Bratby, 1980). It is used as pretreatment in order to increase the size of particles to improve solid-liquid separation processes such as filtration or centrifugation. The formation of flocs is induced by the addition to wash water of inorganic metal salts or synthetic polymers such as ferrisulfate, aluminum chloride and polyaluminum sulfate (Lehto et al., 2014), and gentle agitation.

7.2.3 Sedimentation

Settling can be used as a mean of reducing solids content for pre-treatment for filtration. Simple rectangular settling tanks can have big footprints, requiring large areas, which are reduced using inclined plate settlers that increase surface loading, that is, the flow rate per unit of surface area where settling is allowed to happen. Sedimentation tanks must be designed in such a way to minimize disturbances that can resuspend solids. A simple settling tank has been reported to remove particles effectively in carrot wash water (Kern et al., 2006).

7.2.4 Flotation

Flotation is a process in which tiny bubbles of air are formed in the tank, which can attach suspended solids and make them buoyant to drive them to the surface. In the dissolved air flotation (DAF) process, pressurized air is injected to water that is released at the surface at atmospheric pressure. The air bubbles attach to the flocs, forming agglomerates (Babaahmadi, 2010). The floating solids can be then removed by a skimming device and the water withdrawn at the bottom of the vessel. It can be preceded by coagulation-flocculation.

7.2.5 Centrifugation

Centrifugation is a process that uses centrifugal acceleration to separate mixtures. The two main centrifuge models in process wash water treatment are the solid bowl centrifuge and the basket bowl centrifuge. In the first, a bowl rotating around a horizontal axis is fed through one extreme; the solids accumulate on the inner surface of the bowl and are driven outside by a conveyor that rotates at a different speed, while the liquid exits over the weirs. The basket bowl centrifuge consists of a perforated bowl that rotates on a vertical axis. Both solids and liquids are impelled toward the periphery; the solids are retained on the inner surface of the walls, forming a cake; and the water is filtered through this cake and removed from the mixture. The system can be stopped to be cleaned when the cake reaches a critical size, or automatic or semi-automatic discharge systems can be used (Mundi, 2013).

7.2.6 Filtration

Several types of filters can be used for process wash water clarification. Sand filters are the classical example, but more sophisticated methods are now available, such as (in order of lowering cut-off) microfiltration, ultrafiltration, nanofiltration, and reverse osmosis; the systems with lower cut-offs generate filtrates that do not need disinfection. Reimann (2002) applied ultrafiltration and reverse osmosis sequentially to wash

water of carrots, finding 5-log reduction for total bacteria. The deposition of solids on membranes causes filter fouling, which is a problem that imposes stopping the system periodically for filter cleaning or replacement. Submerged microfiltration systems have shown promising results (Nelson et al., 2007).

7.2.7 Membrane Bioreactors

Membrane bioreactor (MBR) is the combination of a suspended growth bioreactor with a membrane separation process (Radjenovic et al., 2008) such as microfiltration or ultrafiltration. The solid materials of wash water are degraded by microorganisms, and then the wastewater is filtered by a sidestream or submerged filtration system. The filtrate is microbial-free water and, therefore, does not require a further disinfection system.

7.3 Ultrasonication

7.3.1 Introduction

Power ultrasound refers to the use of sound waves at frequency about the audible range and high power dissipation levels for different applications including water treatment. Passage of ultrasound through a liquid medium, under such conditions, creates bubbles or voids that undergo cycles of growth and collapse under the influence of a fluctuating pressure field, finally collapsing and creating significant effects locally in the reactor. The important effects that can result in intensification of different chemical and physical transfer processes include generation of local hot spots, high shear rates, and liquid circulation associated with intense turbulence (Gogate, 2008). The schematic representation of the cavitation phenomena due to passage of ultrasound and different stages of cavity growth and collapse is given in Figure 7.2.

With specific reference to the application of inactivation of the microorganisms leading to treatment of wash water, the following simultaneously acting effects of cavitation induced by ultrasound contribute to the overall disinfection (Mason et al., 2003):

1. Physical effects such as generation of intense turbulence, shear and liquid circulation currents often described as acoustic streaming.
2. Chemical effects such as generation of active free radicals and local hot spots (conditions of high temperature and pressure) that can induce pyrolytic dissociation reactions.
3. Heat effects based on local conditions of significant temperature.

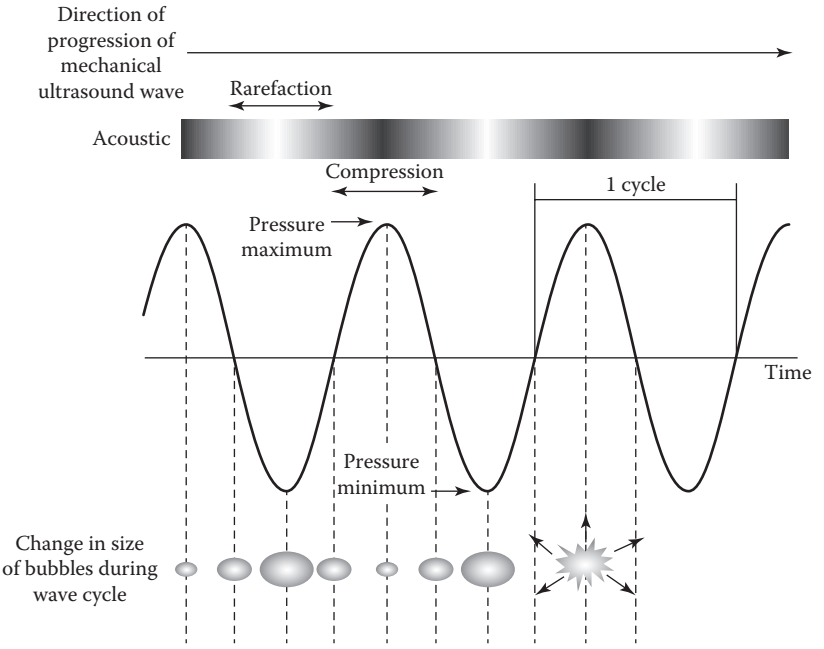

FIGURE 7.2
Schematic representation of ultrasound-induced cavitation.

4. If ultrasound is used in combination with chemical treatment such as chlorine, hydrogen peroxide, or ozone, the utilization of oxidizing chemicals is enhanced based on the intense turbulence reducing the mass transfer barriers and also giving enhanced penetration of the chemicals through the microbial cell wall.
5. Deagglomeration of clusters of microorganisms in the solution leading to enhanced efficiency for disinfection.

7.3.2 Ultrasonic Equipment

Ultrasonic equipment usually involves use of a transducer to transmit the sound waves into the medium. In general, ultrasonic transducers are designed to convert the supplied electrical energy into mechanical vibrations before transmitting high frequency sound waves into the liquid medium. The operation can be of two different types: direct contact where the transducer is directly in contact with liquid and indirect contact where the transducers are attached to the wall. Direct contact, though producing intense cavitation leading to beneficial throughput, has a drawback of possible contamination of the processed liquid, which may not be allowed in processing of food products being used for direct human consumption. Careful optimization of the processing conditions is required so as to avoid erosion of the

transducers in contact with liquid, at the same time giving desired intensities for disinfection (Mawson et al., 2014).

A design used commonly for laboratory-scale characterization is the ultrasonic horn, which gives the advantage of delivering large amounts of power directly to the processing liquids. The ultrasonic horn typically operates in direct mode operation and has a restriction of the zone of influence where the maximum activity is observed very close to the horn tip. The liquid circulation generated due to the intense dissipation of energy helps in providing efficient mixing especially at smaller scales of operation, and the levels of turbulence generated can be sufficient for inducing particle breakage or deagglomeration of the microbial flocs present in the wash water. For large-scale operations, it would be possible to combine a number of horns using a flow loop (Figure 7.3), and various combinations of frequencies may also be used to intensify the operation. Bubble dynamics studies have confirmed that using multiple-frequency operation results in more intense cavitation compared with single-frequency operation at similar levels of power dissipation (Prabhu et al., 2004). Utilizing a number of ultrasonic horns operating at multiple frequencies in the flow loop also helps in achieving high cavitational intensity, which may be necessary if the microbial loading in the wash water is significant or even if the system to be treated is complex, for example, involving large solid or microbial floc loadings.

Ultrasonic bath or flow cells with varying configurations, such as rectangular or hexagonal, offer the possibility of efficient pilot-scale operation in an indirect mode of operation and can also be effectively scaled up to large-scale units. The flexibility of using a large number of transducers depending on the scale of operation also allows for controlling the cavitational intensity,

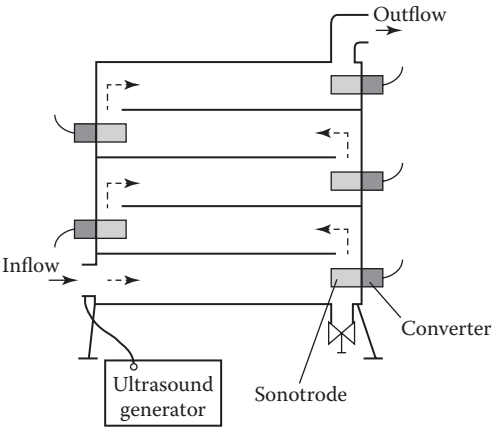

FIGURE 7.3
Schematic representation of flow systems based on ultrasonic horn. (Reprinted with permission from *Ultrasonics Sonochemistry*, 14, Nickel, K. and Neis, U., Ultrasonic disintegration of biosolids for improved biodegradation, 450–455, Copyright (2007), with permission from Elsevier.)

which can give an energy-efficient operation. Another key advantage due to the use of multiple transducers in the case of flow cells is that significantly uniform cavitational activity distribution can be obtained compared with conventional horn or bath-type reactors, leading to better prospects at large-scale operation (Gogate et al., 2011).

The sonochemical reactor designs can be modified so as to achieve combined operation of ultrasound and ultraviolet irradiations that can give intensified processing with a high degree of disinfection. A simple design for the combined operation can be based on a ultrasonic bath with transducers attached to the bottom or equipped with a longitudinal probe coupled with a ultraviolet tube provided at the top of the reactor (Khandpur and Gogate, 2016). This simple design would be more suited for batch processing. A typical configuration suitable for continuous processing can be a flow cell type where the walls of the reactor would be equipped with ultrasonic transducers, whereas the central core can have ultraviolet light tubes that can result in simultaneous exposure of the wash water to both forms of radiations (Gogate et al., 2003).

7.3.3 Factors Affecting Ultrasound Treatment

The effectiveness of a power ultrasound treatment is influenced by a number of factors, including the frequency and intensity of ultrasound, the geometry of the equipment, the exposure/contact time with the microorganisms, the type of microorganisms, medium physicochemical properties, as well as the temperature and applied pressure. Some guidelines about the optimum selection of these parameters have now been provided for better understanding of the operational aspects (Gogate and Kabadi, 2009; Gogate et al., 2011).

Frequency of ultrasound has been found to have a great impact on microbial destruction since the maximum bubble size before implosion is based on frequency of operation. In general, an increase in the ultrasonic frequency results in enhanced chemical effects and reduced physical effects such as shear and turbulence. For water treatment application, since physical effects are dominating, typically low frequency operation (20–100 kHz) is recommended. It is also important to note that variable frequency operation using multiple transducers can help in intensifying the cavitational activity and hence the processing rates.

Power dissipation into the reactor typically affects the cavitational collapse intensity and hence the extent of microbial inactivation. Typically optimum power dissipation levels exist, and it is recommended to dissipate power through higher areas of irradiation so that higher cavitationally active volume can be maintained. Power dissipation per unit treatment volume can be a useful scale-up criteria provided that adequate adjustments in terms of intensity of irradiation are also performed. Use of multiple transducers will allow maintaining the required intensities along with higher active volume, leading to better performance at large-scale operations.

Temperature is an important parameter for sonication as it has a great influence on several properties of the liquid medium, such as viscosity, surface tension, and mainly vapor pressure. Although an increase in temperature leads to an increase in the number of cavitation bubbles, intensity of the collapse of these bubbles is hindered by the increased vapor pressure. Temperature has also a favorable effect in terms of reduction in viscosity and can lead to microbial inactivation due to heat effects. Overall, an optimum temperature is likely to exist that needs to be established for the specific system under investigation.

External pressure is also a critical factor affecting the intensity of cavitation. An increase in pressure leads to an increase in both the cavitation threshold and the intensity of bubble collapse. At high pressure, the time required for bubble collapse decreases and the intensity of implosion increases, leading to more inactivation at high pressures. However, above a certain pressure, the ultrasound field is incapable of overcoming the combined forces of pressure and cohesive forces of liquid, leading to reduced cavitation; hence, optimum pressure needs to be maintained.

The medium physicochemical properties also significantly affect the generation of cavitation as well as its collapse intensity. Typically low viscosity, low surface tension, and presence of dissolved gases or solids at optimum loadings will enhance the cavitational effects and lead to a better degree of disinfection.

The presence of solid particles (either inert or as catalyst for dissociation of chemicals either purposefully added or generated *in situ*) in the system can ease the process of cavity generation based on the introduced heterogeneity; hence, the overall cavitational activity in the reactor can be intensified, giving enhanced disinfection rates. It is important to note that the solid loading would be an important factor in deciding the degree of intensification as the excess presence of solids can lead to scattering effects, giving reduced transfer of energy into the liquid. Also, the extent of intensification would be dependent on whether the solid is only enhancing the cavitational activity or also playing the role of catalyst.

7.3.4 Applicability of Ultrasound for Disinfection

Bacterial cells differ in their sensitivity to ultrasound treatment, which is typically based on size, shape, and species (Piyasena et al., 2003). Typically, Gram positive cells have a thicker and tightly adherent layer of peptidoglycans and, hence, can be more resistant to ultrasound compared with Gram negative cells. Also, aerobes show more sensitivity than anaerobic bacteria. Generally, bacterial spores are the most resistant microorganisms to any physical or chemical treatment and, hence, extended treatment times using ultrasound would be required. Furthermore, physiological condition of cells also affects the rate of inactivation, with younger cells being more sensitive than older cells. Depending on the presence of microorganisms, suitable

adjustment in the intensity can be done based on the different operating parameters, giving the desired intensity of treatment. Also, ultrasound can be effectively combined with other treatment techniques, such as chemical, temperature, pressure, as well as UV light, to give better treatment efficiencies, and most of the time synergistic effects.

Ultrasound can be used in different approaches, such as pre-treatment to conventional temperature or pressure treatments or in simultaneous operation with pressure/temperature treatment. Both these approaches enhance the efficacy of treatment and reduce the temperature and/or pressure requirements so that the overall treatment costs are reduced. Ultrasound can also be coupled with chemical treatment wherein again the advantages will be enhanced efficacy and reduced requirements of chemicals. Combination with UV light can be effective, leading to synergistic results due to the similarity of controlling mechanisms coupled with the fact that the turbulence generated due to the cavitational effects can give enhanced penetration of the UV irradiations and reduced mass transfer resistances.

7.3.5 Experimental Evidences Based on Literature Overview

There have been many studies reporting the use of ultrasound for water treatment either operated alone or in combination with other approaches for intensification. We now discuss a few illustrative literature reports to give an idea about the efficacy and the effect of operating parameters.

Seymour et al. (2002) investigated the effectiveness of ultrasound for the microbial decontamination of minimally processed fruits and vegetables, which are known to have problems related to the contamination of foodborne pathogens. The model foodborne pathogen analyzed in the work was *Salmonella* Typhimurium, which is generally found attached to fresh vegetables such as lettuce. Various treatment approaches, such as only water, combination of water with chlorine, combination of ultrasound and water, as well as combination of chlorine, water, and ultrasound, were compared for the efficacy of inactivation. It was reported that maximum 2.7 log reduction was obtained for the combination of ultrasound with chlorinated water, which was about 2 times higher compared with only chlorinated water and combination of water with ultrasound. It was also established that the physical effects in terms of turbulence and high-intensity liquid streaming (circulation) aid in removal of the cells attached to the surface of fresh produce, giving a higher degree of disinfection and safety to the fresh produce. Large-scale trials were also demonstrated at an operating capacity of 40 L, and it was reported that combination of chlorine and ultrasound yielded the best results for *E. coli* decontamination efficiency. It was also demonstrated that the frequency of ultrasound does not have any significant effect on the treatment (25, 32–40, 62–70 kHz) attributed perhaps to all low-frequency values being investigated in the work.

Dadjour et al. (2005) investigated the ultrasound-induced disinfection of *E. coli* in an ultrasonic reactor operating at 39 kHz as the frequency of

irradiation and maximum power dissipation of 200 W. The effect of presence of TiO_2 particles on the extent of disinfection was studied, and it was reported that the concentrations of viable cells reduced to only 2% of the initial value in the presence of TiO_2 at loading of 1 g/L after a 30 min treatment, whereas only 13% reduction was observed in the case of only ultrasound in similar time. Comparison of the catalytic action of TiO_2 and Al_2O_3 under similar conditions revealed that the rate of cell killing was significantly higher in the case of TiO_2 (about 3.5 times higher). The observed enhancement in the disinfection efficacy can be attributed to the surface cavitation induced by the heterogeneity introduced by the solid particles coupled with the catalytic action leading to production of enhanced quantum of free radicals (especially in the case of TiO_2, which also explains the higher disinfection).

Salleh-Mack and Roberts (2007) also investigated the application of ultrasonic irradiation for the inactivation of *E. coli* at operating frequency of irradiation as 24 kHz and maximum intensity of 85 W/cm^2, with understanding of the effect of different operating parameters. Ultrasonic irradiation was reported to give the desired 5 log reduction of *E. coli* in only 10 min of treatment. It was also reported that high temperature operation led to better efficacy for disinfection, attributed to the combined mechanisms of heat and cavitational effects. Study of effect of pH revealed that acidic conditions with optimum pH of 2.5 were the best, which can be explained on the basis of ease of hydroxyl radical formation and higher oxidation potential values. However, presence of soluble solids over the loading range of 0–16 g/100 ml of solution did not yield significant benefits.

The application of ultrasound in combination with peracetic acid (3.2 mg/l) in artificial wash water with a chemical oxygen demand of 500 mg O_2/l achieved > 6 log reduction for *E. coli* O157:H7 and *Salmonella* after 11 min, even though for *L. monocytogenes* the reduction was only 1.7 log after 20 min. For recycling purposes, the application should not need to add peracetic acid as such but it would use the residual concentration that could remain from its use in the washing tank (Gómez-López et al., 2015). Ultrasonication efficacy under these conditions have been proved to be insensitive to changes in water hardness and chemical oxygen demand, which makes it suitable to apply in wastewater treatment, where the water characteristics can be different from batch to batch and dynamic within batches (Gómez-López et al., 2014).

7.4 UV Light Methods

7.4.1 Introduction

UV light is the portion of the electromagnetic spectrum in the range 200–400 nm. The germicidal action of UV light is mainly produced by the UV-C light

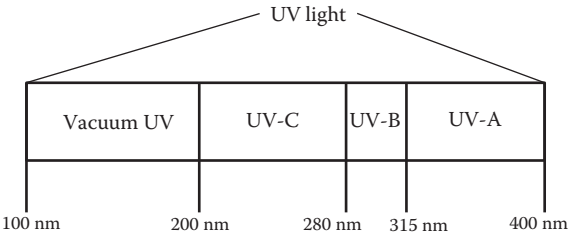

FIGURE 7.4
Different types of UV light as function of wavelength.

(200–280 nm) (Figure 7.4). The treatment of water with UV light is based on the laws of photochemistry. The first law of photochemistry, also known as the Grotthus–Draper law, states that only light that is absorbed by a molecule can produce a photochemical change in that molecule. The second law of photochemistry, also known as the Stark–Einstein law, states that only a photon is absorbed by each molecule undergoing a photochemical reaction. A third law of photochemistry has been recently proposed by Bolton (2004); it states that "the energy of an absorbed photon must be equal to or greater than the energy of the weakest bond in the molecule." These laws rule the photochemical degradation of compounds present in wash waters as well as the microbial inactivation by UV light, which occurs due to the photochemical modification of DNA, which yields mainly thymine dimmers.

Two light sources must be distinguished based, low-pressure mercury lamps and medium-pressure mercury lamps. The first emits a quasi-monochromatic output at 253.7 nm, while the second produces a polychromatic output. An emerging option is the polychromatic emission from pulsed xenon lamps, which deliver a very intense light at intervals (Gómez-López et al., 2007).

7.4.2 First Law of Photochemistry

The first law of photochemistry has several practical consequences for water treatment. The law does not imply that a photochemical reaction must occur whenever a photon is absorbed. Only part of the total absorbed photons causes a photochemical reaction; the fraction of molecules that reacts is called the quantum yield. Photons must contact the target molecule to induce a photochemical change; therefore, any hurdle between them must be avoided, hindering its application in washing tanks but being still suitable for water reconditioning. This law must be taken into account in the design of UV reactors and process optimization, which are function of water quality and fluid dynamics inside the reactor. The efficacy of the UV process decreases with the amount of suspended solids; therefore, any suitable method to remove them, such as filtration, will act in favor of UV effects. This is also reduced by UV absorbing substances present in the water and

will be a function of the transmittance of the water to the used wavelengths, which implies that a water body that is clear to human vision is not necessarily UV-transparent. In this context, the UV reactor design must take into account the use of the required retention time and appropriate flow regime in order to assure a homogeneous treatment of the whole water body with the required exposition time.

7.4.3 Second Law of Photochemistry

The second law of photochemistry needs some additional terms and definitions to better appreciate its implications for UV water treatment. These, as many others, are well defined by IUPAC (1996). The most important is fluence (J/m^2), which is the amount of energy of all wavelengths from all directions passing through an infinitesimally small sphere divided by the cross-sectional area of the sphere. It is the time integral of fluence rate, which is the rate that the energy incident on the aforementioned sphere divided by its cross-sectional area (W/m^2). A consequence of the second law is that the photochemical process will depend on fluence, and, consequently, the same effect will occur at a high fluence rate during a short exposure time than at a low fluence rate during a long exposure time. Fluence must be the used term rather than UV dose. It is the unit that characterizes a photochemical process, and its correct dosimetry is of paramount importance; it allows inter-experimental comparisons in research and allows scaling up to industrial processes. A method for fluence measurement in liquid samples that has become standard and the basis of regulations was set by Bolton and Linden (2003), which has been recently improved (Bolton et al., 2015) and the latter adapted to pulsed light technology (Gómez-López and Bolton, 2016).

7.4.4 Proposed Third Law of Photochemistry

Implications of the proposed third law of photochemistry can be realized, taking into account that not all photons impinging on a target are absorbed, but there is an absorption spectrum characteristic of each molecule; the absorption spectrum of DNA is commonly used for microbial inactivation calculations. However, not all the absorbed photons cause reactions, only those with enough energy. UV photons are more energetic than visible light photons as described by Planck's law. The use of the absorption spectrum of DNA is not always accurate since the action spectrum of *Bacillus subtilis* spores differs from it, which is relevant because these spores are commonly used as surrogates for biodosimetry of industrial UV reactors.

7.4.5 Photoreactivation

Beside the laws of photochemistry, the disinfection of water by this technology must take into account that microbial inactivation by UV light is

not a synonym for killing. Part of inactivated microorganisms can undergo photoreactivation upon exposition to visible light, so that the actual microbial population in non-light protected water bodies will be higher than that experimentally determined based on classical culture methods. Furthermore, the generation of viable but non-culturable microorganisms is an increasing concern in the field of pulsed light technology since it is unknown how relevant is this phenomenon (Rowan et al., 2015).

7.4.6 Experimental Evidence

The efficacy of UV light on vegetable wash water disinfection was tested at laboratory scale by Ignat et al. (2015), who presented results as an indicator of the potential use of UV-C (253.7 nm) to disinfect wash water, reporting >5 log reductions in counts of *Salmonella enterica* and *L. monocytogenes* inoculated in water and treated with an incident fluence of 0.01 J/cm^2 on a 0.4 thick water layer. In simulated washing cycles with lamb lettuce up to five, still >3 log reductions were obtained in native microflora in the wash water. Using a similar experimental set-up, Manzocco et al. (2015) reported >6 log reductions in *S. enterica* and *L. monocytogenes* upon exposure to fluence >0.7 J/cm^2. The use of pulsed light treatment directly in the washing tank has been proposed as a method to decontaminate fruits, with the alleged advantage of avoiding shadows and heating; the latter is an unwanted side effect of this treatment (Huang and Chen, 2014, 2015). One can suppose that this method would reduce the potential presence of foodborne pathogens in wastewater.

The efficacy of ultraviolet irradiations for disinfection can be further enhanced using a combination with photosensitizers such as zinc oxide. Ercan et al. (2016) investigated the combined application of ultraviolet light (UV-A) and zinc oxide (ZnO) for inactivation of *E. coli* BL21 and T7 bacteriophage present in the wash water (simulated). The individual operations of UV-A (9.53 J/cm^2) and 1 mM ZnO resulted in 3.9 and 0.7 log CFU/mL reductions respectively for the case of bacteria in the logarithmic phase in a treatment time of 60 min, whereas the combined treatment resulted in 6 log CFU/mL reductions. The extent of reduction was also reported to be dependent on the growth phase of bacteria, with the stationary-phase bacteria established to be more resistant resulting in only 3.5 log CFU/mL reduction for the combined treatment. Presence of any organic matter in the wash water also resulted in reduced inactivation rates, perhaps attributed to competing mechanisms for the oxidants formed in the process. Approximately 2-log reductions were observed at the maximum organic load investigated in the work. The reported intensification with ZnO is very important as this can serve as a replacement for the more commonly used chlorine-based sanitizers that offer significant limitations, including safety hazards due to formation of carcinogenic chlorinated organic matter as well as possibility of over-exposure to chlorine of the workers.

Selma et al. (2008) investigated the disinfection efficacy of ozone (O_3) and UV-C illumination (UV), operated individually as well as in combination (O_3-UV), for the reduction in microbial flora present in wash waters of different vegetable produce, such as fresh-cut onion, escarole, carrot, and spinach. The work also dealt with understanding the influence of physicochemical properties of water on decontamination efficacy. It was reported that both O_3 and UV, operated individually, showed effectiveness for microbial inactivation with 4.0 and 5.9 log CFU/mL reduction respectively in 60 min of treatment. The combined operation of O_3-UV was reported to be more effective compared with the individual operations, with maximum microbial reduction as 6.6 log CFU/mL in same treatment time. Use of ozone also helped in reducing the turbidity of wash water as per demonstrated results for both operations involving ozone as O_3 and O_3-UV treatments. It was conclusively established that the combination approach of O_3-UV can be an effective alternative to the commonly used sanitizers, such as chlorine, and can also allow use of lower quantum of fresh water as well as lower sanitizer doses.

Massé et al. (2011) investigated the application of UV irradiations to inactivation of bacteria in water to be used for washing of milking equipment in dairy farms. Two ultraviolet technologies, based on presence or absence of an automatic sleeve cleaning device, were evaluated, and it was reported that no fouling was observed during 6 months of continuous operation of the self-cleaning UV system. It was also reported that automatic cleaning was essential as the dose provided by a non-self-cleaning UV lamp decreased by 41% and 96% after 30 and 60 days of continuous operation respectively, possibly attributed to the fouling induced by water hardness and iron levels. It was also reported that both UV technologies were efficient in disinfecting water containing high pathogen concentrations, with a dose of 136 mJ/cm^2 completely deactivating initial load of 1090 and 595 CFU/100 mL of total and fecal coliforms respectively.

7.5 Feasibility and Economic Implications of the Recycle of Vegetable Wash Water

A significant amount of water is used in washing of fresh-cut vegetables, and its recycle afterward in a suitable integrated treatment system would mean considerable reduction in the requirement of fresh water. The integrated treatment system must include advanced processes like the ultrasound and ultraviolet irradiation-based treatments in addition to the conventional filtration- and adsorption-based approaches, and the discussion presented in this chapter is important for construction of such integrated systems. Recycle of wash water appears indeed feasible especially in countries where water scarcity is an important national issue. The optimization of the integrated

treatment systems as well as the fresh cut sanitation systems in terms of the operating parameters and the reactor configurations would lead to reduction in the treatment costs, leading to economical operations (Gil et al., 2009). The additional advantages of reducing the consumption of natural resources and protecting the environment would always be significant in addition to the possible economic benefits. The overall cost-benefit analysis for the application of the disinfection and recycle system should always consider water savings (for the fresh water requirement), system installation (one-time capital cost), and the operating cost for treatment as well as the environmental impact. The water treatment systems should be integrated into a complete water management system during the cost-benefit analysis. At this stage of the knowledge database, benefits of installing water treatment systems and recycle appear to be immense but commercial installations are currently hampered due to the possible abundant availability of water and lack of awareness among the consumers. It is very optimistic that, in future years, the trends will change and there would be a mandate for using the recycled water to maximum extent.

7.6 Potential Safety Risks due to Water Recirculation

Nonetheless, beside the need for proper disinfection, recent research warns about the potential accumulation of allergenic proteins after successive water recycling (Kekaert et al., 2012). Similarly, recycled water can carry contaminants that can accumulate after several rounds of recycling. Hamilton et al. (2006) reported the presence of the agrochemicals chlorphyriphos and linuron in wastewater from carrot-washing operations in Australian facilities.

Reconditioned vegetable wash water must be evaluated to guarantee its safety. The concerns about the reuse of water in the food industry have attracted the attention of international organizations such as the Codex Alimentarius Commission, which proposed several guidelines for the hygienic reuse of water in food plants (Codex, 2000), and the International Life Sciences Institute, Europe (ILSI Europe, 2008).

Hazard analysis critical control point (HACCP) is a tool that should be used to continuously monitor the safety of reconditioned vegetable wash water and its application as recommended by the international organizations. A generic and very comprehensive HACCP plan for water reuse in the food industry, including vegetable wash water, has been reported by Casani and Knøchel (2002). A recent study has also identified centrifuge water as the most suitable point to detect microbial contamination of the vegetable (Castro-Ibañez, 2016), which gives a good idea about the source of water contamination.

7.7 Concluding Remarks

Given the current concerns about the use of chemicals for water treatment, some physical methods can be useful for process wash water reconditioning to reduce the use of water and wastewater production and to avoid or minimize the use of chemical substances. The effective use of combined methods can also allow lower use of fresh water based on the efficient recycle of the used water and can give much higher efficacy of treatment compared with individual operations. During the reuse, the safety of reconditioned water must be continuously monitored to minimize risks.

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8

Food Safety and Food Security Implications of Game

Kashif Nauman and Peter Paulsen

CONTENTS

8.1 Introduction

Since ancient times, wild animals have been used as a food source. While meat from these animals has always contributed to human nutrition, nowadays it is either as a cheap or affordable source of protein and fat in the form of bushmeat or it be processed into luxury foods in developed regions. Humans can be exposed to health hazards during the handling of wild animals and during the processing and consumption of the meat of these animals. Processing will also vary in qualitative as well as in quantitative terms from region to region. Codes of good practice and surveillance systems should help to control these hazards, and the farming of "wild" animals will introduce a level of biosecurity comparable to that of extensively

kept (ranched) domestic animals. The wildlife—domestic animal interface has been studied extensively in terms of transmission of infectious agents or of antibiotic resistance. Likewise, wild animals can be used as sentinels or bioindicators for biotic and abiotic hazards in the ecosphere. The role of wildlife as a reservoir for emerging infectious diseases, and their impact on human health as well as on trade and food security requires a close observation of wildlife trade and translocation not only in a regional, but also in a global context.

8.2 Definitions of "Game," "Game Meat," Contribution to Food Supply and Legislative Framework

Meat from wild animals has contributed to protein and fat supply in human nutrition since ancient times (Ahlet al. 2002). Nowadays, relying on meat of non- or semi-domesticated animals is still a necessity in some regions of the world, whereas in others, meat from wild animals is considered a delicacy, and appraised by consumers concerned about animal welfare and the ecological aspects of modern intensive animal husbandry (Hoffman and Wiklund 2006). The favorable composition of meat, especially from wild ruminants, has been studied in great detail (see e.g., Valencak and Gamsjäger 2014), and it has been argued that humans are better adapted to that meat than to the meat from domesticated high-bred farmed animals (Cordain et al. 2005). However, the free-ranging life of wild animals complicates the assessment of which biological, chemical, and physical hazards the animals have been exposed to before being utilized as food, thus compromising biosecurity.

An unambiguous definition of "game meat" is hampered by various facts, and often done on a convenience or commodity basis. Basically, animal species and the mode of living/rearing need to be considered. It could include all non-domesticated species or only those which are hunted (Hoffman and Cawthorn 2014). From a traditional European view, this comprises: wild ruminants, wild suids, birds and some rodent species. However, in other regions of the world, kangaroos, reptiles (crocodiles), bats, and various other animals are hunted for local supply or for export. As some of these species can be free-roaming or can be kept in herds in enclosures under health supervision and with supplementary feeding, the distinction between "free living" and "farmed" or "ranched" game may sometimes be arbitrary, even more so, as extensively farmed or feral domesticated animals may experience more "freedom" than farmed "wild game." For example, reindeer farming as practiced by the Samiin Sweden holds a somewhat intermediate position (Wiklund 2014). Complications arise, especially in Europe, when the hunting industry tries to increase the harvest of "wild game" by feeding and other artificial means, whereas, on the other side, abundance of

wild animals in protected areas or in the periphery of large cities increases, prompting active population management (Gortazar et al. 2016). However, since wild animals or the products thereof can be traded, clear definitions are needed. For example, the EU distinguishes "wild game" from "farmed game." The latter comprises farmed ratites and farmed land mammals other than domestic ruminants (including buffalo and bison), pigs, and solipeds. "Wild game" are (a) wild birds that are hunted for human consumption and (b) wild ungulates and lagomorphs (i.e., rabbits, hares, and rodents), and other land mammals that are "hunted for human consumption and are considered to be wild game under the applicable law in the Member State concerned." This includes mammals living in enclosed areas under conditions of freedom comparable to those of "wild game." Even this clarification is not unambiguous, when it is considered that such areas can be as small as approximately 110 hectares, but can also be 10000 hectares and more in size. The distinction of "small" (wild birds and lagomorphs) from "large" wild game (species other than small wild game) has consequences for the mode of postmortem handling and meat inspection (Reg. (EC) No. 853/2004). Whereas farmed game is subject to veterinary health checks before and to meat inspection after slaughter, hunted wild animals will be examined for abnormal behavior and signs of disease before killing and in the course of evisceration by trained persons (i.e., qualified hunters or game wardens). A written record of the result of this examination accompanies the eviscerated (in the case of large wild game) or uneviscerated (common in small wild game) carcass on its way to the approved game handling establishment, where veterinary meat inspection of the carcass is conducted, taking into consideration the written information issued by the trained person. The latter document also assures traceability. Besides this approved game meat chain, the local supply of small quantities to consumers or to local retailers are governed by national legislation of the EU member states (see e.g., Citterio et al. 2011; Fettinger et al. 2011; Herenyi et al. 2014), whereas no special legislation applies to own private consumption. There are, however, textbooks that can serve as guides to good hygiene practice in game meat production and processing (Laaksonen and Paulsen 2015). EU legislation on this subject is not only the framework for intra-community trade, but also for export to third countries. Likewise, inspection systems warranting an equivalent level of food safety are required for meat from wild game imported into the EU.

In the US, meat from wild animals is non-amenable to mandatory Food Safety and Inspection Service (FSIS) inspection under the Federal Meat Inspection Act, but considered as "exotic meat" which may be subject to voluntary meat inspection—although the lack of information on the antemortem condition and on the hygiene during harvesting and transporting of the animal will not allow for a definitive decision on the fitness of the meat for human consumption. Thus, as a rule of thumb, hunted wild animals may be used for private consumption but not be sold, whereas wild animals kept on farms, under the appropriate regulation, may be inspected and their meat

be sold (Amann undated; Harris and Ling Tan 2004). The conceptualization of an informal and a formal game meat chain becomes even more clear in countries exporting large quantities of game meat, for example South Africa (Bekker et al. 2011).

In daily use, the term "bushmeat" denotes meat from various wildlife (including primates), in informal food chains, sometimes obtained by illegal activities. "Venison" is mostly used for meat from wild deer, either free-ranging or farmed (Hoffman and Cawthorn 2014). The array of wild animal species used for meat production is extremely wide, and sometimes species have been introduced deliberately for recreational hunting or invasive species or feral animals are hunted. For example, in New Zealand, chamois (introduced in 1907) and feral goats and pigs can be hunted and their meat be used for human consumption (New Zealand Department of Conservation undated), whereas the potential of Egyptian goose and common warthog—both considered agricultural pests in South Africa—to serve as food has been studied recently by Geldenhuys (2014) and Swanepoel (2015). For Tierra del Fuego, the necessity to control wild animal populations, either autochthonous (Guanaco) or introduced (beaver), and the benefits of processing them into food has been discussed by Gonzalez et al. (2004) and Hofbauer et al. (2005), but there are many more examples for immigrant, introduced, or expanding species. In any case, the possible role of meat from wild animals as a part of the future food security solution (Hoffman and Cawthorn 2013; Vagsholm 2014) requires that wildlife be included in One Health concepts.

Game meat consumption is only one facet of the consumption of food from animal origin. In Western Europe, the overall contribution to meat supply may be in the range of 1%, or approximately 0.5 kg per head and year. However, for hunters or their families, "high consumption" could amount to 18.2 kg per person and year. Game meat consumption data for some European countries has been collated by Laaksonen and Paulsen (2015), and is 0.2–0.5 kg per person and year for the average consumer, whereas estimates for hunters and their families are in the range of 5–30 kg. Such data is particularly useful, when exposure assessment studies are to be conducted, and not only the "average consumer," but also "high" and "extreme" consumption scenarios are to be studied.

8.3 Game Meat Production Systems, Foodborne Disease and Hygiene Issues

Game meat hygiene and meat safety issues are closely linked to the production system, starting from the presence of biotic (bacteria, viruses, parasites) and abiotic (e.g., environmental pollution) hazards in the environment of the animals, the exposure of the wild animals to these hazards and aggravating

factors, such as stocking density or overcrowding and other factors that compromise the immune status and allow the spread of disease. Consumers perceive wild animals often as "healthy" and, consequently, their meat is regarded as free from noxious or toxic agents (Hoffman and Wiklund 2006). In fact, game meat is rarely reported or identified as the causative agent for outbreaks of foodborne disease (see Mei Soon and Baines 2013), and reports often relate to local production and traditional preparation techniques involving no or low heat treatment (Awaiwanont et al. 2014). Most often, *Trichinella* outbreaks due to consumption of wild boar meat products are reported, but this is not an exclusive issue for wild animals, but rather a combination of low biosecurity plus lack of meat inspection and "unsafe" meat preparation practices, such as barbecuing or short-time fermentation. On first view, this conflicts with the multitude of hazards detected in wild animals or products thereof (see Chapter 9). However, like in meat production from farmed animals, the actual mode of rearing, harvesting, processing and, ultimately culinary preparation constitutes the sequence of filters which can turn the hazard into a negligible or into a relevant risk. The meat production systems can be broadly divided into three groups. The first is similar to that for extensively farmed domestic animals, including herd health management, slaughter/killing at suitable premises and processing of the carcass in approved plants. In EU terms, this would be the "farmed game" production scheme. This setting differs mainly in two aspects from regular farming. First, that it may be necessary to kill animals from some distance (by a shot in the head or neck) instead of captive bolt stunning or electrical stunning, which requires close contact to the animal, and second, that evisceration may be done on the spot, whereafter the carcass is moved in a suitable room where skinning and cooling are carried out. This system allows inspection antemortem and postmortem and should not pose other problems that are encountered in usual slaughter. This applies also to the shortcomings in slaughter and meat hygiene, for example that symptomless carriers of pathogens cannot be detected. On the other hand, traditional hunting for self-subsistence includes no formal assessment of the fitness of the meat for human consumption. The mode of killing, for example inexpert shots in the abdomen, can release gut content and the enteric bacteria can spread on the peritoneal surfaces or be translocated via the bloodstream. Admittedly, rapid removal of gut content (before bacteria attach to meat surfaces) and rapid cooling would slow down or stop multiplication of usually mesophilic contaminant bacteria. However, such procedures are not always easy to perform or the infrastructure is lacking (e.g., clean water). It can be argued that such meat, often termed "bushmeat" is not intended for sale; however, even when consumption is restricted to hunters´ families, outbreaks can occur. It can be assumed that the mode of culinary preparation is the key factor in this setting. The third, intermediate form is represented by for example hunting wild animals in Europe, where the antemortem inspection is assigned to hunters and the first inspection upon evisceration to trained hunters (see section xx), followed by the official

veterinarian. Guides to good hygiene practice complement the inspection scheme (see e.g., Laaksonen and Paulsen 2015). The main pillars in such guides are centered around the hygiene issues in wild game production (Soon Mei and Manning 2013), namely (1) the correct placement of the shot, assuring that the stomach or bowels are not ruptured, and that the animal is killed without avoidable stress and pain; (2) the removal of inner organs without effluents from the intestinal tract contaminating meat surfaces; (3) the transport to cooling facilities within suitable time (often, a maximum of 2–3 h from killing to evisceration and 6–12 h to the onset of cooling is recommended, but this will largely depend on ambient temperature) (see Paulsen 2011), and (4) the correct placement of the carcass in the cooling cabinet. Whereas these steps (1) to (4) are referred to as "primary production" and are the responsibility of hunters, skinning and cutting are processes done in approved plants, operating either under national or EU approval. Similar schemes will apply for other regions of the world.

8.4 Hazards Associated with Game Meat

The identification and characterization of hazards and the evaluation of their impact on food safety along the game meat chain follows the same basic rules as for farmed domestic animals. It is generally accepted that wildlife is a reservoir for emerging or re-emerging infectious diseases, and must not be disregarded when shared infectious agents are to be totally eradicated (Gortazar et al. 2014). Few formal risk assessments for game meat have been carried out on a qualitative basis (Coburn et al. 2003, 2005) or on a quantitative basis for contaminants (e.g., BfR 2010). However, the concept of ranking or prioritizing hazards in wildlife instead of just reporting them has recently gained much attention (Ciliberti et al. 2015; Gortazar et al. 2016; Ruiz-Fons 2015; Wiethoelter et al. 2015).

8.4.1 Biological Hazards

A number of studies have reported bacterial numbers (as total viable bacteria, Enterobacteriaceae, Coliforms) and frequencies of pathogenic bacteria on carcass surfaces and meat cuts from wild game, but due to different settings and methods of sampling, comparisons need to be done with caution. Gill (2007) concluded in his review, that the general microbiological condition of carcasses from farmed game or from birds slaughtered at abattoirs can be quite similar to the microbiological condition of carcasses from domestic animals or birds, whereas in hunted game, a number of factors can compromise food safety (e.g., placement of shot, incorrect evisceration, insufficient cooling); prevalence of enteric pathogens in meat from wild or

farmed game can be less than for meat from intensively kept domestic animals. Reviews with a broader scope, including other exposure pathways than meat, report a variety of agents for deer (Böhm et al. 2007) and wild boar (Meng et al. 2009). The number of studies on the microflora of game has multiplied since then, and the role of wild game as inapparent carrier of zoonotic agents is now considered more critically. In 2013, the European Food Safety Authority (EFSA) published an assessment on biological hazards and meat inspection of farmed game (EFSA 2013). The assessment considered reports from all over the world, and focused on the question if a given hazard could have substantial impact on human health, and if this hazard could be detected with meat inspection procedures already in place. For wild boar, *Bacillus cereus, Clostridium botulinum, Clostridium perfringens, Listeria monocytogenes, Staphylococcus aureus, Campylobacter* spp., *Salmonella* spp., pathogenic VTEC, *Yersinia enterocolitica, Toxoplasma gondii, Trichinella* spp., and Hepatitis E virus were pathogens of concern. The latter agent is obviously an emerging issue, but actual knowledge on public health is still limited. The highest priority was assigned to *Salmonella* spp. and *Toxoplasma gondii,* whereas *Trichinella* spp. was considered a relevant, but easily manageable hazard. Likewise, *Toxoplasma gondii* was considered a priority issue in farmed deer. For wildlife, similar multi-stage rankings have been conducted (Ciliberti et al. 2015; Gortazar et al. 2016; Ruiz-Fons 2015; Wiethoelter et al. 2015), but sometimes with a different focus. For example, Wiethoelter et al. (2015) assessed the relative interest of scientists in pathogens in wild animals (in terms of publications), and among her "top ten," only four, namely salmonellosis, trichinellosis, echinococcosis, and toxoplasmosis are typical foodborne diseases, whereas the others are more contact or occupational diseases. From a European perspective and referring to ruminants, Ciliberti et al. (2015) ranked *Salmonella*, *Coxiella burnetii*, Foot and mouth disease virus, *Mycobacterium bovis*, Bluetongue virus and European-tick-borne-encephalitis virus as "top six." Likewise, Ruiz-Fons (2015) prioritized for suids and on a global scale ranked *Salmonella*, *Campylobacter,* VTEC, *Leptospira* sp., Hepatitis E virus, swine influenza virus and Japanese encephalitis virus. The most recent, detailed multispecies ranking considering implications on human and animal health, and maintenance of the agent in wildlife, by Gortazar et al. (2016), identified *Mycobacterium tuberculosis* complex, *Coxiella burnetii*, pathogenic *E. coli*, highly pathogenic avian influenza virus and tick-borne agents as the "top-five" pathogens; however, a much higher number was considered "highly relevant" by the authors. Obviously, a strict "foodborne disease" oriented ranking would yield a different result, but food security (in terms of livestock production), human health (in terms of contact and occupational diseases), food safety and animal health should not be seen as separate issues. Thus, it not surprising to note that each ranking includes at least one typically meat transmitted agent and agents that can be acquired when handling game or processing or preparing game meat. Assessments like these are valuable since they help to focus from the multitude of potentially

present pathogens to those which need to be managed specifically or by common-sense hygienic procedures and precautions.

8.4.2 Chemical Hazards

Residues in organs and tissues from game have received much attention in their function as bioindicators, for example after release of radioactive isotopes, heavy metals, and persistent organic pollutants in the environment. More recently, the bullet-derived lead contamination of edible tissues of shot wild game has been studied. As regards food safety, the assessment of such contamination will not only depend on the nature and magnitude of the contaminant, but largely on the amounts of game meat actually consumed and the relation of this intake to other sources.

8.5 Food Safety and Food Security Aspects of International Trade with Wildlife

8.5.1 Extent of Trade and Monetary Impact

Wildlife trade is not always done on a legal basis. In fact, illegal trade with wildlife or products thereof is considered one of the fastest growing branches of illicit trade worldwide (Stoett 2002) after drugs, arms, and human trafficking (Good 2014; UNEP 2014). Both animals and plants are involved, either live or as dead specimens, parts, or tissues (such as ivory or leather) or derivatives (such as traditional medicines) (Engler and Parry-Jones 2007). In general, there are six categories of commodities including foods, clothing, and fashion (skins, wool, fur, feathers, and jewelery (pearls, corals, and teeth)), traditional medicines, ornaments, industrial resins and extracts, and household items. The demand for wildlife and derived products is perceived by a number of different consumer groups; in the US, exotic companion animals are high in demand, whereas in China, some products are used in traditional medicine, or, as ivory, are processed into decorative objects. The implications of economic constraints or tradition in the use of meat from wild animals have been studied by Kamins et al. (2014) for fruit bats in West Africa and reviewed for a variety of pathogens in Thailand by Awaiwanont et al. (2014). Although these are regional issues, tourism and trade may help to spread the agents.

Worldwide, an estimated 40,000 primates, 4 million birds, 640,000 reptiles, and 350 million tropical fish are traded live each year (Karesh et al. 2005). TRAFFIC estimates that from 2000 to 2005, 3.4 million lizards, 2.9 million crocodiles, and 3.4 million snake skins—all species listed under the Convention on International Trade in Endangered Species of Wild Fauna and

Flora (CITES)—were imported into the EU, along with 300,000 live snakes for the pet trade plus 424 tons of sturgeon caviar—more than half of all global imports. The number of recorded transactions of endangered flora or fauna is steadily increasing, and currently in the order of magnitude of 1 million/year (CITES 2016).

In monetary terms, estimates for the volume of legal and sustainable trade range from 6 billion USD (Check 2004, 10) billion USD (Schmidt 2004), or 20 billion USD (Alacs and Georges 2008) to 15–160 billion USD (Warchol 2004). Much higher estimates for global import value are given by Lawson and Vines (2014) and TRAFFIC (2008b), with 300 and 323 billion USD, respectively. As Europe is the biggest importer of wildlife species, its import bill statistics, according to TRAFFIC (2008a) indicate that EU imports alone were equivalent to €93 billion in 2005 and this increased to nearly €100 billion in 2009.

The financial aspects of illegal, informal, and unsustainable wildlife trade are not easy to estimate. Warchol (2004), McGrath (2012), World Wildlife Fund (WWF) (2014) and Voice of America (VOA) (2009) estimate around 6, 19 and 20 billion USD, respectively, but much higher figures of 50–150 billion USD are assumed by the United Nations Environment Programme (UNEP) (2014). Due to weak controls in law enforcement and inconsistencies in recording offences (Sollund and Maher 2015), it is quite difficult to get realistic data of this part of such a lucrative market. Due to this irruptive and unsustainable use of wildlife, the International Union for Conservation of Nature (IUCN) red list of threatened species records a significant increase in the number of animals and plants in the critically endangered, endangered, and vulnerable categories (Engler and Parry-Jones 2007).

Statistics on the illegal trade of wildlife indicate that the EU is the main destination or transit region. On an average 2500 seizures of wildlife and associated products that are made every year, these figures are roughly constant since 2007 (European Commission 2014) and correspond to €449 million (TRAFFIC 2014). Between 2003 and 2004, EU enforcement authorities made more than 7000 seizures, totaling over 3.5 million CITES listed specimens, while between 2005 and 2009 EU enforcement authorities made over 12,000 seizures of illegal wildlife products (TRAFFIC 2008a).

In addition to this, there is massive trade in wildlife and associated products on both a local and regional scale. Wildlife and wildlife products used locally, that is consumption of meat, are now exported thousands miles after passing through a complex chain of processing and trade channels or are indirectly used for scientific or tourism purposes (Chomel et al. 2007).

This wildlife trade pattern shows continuous growth every year particularly in illicit trafficking due to the lucrative market available and as it is less dangerous than other smuggling options like drug trafficking. On one hand, legal trade is providing conservation incentives, food security, socioeconomic benefits to communities, luxury and aesthetic satisfaction to buyers by legitimate business, and wildlife tourism, which is a multibillion dollar industry in developing and developed countries (Chomel et al. 2007).

But on the other hand, illegal trade can cause biodiversity loss (Baker et al. 2013) overexploitation of endangered, rare, and protected wildlife species populations, replacement of legitimate subsistence hunting, disbalance of ecosystems and nature, introduction of invasive species, violation of welfare codes, and particularly, introduction of emerging infectious diseases (EIDs) due to a lack of checks for diseases and a lack of health certificates. However, illegal wildlife trade is also encouraged by poverty, poorly managed borders, corruption, weak regulations and enforcement, which is fueling the illegal trade situation (UNEP 2014).

8.5.2 Wildlife and Emerging Infectious Diseases (EID)

Globalization has resulted in an unprecedented volume of trade in animals and animal products, which has created new pathways for the supply of wildlife and wildlife products and the resultant release of pathogens of importance to humans, domestic animals, and wildlife animals (Chomel et al. 2007; Karesh et al. 2005; Smith et al. 2009). In wildlife trade, each shipment represents a new area of origin and as a result, a new threat to help in the spread or emergence (or re-emergence) of diseases (Travis et al. 2011). The vast majority (92%) of the trade is designated for the commercial pet trade; nearly 80% of shipments originate from wild populations where no mandatory testing for pathogens is present. More than 69% of shipments contain animals from Southeast Asia, which is the epicenter of many zoonotic diseases (Smith et al. 2009).

Wildlife trade has assisted in the introduction of exotic species in new regions (Smith et al. 2009), which not only causes human diseases but also has effects on international trade, on the native wildlife population, on the rural livelihood of communities and on the ecosystem (Chomel et al. 2007). Wildlife trade can act as a reservoir to facilitate transmission of zoonotic diseases and represents a threat to human health and economies (Greatorex et al. 2016; Morse et al. 2012). Animals, in particular wild animals, are thought to be the source of >70% of all emerging diseases (Kuiken et al. 2005). In these diseases, 60% of emerging diseases are zoonotic and 72% originate from wildlife (Jones et al. 2008).

Animal consumption-based food systems, ranging from the harvest of free-ranging wild species to the in situ stocking of domestic or farmed wild animals, have been implicated in the emergence of many of these viruses, including human immunodeficiency virus (HIV), Ebola, severe acute respiratory syndrome (SARS), and highly pathogenic avian influenza (Murray et al. 2016). Trade that brings wildlife into close proximity with humans and domestic animals provides an interface for pathogen transmission. This interface can contribute to disease emergence, as illustrated by the role of wildlife trade in the spread of a range of diseases including SARS, monkey pox, and highly pathogenic avian influenza H5N1. The majority of organisms which act as causative agents in EID are bacteria (54.3%) while viral

pathogens constitute 25.4%, protozoa 10.7%, fungi 6.3% and helminths represent 3.3% (Jones et al. 2008).

In human–wildlife–domestic animal interaction, many EIDs are reported in the literature including Aleutian disease (McDonald et al. 2004), Alveolar *Echinococcosis* (Lee et al. 1993), Bovine tuberculosis (Pérez et al. 2001), Brucellosis (Dobson and Meagher 1996), Ebola virus (Leroy et al. 2004), Foot and mouth disease (Samuel and Knowles 2001), Hendra virus disease (Escutenaire and Pastoret 2000), Hepatitis E virus (Takahashi et al. 2004), Influenza H5N1 (Wang et al. 2006), Leishmaniosis (Rotureau 2006), Leprosy (Rojas-Espinosa and Lovik 2001), Leptospirosis (Szonyi et al. 2011), Malignant catarrhal fever (Aguirre 2009), Nipah virus (Epstein et al. 2006), Psittacosis (Moroney et al. 1998), SARS (Bell et al. 2004), Simian foamy virus (Wolfe et al. 2005), *Trichinella* (Ancelle et al. 2005), Tularemia (Wobeser et al. 2009), *Yersinia pestis* (Zietz and Dunkelberg 2004), and many others with domestic animal–wildlife interaction, and among wildlife and captive wildlife interaction (Daszak et al. 2000). Only a fraction of these agents or diseases is transmitted via food. However, the handling of animals and the processing of the carcass or meat should not be disregarded as a pathway for disease transmission. For example, in 2004, 46 persons died of rabies transmitted by vampire bats, mainly in Brazil (22 cases) and Colombia (14 cases); only 20 human cases of rabies were transmitted by dogs in all Latin America (Schneider et al. 2007). A similar trend was observed in 2005.

8.5.3 Outbreaks Caused by Wildlife Imports

In 2003, monkey pox virus caused an outbreak in 71 humans. The causative agent was identified in prairie dogs housed alongside imported African rodents from Ghana (Hutson et al. 2007; Giulio et al. 2004). *Francisella tularensis* antibodies were detected in one person (Avashia et al. 2004), as a result of an infection acquired from commercially traded prairie dogs. In 1998, *Chlamydia psittaci* caused psittacosis in 15 custom officers with one officer admitted to hospital (De Schrijver 1998) after exposure to the parakeets illegally exported by an Indian sailor. Highly pathogenic H5N1 influenza virus was detected in smuggled Thai eagles in Belgium (Van Borm et al. 2005), tigers and leopards (Keawcharoen et al. 2004), and birds in traditional wet markets (Hayden and Croisier 2005). In the same manner, trade and consumption of bats facilitated the spread of SARS (Li et al. 2005).

Exotic birds and animals, which are exhibited on different platforms—even after quarantine measures—are also associated with almost 25 outbreaks in the period 1999–2000 (Bender and Shulman 2004). Likewise, 65 cases of salmonellosis were reported in a zoo (Friedman et al. 1998), and in a circus in Illinois, 12 handlers acquired tuberculosis from the exotic elephants (Michalak et al. 1998), and in another zoo in Louisiana, 7 animal handlers got tuberculosis (Stetter et al. 1995).

8.5.4 Wildlife Trade and Animal Welfare

In the entire supply chain of wildlife and products, which starts from the killing or capturing, violation of animal welfare rights may occur. This condition becomes worse during transportation, particularly in illegal trafficking. Obviously, the frequency of fatalities among captured animals, even before they are transported, can be in the range of 50%–100%, as reported for birds in Senegal and Indonesia (up to 100%), ornamental fish in India and Hawaii (up to 85%), and chameleons in Madagascar (up to 50%) (Nowak 2016). Smugglers use every possible way to get entry into the market to fetch maximum benefits at the cost of welfare and ultimately, death of animals.

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9

Managing Food Supply Chains for Safety and Quality

S. Andrew Starbird and Vincent Amanor-Boadu

CONTENTS

9.1 Introduction

As populations grow, food safety becomes increasingly important, food production becomes more intense and dispersed, consumer preferences change, and international trade opens new opportunities for commerce. Scientific advances in growing, storage, handling, processing, and preparation add to the complexity of the network that moves food from producers to consumers. Ensuring the availability of safe and wholesome food is a growing challenge for companies and governments. An important tool in meeting this challenge is effective and efficient supply chain management.

In this chapter we show how supply chain management contributes to meeting the world's needs for safe food. We begin by defining a food supply chain and how we measure food safety in the context of a supply chain. We continue with a discussion of the regulatory environment for food production and distribution. More so than for other products, food is subject to considerable governmental control and oversight. The next section focuses on consumer preferences and how they impact the management of the supply chain and the delivery of safe food. Finally, we present some of the more technical economic challenges of food supply chain management and discuss the most common strategies for managing safety in light of those challenges.

9.1.1 Food Supply Chains and Their Management

A supply chain is a system of processes involved with moving a product or delivering a service from producers to consumers. In a food supply chain, these processes include harvesting from geographically distributed farms, transportation to aggregation points for processing and packaging, and distribution to retail food stores or food service firms.

Managing supply chains for food is different than managing the supply chains for other products. Food has special characteristics that make the supply chain more complex. For example, agricultural production is seasonal, exhibits significant uncertainty, and results in perishable end-products. In addition, long distances between production locations, processing, and retail distribution increase the risk of quality and safety errors.

Consumer perceptions of food add to the complexity of the supply chain. Unlike most products, food is consumed daily and its preparation is tied to cultural and family tradition. When and how food is consumed has a significant impact on the distribution systems. In-home preparation and consumption requires a significantly different supply chain than food service preparation and consumption. Those distribution channels must be monitored and managed differently.

Finally, more so than most products, food production, quality, and safety is significantly impacted by government agencies. Almost every nation has a government authority responsible for monitoring and, in some sense, controlling the production of food. The goals of government involvement include supporting domestic food production and security, ensuring food safety, and monitoring the environmental impact of production and distribution. In some countries, government is actively involved in evaluating and accrediting food service providers.

9.1.2 Types of Food Supply Chains

Food supply chains, like all other supply chains, are organized to achieve specific objectives, one of which is to maximize the overall value created. The supply chain's objectives are often determined by the initiating firm, which sees the organization of the supply chain as a means to achieve its goals. In addition, the objective of the supply chain is motivated by one or more drivers, which we have organized into four groups: Product or service; Customer; Corporate Social Responsibility; and Regulation. The objective and the driver together define the food supply chain type that is organized by its participating firms.

Consider a beef processor seeking to control the cost of procuring cattle. Such a processor would be interested in developing closer relationships with its upstream cattle suppliers, who could be aggregators or cattle ranchers, in ways that contribute to cost reductions in both acquisition and variability in the animals, since variability can have a significant influence on yield, processing speed, food safety, and other costs. Focusing only on cattle price without considering variability could adversely affect the achievement of cost control objectives. A *cost control supply chain*, then, is influenced by the product that is being procured, and to a limited extent, by the customers for whom the product is being developed.

Like cost control supply chains, *logistics supply chains* are product-driven and organized around partners who handle the transportation and storage of food products. They may, thus, involve transportation companies and warehouse providers. Their purpose is to minimize the exchange costs associated with products as they move from manufacturers through wholesalers and distributors to retailers and food service providers. These exchange costs include physical transfer and movement of products from one point to another, controlling product climate (temperature, humidity, pressure,

etc.) and preventing loss in transit and in storage. As such, the nature of the product is critically important in the structure of logistics supply chains. The location and nature of the customer also drive the motivation and operations of logistics supply chains.

Alternatively, a *fair trade food supply chain*, for example, involves food producers in a developing country supplying their products to a processor in a developed country who distributes it with the *appellation d'origine* mark so buyers can differentiate and pay a premium price. Consumers of the products in a fair trade food supply chain are willing to pay a premium price because they believe a portion or all of their premium goes towards a good cause—the education of farmers' children, for example. The product, the customer, and participating companies' social responsibility positions are critically important drivers of fair trade food supply chains.

In the past, traceability was used by food companies to differentiate themselves in their ability to assure their customers that the credence attributes of importance to them are authentic. With new food safety regulations in the United States China, the EU, and other countries, traceability has become mandatory for food companies, especially those involved in the importation of food ingredients or finished products. While the product may be important, customers and regulation are critically important drivers of *traceability-focused food supply chains*. For certain foods, such as those considered under fair trade or process-verified systems, corporate social responsibility may also be a critically important driver of traceability-focused food supply chains.

Environment can be defined broadly to cover the climate and natural resources, such as water and energy, and it can be defined narrowly to encompass the communities in which people work and live. Driven more by customers, by corporate social responsibility, and to a lesser extent, by government regulation, *environment-focused food supply chains* typically seek to procure, process or manufacture and distribute their products in ways that minimize their total carbon footprint. They achieve this objective through effective utilization of energy, water, and other natural resources. Food retailers, such as Wal-Mart Stores, Inc. and Whole Foods Market in the United States, Tesco PLC in the UK, Carrefour in France and ICA and COOP in Sweden are working with their suppliers to achieve environmental objectives that also improve their profitability. They are developing "green labels" to differentiate their sustainability products from their own products that do not qualify as "green" and from those of the competition. Their objective may also include fostering healthy workplaces, that is, those that are respectful of and fair to their employees. Participants in environment-focused food supply chains choose not to purchase inputs or services from suppliers violating their environmental values.

Local foods supply chains focus on local products and services and use only locally sourced ingredients and other inputs in their food manufacturing and distribution systems. Objectives of local foods supply chains may include environmental goals, like reducing the community's carbon footprint, in addition to objectives related to social responsibility, such as local economic

development. The participants in this supply chain expect each other to use locally produced inputs to produce, package, and distribute their food products. The definition of "local" differs across supply chains depending on their products and seasonal effects on supply. Customer preferences and corporate social responsibility are critically important to the structure and conduct of local foods supply chains but the nature of the product or service is also an important driver.

Any of the seven types of food supply chains described in Table 9.1 may fit into the generic food supply chain presented in Figure 9.1. While the figure shows the complete supply chain—from input suppliers, through producers and manufacturers to distributors and retailers—the boundaries of the different types of supply chains will differ according to their specific objectives. For example, while a *food safety supply chain* driven by the perceived food safety risks of genetically modified foods could include seed input suppliers to ensure ease of verification, the cost control supply chain example provided above will not include suppliers by default. A food distribution company that chooses to import finished food products may structure a supply chain to include the food processor or manufacturer, the wholesaler or distributor, and the retailers who sell the products to consumers. For such a company, neither farmers nor food service may be of interest to the supply chain developed. The foregoing describes the internal operations of the supply chain—players, chain length, product, information flow, rewards, and costs.

Every supply chain has an external environment, defined to encompass government and industry competitors and collaborators. Third-party certifiers and auditors of specific activities performed by food companies and their partners are becoming increasingly important. Some of these are mandated by regulators and others are used by firms to differentiate themselves in their markets. It is also becoming increasingly obvious that civil society and non-governmental organizations (NGOs) are influencing the structure

TABLE 9.1

Types of Supply Chains and Their Primary Drivers

	Supply Chain Driver			
Supply Chain Type by Objective	**Product or Service**	**Customer**	**Corporate Social Responsibility**	**Regulation**
Cost management	+++	+		
Logistics	+++	++		
Food safety	+	+++	+	+++
Traceability	++	+++	+++	+++
Local food	++	+++	+++	
Fair trade	+++	+++	+++	
Environment (resource conservation)	+	+++	+++	++

+ Important driver; ++ very important driver; +++ critically important driver.

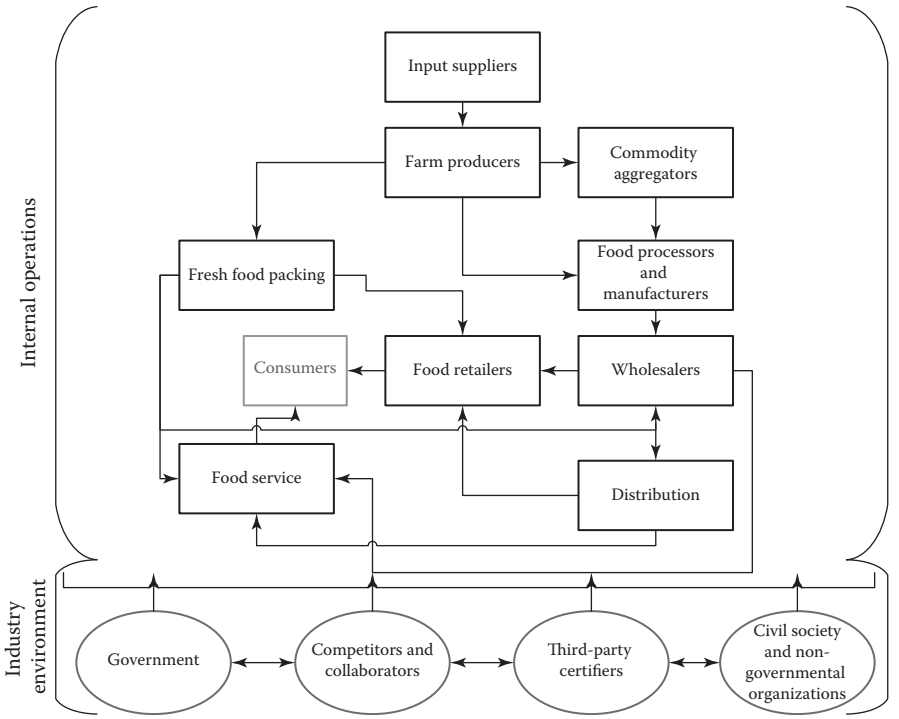

FIGURE 9.1
Food supply chain framework.

and conduct of food supply chains. The activities of some NGOs, including Consumers Union, Organic Consumers Association and Sierra Club, have been very vocal and prominent in their advocacy against genetically modified foods for their safety. Their activities have affected the formation of certain types of food supply chains and who can and cannot participate in these supply chains. As such, their operations contribute to the external environment firms consider when developing their supply chains.

9.1.3 The Challenges of Defining Food Safety and Quality

Each stage of the food supply chain has the potential to impact food quality and safety. Food quality generally refers to aesthetic elements of foods that enhance the pleasure of consumption: external characteristics, such as smell, size, taste, consistency, shape, gloss, and color; and internal characteristics, such as chemical, physical, and microbial state. Food safety, on the other hand, refers to contamination by biological, chemical, or physical agents that have the potential to cause illness in people. Methods for handling, preparing and storing food impact the potential for contamination.

Food safety and food quality are intertwined in many ways, sometimes making it difficult to talk about them independently. In a scientific sense, one might think that food safety is subset of food quality—one of its many dimensions. In an economic and management sense, there are important differences between safety and quality. While food safety is the norm or default expectation (i.e., food is expected to be safe), food quality is not—consumers expect differences in food product quality across suppliers. These differences in quality may be reflected in different product prices. That price difference reflects the consumer's perception of the product's safety as well.

Contaminated food products that present food safety risks may look, smell, feel, and taste just like uncontaminated food products, explaining why food safety hazards are generally unobservable by consumers. Some contamination by certain known pathogens (bacteria, viruses, etc.) and chemicals can be measured using scientific instruments or by conducting specific laboratory tests. However, it is impossible to know if an as yet unknown contaminant is in a particular food product since there are no tests for unknown contaminants. The only condition that will allow food contaminated by an unknown contaminant to be detected is if such contamination should change the physical characteristics (quality) of the food product or cause a consumer to experience an adverse health effect. Given that food suppliers would not, in their own interest produce products they know to be unsafe, it is plausible to assume that food safety hazards then are *accidents*, occurring as a result of unintentional human errors or system failures.

9.1.4 Chapter Outline

The management of food supply chains is influenced by government regulation, consumer perceptions of safety and quality, and by economic systems. Governmental regulations are used as a mediating force to enhance and standardize the food safety behavior of agri-food supply chains' stakeholders. These regulations are very robust in the United States and in the EU and are only recently evolving in developing countries, such as China. In the next section, we explore the effectiveness of regulations in moderating agri-food chain behavior within the context of food safety hazard risks.

In the following section, we explore how consumer perceptions of food safety affect the supply chain for food. We discuss how these perceptions are formed and what defines them, and we argue that while some of these perceptions are strong enough to produce regulatory policies that alter the cost structures facing agri-food supply chains, they may not always achieve their purpose of improving safety. In the final section of the chapter, we explore the economic and management system that has evolved in food supply chains to minimize consumer risk and mitigate the incidence of foodborne illness. We focus on three policies—labeling, contracting, and traceability—as they are used to enhance food safety.

9.2 Regulation of Food Safety and Food Supply Chains

Unlike most of the other food quality dimensions that provide implicit, explicit, intrinsic, or extrinsic cues that consumers can ascertain through their senses prior to consumption (Chamhuri and Batt 2015), food safety can only be assumed by consumers. If the safety of a food product should rise to the surface prior to consumption, it is highly probable that one of the quality cues would have triggered it, for example, odor from decomposition, or unhygienic production or sale surroundings. In general, food safety hazards become observable only after consumption, when they produce adverse health effects.

Consumers' inability to ascertain the safety of food products they are purchasing creates an information gap in the market exchange process which can lead to significant social costs. For example, food processors in the United States in the first third of the last century used various poisonous chemicals to adulterate food products or mask their decomposing odor from unsuspecting consumers. This practice continued for more than 30 years after the passage of the Food and Drug Act (1906) and the Meat Inspection Act (1906). Yan (2012, pp. 705–729) reveals similar activities in present-day China, where food processors and purveyors are using toxic chemicals, such as formaldehyde and its derivatives, to whiten sea food and grains or other toxic chemicals to preserve food or color them to improve their appeal. These actions are aimed at misleading consumers about the quality cues they receive without revealing the embedded safety hazards. This is akin to the information asymmetry problem that exists when purchasing a *shiny* used car that is *actually* a lemon (Akerlof 1970, pp. 488–500). The principal difference is that the food safety risk posed here can have extensive economic and social cost implications depending on how many consumers become exposed. We discuss U.S., EU and China food safety regulations and the lessons that may be drawn from them as countries look to build robust and effective safe food systems.

9.2.1 Food Safety Regulation in the United States

A study commissioned by the U.S. Congress in 1902 revealed widespread adulteration of food, sometimes with poisonous chemicals to improve color or mask odor or as a preservative. The findings supported the U.S. Congress passing two laws in the middle of 1906: the Food and Drugs Act; and the Meat Inspection Act.* Their focus was on prohibiting misbranded and adul-

* It is important to note that the U.S. FDA does not have oversight over all food and food products in the United States It shares this responsibility with the U.S. Department of Agriculture (USDA).USDA's Food Safety and Inspection Service (FSIS) is responsible for meat (excluding meat of game animals), poultry, and eggs product safety and FDA is responsible for all other food not under FSIS mandate.

terated food and drug products in interstate commerce. Legal challenges to the laws provided the foundation for using science to regulate food safety in the United States For example, in the 1914 *U.S. v. Lexington Mill Elevator Company* case (232 U.S. 399) on food additives, the U.S. Supreme Court ruled that for bleached flour with nitrite residues to be banned, the U.S. government would need to establish it as a hazard to public health. The burden, evidenced from the ruling, was on the government to demonstrate that the food was *unsafe*, not on the company to establish that the food was *safe*. A decade later, the Court ruled that the Food and Drugs Act condemns "every statement, design, or device on a product's label that may mislead or deceive, even if technically true."* This ruling focused on information accuracy, not technical veracity, in communicating with consumers, classifying any label inaccuracies as misbranding.

Despite the aggressive pursuit of safety enforcement by the Food and Drug Administration (FDA), adulterations and violations continued, causing deaths and injury across the country. It came to a head in 1937 when 107 people, mostly children, died from *Elixir of Sulfanilamide*, made from the poisonous solvent diethylene glycol. This tragedy paved the way for the passage of a more effective law that had been lingering in Congress for more than 5 years to be passed in 1938: The Federal Food, Drug and Cosmetic Act. This law guided U.S. food safety enforcement for more than 70 years, until its overhaul in 2011 with the introduction of the Food Safety and Modernization Act (FSMA). The need for the FSMA was justified by the increasing share of imports in food consumed in the United States, the increasing complexity of the nature and operations of food supply chains, and the increasing proportion of at risk population (children and the aged) in the country. Instead of responding to food safety hazards—which it had done over its 100-plus years—the FSMA provides the U.S. Food and Drug Administration with new and specific enforcement tools to prevent food safety hazards from happening. It recognized the essentiality of thinking about safety across the whole food supply chain and bringing a focus to stakeholders' safety responsibility from farm to table.

FSMA's four main components are: (1) Import Safety; (2) Inspections, Compliance, and Response; (3) Prevention; and (4) Enhanced Partnerships. The Import Safety component puts the onus on importers to guarantee that their foreign suppliers have adequate preventive controls in their plants to ensure that production and products are on a par with U.S. standards. Importers are responsible for determining known and anticipated biological, chemical, and physical hazards for the food products they import, assessing the risks presented by these hazards using hazard analysis based on illness, scientific reports, and other data. The FDA has authority to inspect the facilities of foreign suppliers to U.S. firms to verify standards and operations. If they deny FDA entry to their facilities, FDA has the authority under FSMA

* U.S. v. 95 Barrels Alleged Apple Cider Vinegar, 265 U.S. 438.

to deny product entry into the United States for firms that deny FDA access to inspect their facilities.

The inspection, compliance, and response component mandates increased and more frequent inspections of food production facilities to increase stakeholders' focus on prevention. The agency now has mandatory recall authority for products deemed hazardous as well as expanded access to company records. FDA also has expanded administrative detention under the FSMA, which allows it to suspend a facility's registration for serious violations. Also, FDA is not expected to take any company's records as final; it may order third-party laboratory testing to verify records.

The novel aspects of the prevention component include the establishment of processes to prevent intentional adulteration and ensure that product transportation did not compromise safety in transit. To achieve this, FSMA mandates the training of transportation personnel in sanitary transportation practices as well as in appreciating such food safety protocols as product climate control, and the prevention of contamination of food from non-food items when transported in the same vehicle.

A key aspect of the prevention of intentional adulteration component is the establishment of a written defense plan. The FSMA strengthens the familiar Hazard Analysis Critical Control Points (HACCP), and extends it beyond the processing plant, making it a supply chain responsibility. Food safety hazards arising from natural toxins, microbiological and chemical contaminants, pesticides, drug residues, allergens, parasites, etc. must all be documented and their critical control points in the facility identified. The company's defense plan must be reviewed at least every 3 years to ensure its currency in dealing with changing conditions. In addition to the defense plan, the protection against intentional adulteration requires companies to undertake vulnerability assessments for each food product manufactured, processed, packed, or stored at a food facility. Companies are expected to identify physical access to all their products and determine the probability of success for someone with intent to contaminate them, revising their defense plans accordingly. Most importantly, employees assigned to vulnerable areas in the plant must receive appropriate training to enable them perform their job adequately. To provide evidence of compliance, facilities are required to keep records of their food defense monitoring, corrective, and verification efforts.

The final component of the FSMA is a direction to the FDA to build an integrated food safety system in partnership with state and local authorities. Until now, food safety rules and enforcement has been a fragmented process with various enforcement statutes, creating uneven food safety standards across the country. It mandates the development of a national agriculture and food defense strategy and developing a consortium of laboratory networks to support its surveillance of the industry.

9.2.2 EU Food Safety Law

A series of high profile food safety failures in the 1990s produced a crisis of public confidence in European governments' ability to ensure food safety. The crisis produced an opportunity to develop new food and feed safety laws that were not only effective but transparent. Envisioning food safety as a total supply chain responsibility, the European Commission developed its new food safety regulations from a "farm-to-table" perspective, aiming to protect and promote consumer health through the enforcement of high food safety standards (Commission of the European Communities 2000).

The general principles for the new General Food and Feed Law were laid in Regulation EC No. 178/2002, adopted by the European Parliament and the European Council in 2002. The General Food and Feed Law established the European Food Safety Authority (EFSA), an independent agency responsible for scientific advice and communicating emerging food safety risks in the food supply chain. Unlike the U.S. FDA, which has oversight on both risk assessment and risk management, EFSA is responsible for only risk assessment. Enforcement of the Food and Feed Law is the responsibility of the Food and Veterinary Office, whose primary role is to ensure that both EU and foreign governments exporting food and feed to the EU have the appropriate procedures in place for ensuring that EU food and feed standards are maintained.

The EU's General Food and Feed Law has three principal tenets: (1) Risk analysis; (2) Precautionary principle; and (3) Transparency. Risk analysis establishes the scientific foundations for food safety. The components of risk analysis are risk assessment, risk management, and risk communication. Risk assessment is the first step in risk analysis and rests on using the best available science to assess risks in an independent, objective, and transparent manner. Risk management depends on the assessment outcomes, and involves determining the best means for preventing, eliminating, or reducing identified risks. The third component involves active and interactive communication among all stakeholders throughout the analysis process.

Perhaps the most controversial of the General Food and Feed Law is its precautionary principle. This principle indicates that under specific conditions, if concern that an unacceptable health risk exists and if available information and data are not sufficiently complete to allow for a comprehensive risks assessment, decision makers may be warranted to manage such risks using the *precautionary principle*, while seeking more data and information to enable comprehensive risk assessment. The precautionary principle is to be applied only as a provisional solution as new data supporting comprehensive risk assessment is collected.

The principle of transparency seeks to ensure that in the making of food and feed policy in the EU, mechanisms are laid out in the law to ensure public consultations are followed. Additionally, public officials have the responsibility to inform the general public whenever food and feed safety systems

are breached if such breaches present reasonable grounds for suspicion to produce adverse human and animal health events.

In order to assure consumers that food and feed available in the EU is safe, the General Food Law mandates traceability of all food and feed throughout the agri-food supply chain. The regulation defines traceability as the ability to trace and follow food, feed, and ingredients through all stages of production, processing, and distribution. This safety requirement facilitates identification of hazard points and an effective process for withdrawing contaminated products from the supply chain. It is not only limited to food produced in the EU but covers all imports coming into the EU. More importantly, it places the onus on companies to be able to identify the immediate supplier of any product they receive and its immediate subsequent recipient. For foods considered higher risk, more stringent traceability requirements are mandated. These include meat and other animal products and sprouts and sprout seeds.

The General Food Law holds the food or feed company responsible for complying with the regulations. Competent national authorities in member states have the responsibility of assuring adequate and effective controls are in place and enforced. When stakeholders of food supply chains identify any breach in their food safety systems, their primary responsibility is to withdraw or recall the product and notify the competent national authority to trigger monitoring to reduce and control the potential risks posed by the breach.

9.2.3 Food Safety in China

China is rapidly moving into an industrial society, currently having the second largest gross domestic product after the United States (World Bank 2016). The rapidity of this progress has created a lot of economic and social good as well as many problems and challenges, including those related to food safety. The outside world's attention to China's food safety challenges was arrested by the 2008 revelation that a Chinese supplier adulterated infant milk products with melamine, causing the death of four infants and making more than 53,000 sick. Around the same time in North America, dozens of cats died and thousands of other pets became sick after eating pet food imported from China that was shown to have melamine in it.

Yan (2012, pp. 705–729) argues that China is evolving from food hygiene to food safety. However, Yan notes that poor enforcement and greed among China's food supply chains' stakeholders might lead China from food hygiene to unsafe food to poisonous food instead. Yan provides numerous examples and cases of these problems, from adulteration and poisonous additives, employing pesticides as food preservatives, and offering fake foods to consumers. In all these, the issue of information asymmetry prevails at consumers' disadvantage, and with that disadvantage comes significant personal and social costs resulting from adverse health effects.

The embarrassment of the intentional adulteration of food and feed caused the Chinese government to enact its Food Safety Law in 2009 and revise it in 2015. The intent of the 2015 revision was to strengthen the regulation and improve oversight along the supply chain. It demanded a record of chain of custody by food manufacturers for incoming raw materials, food additives, other chemicals and related inputs. These records must include purchase date, supplier's name and contact information, specification, quantity, production date, and batch number. These records must be kept for between 6 months and 2 years.

Given the extensive problems presented by additives in compromised foods in or from China (Yan 2012, pp. 705–729), it is not surprising that food additives were high on the regulators' mind when developing the China Food Safety Law of 2015. Article 60 of the Law requires distributors to inspect suppliers' license and quality certificates when purchasing food additives, and to record all relevant information about the supplier and the product. It is interesting that the law also covered online retailers, requiring them to operate under the same standards as bricks-and-mortar stores.

China's Food Safety Law (2015) is overseen by five regulatory bodies. The China FDA is the main regulator of all activities related to food production, sales, distribution, and catering. The National Health and Family Planning Commission monitors and assesses food safety risks and develops national food safety standards. The Food Safety Commission, with responsibilities similar to those of EFSA—conducts food security analyses, studies, and coordinates food safety plans and activities. It is also responsible for proposing food safety policies and overseeing the implementation of the food safety regulations. Food imports and exports are under the authority of the General Administration of Quality Supervision, Inspection and Quarantine and animal production and management of genetically modified foods are regulated by the Chinese Ministry of Agriculture.

A forum hosted by the Brookings Institution in May 2016 observed that the new regulations are only a beginning to the long journey to improving food safety in China (Fu 2016). Among the necessary steps to get food safety challenges addressed are a greater coordination of the activities of the various government agencies with food safety regulatory responsibility, including the China FDA, the Ministry of Agriculture, and the National Health and Planning Commission. The highly fragmented Chinese food industry needs to be better organized to facilitate effectiveness in imposition and enforcement of food safety standards across the food supply chain. Addressing this fragmentation will enable the implementation of traceability programs that will begin to weed out non-conforming firms.

9.2.4 Lessons for the Future

The emphasis on regulating food safety from a supply chain perspective is critical in a world that is increasingly connected from agricultural inputs

produced in North America and used on farms in South America to produce soybeans that are shipped to China for processing, and exported as industrial food ingredients to North America and as part of food products exported to Africa for consumption. The increasing number of bilateral and multilateral trade agreements across all continents suggests that harmonization of food safety rules would begin to be seen within these trade blocs, as seen in the EU. To be part of these trade blocs and benefit from the market access opportunities, countries large and small will be required to have food safety systems in place that span their whole supply chains to ensure transparency and effective traceability. There is a lot to learn from the programs the United States and EU have developed and a lot to learn from the implementation challenges that China is experiencing.

9.3 Consumer Perceptions of Food Safety

We have argued that food safety is an intricate component of food quality, and that food safety hazards are difficult to identify *ex ante* under most circumstances. Yet, consumers often have perceptions about the safety of certain food products or the processes used to produce some foods. How do they come to form their perceptions and how do these perceptions inform their choice decisions and demand for food products? Answering this question is the purpose of this section. We begin with unpacking the concept of perception and its antecedents and how they influence consumer decisions and choices. We organize food safety hazards into three groups based on the duration and intensity of their adverse effects, arguing that the majority of food safety failures occur with food products that were either perceived to be safe or about which the consumer had no safety concerns. Based on these, we show that the real challenge to food safety is the lack of knowledge about food safety and suggest the need to enhance processes and initiatives that bring food safety to the fore of consumer choices.

9.3.1 Constructing Perception

Judd (1909, p. 44) argues that perception is "compact, immediate process dependent for its explanation upon the present conditions here and now." Judd's statement was in response to reviewers who argued for the inclusion of "past experience" in the formation of perception. Nearly a century later, Lomas (2002, pp. 206, 221–222) shows that "perception delivers conscious, conceptual information … perception does not deliver atomistic bits of information with which to reason, but provides complexes of information." Consumers, as an economic agent, employ all information—both real and imagined—available to them at the time of a decision to

ensure their choices framed by their perception offer them the highest satisfaction.

In conceptualizing perception, Slovic and Peters (2006) introduce "affect heuristic"—developed from feelings of dread—as different from objective risk assessment. For example, while the average dose of medical X-rays per U.S. resident is estimated at about 40 mSv/year, about 20,000 times higher than the average radiation within 20 miles of a U.S. nuclear plant (Wilson and Crouch 2001), the dread of living within 20 miles of a nuclear plant engenders a higher perception of risk. Similarly, the dread about novel foods and production techniques can lead to perceptions of higher risks than their objective risk assessments would suggest.

Affect is not independent of consumers' risk-benefit assessment. Alhakami and Slovic (1994) observed a negative correlation between an activity's perceived risks and benefits and the intensity of the affect associated with the activity. Risk perception, therefore, is influenced not only by what consumers think but by how they feel about the risk (Slovic and Peters 2006). Favorable feelings about an activity lead to judging its risks as low and its benefits high, and unfavorable feelings lead to judging risks as high and benefits low.

The foregoing indicates that perceptions emerge through and from complicated sense-making processes which allow people to identify, collect, organize, and interpret information to understand and make sense of their environment (Weick et al. 2005). Their formation is influenced by information about not only the product or service but the processes that support its production, processing, handling, distribution, storage, and delivery—that is, its supply chain—as well as consumer feelings (affect) about them. This suggests that consumer characteristics influencing affect development act in concert with product characteristics and available information to define benefits and costs and the associated dread which produces unfavorable or favorable perceptions.

9.3.2 Perceptions and Food Safety Risks

The affect–judgment relationship described above explains some of the perceptions about genetically modified foods in Europe, where with high unfavorable feelings, risks are judged to be high and benefits low (Wohlers 2010). These perceptions emanate solely from affect given that there is no history of any harm associated with genetically modified foods. Yet, more than 250 food-related diseases caused mostly by bacteria, viruses and other pathogens affect about 17% of Americans annually (CDC 2015) and are implicated in the death of an estimated 1.5 million children (World Health Organization 2015).

We identify three categories of food safety risks based on the time lapse between exposure and the advent of adverse effects linked to the exposure: food poisoning; foodborne disease; and food-induced illness. Food poisoning generally occurs within a few hours of consuming food contaminated

by disease-causing pathogens or poisonous chemicals. It is often characterized by *violent* abdominal pain, nausea, diarrhea, vomiting, and so on. The symptoms of discomfort and pain often disappear within 24–48 h, and often the incidence does not require a doctor's office visit or hospitalization. About 99% of food safety hazard events in the United States fall into this risk group (CDC 2016).

Foodborne diseases begin with symptoms akin to those of food poisoning and persist beyond food poisoning's 48 h, causing illness and complications that may last several weeks and require hospitalization. Foodborne diseases account for more than 26% of all hospitalizations related to known and unspecified pathogens in the United States (CDC 2016).

Food-induced illnesses have a much longer lag between exposure and attributable adverse effects. The duration of the lag is determined by the intensity of the exposure and the accumulative nature of the exposure in the body. Exposure to pesticide residues on fruits and vegetables or consumption of foods with color-enhancing or shelf life-extending chemical additives may only present adverse effects after long periods of consumption. Pesticides, food additives, and certain pathogens are, therefore, regulated in many countries to minimize their public health risks. Products may be banned when potential risks are deemed unacceptable. Such was the case when the U.S. Supreme Court ruled that the U.S. Environmental Protection Agency apply the Delaney Clause to set a "zero risk" standard for all pesticides with carcinogenic risks (Viswanathan 1993). When perceived risks, no matter how small, engender high levels of dread causing high unfavorable perceptions about particular products or technologies, they engender aggressively limiting public policies, as have been seen with respect to the precautionary principle guiding genetically modified foods regulations in the EU (Amanor-Boadu 2004).

Obviously, food products causing food poisoning or foodborne illnesses are hardly perceived as unsafe by consumers prior to consumption because they are familiar and present no dread. They are, therefore, referred to as "preventable" public health problems because a little care by consumers would make these events less likely. Food-induced illnesses, on the other hand, engender high levels of dread because they are often associated with unfamiliar food products or food production processes. This dread can cause strong unfavorable consumer perceptions that can produce regulatory environments that agri-food supply chains may find debilitating.

9.3.3 Shaping Perceptions about Food Safety

Perceptions about food safety may be defined as a function of the affect and consumer characteristics, including demographic and psychographic characteristics, and social, religious, cultural, and institutional attributes (Breakwell 2000; Fife-Schaw and Rowe 1996; Sparks and Shepherd 1994). Gender, age, ethnicity, having children at home, income, education, and geographic

location are some of the demographic characteristics that have been found to influence perceptions about food safety. For example, in assessing food safety perceptions about four production methods—irradiation, antibiotics, hormone treatment, and pesticides—in the United States Nayga (1996) found that age, education, gender, ethnicity, and whether the respondent was living in a non-metro area influenced safety perceptions. Both education and income were found to have favorable and statistically significant effects on perceptions about the safety as did residing in a non-metro area. Likewise, race was shown to have an effect, with black respondents presenting unfavorable perceptions about meat from animals treated with antibiotics and with hormones that were different and statistically significant from white respondents. However, employed and unemployed respondents as well as respondents with and without children under 18 years in their households did not differ in their food safety perceptions about the four production technologies. Demographic characteristics were not statistically significant in determining consumers' willingness to pay for food safety in Vietnam with the exception of urban residence, which was found to have a positive influence (Mergenthaler et al. 2009). Tonsor et al. (2009) show that consumer demographics, country of residence and trust in alternative food safety information sources influence risk perceptions in Canada, the United States and Japan. They found risk perceptions to be higher for females in Canada and the United States than in Japan while higher income consumers in all three countries had lower risk perceptions than their lower income counterparts. Consumers relying on credence attributes for food safety information expressed higher unfavorable perceptions while those depending on professionals, such as physicians, exhibited more favorable perceptions.

Affect, as seen from above, is determined by the nature and depth of the dread felt about the potential risks. Fife-Schaw and Rowe (1996) and Wåhlberg and Sjöberg (2000) projected these determinants of affect into three dimensions: Familiarity; Control; and Natural. Affect's strength in this function is influenced by consumers' familiarity with the product—that is, newness—and their confidence in reassurances about the product's safety from competent agents, which is itself influenced by their confidence in the agents' knowledge and independence. It is also influenced by the extent to which consumers believe they have control over their exposure to the risks—voluntariness of exposure. This may explain why consumers hardly see food safety problems with the food they prepare at home even though home-prepared meals present the most hazards. Naturally occurring risks are perceived differently than risks emanating from human action, with the latter receiving less unfavorable perceptions (Brun 1992). High trust in one's own ability to control potential risks or in the ability of public institutions to do so often reduce unfavorable perceptions about these risks while low trust exacerbates unfavorable perceptions (Frewer 1999).

European food safety hazards in recent decades (Bovine Spongiform Encephalopathy (BSE) or Mad Cow Disease), dioxin-contaminated animal

feed in poultry and swine, and Escherichia coli 0157, Salmonella, Listeria, etc.) have contributed to public distrust of the governments' ability to ensure food safety. While these hazards were not novel, the inability of existing regulatory systems to manage them was the primary source of the crisis in confidence, exaggerating the lack of consumer confidence in the regulatory system's ability to ensure the safety of novel foods and food production processes, such as genetically modified foods, whose risks were perceived as generally unknown and uncontrollable (Grunert 2005). In contrast, without any high profile food safety event in the United States consumer trust in government's ability to ensure safety has largely gone unchallenged, with 80% of consumers indicating confidence in the safety of the food they purchase from grocery stores (Kinsey et al. 2009).

Because affect emanates from a sense-making process, it can be shaped by exposure to information (Slovic and Peters 2006; Breakwell 2000). The information may be intrinsic or extrinsic to the product and/or implicit or explicit in its delivery. For example, it may be a message crafted to provoke confidence in safety and quality through associations with known and accepted quality and safe brands (the idea of *German engineering*). This is not dissimilar to using third-party organizations, perceived to offer transparency and legitimacy for food safety issues, to certify the safety or quality of a product (Almeida et al. 2010). The trustworthiness of the information source and the content of the presented information, that is, the message and the messenger, both influence the shaping of consumer perception (McCluskey and Johan 2004; Breakwell 2000). To this point, Mergenthaler et al. (2009) found that while general media predictors in Vietnam had a statistically significant positive effect on consumers' willingness to pay for food safety, specific food safety stories had a negative but statistically insignificant effect. Redmond and Griffith (2005) reported UK consumers expressing a high preference for medical doctors and cooking shows as a source of food safety information.

9.3.4 Securing Competitiveness through Effective Perception Management

We have, thus far, explored perception and the factors that influence its formation and identified affect and consumer characteristics as its determinants. We noted that affect is influenced by dread about a potential food safety hazard, and that of the three food safety hazards—food poisoning; foodborne disease; and food-induced illness—consumers formed perceptions mainly about food-induced illnesses, which have much lower probabilities of occurring but presented the highest levels of dread. The others present no concern because foods that often cause them are assumed safe and within consumers' control. Yet, the majority of food safety hazards are a result of consuming foods that were deemed safe by the consumer. Why?

Expert assessments of food safety risk events show that the majority of them occur in the home (World Health Organization 2015). Therefore, consumer knowledge about safe food handling at home presents higher risks for agri-food supply chains. Ergonul (2013) in Turkey, Worsley et al. (2013) in Australia, Burke et al. (2016) in Canada, and Gong (2016) in China found poor knowledge about food handling practices in the domestic environment. Henley et al. (2012) found poor food safety knowledge and practices among Asian, Hispanic, and African–American consumers in the United States In a study involving more than 500 students in 18 UK schools, Lange et al. (2016) found student knowledge about food safety and handling to be inadequate, especially regarding cleaning and reheating. The situation seems to be no better for food handlers in restaurants and institutions. Covering three European countries, Smigic et al. (2016) found inadequate knowledge about temperature control, sources of contamination, and foods most at risk of contamination among restaurant workers. Similarly, Kunadu et al. (2016) found food handlers in hospitals, schools, and prisons in Ghana had undertook multiple freeze-thaw cycles of frozen food and exhibited inadequate hand washing after coughing or sneezing during food preparations.

The foregoing shows that the consumer perception about food safety is misplaced in the broad scheme of events. Given the high cost of food safety events and given that the majority of these costs occur with products that consumers do not even think about within safety contexts, it is imperative to address this knowledge gap among consumers. Given that research shows this gap exists because of inadequate knowledge in countries developed and developing, agri-food supply chains and their supporting institutions—research universities, professional associations, trade associations, government, and non-governmental organizations—need to undertake extensive investments in improving food safety knowledge across the globe. The urgency of this is underscored by the global nature of food supply and how unnecessary costs affect the overall competitiveness of agri-food supply chains and the wealth of their customers.

9.4 Supply Chain Economics and Food Safety Management

In the first section of this chapter, we showed how regulations are used to address market failures in food safety and assessed the regulatory processes in the United States EU, and China. We discussed consumer perception of safety and quality and its impact on food supply chains in the previous section, arguing that food supply chains can influence perceptions by understanding their consumers. With regulatory agencies increasingly moving towards addressing food safety as a supply chain problem, in this section we discuss the challenges associated with the measurement of food safety, the

challenges of managing safety when there is imperfect information available, and the use of labeling, contracting, and traceability to manage and enhance safety through the food supply chain.

9.4.1 Credence, Experience, and Search Attributes

In marketing, product design, and supply chain management, we often distinguish between credence, experience, and search attributes of goods and services. As we have discussed, credence attributes are attributes that the buyer cannot evaluate with certainty even after the product is purchased and consumed. In contrast, experience attributes are those that the buyer can evaluate, but only after a good or service has been purchased and consumed. Examples of an experience attribute are the taste of a fine wine, the quality of a musical performance, and a ticket for airline travel. The quality of these goods and services can be evaluated after they have been experienced, but not before. Finally, search attributes are attributes that buyers can confidently evaluate before they purchase. The time and duration of a performance, the size of bottle of wine, the color and features of a new car are all search attributes.

Food products have credence, experience, and search attributes. Credence attributes include origin and production process (e.g., Kosher, Halal, fair-trade). Experience attributes include taste and shelf life (freshness), and search attributes for food include size, color, smell, and packaging. Food safety is a complex attribute that exhibits characteristics of both credence and experience attributes. If an unsafe food product causes an illness, and the illness can be traced directly to the food product, then safety is an experience attribute. It is far more common, however, that foodborne illnesses and food-induced illnesses are untraceable and safety is, therefore, a credence attribute. See Painter et al. (2013) and Gould et al. (2013) for a description of the challenges of tracing the cause of foodborne illnesses and estimates of the proportion of illnesses that can be traced.

With few exceptions, therefore, food safety is a credence attribute. Credence attributes have been the focus of a number of studies related to the markets for food products. Moser, Raffaelli, and Thilmany-McFadden (2011) review research on consumer preferences in fruits and vegetables and find that credence attributes related to health, sight and smell, pesticide residue, and local production are most important. Sogn-Grundvag et al.(2014) examine the interplay of private labeling and credence attributes on fish in the UK and find that there is a price premium for line-caught fish as well as home country of origin—both credence attributes associated with process and origin. According to Lassoued and Hobbs (2015), confidence in the presence of credence attributes is a primary factor in explaining food brand trust by consumers. In recent years, consumers have grown more interested in organic foods, a credence attribute associated with process, and research shows that consumer perception of organic food attributes has both hedonic (consumed

for luxury purposes) as well as utilitarian (to meet practical needs) aspects (Lee and Yun 2015).

These studies reveal the importance of credence attributes in the market for food and the perceptions of consumers. Credence attributes are especially important in food supply chains because information about quality is unequally allocated among buyers and sellers. As we show below, the imperfectly allocated information leads to significant market inefficiencies.

9.4.2 Asymmetric Information, Moral Hazard, and Adverse Selection

The existence of credence attributes is a complication, but not a significant economic issue, if buyers or sellers are equally ignorant. Credence attributes become an economic and management issue when the allocation of information about credence attributes is asymmetric. A large part of economic theory is dependent on the assumption of perfect information, that is an equal (symmetric) allocation of information about price, utility, quality, and production methods among buyers, sellers, and agents. When that assumption is not true, there is a potential for two problems in a supply chain: moral hazard and adverse selection.

In a food supply chain, moral hazard exists when safer foods are more expensive to produce than less safe food and the seller decides to produce and deliver less safe food to the buyer (Starbird 2005b). The moral hazard exists only if safety is a credence attribute and the seller cannot be held accountable for downstream safety failures (see Amanor-Boadu and Starbird (2005) for a discussion of the value of anonymity in a supply chain). Adverse selection is a problem when the lack of safety is a state of nature, rather than a choice, for the seller. In other words, sellers are classified as unsafe or safe, and cannot change their nature before a transaction occurs. In markets like these, high quality (safer) producers are driven out of the market and only low-quality (less safe) producers are left (Akerlof 1970).

The economic impact of asymmetric information in food supply chains, and how best to manage when asymmetric information is present, is the subject of many studies regarding food safety. For example, McCluskey (2000) examined the impact of asymmetric information on the supply chain and marketing of organic foods. She concludes that repeat-purchase relationships and third-party monitoring are necessary conditions for high-quality organic foods to remain available. Golan et al. (2001) showed that federal rules regarding food labeling could alleviate problems of asymmetric information—but are rarely effective in correcting undesirable exogenous impacts of food production and distribution. Asymmetric information about vaccinations and its impact on cattle auctions is the subject of a study by Chymis et al. (2007). They find that third-party certification could reduce the transfer cost of cattle in this kind of market. Finally, Hobbs (2004) examines the role of traceability systems in resolving the impact of asymmetric information on food safety and food quality.

These studies, and many others, describe the significant impact of asymmetric information about credence variables on the market for food. They also touch on some of the management strategies that have evolved to address an asymmetric allocation of information about food quality. Three strategies have emerged: labeling, contracting, and traceability.

9.4.3 Mitigating Asymmetric Information: Labeling, Contracting, Traceability

One of the greatest challenges for managers and regulators is finding ways to mitigate the impact of asymmetric information on food safety. Over the last several decades, labeling, contracting, and traceability have emerged as effective strategies for overcoming the problem. These strategies are complements rather than substitutes, and they are often employed simultaneously.

9.4.3.1 Labeling

Labeling food to identify its origin, production process, nutritional content, and expiration date is common in most retail markets. Further up the supply chain, the results of chemical and biological analyses are often a part of labeling. Labeling for food safety is problematic because the definition of safe depends on the level of contamination by multiple (potentially) pathogenic agents. For many pathogenic agents, the acceptable level of contamination is defined by government standards which are assumed to be met by all food in the supply chain. For other contaminants, standards are defined by third parties, or by community expectations. As discussed in the section on consumer perceptions, the default assumption of food consumers is that products are safe.

Instead of using a label of "safe," food producers typically use proxy variables related to safety. GMO content, organic production processes, and country of origin labels impact consumers' perceptions of food safety. Food labels like these can have a significant economic impact and are subject to considerable debate. For example, Roe and Sheldon (2007) found that discrete labeling for a government mandated standard can distort quality in a market. When this happens, labeling can impact the distribution of welfare among consumers. The establishment of government standards is also an important issue in evaluating the benefits of labeling in a food supply chain. Sheldon et al. (2015) show that there is a potential for regulated firms to influence the standards set by government, especially for novel food attributes. The impact of labels in a market for goods with credence attributes is the subject of work by Bonroy and Constantatos (2008). Their findings suggest that labeling impacts monopoly power and firm profitability in markets where firms are either high- or low-quality producers. A study specific to labeling beef (Lim et al. 2013) showed that consumers' willingness to pay

was significantly influenced by country of origin and by whether the product had been tested for bovine spongiform encephalopathy (BSE) and was traceability-enabled.

Labels have the potential to offset the economic inefficiencies caused by the existence of asymmetric information in a food supply chain. The potential is limited, however, when quality standards are inconsistent or unclear, and labels are not mandated. Food safety labels are rare because safety is impacted by many different agents, any one of which can result in food being unsafe. Far more common in food supply chains are labels related to individual pathogenic (e.g., BSE) and allergenic agents (e.g., peanuts).

9.4.3.2 Contracting

Contracts are used extensively in food supply chains to facilitate the exchange of goods, services, information, and money. Under certain circumstances, contracts can be used to motivate suppliers to deliver safe food. For this strategy to work, safer foods must be more profitable than less safe food, which implies that the downstream cost of unsafe food can be, at least partially, allocated to the supplier of the unsafe food.

With carefully constructed contracts, "The objectionable effects of an imperfect allocation of information can be partially corrected by an equitable allocation of cost" (Starbird 2005a). The provisions of a contract that can effectively segregate safe and unsafe producers are the magnitude of the cost of a food safety failure and how much of that cost can be allocated to suppliers, which is a measure of traceability (Starbird and Amanor-Boadu 2007). These results are similar to those of Resende-Filho and Hurley (2012) who found that the size of contingent payments for safe food and the "precision" of a traceability system (the ability to identify the supplier source of unsafe food) are, effectively, substitutes in motivating suppliers. This trade-off means that managers should think carefully and evaluate the impact on safety before investing in more precise traceability systems.

9.4.3.3 Traceability

Traceability systems track the flow of food through the supply chain and are perhaps the most thoroughly studied system for improving safety in a food supply chain. Most studies recognize that traceability systems are only effective to the extent that they complement management practices that support safety. Some, however, show that the existence of a traceability is enough to motivate suppliers to delivery safer food (Pouliot and Sumner 2008).

Traceability systems have multiple functions including improving supply-side management, increasing safety and quality control, and facilitating the marketing of foods with credence attributes (Golan et al. 2004). Hobbs (2004) identifies traceability functions that are more closely related to food safety in a supply chain. These include identifying contaminated products that reach

consumers, the allocation of liability for a food safety failure, and quality verification to avoid foodborne illness outbreaks.

To be effective, the traceability system must be complemented by a number of management, marketing, and quality control methods. For example, Aung and Chang (2014) suggest that the food traceability system is a link between tactical efforts like Good Management Practices (GMP) and Good Hygiene Practices (GHP) and a Hazard Analysis and Critical Control Points (HACCP) plan, and strategic efforts such as Total Quality Management (TQM). Traceability becomes a tool for implementing these supply chain management policies directed toward food safety. Similarly, Bosona and Gebresenbet (2013) identify traceability systems as a critical component of a comprehensive logistics management strategy. They provide detailed recommendations for making a firm's traceability system more effective in supporting logistics as well as quality control systems.

The effectiveness of traceability systems in supporting supply chain management depends on the match between the information tracked and the information needed. Golan et al. (2004), provides a useful way of categorizing traceability systems in terms of the data collected: depth, breadth, and precision. Depth is a measure of how far back in the supply chain traceability extends. Deeper traceability systems connect more stages, firms, and agents in the system, but they are also more costly. Breadth refers to the number of product attributes that are tracked. In the context of food safety, a broad traceability system would track multiple sources of foodborne illness. Finally, precision is a measure of the exactness in the traceability system. Smaller production units, shorter intervals of time, and more accurate tests contribute to the precision of the traceability system. One could argue that the best depth, breadth, and precision of a traceability system changes over time, making the challenges of food supply chain management even greater.

9.4.3.4 Techniques for Labeling, Certification, and Traceability

Fundamentally, labeling and certification are methods for communicating information to buyers about the quality and safety of a food product. In a food supply chain, the method of labeling depends on the type of product and what information is being communicated. As we have already noted, consumers assume that food in a market is "safe" and so that attribute rarely requires a label.

Most food labels are related to process and product attributes that impact quality. Process attribute labels include "all-natural," "GMO-free," and country of origin labels. Product attribute labels are usually related to nutritional content, weight, or volume. Certification is a subset of labeling that is based on a threshold standard for the presence or absence of an attribute. Food products that meet established standards are certified as "organic" or "nonfat," for example. Labeling is more general than certification and includes

product attributes that are measured and reported without comparison to an explicit standard, like weight and volume.

The most common type of labeling in food supply chains is related to nutritional content. Some nutritional labels are required by law and some exist to support marketing efforts. For example, nutritional facts related to calorie, fat, cholesterol, sodium, carbohydrate, and protein content are required by the U.S. government. Additional nutritional claims like "no artificial colors or flavors," "no preservatives," or "organic" are not required by law, but the use of those labels (which imply certification) is carefully regulated. Other common labels for retail food products include "expiration date" and "safe handling instructions."

Two important labeling techniques that support food supply chain management are Universal Product Codes (UPCs) and Global Trade Item Numbers (GTINs). UPCs and GTINs are standardized, machine-readable labels used to identify products and services and their origin. They can be used to track suppliers and manufacturers of food products and, therefore, facilitate traceability. The most common use of UPCs and GTINs is, of course, to facilitate transactions by encoding information about quality and quantity that can be converted to a price by point-of-sale (POS) software.

As with other aspects of the food supply chain, there are a number of technological advances on the horizon for labeling and traceability. One intriguing possibility is the development of unique and edible DNA barcodes. Recent tests show DNA barcodes are a promising way to transfer information in the supply chain for apples and for olive oil, without impacting product quality (Beck 2016). Similarly, the growing Internet of Things (IoT) provides opportunities for integrating new automatic tracking and tracing technologies into the food supply chain. Zhou and Piramuthu (2015) examine the potential for using IoT techniques to support physical flow traceability, business process traceability, and performance traceability.

In this section, we explored how the nature of food safety creates challenges for effective food supply chain management. First, we showed that safety is a credence attribute of food because it is difficult if not impossible to observe before purchasing and consuming a food product. Second, we described how the existence of a credence attribute like food safety can lead to asymmetric information and, therefore, economic inefficiency in supply chain transactions. Finally, we discussed three methods that have developed for mitigating the impact of asymmetric information and supporting the efforts of managers to provide safer food: labeling, contracting, and traceability.

9.4.4 Monitoring and Inspection for Food Safety and Quality in a Supply Chain

In general, monitoring food safety systems occur in process. Monitoring for food safety and quality can be categorized by whether the measured variable

is environmental, a process attribute, or a product attribute. Environmental variables that impact food safety and quality include temperature, humidity, and transit time. Because these variables have the potential of affecting safety and quality, they are monitored in process. Food manufacturers want to ensure that the appropriate temperature for the production of a particular product is maintained at all the critical control points. They also want to ensure proper storage of finished products and ingredients as well as the overall cleanliness of the processing environment. Whether process attributes, such as country of origin, Halal or Kosher, exist is monitored in process since they cannot be effectively verified post production. On the other hand, product attributes, such as moisture content, presence or the minimum residue levels of specific chemicals in products, and shelf life studies can be monitored and inspected to ensure safety and compliance with safety regulations. Thus, monitoring and inspections are used to ensure food safety and identify where food safety risks occur. When inspected food products are cleared as safe and leave the manufacturer's facility, then any failure can be attributed to post-plant handling, unless there are errors in the inspection. We discuss these in the next subsections.

9.4.4.1 Monitoring for Food Safety and Quality

The nature of the variable (environmental, process attribute, or product attribute) determines the system for monitoring the variable. Some variables impact all stages of the food supply chain (e.g., temperature) and must be monitored continuously. Some variables are determined at the beginning of the supply chain (e.g., country of origin) or can only be measured at the end of the supply chain (e.g., shelf life). Temperature is a good example of an environmental variable that is so important to safety and quality that it is often continuously monitored over the entire food supply chain. The activities in the supply chain that maintain a predetermined temperature range are referred to as the "cold chain." Cold chains are common in both the food and pharmaceutical industries and an unbroken cold chain provides assurance of the freshness, shelf life, and safety of food products.

The party responsible for monitoring environmental, process attribute, and product attribute variables depends on regulatory and market requirements. For variables that have a high risk of causing illness (e.g., contamination by biological pathogens), testing and reporting is often by government agencies, or by independent third parties. In the United States federal government agencies like the Center for Disease Control, the U.S. Department of Agriculture, and the Food and Drug Administration collect information about attribute and outcome variables that influence safety. State and local governments are also involved in monitoring safety and quality.

New technology is creating new opportunities for inexpensive, accurate, and timely monitoring of safety and quality variables. Kuang et al. (2015) develop a framework for adapting "Big Data" analytical techniques to food

safety monitoring. Big Data refers to the combination of information systems technologies with specialized computational software and new data capture and collection methods. These techniques have the potential to increase the accuracy and speed with which environmental, process attribute, and product attribute variables are monitored and controlled.

The ability to monitor environmental, process attribute, and product attribute variables depends on methods for data collection. In the food supply chain, data regarding these variables are often gathered through sampling inspection. Sampling inspection is required when it is impossible or impractical to inspect every item in a supply chain transaction. In food supply chains, sampling inspection is common because the volume of product is very large or the measurement of important variables is time-consuming or destructive.

9.4.4.2 Food Inspection and Food Safety

Although both regulatory agencies of governments and firms may have responsibilities in food safety monitoring, it is often the primary responsibility of the food manufacturer. On the other hand, inspection often lies within the domain of the government even though firms may conduct inspections for food safety on their own accord. In meat processing, for example, the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) has the responsibility of inspecting poultry and beef products for safety before they are allowed into the market. The safety of fish products is inspected by the Food and Drug Administration. Both of these agencies follow HACCP inspection protocols, assuring themselves that no compromised product has been produced or shipped from the plant and that the HACCP system is effectively controlling relevant food safety hazards.

The effectiveness of inspection is influenced by the sampling methods used by the inspection agency, as such sampling inspection reduces food safety risks, but does not eliminate uncertainty about food safety and quality. Because of sampling and testing error risks, safe products may fail inspection and unsafe products may pass inspection in many supply chains. A number of studies examine the impact of inspection errors on the behavior of suppliers and buyers. For example, Amanor-Boadu and Starbird (2005) show that when potential benefits are significantly lower than costs associated with inspection errors, participants of food supply chains structure them to minimize inspection costs without necessarily maximizing safety. Additionally, Khan et al. (2014) show that supply chain inspection errors support investment in relationship management, improved product and process design, and in workers' training programs. Finally, Starbird (2007) explored how contracts can be designed to exclude unsafe suppliers when inspection errors create uncertainty about food safety. The impact of inspection errors on supply chain management disappears as the accuracy of inspection increases. Fast and accurate tests will improve management decision-making, and enhance the quality and safety of food moving through the supply chain.

9.4.5 When Food Safety Fails: A Note on Food Product Recall

A food recall becomes necessary whenever a food safety-compromised product is available to consumers. The purpose of a food recall is to reduce or prevent public safety risks by removing compromised products from the market. Specifically, recall actions stop delivery and sale of compromised products, inform the appropriate regulatory agencies, and properly and in a timely manner remove compromised products from the market.

There are three recall classes defined by the potential risk of the compromised product to human health. Class I recall is when there is a reasonable probability that using or being exposed to the compromised product will cause serious adverse health effects or death. Class II recall is when the use of or exposure to the compromised product may cause temporary or medically reversible adverse health effects. Under Class II recall, the probability of a serious adverse health effect is considered remote. We have a Class III recall when the use of or exposure to the compromised product is not likely to cause adverse health effects.

Food manufacturers and their supply chains are required by regulators, such the U.S. Food and Drug Administration, to have recall plans and processes available so that, should the need arise for a recall to be implemented, it can be done smoothly and quickly. The plan requires a number of prior processes, including a recall team and the principal contact person in case of a compromised product triggering the need for a recall. It is also a good practice (but mandatory in other places) that food manufacturers develop a complaint file for each incident that could trigger a recall. The complaint file allows any adverse event related to the company's products to be logged with information on the complainant's name, address, phone number, and description of adverse health effects for affected people with their ages as well as the adverse health effects, when and where the product was purchased, and how the customer became exposed to it. Completing this document is necessary for the company to contact the appropriate government agencies to discuss the class of recall to be initiated and other corrective actions.

The other prerequisite process needed is a traceability system (lot and batch codes and quantities, processing location and distribution locations, chain of custody between manufacturer and retailer, etc.). This system allows products to be linked to suppliers and the products to be linked to ingredient suppliers as well as reconcile production with distribution to ensure accurate control over compromised products. Additionally, accurate sales records from retailers and distributors helps expedite the recall and increases its effectiveness in removing compromised products from the market and from people's homes. The effectiveness of corrective actions increases when such information is available. An overview of the generic recall process is presented in Figure 9.2.

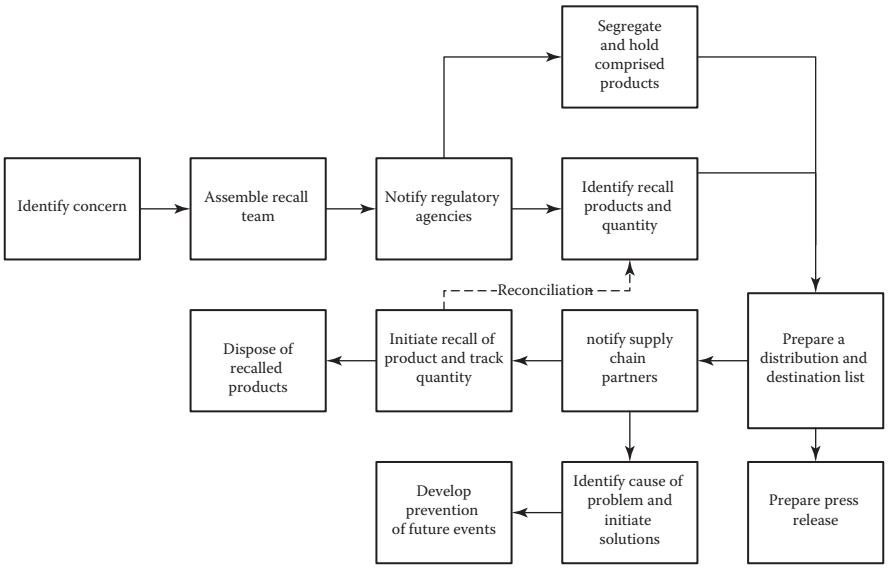

FIGURE 9.2
Generic recall process flow.

9.5 Final Thoughts

In this chapter, we discussed the relationship between supply chain management and food safety. This relationship is complex for many reasons. One reason is that consumer perception of food safety and quality depends on individual preferences as well as social and cultural expectations. Another is that government regulation associated with food safety significantly impacts management flexibility and decision-making. Finally, the nature of food safety, as a credence attribute, can result in economic dysfunction and inefficiencies. Managers and the market have developed a number of strategies for mitigating these inefficiencies including labeling, certification, contracting, and traceability. And when these fail and a safety-compromised product enters the market, food recall processes have been developed to ensure that public health risks are minimized.

Despite the difficulties described above, firms, consumers, and governments are working closely to improve the safety performance of food supply chains. Many significant advances have been made in the last two decades and many more are expected in the years ahead. International trade agreements and globalization would support the development and harmonization of food safety standards across countries, reducing risks from food consumption and improving consumer well-being.

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Retail Food Safety: Concerns, Regulations, Remedies

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CONTENTS

10.1 Introduction

Retail food safety is an ever-increasing concern along the farm-to-consumer continuum. The focus of this chapter is on products sold at grocery stores, regardless of size, from local markets to international retail giants such as Walmart. The variety and types of products offered at retail markets have changed dramatically. A typical large supermarket carries more than 42,000 items (FMI, n.d.). In 1998, a typical grocery store in the United States carried 345 produce items compared with just 173 in 1987 (Dimitri et al., 2003). From a microbial food safety risk perspective, select perishable items present a greater concern and these are typically found in four departments: produce, deli, meat and poultry, and seafood. For example, the consumption of fresh produce is part of a healthy diet, but pathogen contamination of fresh produce has resulted in serious public health consequences. Outbreaks in recent years have been linked to cantaloupes, tomatoes, spinach, lettuce, and mangoes. There is no exclusion when it comes to fresh fruits and vegetables that

can be contaminated by foodborne pathogens. Regulatory agencies, retailers, and consumers must be proactive in implementing strategies to ensure the safety of highly perishable food items. The challenge to ensure the safe handling of food at retail outlets can be more onerous for non-chain operations, and can have a greater impact on lower socioeconomic groups.

10.2 Consumer Awareness and Socioeconomic Groups

The safety of the food supply affects consumers in developed and developing countries. The World Health Organization (WHO) estimates that nearly 1 in 10 people become ill from eating contaminated food and nearly 420,000 die (WHO, 2015). In the United States, more than 9 million episodes of foodborne illness have occurred, resulting in 1351 deaths (Scallan et al., 2011). The quality and safety of food available to consumers can depend on socioeconomic status, which can be influenced by the retail stores that are available for that population to purchase food (Quinlan, 2013). Typically, retail outlets available to low-income individuals are small independent retailers rather than large supermarkets (Gillespie et al., 2010). Research indicates that small non-chain store owners often self-supply (Andreyeva et al., 2011), that is, they purchase products from a supermarket or warehouse and transport them themselves to their store. The study showed that fresh fruits and vegetables were self-supplied by 78% of small retailers interviewed in the study. The products were being transported in unrefrigerated personal vehicles, representing an unsafe step in the farm-to-consumer continuum. Clearly, there are many factors that can contribute to food safety issues, and the size and location of an operation are additional variables. Small operators may lack the knowledge and resources to develop and implement practices that prevent the unsafe handling of food, resulting in greater numbers of food code violations (Darcey and Quinlan, 2011). Regardless of store size, perishable products offered to the consumer are from similar food categories, namely produce, meat and poultry, seafood, and deli.

10.3 Food Waste

Retail grocery stores often dispose of edible products. Food waste has become a global challenge in developing and developed countries. There are generally three ways to handle foods of low or defective quality—donate, recycle, or dispose. In the United States, retailers account for over half of all food waste through the disposal of products that are low in quality but still

edible. In France, regulations indicate that grocery stores and supermarkets must donate unused foods approaching their best before date. In the United States, few retailers donate the food to charity, partially due to the fear of liability and potential unforeseen safety issues associated with products that are of less than optimal quality. Food waste is often associated with perishable products, which is attributable to the lack of infrastructure to ensure proper transportation, refrigeration, sanitization, and pest control, which precipitate a loss of product quality and, in the worst-case scenario, microbial safety hazards. In addition, the improper handling and management of foods intended for donation or repurposing may result in them comingling with products intended for sale to the consumer. Clear safe handling guidelines are needed for handling foods at the retail level that are suitable for donation. Otherwise, foods of high nutritional value will continue to be disposed rather than donated to organizations or individuals.

10.4 Retail Departments and Microbiological Safety

10.4.1 Produce Department

Globally, a movement has emerged for the increased consumption of fresh fruits and vegetables as they provide valuable nutrients to the diet and reduce the risk of certain metabolic diseases and some cancers (USDA, 2010). Surprisingly, the consumption of fresh fruits and vegetables by adults in the United States has declined in recent years (Moore and Thompson, 2015). This is related to changes in the intake of fruit juices and certain vegetables (green beans, corn, and tomatoes) (Produce for Better Health Foundation, 2015). In retail markets, fresh-cut fruits including cantaloupe, watermelon, and strawberries are available in a ready-to-eat (RTE) format. The convenience of these types of foods is appreciated by consumers, but they can present significant microbiological hazards if not prepared and managed properly. A greater number of retail grocery stores are preparing and packaging these types of items in-house. There are benefits and risks associated with preparing these products in-house, which, when not properly considered, can negatively impact human health. Produce (fresh and fresh-cut fruits and vegetables) will be used to highlight the challenges faced by retailers to ensure the microbial safety of products prior to being handed over to the consumer. Common practices and government regulations associated with those practices are underscored. Measures that can be implemented to improve microbial safety are also considered.

The number of outbreaks and cases of foodborne illness linked to the consumption of contaminated produce continues to escalate globally (Critzer and Doyle, 2010; Herman et al., 2015; Hoelzer et al., 2012; Teplitzki et al., 2011).

Fresh and fresh-cut fruits and vegetables are not processed in ways that will effectively eliminate human pathogens. Regardless of whether a country is developed or developing, challenges exist in devising and implementing measures that will improve the safety of produce. The types of microorganisms that can be associated with produce are varied and may include bacteria (e.g., *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella*), viruses (e.g., hepatitis A virus and norovirus), and parasites (e.g., *Cryptosporidium parvum* and *Cyclospora cayetanensis*). The sources of these pathogens and other microbes include contaminated agricultural water, soil amendments, contaminated harvesting equipment, field workers, processing plants, and retail handling.

Surprisingly, very little research has focused on safety issues associated with the practice of crisping and misting by retail establishments. The market penetration of these practices in the United States was estimated to be 80% in 2006 (*The Produce News*, 2006). Brochures and training materials from industry commodity institutes (e.g., IGA Institute, 2005) and government offices (California Department of Public Health, 2011; Defense Commissary Agency, 2012) promote crisping and misting as strategies to extend produce shelf life. Crisping involves the soaking or partial immersion of a commodity (lettuce, spinach, celery, etc.) in water to improve its appearance. For example, heads of lettuce may be placed into a large sink or tub filled with water and soaked for 5–15 minutes. Leafy greens are particularly vulnerable to dehydration during transportation/shipping; a 15% increase in the weight of such commodities may occur following crisping. The increase in commodity weight is of course associated with water uptake. Since sanitizer may or may not be added to the soak water, there exists the very real potential for cross-contamination to occur during soaking. After removal from the crisping water, the product is placed onto crisping racks to drain, permitting water run-off that may come into contact with other products, potentially resulting in cross-contamination, and is then held at refrigeration temperature until placed for sale.

In addition to leafy greens, fresh-cut fruits, such as cantaloupe, for example, prepared at retail markets are typically soak washed. The microbial safety of cantaloupe is a global concern as outbreaks linked to cantaloupe have been reported in a number of countries including the United States, Australia, and Canada. According to the Centers for Disease Control and Prevention (CDC), at least two outbreaks linked to cantaloupe have occurred in the United States every year from 1999 to 2014 (CDC, 2016). A cantaloupe-related *Listeria* outbreak in 2011 is considered one of the deadliest multistate outbreaks in the United States to date, resulting in 147 illnesses and 33 deaths (McCollum et al., 2013 and Huang et al., 2015). The severity of cantaloupe-linked outbreaks worldwide highlights the importance of improving post-harvest processing practices, especially those at retail establishments.

The safety of fresh produce remains a challenge for the food industry. As technologies to enhance the safety of fresh produce become more readily

available, they should be utilized by all food establishments. As specified in the United States Food and Drug Administration (USFDA) Food Code under Section 3-302.15 Washing Fruits and Vegetables, "raw fruits and vegetables *shall* be thoroughly washed in water to remove soil and other contaminants before being cut, combined with other ingredients, cooked, served, or offered for human consumption in ready-to-eat form." Further, under paragraph 3-302.15(B), it says "Fruits and vegetables *may* be washed by using chemicals as specified under § 7-204.12."

Washing, in this context, is required but using treated water is optional. It is well documented that raw agriculture commodities (RACs) may be contaminated with pathogens and, when soaked or submerged in water, there is a risk of cross-contamination. Various chemical sanitizers are available that can minimize and/or prevent cross-contamination and, to a lesser degree, reduce the pathogen load on fresh produce. Therefore, when produce is washed, crisped, rehydrated, or processed by soaking or submersion, the water used for these purposes should be chemically treated to minimize the risk of cross-contamination and improve public health. This would be a prudent practice.

The use of chemicals for the washing, treatment, storage, and processing of fruits and vegetables is specified in the "USFDA Food Code, Section 7-204.12 Chemicals for Washing, Treatment, Storage and Processing Fruits and Vegetables, Criteria" as follows:

(A) Chemicals, including those generated on-site, used to wash or peel raw, whole fruits and vegetables shall

1. Be an approved food additive listed for this intended use in 21 CFR 173, or
2. Be generally recognized as safe (GRAS) for this intended use, or
3. Be the subject of an effective food contact notification for this intended use (only effective for the manufacturer or supplier identified in the notification), and
4. Meet the requirements in 40 CFR 156 Labeling Requirements for Pesticide and Devices

The criteria for using chemicals for the washing, treatment, storage, and processing of fruits and vegetables are designated in the Food Code as priority items. Sufficient controls are already prescribed to ensure the safe and effective use of these chemicals.

Washing raw fruits and vegetables can remove soil and other contaminants. Many food establishments use soaking or submersion as an approved, effective technique for washing produce. This method is often preferred for a variety of reasons, including: (1) the contact time is better controlled; (2) all surfaces come in direct contact with the water; (3) it reduces the amount of waste water; (4) it allows for simultaneous washing and rehydrating; (5) it

helps minimize shrink and extends shelf life; (6) it improves the appearance of the product; and (7) when chemicals are added, it can provide an antimicrobial treatment for the reduction/prevention of cross-contamination.

It is well documented that pathogenic microorganisms may be present on the exterior surfaces of raw fruits and vegetables (FDA, 2009). The "Food Code, Annex 3, Chapter 3, Section 3-302.15, Washing Fruits and Vegetables" states that "more recent studies have demonstrated washing to fall short of their [pathogens] complete removal." There is currently no readily available treatment that can ensure the removal or destruction of all pathogens on RACs with the possible exception of irradiation. Using chemically treated water to wash and/or process fresh produce can impact public health by minimizing the risk of cross-contamination and reducing pathogens if they are present.

In January 2014, the Food Marketing Institute published, in collaboration with the Produce Marketing Association and United Fresh Produce Association, the *Produce Safety Best Practices Guide for Retailers*, which advises retailers to use sanitizers when soaking/submerging fresh produce. The following guidance was provided to retailers regarding crisping fresh produce:

- If a bath is used, follow sanitizer recommendations.
- If using a bath, an appropriate sanitizer should be used in compliance with label directions (FMI, 2014).

Treating produce wash water in the processing sector has been extensively studied. The Food and Drug Administration (FDA)'s *Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables* (FDA, 1998) specifically addresses this issue. In Chapter 2, Section 2.2 states, "antimicrobial chemicals in processing water are useful in reducing microbial build-up in water and may reduce microbial load on the surface of produce. Thus, antimicrobial chemicals may provide some assurance in minimizing the potential for microbial contamination" (FDA, 2009).

The failure to add antimicrobial chemicals in processing water has also been cited as a contributing factor in foodborne outbreaks attributed to fresh produce. For example, the U.S. House of Representatives' Committee on Energy and Commerce published a "Report on the investigation of the outbreak of *Listeria monocytogenes* in cantaloupe at Jensen Farms" (January 10, 2012), which states that FDA officials found several deficiencies in Jensen Farms' practices including NOT using an "antimicrobial solution such as chlorine in the water used to wash the cantaloupes."

The FDA's "Analysis and evaluation of preventive control measures for the control and reduction/elimination of microbial hazards on fresh and fresh-cut produce, a report of the Institute of Food Technologists for the Food and Drug Administration," published September 30, 2001, provided this summary in Chapter 5, Section 1:

> It is well established that pathogenic microorganisms associated with whole or fresh-cut produce can cause disease outbreaks, thereby demonstrating the need for improved mitigation efforts to reduce risks associated with these products. The best method to eliminate pathogens from produce is to prevent contamination in the first place. However, this is not always possible and the need to wash and sanitize many types of produce remains of paramount importance to prevent disease outbreaks. It should be noted that washing and sanitizing are unlikely to totally eliminate all pathogens after the produce is contaminated. Therefore, it is important to use washing and sanitizing protocols that are efficient. (https://www.fda.gov/Food/FoodScienceResearch/ucm091363.htm)

The technology and/or products used to treat the water for washing, crisping, rehydrating, or processing fresh produce by soaking or submersion are not proprietary. Several antimicrobial compounds are readily available to the industry. No one product or supplier is advocated. Food establishments have the opportunity to select a water treatment that is most appropriate to their circumstances. A comprehensive review of these chemicals can be found in the FDA's Preventive Control Measures for Fresh & Fresh-Cut Produce, Chapter V., Methods to Reduce/Eliminate Pathogens from Produce and Fresh-Cut Produce (https://www.fda.gov/Food/FoodScienceResearch/ucm091363.htm).

Practices used by retailers to prepare fresh fruits and vegetables in a RTE form must be adequate to ensure the safety of those products being sold to consumers. The challenges faced by retailers is not confined to the produce department and extend across all departments in which a product is handled prior to sale to consumers.

10.4.2 Deli Department

Deli sales generally account for 5% of total supermarket sales (Progressive Grocer, 2015). Deli meats include a range of meats that are typically precooked or cured such as meat loaves and sausages (salami, pepperoni, roast beef and ham). Such products are typically sliced to order and served cold. Therefore, the contamination of a product at a retail store presents a human health risk and may result in foodborne illness. In recent years in the United States, *Listeria* outbreaks have been linked to foods (e.g., ice cream, bagged salad and soft cheese) other than deli meats (CDC, 2017). A *Listeria monocytogenes* risk assessment suggests that approximately 83% of human listeriosis cases are linked to RTE deli meats that have been contaminated at the retail level (USFDA, FSIS, 2010). The concern associated with the retail contamination of deli meats is supported by research. In one study, 9 out of 30 delis showed low *L. monocytogenes* prevalence for all surfaces sampled (Simmons et al., 2014). The study also showed the persistence of a given *L. monocytogenes* strain within a deli. *L. monocytogenes* on food contact surfaces can result in

the cross-contamination of a product. Of particular concern is the transfer of *L. monocytogenes* from the slicer to deli meat during mechanical slicing (Sheen and Hwang, 2008). It is unlikely that a single measure will control and reduce *L. monocytogenes* in deli environments as the enhanced training of personnel, improved sanitation practices, and stringent temperature control are all important.

10.4.3 Meat and Poultry Department

Regulatory agencies in each country have established acceptable levels of certain microorganisms that can be associated with raw meat and poultry products. In the United States, the Food Safety and Inspection Service (FSIS) has established performance standards for *Salmonella* and *Campylobacter* in raw chicken parts and not-ready-to-eat comminuted chicken and turkey products. In essence, this means that such products sold at retail markets may be contaminated with those pathogens. A recent report by the FDA National Antimicrobial Resistance Monitoring System (NARMS) indicates that the prevalence of *Salmonella* in retail poultry is at its lowest level since 2002. In ground turkey, the prevalence of *Salmonella* has declined from a high of 19% in 2008 to 6% in 2014. In retail chicken over the same time period, the prevalence has dropped from 15% to 9%. (www.fda.gov/AnimalVeterinary/NewsEvents/CVMUpdates/ucm498038.htm). In the United Kingdom, the monitoring of the *Campylobacter* contamination of fresh chickens at retail markets found that 9.3% of chickens tested positive for the highest level of contamination (FSA, 2016). Overall, *Campylobacter* was present on 50% of chicken samples, down from 71% in the previous year. Controlling *Campylobacter* contamination at the farm level is an important step in reducing the sale of contaminated meat at retail markets (Skarp et al., 2016). Although these results are encouraging, consumers at retail markets remain at risk through the handling of products presented for sale even when they are packaged. The outside of the packaging materials may have been cross-contaminated during the packaging process. Consumers must be educated in safe handling practices, even in retail stores (Ventura da Silva, 2009). Large retail chains often have hand sanitizer and wipes available for patrons in meat and poultry departments as an added safety measure.

10.4.4 Seafood Department

Seafood is an important part of a healthy diet for people throughout the world (Iwamoto et al., 2010). Seafood consumption is associated with many potential health benefits, including reducing the risk of heart disease. Seafood includes finfish (e.g., salmon and tuna), mollusks (e.g., mussels, oysters, and clams), and crustaceans (e.g., lobster, shrimp, and crab). Certain types of seafood are inherently more risky than others owing to how they feed. Mollusks feed by filtering volumes of seawater, which can result in the

accumulation and concentration of human pathogens when present. In the Unites States in 2013, the food categories most commonly implicated in outbreaks were fish (50 outbreaks, 24%), mollusks (23, 11%), chicken (21, 10%), and dairy (21, 10%) (CDC, 2013). Similar to other foods, seafood may become contaminated during any step—handling, processing, and transportation. During the mid-1990s in the United States, a seafood hazard analysis critical control point system was implemented (Iwamoto et al., 2010). This regulation does not cover retail establishments. The importance of maintaining appropriate temperature control cannot be overstated for seafood, particularly for a product that is offered as "fresh." Such a product is typically maintained on ice in chill cabinets. Consumers can cross-contaminate products during the handling of groceries when shopping or in the home during storage and preparation.

10.4.5 Summary

Retailers have a daunting task to ensure the safety of foods presented to customers. Each category of foods has unique characteristics that may require specialized handling. Sound sanitation programs, temperature control, and adherence to appropriate regulatory measures are a few factors that can enhance the safety of highly perishable products sold by retailers. Consumers play a key role, too. Consumers should be educated in proper handling methods that will prevent cross-contamination and limit microbial growth. A wealth of information is available on food handling practices in the United States, Canada, and Europe. A paucity of information is available for other regions of the world. In many instances, this relates back to the resources available to each country's regulatory agencies.

10.5 Retail Food Handling in Europe and China

The European Union (EU) food and drink industry makes a significant contribution to the economy of the EU. It generates €1244 billion in turnover based on 2013 data, making it the largest manufacturing sector in the EU. There are 289,000 enterprises throughout all member States of the EU, employing 4.24 million workers (Anonymous, 2015). A large market contribution and a high number of employees impose a challenge to food safety in all food operations sectors. The diversity in business format size as well as employment form impose an additional burden on the EU for addressing food safety issues (Reynolds and Cuthbertson, 2014). The flexibility of employment opportunities permits non-professionals to become involved, particularly through part-time employment positions; this makes the

training and education of workers more challenging and requires greater resources (Giaccone and Nunzio, 2012).

The European Commission (EC) developed integrated approaches to improve food safety from the farm to the table in a white paper document. The document outlines comprehensive actions covering all sectors of the food chain, including retail sales. In 2002, the European Parliament and the Council passed Regulation (EC) No 178/2002, which laid down the general principles and requirements of food law in Europe (known as the General Food Law). It sets out the general principles, requirements, and procedures associated with food and feed safety. The European Food Safety Authority (EFSA) is an independent agency that was legally established by the EU under the General Food Law and is responsible for risk assessment (science) and risk management (policy support). The EFSA provides science-supported opinions associated with a variety of timely issues in the area of food and feed safety. For example, sodium propionate (E 281) was a food additive approved for use in several food categories (bakery and cheese products) up to 3000 mg/kg. Recently, the panel of the EFSA concluded that it would be safe to add up to 5000 mg/kg of sodium propionate into meat preparations, processed meat, and fish, for the purpose of preservation (EFSA ANS Panel, 2016).

Although China is a developing country, it is one of the largest retail markets in the world (USDA, 2014). In contrast to developed countries, such as Canada and the United States, and EU nations, China is in a transitional stage of urbanization. The growing number of urban households and the increasing income of those households are two key factors accounting for the continued retail growth (Fu, 2016). In other words, China is undergoing a transition, and so is retail food safety management. Food safety is a major concern for most Chinese people in the wake of major scandals that have impacted a variety of foods.

The first comprehensive China Food Safety Law was passed by China's National People's Congress (NPC) Standing Committee in 2009. To better address food safety issues, the Chinese government looked to other nations and gradually modified and improved the legislation and implementation of food laws. The latest update was in 2015, and an unofficial translation of the law is available on the website of the Foreign Agricultural Service, United States Department of Agriculture (Fu, 2016; USDA FAS, 2015). Since urbanization in China is ongoing, it represents one of the greatest obstacles to food safety as there are many small local retailers that lack the training and resources to address food safety issues. China's new food safety laws are laudable and expand the requirements and inspections that unfortunately result in increased expenses for all food retailers. One outcome of the new regulations may be the consolidation of food companies from processors to retailers as small operators may lack the resources to accommodate the new regulations. Moreover, the Chinese government is supporting the aggregation of production units at the farm level, enabling the cooperation

between small farmers and larger retailers. For example, participating farmers can receive approved pesticides and instructions on how to use them appropriately. Importantly, farmers involved in such programs can directly sell their products to large retailers to improve profits. The establishment of farmer–retailer relationships can improve food safety, both chemical and microbial, as they improve product traceability. The model has the potential to widely impact food safety throughout China, but will require time to implement and realize the benefit to all consumers, domestic and international, through trade.

10.6 Food Safety Training

Training programs for workers in retail and other sectors of the food industry differ throughout the world. Often such programs are designed to address key issues within a country and specific foods intended for the global market. The United States Food Safety Modernization Act (FSMA) aims to improve food safety across the entire farm-to-table continuum, even the animal feed sector (FDA, 2015). The FSMA does not include regulations for retail food stores, but does address foods intended for sale to consumers in retail outlets. To better implement FSMA, especially under the Preventive Controls for Human Food, training and education is paramount with food safety plans being developed by qualified individuals. Under FSMA, current good manufacturing practices (cGMPs) are updated for human and animal foods. The education and training of workers is central to cGMPs and meeting FSMA requirements. Specifically, "Management is required to ensure that all employees who manufacture, process, pack or hold food are qualified to perform their assigned duties" (FDA, 2016a). In addition, "Such employees must have the necessary combination of education, training, and/or experience necessary to manufacture, process, pack, or hold clean and safe food" (FDA, 2016a).

"Training" and "education" are not the same. Training focuses more on teaching managers/employees a specific task or skill in order to bring it to a desired level of proficiency. On the other hand, "education" concentrates more on passing knowledge to workers that are involved in tasks associated with food production and processing. In other words, training is teaching managers and food handlers *how* to ensure food safety, while education is more likely to explain *why* a specific skill, task, or procedure is necessary for a given safety issue (Faber et al., 2014).

Retail stores must be held responsible for identifying and providing baseline training for new employees in order to keep food products safe before selling them to the consumers. Poor personal hygiene is one of the major factors contributing to foodborne illness at the retail level. Therefore, baseline

training should include but not be restricted to handwashing and other good personal hygiene practices, and proper temperature/time control and food handling practices. There are various approaches to identifying the baseline training that occurs at a specific food operation. Kim et al. (2013) utilized the Delphi survey technique to identify the baseline training for deli operations by running an expert panel. The Delphi method basically contains multiple-rounds of feedback that allow panelists to reevaluate their initial decision. Strengthening the baseline training for a specific food category facilitates new employees in incorporating the learned knowledge into their daily operations.

However, due to the diversity and complexity of food products being sold in retail stores, safety training needs to go beyond the transitional teaching style. It is suggested that food safety issues should be elevated to the level of "organizational culture" (Yiannas, 2008). Organizational culture in food safety refers to the shared beliefs on food safety, the shared attitudes towards food safety, and the willingness to discuss and share opinions on food safety issues of the individuals within the organization. In general, the traditional teaching style focuses more on whether employees understand and master food safety practices, but associating food safety with organizational culture highlights the active participation of employees, which is a form of two-way communication. For instance, it is beneficial to consider connecting with the emotions of deli employees when conducting food safety training, such as by telling an actual story of individuals who have suffered from food-borne illnesses (Kim et al., 2013). This helps employees realize their roles and importance in addressing food safety issues.

One of the key foundations in creating a strong food safety culture is a qualified leader with a strong commitment to food safety. A new term introduced in the FSMA is "qualified individual." It refers to "someone who has successfully completed training in the development and application of risk-based preventive controls or is otherwise qualified through job experience to develop and apply a food safety system" (FDA FSMA, 2016). A similar term is included in the FDA Food Code—"person in charge"—which refers to "a certified food protection manager who has shown proficiency of required information through passing a test that is part of an accredited program or responding correctly to the inspector's questions as they relate to the specific food operation" (FDA, 2016a). The kind of knowledge required for the person in charge as well as their responsibilities in each specific task of food operation, such as describing the relationship between personal hygiene and the prevention of foodborne diseases, describing the symptoms associated with the diseases transmitted through food, properly maintaining the required temperatures and times of storage and food operation, identifying the source of water used, and identifying the critical control points (CCPs), are indicated under Chapter 2 of the FDA Food Code.

Many options exist through which a person can receive the appropriate training required under FSMA and the Food Code. In the United States, the FDA offers various training options and delivery formats. For example,

public–private alliances for providing training include the Produce Safety Alliance, Food Safety Preventive Controls Alliance, and Sprout Safety Alliance, which are operated by academic institutions (i.e., Cornell University and Illinois Institute of Technology's Institute for Food Safety and Health [FDA, 2016b]). The United States was used as an example. Specific regulations and training requirements differ for each country, but all are intended to utilize the most current and relevant science to support approaches that will improve the safety of the food supply.

Food safety consumer education coupled with employee training and education programs are required to mitigate and prevent cases of foodborne illness. Comprehensive regulations and guidelines have been established and must be linked with evaluation programs to determine the current state of compliance and employee handling practices in retail establishments. To document the compliance status for the 1997 FDA Food Code, USFDA regional retail food specialists collected data during a site visit. Nine distinct facility types were evaluated through direct observations and discussion with management and employees. The compliance status was categorized into four groups—in compliance, out of compliance, not applicable (meaning the behavior or practice did not apply to the establishment), or not observed (meaning the behavior or practice was applicable but not observed during the visit). These types of evaluation programs are costly and time-consuming, but serve to verify whether regulations and guidelines are being implemented or considered in efforts to ensure food safety.

The processing and handling of foods at retail markets can have a profound effect on the safety of food presented for sale to consumers. Certain foods represent a greater food safety risk when not handled and stored appropriately. Foods that prior to consumption will not undergo a process that will inactivate foodborne pathogens when present are of greatest concern. Such foods include deli meats and fresh and fresh-cut fruits and vegetables. Regulatory agencies have established regulations and guidelines for retailers to minimize contamination during in-house processing and handling. In conjunction, consumers must also be cognizant of practices that support safe food handling during transport to and storage at home.

10.7 Current Issues in Food Retail Markets

Food labeling is one of the most important ways for the food manufacturer to communicate with the consumers. Food labels contain a lot of information about the product, the manufacturer, and, in some instances, cooking and storage requirements. In the United Sates, it is mandatory for most prepared foods (i.e., canned foods, cereal, snacks, frozen foods, and drink) to be in compliance with the food labeling program. The language of the principal display

panels and health claims are strictly regulated under 21 CFR 101. However, for raw produce (vegetables and fruits) and raw fish, nutritional labeling is voluntary. The USFDA assesses the level of food retailers in implementing the voluntary nutrition labeling of raw fruits, vegetables, and fish based on the percentage of those commodities displayed and labeled. To determine the level of implementation by food retailers, the USFDA evaluates a representative sample of 2000 stores, considering the store type and size.

A new area in the retail food market is internet sales. According to a report from the Nielsen Company (2015), one-quarter of global respondents have purchased grocery products on the Internet and had the products delivered directly to their home. Among these respondents, more than half (55%) plan on continuing to purchase grocery items online. Given the increasing interest in online sales by consumers, the translation of the current regulations for internet retailers is needed to ensure proper safe food handling.

Today, an increasing percentage of retailers require their suppliers and distributors to provide certification demonstrating the effective implementation of food safety control systems, including Safe Quality Food (SQF) and ISO 22000/PAS220. The SQF Program is recognized by the Global Food Safety Initiative (GFSI). It considers both food safety and food quality issues. Retailers can enhance the impact of SQF certification through feedback they receive concerning the quality and safety of products sold. ISO 22000 is a family of globally accepted food safety management standards to help organizations in the food chain identify and control food safety hazards.

Organic foods continue to increase as a segment of the retail market. Organic commodities are now available in nearly 20,000 natural food stores and nearly three-quarters of conventional grocery stores (USDA, 2016). In the United States and other countries, violations of organic regulations are subject to enforcement actions, including financial penalties or the suspension/revocation of organic certification. Recalls of organic foods suspected of bacterial contamination by *Salmonella* and *Listeria* increased more than twofold during the first half of 2015, as compared with the previous years (Strom, 2015). The restrictive usage of chemical antimicrobial agents on organic products can create challenges in controlling contamination and the cross-contamination of products in the farm-to-table continuum. Retailers must be cognizant of regulations associated with organic commodities and employ appropriate strategies to mitigate or prevent contamination in retail operations.

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11

Sustainability of Food Security in the New Era

Taylan Kiymaz and Hami Alpas

CONTENTS

11.1 Introduction

The latest estimates of the Food and Agriculture Organization (FAO) indicate that about 800 million people in the world—just over one in nine—were undernourished between 2014 and 2016. From 1990 to 1992, the number of undernourished people declined by 216 million globally, a reduction of 21.4%, compared to a 1.9 billion increase in the total population over the same period. Changes in large and populated countries, namely China and India, play a significant role in the overall hunger reduction trends in developing regions. Rapid progress was achieved during the 1990s, when developing regions experienced a steady decline in the number of undernourished people.

The decline in hunger can be considered a great success under volatile commodity prices, higher food and energy prices, rising unemployment, and the global economic recessions that occurred in the late 1990s and after 2008. Increasingly extreme weather events and natural disasters have caused significant social and economic damage and increased the risk of food insecurity. Moreover, political instability and civil disturbances have increased the

number of displaced people. These developments have increased food insecurity in some of the most vulnerable countries, particularly in sub-Saharan Africa, while regions like Eastern and South-Eastern Asia have minimized the adverse effects.

Moreover, for alleviating poverty and reducing malnutrition, economic growth is necessary for all countries. It is critical for sustainably increasing employment and incomes, especially in low-income countries. Across the developing world since 1990–1992, economic growth has brought a strong and persistent reduction in hunger. Increases in the incomes of the poor are associated with a higher intake of dietary energy and other nutrients (FAO et al. 2015, p. 17).

On the other side, the term nutrition and its security emerged at the beginning of 1990s to emphasize both macro- and micronutrient requirements in the diet. Nutrition policy formation started to become a priority area in most of countries.

World food security is under threat because of the rapid growth of food demand and the deterioration of natural resources, which is increasingly unpredictable due to climate change. An integrated approach to food security and the environment should take into consideration the food, water, energy, environment, and climate nexus, while reorienting food production, distribution, and consumption without harming food safety in the food chain, which is very complex in nature and vulnerable. Food security will require not only increasing agricultural productivity but also minimizing environmental impacts and increasing natural resource efficiency. Furthermore, the dilemma of balancing food safety and sustainability exists and should be taken into consideration by policy makers.

Inadequate food security and nutrition are likely to have negative consequences for the livelihoods and economic capabilities of vulnerable populations. Sustainability can be considered a precondition for long-term food and nutrition security. The environment, and especially climate and the obtainability of natural resources, are a precondition for the availability of food as well as the preservation of biodiversity. Moreover, the sustainability of food and nutrition security can be evaluated under the framework of sustainable development. Sustainable development involves meeting the needs of present generations without jeopardizing the ability of future generations to meet their own needs. Food security and nutrition bring an essential dimension that is intertwined with sustainable development in that sense.

Additionally, interdependencies exist between food security and nutrition and many other parts of a broad sustainable development agenda, including economic growth, population dynamics, decent employment, social protection, energy, water, health, sanitation and food safety, natural resource management, and the protection of ecosystems (United Nations 2013a, p. 1).

The global human population is estimated to grow from 7.3 billion in 2015 to 9.3 billion by 2050. Forty-seven percent of the population growth will be

in sub-Saharan Africa, where agricultural productivity and soil quality is exceptionally low and where reliance on imports of basic staples is already high. Moreover, at least 3 billion people will enter the global middle class by 2030, and they will demand more resource-intensive foods such as meats and vegetable oils (Hanson 2013, p. 4). To sufficiently feed all people by 2050, worldwide food security has to be guaranteed in a way that is driven by the factors of sustainable development. In that respect, the 2030 Agenda for Sustainable Development and the Sustainable Development Goals (SDGs), adopted at the United Nations (UN) Sustainable Development Summit on 25 September 2015, provide a guide through which to foresee the future food security situation.

In this study, the development of the definitions of food security and sustainability are first given along with the interdependencies between these two concepts in the literature. Then, the coherence of food and nutrition security with the sustainability approach is addressed. Among other factors, climate change is considered to be a primary threat to sustainable food security. Lastly, the future sustainability of food and nutrition security is considered in the framework that is put forward through the UN'S SDGs.

11.2 Methodology

In this study, a literature review is conducted to identify the different dimensions of the interaction between sustainability, food security/insecurity, and agricultural and food production. The concepts of sustainability and food security are discussed first. Then, the relationship between these concepts is addressed considering agricultural production and the impacts of climate change. Following this, the SDGs are assessed in the scope of sustainable food security.

11.3 Food Security and Sustainability Concepts

The evolution of two concepts, namely food security and sustainability, is believed to help understand the linkage between the two. Sustainable development is another complementary concept that captures the dynamic structure and development needs of food security through time.

Food security has evolved since the 1970s and the concept has been gradually broadened. Initially, it focused mainly on the availability of food and on ensuring the food supply with regard to availability and the global and

local price stability of basic foods. This was the outcome of the extreme instability of agricultural commodity prices in the early 1970s. The occurrence of hunger and food crises required a new definition of food security to recognize the needs of vulnerable people. After the World Food Conference of 1974, food security was defined as "the availability, at all times, of adequate world food supplies of basic foodstuffs to sustain a steady expansion of food consumption and to offset fluctuations in production and prices." In 1986, when the World Bank published its seminal report "Poverty and Hunger," a timescale for food security was introduced by distinguishing between chronic food insecurity and acute, transient food insecurity. These concerns were reflected in an extension of food security to include the "access of all people at all times to enough food for an active, healthy life."

A redefinition of food security was put forward before the World Food Summit held in 1996. Food security, at the individual, household, national, regional, and global level, is achieved "when all people, at all times, have physical and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life." In the mid-1990s, as the term "food security" evolved, the terms "nutrition security" and "food and nutrition security" also emerged in order to combine all of the necessary elements.

By 2009, the term food security and nutrition had become one of the strategic objectives of the FAO. The last revision of the definition of food security came during the 2009 World Summit on Food Security. Stability, a fourth dimension, was added as the short-term time indicator of the ability of food systems to withstand shocks, whether natural or manmade. This new fourth dimension introduced the concept of constancy in food security. Therefore, the food security concept was expanded to include not only the availability of food but also explicitly the accessibility of food, its utilization, and lastly the stability of all these dimensions (Berry et al. 2015, p. 1).

Furthermore, the history of the emergence of sustainability and sustainable development provides a better understanding of the relationship between sustainability and food security. In 1972, the Conference on the Human Environment in Stockholm was devoted to environmental issues. During that conference, the relationship between the environment and development was addressed, as "although in individual instances there were conflicts between environmental and economic priorities, they were intrinsically two sides of the same coin." Another result of the Stockholm Conference was the creation of the UN Environmental Program (UNEP), which has the mission "to provide leadership and encourage partnership in caring for the environment by inspiring, informing, and enabling nations and peoples to improve their quality of life without compromising that of future generations." It also adopted the "Stockholm Declaration on the Human Environment," which included the integration and coordination of

development planning to allow for environmental protection. However, a more integrated perspective that incorporated both economic development and environmental sensitivities was needed as the results of the Stockholm Conference were not satisfactory.

In 1983, the UN General Assembly created the World Commission on Environment and Development, which was later known as the Brundtland Commission, named after its Chair, Gro Harlem Brundtland. In 1987, the Commission published the Brundtland Report, entitled "Our Common Future." It built on what had been achieved at Stockholm and provided the most politically significant of all definitions of sustainable development: "sustainable development is development that meets the needs of the present without compromising the ability of future generations to meet their own needs." The next event was the UN Conference on the Environment and Development (UNCED) that was held in Rio de Janeiro, Brazil, in 1992. The key outputs of the Conference were the Rio Declaration, Agenda 21, and the Commission on Sustainable Development, all of which are concerned with sustainable development. The commitment of leaders from around the world to sustainable development was clearly articulated in Agenda 21, the key document of the summit (Bac 2008; UN 2015). The three Rio Conventions—on biodiversity, climate change, and desertification—derive directly from the 1992 Earth Summit. Each instrument represents a way of contributing to the SDGs of Agenda 21. The three conventions are intrinsically linked, operating in the same ecosystems and addressing interdependent issues.

In Agenda 21, the environmental and development themes are quality of life, the efficient use of natural resources, the protection of the global commons, the management of human settlements, and sustainable economic growth. Agenda 21 recognizes that the persistence of severe poverty in several parts of the world alongside a standard of living based on the wasteful consumption of resources in other parts is not a sustainable model, and that environmental management must be practiced in developing and industrial countries alike. During the 1992 conference, it was agreed that to implement Agenda 21, countries should prepare a national sustainable development strategy. The UNCED process attempted to provide guidance in implementing sustainable development by laying out a set of principles and a plan of action based on the concept (Prizzia 2007; UN 2015).

Later, in the 1997 Kyoto Conference on Climate Change, developed countries agreed on specific targets for cutting their emissions of greenhouse gases under the Kyoto Protocol. The United States proposed to only stabilize emissions, as opposed to cut them, while the European Union called for a 15% cut. At the end of negotiations, industrialized countries were committed to an overall reduction in the emission of greenhouse gases of 5.2%, or below 1990 levels for the period 2008–2012. Although 84 countries signed the Protocol, indicating their intent to ratify it, many others were reluctant to take that step.

In September 2000 at the Millennium Summit held in New York, world leaders agreed on the Millennium Development Goals (MDGs), most of which have the year 2015 as a timeframe and use 1990 as a benchmark. They represent a more practical expression of the principle of equilibrium between the economic, social, and environmental pillars of sustainable development. Some of the targets include: halving the proportion of people living on less than a dollar a day and those suffering from hunger; integrating the principles of sustainable development into country policies; and reducing by half the proportion of people without access to safe drinking water (Bac 2008, p. 579).

The World Summit on Sustainable Development (WSSD) negotiations in Johannesburg in 2002 demonstrated a major shift in the perception of sustainable development away from environmental issues toward social and economic development. This shift was driven by the needs of developing countries and was strongly influenced by the MDGs. The focus of the Johannesburg Summit was on the indivisibility of human dignity and through decisions on targets, timetables, and partnerships, a commitment was made to speedily increase access to basic requirements such as clean water, sanitation, adequate shelter, energy, health care, food security, and the protection of biodiversity. This summit complemented the MDGs by setting a number of additional goals, such as halving the proportion of people lacking access to basic sanitation; minimizing the harmful effects of chemicals; and halting the loss of biodiversity. Some authors consider the summit to be "progress in moving the concept [of sustainable development] toward a more productive exploration of the relationship between economic development and environmental quality" (Bac 2008; UN 2002).

At and since the Rio Summit, sustainable development has found its most prominent anchor around the issue of climate change. Measures to address climate change, both mitigation and adaptation, are linked to sustainable development. The Fourth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC), in 2007, pointed out the iterative relationship between climate change and sustainable development, and demonstrated that the two can be mutually reinforcing. In many respects, the UN Framework Convention on Climate Change (UNFCCC) has become an international proxy for discussions around sustainable development, and a potential means to channel the required funding and technology from developed to developing countries.

Sustainable development has also been integrated into the operations and governing mandate of many prominent international organizations. These include the World Bank, which has affirmed a commitment to "sustainable globalization" that "enhances growth with care for the environment"; the International Monetary Fund (IMF), with a commitment to "sustainable economic growth"; as well as the World Trade Organization (WTO), which endeavors to contribute to sustainable development through the pursuit of open borders and the removals of barriers to trade. Sustainable

development is also a prominent component of the MDGs, which have been widely endorsed by national governments and the world's foremost development organizations since they were adopted at the Millennium Summit in 2000. Sustainable development has also gained currency in the private sector—often in the form of the corporate social responsibility (CSR) agenda. Several voluntary initiatives have been formed including the World Business Council on Sustainable Development (WBCSD), Global Compact, Equator Principles, Global Reporting Initiative, and Extractive Industries Transparency Initiative. In addition, various major international non-government organizations (NGOs), such as World Wide Fund for Nature (WWF), Oxfam International, and Friends of the Earth, have increased the scale and sophistication of their involvement in sustainability principles (Drexhage and Murphy 2010, p. 10).

Rio+20 in 2012 resulted in an outcome document, "The Future We Want", in which states reaffirmed their commitment to all previous agreements, plans, and targets on sustainable development. They also committed to developing a suite of SDGs, building on the priorities identified in Agenda 21 and in the Johannesburg Plan of Implementation, and decided to replace the Commission for Sustainable Development with a "high-level political forum" to progress the implementation of all plans and targets.

Establishing post-2015 goals was an outcome of the Rio+20 summit in 2012, which mandated the creation of an open working group to come up with a draft agenda. Unlike the MDGs, the UN has conducted the largest consultation program in its history to find out what the SDGs should include. The final wording of the goals and targets was agreed in August 2015. The SDGs emerged as a new, universal set of goals, targets, and indicators that UN member states will be expected to use until the end of 2030. The SDGs follow and expand on the MDGs. There are 17 goals and within these goals are 169 targets that are thought to be prioritized on a national basis according to the needs of different nations.

The importance of the coherence between these two concepts is addressed in the following section.

11.4 Coherence of Sustainability and Food Security

There has been an increasing consensus that sustainability is relevant to food security. However, its position in the food security framework has to be defined more clearly. It could be incorporated into the dimensions of availability, for the long-term sustainability of food production, and access, for the long-term sustainability of consumption. It can also be held that sustainability represents the extension of the timeframe of stability, or a relevant substitute for stability.

Sustainability can be considered a precondition for long-term food security. The environment, and especially climate and the obtainability of natural resources, are a precondition for the availability of food as well as the preservation of biodiversity. Economic and social sustainability are necessary for the accessibility of food to people. Social sustainability is also a determinant of the utilization of food. Together, these three dimensions of sustainability—social, economic, and environmental—also ensure the stability of the systems on which food security depend.

During the preparations for Rio+20, the international community identified two main objectives: (1) to integrate better the dimensions of sustainable development; and (2) to make them more concrete and operational. The FAO, Rome-based agencies, as well as numerous governments and stakeholders, emphasized the importance of food security and nutrition as an integral part of sustainable development.

According to the High Level Task Force on Global Food Security (United Nations 2012), achieving food and nutrition security involves: (1) ensuring the consistent availability and accessibility of sustainably produced, nutritious, and safe food; and (2) reducing and/or eliminating losses and waste in food production, processing, and consumption. Food production and availability should be increased in ways that are environmentally, socially, and economically sustainable.

According to Berry et al. (2013, p. 5), food security and nutrition, in its four dimensions, interacts with sustainability in its three dimensions. In other words, sustainability in the use and management of natural resources is a condition for food security, now and in the future. Economic development and social development are crucial to the eradication of poverty, malnutrition, and hunger. On the reverse side, hunger and malnutrition are long-term burdens on societies, hindering economic and social development as well as the sustainable management of resources. This approach is emphasized in the outcome document of the Rio+20 Conference, which reaffirmed the "commitment to enhancing food security and access to adequate, safe and nutritious food for present and future generations," and recognized "the need to maintain natural ecological processes that support food production systems" (FAO 2012). Food security and nutrition for present and future generations is thus both an integral part and goal of sustainable development.

A comprehensive approach to food and nutrition security requires: (1) taking into account the interconnectedness and interactions between food and nutrition security dimensions—availability, access, utilization, and stability; (2) addressing the full spectrum of food and nutrition security, including food production, sourcing, and distribution; (3) integrating cross-cutting issues—such as gender equity, ecosystems and natural resources management, and climate change mitigation and adaptation—into the food security policy cycle; and (4) ensuring multi-sectoral engagement and the coordination of sectoral policies (e.g., agriculture, trade, health, education, and nutrition) (Capone et al. 2014, p. 15).

Furthermore, the inclusion of sustainability recognizes that the health and well-being of current and future generations is the goal of sustainable food security. The four dimensions of food security are interrelated and interdependent, such that food insecurity may occur when there is a disruption at any level. There is potential tension between environmental sustainability and the other dimensions of food security. For instance, when higher agricultural chemical inputs are used to keep production constant, stability over time is not necessarily compatible with sustainability. If food production is not sustainable from an environmental perspective, then it will not be stable over time (Berry et al. 2015, p. 6).

On the consumption side, as another important factor, food waste and losses are two of the most severe social, economic, and ecological pathologies facing the world. The connection between food security and food system sustainability—including the reduction of food losses and waste—has been emphasized recently at the global level in many strategic documents. Food loss and waste varies depending on the type of food, country, and season. Food losses and wastage occur in harvesting, transport, storage, packaging, processing, wholesale, and retail trade, and where the food is consumed. Losses in the first part of the food chain that are due to poor harvesting, transport, or storage are more important in developing countries. In industrialized countries, most losses occur at the retail and consumer level as food is wasted. In food production, estimates of the proportion of cereals wasted at farm level and post-harvest due to poor storage range from 8% to 22%, and reach nearly 100% in some situations for the post-harvest loss of horticultural produce. In marine fisheries, over 40% of production is wasted on account of bycatch. Fruits and vegetables, plus roots and tubers, have the highest wastage rates of any food. Global quantitative food losses and waste per year are roughly 30% for cereals, 40%–50% for root crops, fruits and vegetables, 20% for oil seeds, meat and dairy, and 30% for fish.

Every year, consumers in rich countries waste almost as much food (222 million tons) as the entire net food production of sub-Saharan Africa (230 million tons). In developing countries, 40% of losses occur at post-harvest and processing levels, while in industrialized countries, more than 40% of losses happen at retail and consumer levels. At the retail level, large quantities of food are wasted due to quality standards that over-emphasize appearance. Moreover, the trend toward a higher proportion of animal food items, fruit, and vegetables in the diet tends to shorten the durability of food and could increase the risk of losses and wastage. Distance to market, a more complex food chain, and changes in the composition and variety of food supplies provide opportunities for more food wastage (Capone et al. 2014, p. 18).

What about the climate change issue within the scope of sustainability and food security? Climate change has not yet been touched on in this study, but it seems to be a main driver of food security and sustainability.

11.5 Climate Change as a Primary Threat

After the historic UN summit in September 2015, in which the 17 SDG targets were accepted by world leaders, the Paris UN Climate Conference 2015 represented an historic opportunity to put the world on course to meet the climate change challenge. It was concluded that the world needs a new sustainable model of growth that is beneficial to all.

Sustainable development and climate change share strong complementary tendencies: they are multi-sectoral, they both require international cooperation to solve the problem, and the problem is interconnected through economic and technological development in increasingly complex networks.

The climate change negotiations could benefit from a broadened discussion, by taking sustainable development into consideration. For example, mitigation could be addressed within a broader discussion of energy and economic growth. Adaptation could benefit from a broader understanding of resilient development and measurement, reporting, and verification (Drexhage and Murphy 2010).

Although there are many challenges to overcoming threats to food security and sustainability in the world, climate change can be counted as the most uncontrollable and threatening one in the long term.

Currently, the production of crops and animal products releases roughly 13% of global greenhouse gas emissions, or about 6.5 gigatons (Gt) of carbon dioxide equivalent (CO2e) per year, without counting land use change (another 11%). Even assuming that there will be some increases in the carbon efficiency of agriculture, emissions could grow to 9.5 Gt of CO2e by 2050. When combined with continuing emissions from land use change, global agriculture-related emissions could reach 15 Gt by 2050. By comparison, to maintain global warming below 2°C, world annual emissions from all sources would need to fall to roughly 21–22 Gt by 2050 according to typical estimates, meaning that agriculture could consume roughly 70% of the allowable budget for all greenhouse gas emissions by mid-century. To contribute its full fair share to meet the 2°C target by 2050, agriculture would need to cut its current emissions, even while boosting food production (WRI 2013, p. 84). Agriculture accounted for approximately 24% of global greenhouse gas emissions in 2010.

Projections for the end of the twenty-first century show that global warming will accelerate with predictions of the average increase in global temperature ranging from 1.1°C to 6.4°C. Climate change does not only imply increased average global temperature. Other effects of climate change include trends toward stronger storm systems, increased frequency of heavy precipitation events, and extended dry periods (FAO 2008a; WRI 2013).

The latest estimates confirm that trends in emissions are likely to lead to increases in world temperature, which could have catastrophic consequences. There is much need for mitigation policies, like the expanded use of

renewable energy sources and improvements in energy efficiency in many parts of daily life (UN 2013, p. 121). The ratification of the Paris Agreement by U.S. and Chinese leaders in September 2016 at the G20 summit may be considered as a first step in this aim as both countries represent 18% and 20% of global carbon dioxide emissions (the driving force behind global warming), respectively (Wikipedia 2016).

11.5.1 Climate Change and Food Security

In the long list of potential damages from climate change, and specifically global warming, the risk to world agriculture stands out as among the most important. In the development of international policy on reducing the impacts of climate change, it is important for policymakers to consider its likely global effects and impacts across countries. Two different results were generally reached in most studies. First, by late in this century, global warming could have at least a modest negative impact on global agriculture in the aggregate, and the impact could be severe if carbon fertilization benefits (enhancement of yields in a carbon-rich environment) do not materialize, especially if water scarcity limits irrigation. On the contrary, there were also optimistic estimates which find that baseline warming by late in this century could have a positive effect on global agriculture in the aggregate (IPCC 2007) given current agricultural practices and crop varieties. In Figure 11.1, the disproportionate effects of climate change are examined in developing

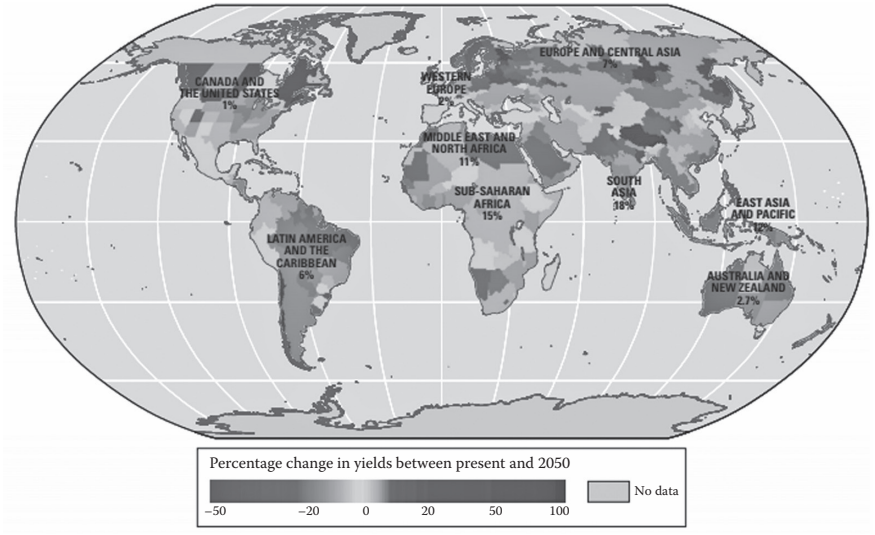

FIGURE 11.1
Crop yield changes due to climate change between present and 2050. (From World Bank, *World Development Report 2010: Development and Climate Change*, Washington, DC, World Bank Group, 2010.)

countries. A warming of 2°C could result in a permanent reduction in annual income per capita in Africa and South Asia, as opposed to minimal losses in high-income countries and a global GDP loss. Developing countries are expected to bear most of the costs of the damages (World Bank 2010).

Unfortunately, science since the 2007 IPCC report has been almost entirely pessimistic. Statistical studies have shown that just a few days of exceptionally high temperatures at the wrong time adversely affect yields of several major crops. Studies have also found that climate change is already adversely affecting overall yields in the northern hemisphere. Droughts and record high temperatures in Russia and the United States during 2011 and 2012 have begun to reveal the consequences of more frequent, highly adverse weather events.

Today, food security and safety, agricultural insurance, less fuel consumption in food logistics, and local food procurement are the more oft-cited old or novel concepts concerning the unknown impacts of the relationship between agricultural production and climate change.

Climate change has implications for food security and food safety. It is widely understood that the risks of global climate change occurring as a consequence of human behavior are inequitably distributed, since most of the actions causing climate change originate from the developed world, but the less developed world is likely to bear the public health burden.

Agriculture, forestry, and fisheries are all sensitive to climate. Their production processes are therefore likely to be affected by climate change. In general, impacts are expected to be positive in temperate regions and negative in tropical ones, but there is still uncertainty about how projected changes will occur at the local level and potential impacts may be altered by the adoption of risk management measures and adaptation strategies that strengthen preparedness and resilience (FAO 2008b).

The possible positive and negative effects of global climate change on agriculture include the following:

- Livestock and crop diseases lowering agricultural output (safety concerns)
- Water logging and open drains affecting farming and livestock herding areas
- Drowning of livestock
- Favored dry lands leading to improved herding zones for livestock
- Increased soil moisture content
- Improved farming practices and water conservation including tree planting

Climate change affects agriculture and food production in complex ways. It affects food production directly through changes in agroecological

conditions and indirectly by affecting the growth and distribution of incomes, and therefore the demand for agricultural produce.

Global and regional weather conditions are expected to become more variable than at present, with increases in the frequency and severity of extreme events such as cyclones, floods, hailstorms, and droughts. By bringing greater fluctuations in crop yields and local food supplies and higher risks of landslides and erosion damage, these events may adversely affect the stability of food supplies and thus food security. Additionally, agriculture accounts for 70% of all freshwater withdrawn from rivers, lakes, and aquifers, and 80%–90% of such water that is actually consumed and not returned. This would worsen the effects of drought when it is realized.

The FAO projects that the impact of climate change on global crop production will be slight until 2030. After that year, however, widespread declines in the extent and potential productivity of cropland could occur, with some of the severest impacts likely to be felt in currently food-insecure areas, which have the least ability to adapt to climate change or finance food imports.

At the global level, the impacts of climate change are likely to be very small; under associated climate-change scenarios (with carbon fertilization), the estimates range from a decline of –1.5% to an increase of +2.6% by 2080. At the regional level, the importance of agriculture as a source of income can be much more important. In these regions, the economic output from agriculture itself (over and above subsistence food production) will be an important contributor to food security (Schmidhuber et al. 2007).

In some of the long-term projections, there are two central results: first, at least modest global agricultural damage can be expected from business-as-usual climate change by late in this century, with output losses about 15% greater if the carbon fertilization effect fails to materialize; and second, the damages will be disproportionately concentrated in developing countries (Cline 2007, p. 25).

Failure to address the environmental impacts would in turn hamper food production in the coming decades in a variety of ways. Land degradation affects approximately 20% of the world's cultivated areas according to various estimates using limited data and different methods. Forest loss is likely to lead to regional drying and warming, causing additional stress on agriculture. Rising sea levels from climate change will also reduce cropland productivity and viable cropland area in some coastal regions. Water stress on cropping is likely to increase due to both the growing water demand and climate change. The recent droughts in different regions of the world are serious cases in that sense.

A World Bank study (2012) estimates that by mid-century, global yields of wheat, maize, and soybeans could decline by 14%–25%, 19%–34%, and 15%–30%, respectively, with a warming of 2.2°C–3.2°C compared with preindustrial temperatures. With a 1m rise in sea levels, almost 11% of South Asia's agricultural land is projected to be vulnerable to flooding. By the end of the century, areas affected by drought disasters are projected to grow

from 15% to approximately 44% of the planet. Regions facing the greatest increases include southern Africa, the United States, southern Europe, Brazil, and Southeast Asia. The evidence remains strong that climate change will have substantial adverse consequences, particularly on sub-Saharan Africa (WRI 2013).

It is hypothesized that ethanol produced from biomass can help mitigate climate change and reduce greenhouse gas emissions by substituting fossil fuel. It is estimated that by 2030, liquid biofuels could supply 3% of the transport sector's energy needs, rising to 5–10% if second-generation biofuels take off. Biofuels can be considered to contribute to climate change mitigation only if their use has produced fewer net emissions of greenhouse gases at the end of the production process than the average emissions from fossil fuel use. Even if there is a net contribution, producing biofuel from purpose-grown crops is not necessarily the most efficient use of available land (FAO 2008b), hence it may create a threat for food security in some regions.

Climate change also has serious effects on food manufacturing and indirectly on trade. Emerging hazards in primary production have a reshaping impact on safety management systems to ensure the safety of the final products. High temperatures can have a direct and detrimental effect on health, growth, and reproduction. These effects are expected to be most dramatic in temperate regions. Climate change may affect zoonoses (diseases and infections that are naturally transmitted between vertebrate animals and man) in a number of ways.

The 2007 IPCC report also emphasizes that increases in daily temperatures will increase the frequency of food poisoning, particularly in temperate regions. Several studies have confirmed and quantified the effects of temperature on common forms of food poisoning, such as salmonellosis. Moreover, there is evidence that temperature variability affects the incidence of diarrheal disease.

Consequently, it is understood that adverse weather effects caused by climate change would bring further environmental problems and deterioration, along with food security concerns. To produce more food, humans would deteriorate the environment and with the more adverse effects of climate change, the sustainability of natural resources would be diminished and growth in agricultural production would be hindered. Food safety concerns will rise in addition to these impacts.

11.5.2 Sustainable Food Procurement

As the frequency and intensity of severe weather increase, there is a growing risk of storm damage to transportation and distribution infrastructure, with the consequent disruption of food supply chains. The risk of rising costs of energy and the need to reduce fossil fuel usage along the food chain have led to a new concept—"food miles"—which should be kept as low as possible to reduce emissions. Food miles refer to the distance the food travels from

where it is grown to where it is ultimately purchased or consumed by the end user. The more food miles that attach to a given food, the less sustainable and less environmentally desirable that food is.

Locally grown and prepared food can cut down on fuel use in "food miles" and make it easier to identify and support environmentally benign food procurement methods. Buying local produce also means that the food is less likely to be associated with the greenhouse gas emissions caused by recent land conversions. Seasonal food need not be imported, does not require energy-intensive conditions such as heated greenhouses, can be produced organically, and reduces the likelihood of energy-intensive methods of storage and transport such as refrigeration and air-freighting.

Local food systems support local economies; for example, farmers' markets positively affect the businesses surrounding them, while also providing a significant source of income for local farmers, helping to maintain the livelihood of local farms. Unlike large industrial farms, small family farms are more likely to spend their dollars in the community on farm-related inputs (e.g., machinery, seeds, farm supplies, etc.); in addition, food grown locally, processed locally, and distributed locally (via the local network) generates jobs and subsequently helps stimulate local economies. This local economic cycle serves the overall food security considering its components.

Researchers have identified the factors driving the rise in food miles as increased global trade, the concentration of power in the hands of supermarkets with centralized systems of distribution, greater car use for shopping (particularly in urban areas), and a rise in packaging and processing (Lawrence 2005).

Large buyers can have great economic benefits to local and regional communities through the market that the buyers provide. Local public procurement markets can contribute to enlarging the space for a more local economy that supports both innovative food production and sustainable consumption practices.

The sustainability challenges faced by contemporary societies include the environmental challenges of water shortages, greenhouse gas emissions, pollution of soil and water, decreasing biodiversity, and waste throughout the supply chain including at the points of consumption. These existing problems are exacerbated by the rising social and economic trajectories of population growth and the increasing concentration of populations in urban areas, notably in developing countries, where consumption trends move toward more protein-intensive (notably meat and dairy) diets.

Even in traditionally developed countries, new social divides are affecting equal access to healthy and quality food for less empowered people. The increasing scarcity and rising costs of carbon-based fuel, and of industrial and phosphorous-based fertilizers, are more immediate as well as longer term challenges. Future scenarios for feeding the world are extremely challenging, with provision at more local levels related to these global trends.

New production and consumption approaches reduce the costs of environmentally sound practices and improve the quality of the natural resource base, including the air, water, and soil—which provide the ecosystem with components on which the ability to continue growing food and maintaining sustainable local communities depend.

As another alternative, organic production methods are usually less energy-intensive than industrial agriculture. They do not use artificial fertilizers, which take an enormous amount of energy and water to produce and result in the emission of the powerful greenhouse gas, nitrous oxide.

Finally, trade policies can have adverse consequences on the environment. Agricultural support programs have led to the use of production methods that are excessively pollutant, as have fossil fuel subsidies. Subsidies have also encouraged the overexploitation of natural resources. Trade and agricultural support reforms can help to limit negative environmental externalities. Trade policy has a role to play in mitigating and adapting to global climate change by increasing incentives to use the most energy-efficient environmental goods and services. In the long run, a fairer international trade system, taking into account the food security and rural development needs of developing countries, will be crucial. For instance, in higher-income countries, agricultural trade distortions need to be eliminated, in particular subsidies and market restrictions, which have devastating consequences for farmers in lower-income countries (UN 2013, p. 108).

11.6 SDGs and Sustainability of Food Security

Among 17 SDGs, some are directly related to agriculture and food issues. The first SDG is to end poverty. According to FAO (2015a), 80% of the world's extreme poor population lives in rural areas where most are dependent on agriculture. Agriculture is the single largest employer in the world and agricultural growth in low-income and agrarian economies is at least twice as effective as growth in other sectors in reducing hunger and poverty. The support and long-term growth strategies for agriculture have to be on the agenda in all countries for a food-secure population. Accordingly, to reach the SDG of zero hunger, tackling food insecurity and malnutrition while promoting sustainable agriculture seems to be an important step in a new era of sustainable development. In that respect, transforming food systems and agriculture, promoting sustainable living and working practices, improving governance, and securing the political will can be accepted as relevant policies.

As another SDG, good health and well-being, food safety, and nutrition have priority. Without safe and nutritious daily food, humans cannot lead healthy and productive lives. Ensuring food quality and safety throughout

the food chain, from production and processing to retail and consumption, would help achieve the primary goals. Additionally, plant and animal health policies provide complementary measures for achieving a healthy society.

SDG number 6 is about access to clean water. Water scarcity, poor water quality, and inadequate sanitation negatively impact food security. Drought also worsens levels of hunger and malnutrition in very poor regions. Crops and livestock already account for 70% of all water withdrawals, and up to 95% in some developing countries. The reduction of freshwater withdrawal for irrigation and livestock through water-saving technologies and education are factors that would help a better food security situation considering the increase in the global population and economic development.

Energy also has a key role in achieving food and nutrition security. Modern food systems are dependent on fossil fuels. These systems consume about 30% of the world's available energy and produce more than 20% of the world's greenhouse gas emissions. Energy prices affect the cost of agricultural inputs and production, and thus influence food prices. To deliver more food using less and cleaner energy, a transformation to renewable energy sources is needed. This is believed to require a systemic approach and global partnership for action. Using a water-energy-food nexus approach, agriculture and food systems would improve energy efficiency, increase the use of renewable energy, and improve access to energy-saving services in food systems.

In relation to another SDG, industry, innovation, and infrastructure, rural development policies focus mainly on tackling food security concerns. The majority of people in developing nations still live in rural areas. Transportation, irrigation, storage facilities, energy, electrification, and communication technology are crucial investments for sustainable agricultural development. Interventions to diversify employment into non-agricultural activities, and provide smallholders with affordable access to technologies and infrastructure to sustainably transform food systems, are the key to poverty reduction. Rural development supports are well-suited to promoting rural jobs and agricultural and food sector investments in order to increase the value added.

As mentioned in previous sections, responsible production and consumption is a major goal for sustainability. A growing global population with deteriorating natural resources and out-migration from agriculture means feeding more people and animals with less water, farmland, and rural labor. Satisfying expected increases in water, energy, and food needs would bring a shift toward more sustainable consumption and production approaches, with more efficient and sustainable agriculture and food systems. Reducing food losses or waste by one-third or more would solve the security problem to a great extent. Consumers in the developed and developing world must shift their diets by making more nutritious and safe choices with lower environmental footprints. Investments targeting rural development and resource-saving technology use as well as agricultural innovation would create expected favorable outcomes in the future.

SDG 13 aims to take urgent action to combat climate change and its impacts globally. Climate change has been found to affect every country and disrupt national economies. Significant impacts of climate change are experienced and are foreseen to be experienced in the future, such as changing weather patterns, rising sea levels, and more extreme weather events. Greenhouse gas emissions from human activities are driving climate change and continue to rise. Increasing temperatures, changing rainfall patterns, and extreme weather events pose a real threat to global food production, but agriculture has a major role to play in responding to climate change. Investments in agriculture and food sectors can simultaneously support climate change adaptation and mitigation while improving rural livelihoods. Adaptation to climate change depends on the ability to understand local climate impacts that will lead to new planting cycles and other mitigation tools. Nationwide mitigation plans are seen as prerequisites to combatting the adverse effects of climate change.

Conservation of life below water is another target for sustainable development. Fisheries and aquaculture offer opportunities to reduce hunger and improve nutrition, alleviate poverty, and generate economic growth along the seashore where the major proportion of the world population lives. According to FAO (2015a), aquaculture is the fastest-growing food sector and has the potential to produce the fish needed to meet the demand for safe and highly nutritious food by a growing population. On the other hand, overfishing must be prohibited, and the expansion of aquaculture production has to be managed to avoid pollution as well as fishery products that are produced unsafely.

Lastly, one of the major SDG topics is protecting ecosystems and life on earth. Inland freshwater ecosystems, particularly forests, wetlands, mountains, and drylands, contribute to biodiversity. They also contribute to local livelihoods, provide clean air and water, and mitigate climate change. Forests and rangelands sustain a range of industries, generate jobs and income, and act as a source of food, medicine, and fuel for residents. Land use changes, including deforestation, result in a loss of valuable habitats, a decrease in clean water, land degradation, soil erosion, and the release of carbon into the atmosphere. Land and agricultural land must be thoroughly protected in order to maintain production and increase economic activity. Both rural and agricultural development projects have significant impacts on agricultural areas and they have to be supported with nations' efforts.

11.7 Conclusion

Integrating various production systems into a single sustainable food and agriculture approach to support and accelerate the transition toward sustainability is gaining more importance (2015b). The approach is based on

five principles that balance the social, economic, and environmental dimensions of sustainable food and agriculture: improve efficiency in the use of resources; take direct action to conserve, protect, and enhance natural resources; protect rural livelihoods and improve equity and social well-being; enhance the resilience of people, communities, and ecosystems, especially to climate change and market volatility; and ensure good governance for the sustainability of both natural and human systems. The efficient use of natural resources without waste, the reduction of carbon emissions with different production methods that do not trigger instability in the climate, and the implementation of measures to increase awareness for the environment will be important policy decisions in food security in the new era.

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12

Nanobiosensors and Their Application in Food Safety

Jamuna A. Bai and V. Ravishankar Rai

CONTENTS

12.1 Introduction

Food safety is a major concern from a public health perspective to prevent foodborne illnesses and food intoxication. Nanomaterials have made the detection of biological and chemical contaminants and toxins easier by increasing sensitivity and signal transduction in biosensing. Their high surface-area-to-volume ratio, high electrical conductivity, magnetic property, surface plasmon resonance, and other unique physicochemical properties have led to their increased application in the detection of analytes. Some of the nanomaterials used in biosensing are: metal nanoparticles (NPs) (5–100 nm), which have unique physicochemical and optoelectronic properties at nano-dimensions; metal nanoclusters, which are ultrasmall NPs (<5 nm) containing less than a few hundred atoms of metals that have a tunable fluorescent emission over the visible and near infrared region; quantum dots (QDs), which are nanoscale semiconductor fluorescent crystals (<5 nm) composed of compounds formed by zinc (Zn) and cadmium (Cd) with telluride (Te) and selenium (Se), and have size-controlled fluorescence, higher fluorescence quantum yields than dyes, and stability against photo-bleaching; carbon nanomaterials, especially carbon nanotubes (CNTs) and graphene; magnetic nanoparticles (MNPs), which have supra-paramagnetic properties (50 nm) and are used to separate target molecules from other compounds; and upconversion nanoparticles (UCNPs), a new class of luminescent materials which transform near infrared radiations into visible radiation used as fluorescent labels (Sharma et al. 2015).

These nanomaterials can be used as a carrier or enhancer, or as a catalyst, reporter, quencher, or separator in biosensors (Figure 12.1). Nanomaterials with a relatively large surface area and a porous nature are used as carriers to load other substances such as probes. Nanomaterial with properties such as a high surface-to-volume ratio and high conductivity, surface plasmon resonance (SPR), quartz crystal microbalance (QCM), and metal-enhanced fluorescence (MEF) is used to enhance signaling and sensitivity. Nanomaterials with high peroxidase activity are used as catalysts. Nanomaterial with SPR and fluorescence properties are used as signal molecules or reporters. Some of these are also used as quenchers in electrogenerated chemiluminescence (ECL) quenching and fluorescence resonance energy transfer (FRET), or inner-filter effect (IFE)-based sensors. MNPs have been used to separate target analytes from complex food matrices (Yang et al. 2016).

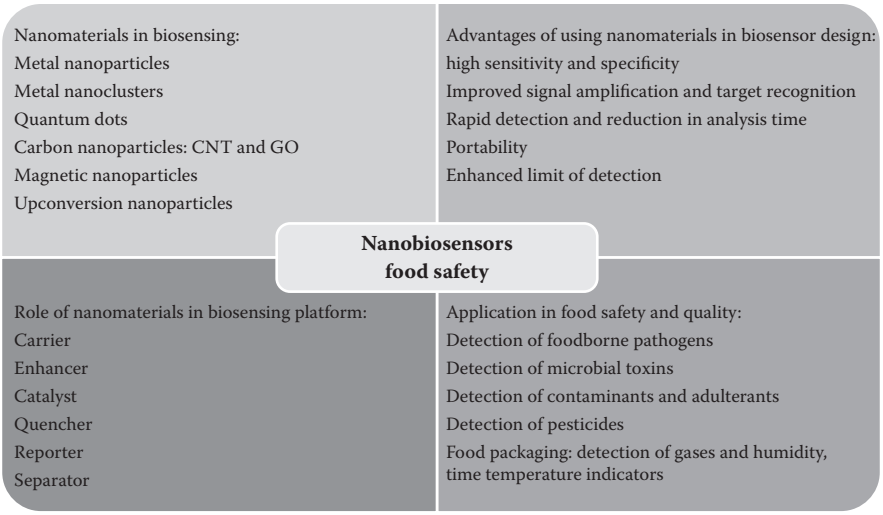

FIGURE 12.1
An overview of application of nanotechnology in biosensor design for food safety.

Nanotechnology has been used to improve optical sensors. The optical sensors use the various properties of light for detection and are classified into luminescent/fluorescent, colorimetric, and surface-enhanced Raman scattering (SERS). The advantage of optical sensors is their ease of use and rapid visual detection and quantification through simple spectrophotometers. One limitation is that samples containing natural pigments, fluorescing substances, or scattering phase-interfaces interfere with measurements necessitating sample pre-treatment. However, these drawbacks are addressed with the use of electrochemical nanosensors. These sensors measure the change in electrical current, potential, conductance, or impedance during the interaction between electrode and analyte. These have good performance with colored and turbid samples, wherein disposable electrodes are used, preventing cross-contamination between samples and saving sample preparation time and cost. The use of nanotechnology has played a key role in enhancing the inherent sensitivity, simplicity, speed, and compatibility with portable measurement devices of the electrochemical nanosensors. Nanotechnology has been used in electrochemical sensors for fabricating electrodes, making design tracers for signal amplification, developing miniaturized sensors for portable devices, and improving the properties of novel recognition elements by using molecularly imprinted polymers and aptamers. It has also been used in designing nanoporous membranes as novel transducing elements (Sharma et al. 2015).

This chapter deals with the use of nanotechnology in improved biosensing and its application in food sectors. The use of nanobiosensors for the detection of foodborne pathogens, fungal and bacterial toxins, adulterants, and

unintentional contaminants such as pesticides will be reviewed. Further, the recent developments and challenges of nanobiosensor application in food safety will be discussed.

12.2 Detection of Foodborne Pathogens

Nanomaterials have been used for developing biosensing platforms to improve the detection of foodborne pathogenic bacteria in a way that is rapid and cost-effective (Table 12.1).

12.2.1 *Escherichia coli*

Oligonucleotide-functionalized AuNPs have been used in the simultaneous detection of Shiga toxin-producing *E. coli* (STEC) strains. An asymmetric polymerase chain reaction (PCR) was used to amplify conserved regions of *stx* genes. Thiol-modified 30 mers were immobilized to AuNPs. This probe was used to capture *stx*1 (119-bp) and/or *stx*2 (104-bp) genes in STEC strains. Gold nanoparticles (AuNP)-oligo probes formed a complex with target DNA due to hybridization and retained the initial red color. While in the presence of non-target DNA, the color changed from purplish-blue due to increased salt concentration. Thus, the optical bionanosensor is based on the direct colorimetric detection of target DNA. The simple and low-cost sensor has been efficiently used to detect STEC strains O26, O45, O103, O111, O121, O145, and O157 from a large number of food samples, such as ground beef and blueberries, after the required enrichment and pooling. The limit of detection (LOD) is <1 log CFU g^{-1} and it can detect in 1 h STEC strains with high specificity (Quintela et al. 2015).

Ni^{2+}-heterogeneous magnetic mesoporous silica (Ni-HMMS) has been used to identify *E. coli* O157:H7 from food samples. Ni-HMMS when coupled with the real-time PCR technique can be used for the high-efficiency magnetic separation of pathogens without DNA extraction. Silica mesopores were incorporated with iron (Fe) particles using a thermal hydrogen reaction. Further, a wet impregnation technique was adopted to functionalize the surface of mesopores with a high concentration of Ni^{2+} ions. The Ni^{2+} ions bind to a cell wall constituent nickel binding membrane protein, NikA present in the pathogens such as *E. coli* O157:H7. The technique has been successfully used with real-time PCR for the detection of 1 Log_{10} CFU mL^{-1} pathogens in food samples (Lee et al. 2014).

Functionalized gold nanorod-based electrochemical sensors have been used for the highly sensitive and specific detection of *E. coli* from a wide range of dairy products. The immunosensor platform comprised of a silica-functionalized gold nanorods, the detection antibodies for the recognition of

TABLE 12.1

Nanobiosensors Used in Detecting Pathogens and Toxins in Food Samples

Target/Analyte	Nanomaterial	LOD	References
Foodborne Bacteria			
Shiga toxin-producing *E. coli* strains	AuNP-oligo probes	<1 log CFU g^{-1}	Quintela et al. 2015
E. coli	Ni^{2+}-heterogeneous magnetic mesoporous silica	1 Log_{10} CFU mL^{-1}	Lee et al. 2014
E. coli	Silica-functionalized gold nanorods	60 CFU mL^{-1}	Zhang et al. 2015
E. coli	Silver nanorods		Chen et al. 2014a
E. coli K12	Carbon QDs	450 CFU mL^{-1}	Weng et al. 2015
Salmonella	MNPs	8×10^4 CFU/mL	Tian et al. 2016
S. Typhimurium	AuNP	7 CFU mL^{-1}	Yuan et al. 2014
S. aureus	AuNP		Chang et al. 2013
S. Typhimurium and *S. aureus*	UCNP MNPs	5 CFU mL^{-1}	Duan et al. 2012a
E. coli, *L. monocytogenes* and *S. enterica*	Qdot 655	3000 cells/well	Bruno et al. 2014
V. parahaemolyticus and *S.* Typhimurium	QD	–	Duan et al. 2013
Campylobacter jejuni	QD	2.5 CFU	Bruno et al. 2009
V. parahaemolyticus	Graphene oxide	2230 cells/ml	Kwun et al. 2014
S. aureus, *V. parahemolyticus* and *S.* Typhimurium	UCNPs	10–25 CFU mL^{-1}	Wu et al. 2014
C. sakazakii	MNPs	3.3×10^3 CFU mL^{-1}	Shukla et al. 2016
Microbial Toxins			
Ochratoxin A	AuNP	20 nM	Yang et al. 2011
Ochratoxin A	AuNP	2 × 10–12 g/mL	Duan et al. 2012b
Ochratoxin A	CNT	24.1 nM	Guo et al. 2011
Ochratoxin A	Graphene oxide	1.9 μM	Sheng et al. 2011
Ochratoxin A and fumonisin B1	UCNP and GO	0.02 $ngmL^{-1}$ for OTA and 0.1 $ngmL^{-1}$ for FB1	Wu et al. 2012
Ochratoxin A	QD	1.9 ng mL^{-1}	Wang et al. 2011
Ochratoxin A	AnNCs	2 pg/mL	Chen et al. 2014b
Ochratoxin A	AuNPs	0.007 ng mL^{-1}	Wang et al. 2010
Ochratoxin A	Iridium NPs	14 pM	Rivas et al. 2015

(*Continued*)

TABLE 12.1 (CONTINUED)

Nanobiosensors Used in Detecting Pathogens and Toxins in Food Samples

Target/Analyte	Nanomaterial	LOD	References
Ochratoxin A	AuNPs	30 pg/mL	Kuang et al. 2010
Ochratoxin A	MNPs	1.39 pg mL^{-1}	Kim and Lim 2015
Ochratoxin A	Silica NPs	0.027 pg/mL	Wang et al. 2016
Aflatoxins	MNPs	0.0012 ng/mL for AF B_1, AF B_2, and AF G_1, and 0.0031 ng/mL for AF G_2	McCullum et al. 2014
Aflatoxins	Mesoporous carbon	3.0 pg mL^{-1}	Lin et al. 2015
AFB1	CdSe/ZnS QDs	0.42 pg mL^{-1}	Ren et al. 2014
E. coli O157:H7 Shiga-like toxins SLT-1 and SLT-2	Fe_3O_4@Al_2O_3 MNPs	–	Kuo et al. 2015
Staphylococcal enterotoxin B	Iron AuNPs	$8.0 \times 107\ M^{-1}$	Temur et al. 2012

E. coli and the signal generating ferrocenecarboxylic acid tags. The nanorods acted as carriers for the immobilization of the antibody and the tags. The high concentration of ferrocenecarboxylic acid tags linked to nanorods increased signal amplification. The LOD achieved with sandwich-type immunoassay was 60 CFU mL^{-1} in 3 h (Zhang et al. 2015).

Silver nanorods were used for the detection of *E. coli* from food samples. The nanorods were deposited on the surface of an anodisc and GA-8 membrane filter (0.2 μm). The filters were used for concentrating bacterial cells and for on-chip surface-enhanced Raman spectroscopy (SERS) detection. The use of a silver nanorod filter enhanced the bacterial SERS signal and thus improved the LOD in comparison to a standard glass silver nanorod. The sensitivity of the nanosensors could be increased by improving the surface morphology of filter substrates (Chen et al. 2014a).

QDs have been used for the inexpensive and efficient labeling of *E. coli* for simple and rapid detection. Carbon QDs have been functionalized with mannose using ammonium citrate. These nanolabels (3 nm), due to the presence of mannose, are able to selectively and specifically bind to the lectin FimH present in the flagella of the *E. coli* K12 strain. The highly soluble mannose carbon QDs were able to detect 450 CFU mL^{-1} of bacterial cells in food samples (Weng et al. 2015).

12.2.2 *Salmonella* Species

Nanosensors based on an immunoassay have been developed for *Salmonella* detection. In the assay, two magnets of varying size were used. The micrometer sized particles were used for capturing while the nano-sized particles

were used for detecting particles. An optomagnetic setup made of low-cost Blu-ray was used for a turn-on readout format. It increased sensitivity by 20 times in comparison to the volumetric magnetic stray field detection device. This was due to the immunomagnetic aggregates that cause steric hindrance and prevent the interior binding sites from interacting with the MNP labels. Such a turn-on competitive immunoassay has high specificity for *Salmonella* detection with an LOD of 8×10^4 CFU/mL in 3 h (Tian et al. 2016).

Nanogold-based aptasensors have been used for the detection of the foodborne pathogen *Salmonella* Typhimurium. Biotinylated aptamer 1, specific to *S.* Typhimurium, is immobilized onto the surface of microtiter plate-wells. The wells were previously modified with streptavidin by the binding of biotin and streptavidin for immobilization. Further, the *S.* Typhimurium, and the aptamer 2-gold NP conjugates, are incubated in the wells, resulting in a sandwich-type aptamer/bacteria/aptamer-AuNPs complex formation. This rapid, highly sensitive, and specific visible aptasensor platform has an LOD of 7 CFU mL^{-1} for the detection of *S.* Typhimurium in samples (Yuan et al. 2014).

12.2.3 *Staphylococcus aureus*

Aptananosensors have also been used to detect *S. aureus* cells. The resonance light-scattering signal of aptamer-conjugated AuNPs in the presence of *S. aureus* cells can be measured. The technique adopted is low-cost and ultrasensitive so that it can rapidly detect a single *S. aureus* cell within 1.5 h (Chang et al. 2013).

Aptamer-conjugated magnetic NPs and UCNPs have been used for the simultaneous detection of *S.* Typhimurium and *S. aureus*. The aptamer-conjugated MNPs are used for the recognition and concentration of bacterial cells while the UCNPs are used as highly sensitive dual-color labels. Aptamer 1 and aptamer 2 are immobilized onto the surface of MNPs, and these capture and concentrate the pathogens. Further, UCNP-modified aptamers 1 and 2 are added to the captured bacteria surface and these form a sandwich-type complex. The LOD of the aptananosensor is 5 CFU mL^{-1} for *S.* Typhimurium and 8 CFU mL^{-1} for *S. aureus*. The luminescent bioassay aptananosensor has high sensitivity and selectivity for the two different pathogens (Duan et al. 2012a).

Highly sensitive aptamer-QD-based lateral flow test strips have been developed for rapid foodborne pathogen detection. The aptamers were coupled to red-emitting QDs (Qdot 655). In this technique, high affinity DNA aptamers with both capture and reporter functions were designed for *E. coli*, *Listeria monocytogenes*, and *S. enterica*. A number of sandwich combinations were tried for the three bacterial lateral flow strips. Of these, the *E. coli* aptamer-lateral flow system had an LOD of 3000 cells for *E. coli* 8739 and 6000 cells for *E. coli* O157:H7 in buffer. The LOD was further improved by using Qdot 655

with the aptamer-lateral flow and 300–600 cells could be detected per test (Bruno et al. 2014).

QDs coupled with aptamers have been used for the sensitive and specific detection of *Vibrio parahaemolyticus* and *S.* Typhimurium. Here, the QDs are used as a fluorescence marker while the aptamers generated by the systematic evolution of ligands by exponential enrichment (SELEX) strategy act as molecular recognition elements. The aptasensors selectively capture the bacteria and detect it with high sensitivity due to the fluorescence of the QDs. The high photostability of the QDs also causes amplified signal intensity. The aptananosensor platform has been applied for the specific recognition of the pathogenic bacteria from complex mixtures such as shrimp samples (Duan et al. 2013).

12.2.4 *Campylobacter*

QDs conjugated with DNA aptamers have been used to develop a portable sandwich assay platform for the rapid detection of *Campylobacter jejuni*. The aptamers were developed for the surface proteins of the pathogen and were used with red QDs. The aptananosensor is highly specific and has LOD of 2.5 CFU in buffer and 10–250 CFU in food matrices. The assay has been validated with a spectrofluorometer and a handheld fluorometer. Using plastic cuvette-adherent technology coupled with a sensitive handheld fluorometer, portable and rapid detection of pathogens can be performed by eliminating the enrichment process (Bruno et al. 2009).

12.2.5 *Vibrio parahaemolyticus*

Graphene oxide-based aptananosensors have been developed for the highly sensitive and rapid detection of *Vibrio parahaemolyticus*. In brief, the assay involves the addition of *V. parahaemolyticus* in Tris–HCl (pH 7) and a hairpin DNA aptamer conjugated with TEX615 in DNA-free deionized water to a graphene oxide NP containing PBS buffer (pH 7.4), followed by 10 minutes of incubation at room temperature (RT). The pathogen and the NP competitively bind to the aptamer. On adding 1,1′-oxalyldiimidazole chemiluminescence reagents, a bright light is emitted and measured. The LOD for the biosensor is as low as 2230 cells/ml, but it is less time-consuming and more economical than the conventional assays (Kwun et al. 2014).

UCNPs and aptamers have been used for the simultaneous detection of *S. aureus*, *V. parahaemolyticus*, and *S.* Typhimurium. The multicolor UCNPs are used as luminescence labels in the assay. They are synthesized through doping with various rare-earth ions to obtain separated emission peaks. The molecular recognition elements, that is, the aptamers for the pathogens, were selected using the SELEX strategy. The NP-coupled aptamers selectively capture and simultaneously quantify the mixture of pathogens on the basis of the peaks obtained. Further, the detection can be improved by the

addition of iron oxide (Fe_3O_4) MNPs to obtain magnetic separation and concentration. The biosensor had LOD of 25, 10, and 15 CFU mL^{-1} for *S. aureus*, *V. parahaemolyticus*, and *S.* Typhimurium, respectively. The use of multicolor UCNPs and aptamers enabled the design of a highly sensitive and specific multiplex nanosensor for the detection of various foodborne pathogens (Wu et al. 2014).

12.2.6 *Cronobacter sakazakii*

Similarly, MNPs have been used for the highly rapid, sensitive, and specific detection of opportunistic foodborne pathogen *Cronobacter sakazakii* in infant formula. MNPs (30 nm) were coated with anti-*C. sakazakii* IgG with a binding efficiency of $86.23 \pm 0.59\%$ to develop immunoliposomes. Using the immunomagnetic concentration and separation technique, the immunoliposomes could rapidly detect 3.3×10^3 CFU mL^{-1} of the pathogen in 2 h without any pre-incubation procedure (Shukla et al. 2016).

12.3 Detection of Microbial Toxins

12.3.1 Ochratoxin A

Ochratoxin A, a toxin produced by *A. ochraceus* and *P. verrucosum*, is one of the commonly occurring food-contaminating mycotoxins. It has been classified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen.

An AuNP-coupled aptasensor has been used for detecting the mycotoxin, ochratoxin A. In the assay, the aptamer in phosphate-buffered saline containing Mg^{2+} ions, undergoes a conformation change in the presence of the fungal toxin. The conformation changes it to a compact rigid antiparallel G-quadruplex structure from a random coil structure. The G-quadruplex structure cannot prevent salt-induced AuNP aggregation, resulting in a visible color change from red to blue. The linear range of the colorimetric aptasensor covered was 20–625 nM and the LOD was 20 nM of ochratoxin A (Yang et al. 2011).

Similarly, a FRET-based aptasensor has been developed for the detection of ochratoxin A. In the assay, a dye-tagged ss-DNA was hybridized with an aptamer conjugated with AuNPs. The interaction between the aptamer-AuNPs conjugate and the dye-labeled ss-DNA causes the fluorescence quenching of carboxyfluorescein (FAM). However, in the presence of ochratoxin A, fluorescence is recovered due to the formation of a quadruplex-ochratoxin A complex, which detaches from the surface of AuNPs. The aptamer has a high binding affinity for the toxin while the AuNPs are highly sensitive and undergo FRET. The relative fluorescence intensity is proportional to

the toxin concentration and has LOD of 2×10–12 g/mL. This sensitive and specific method has been successfully used to detect mycotoxin in maize samples and is validated using a commercially available enzyme-linked immunosorbent assay (ELISA) method (Duan et al. 2012b).

Single-walled CNTs have been used to develop an aptasensor for ochratoxin A detection. The CNTs quench the fluorescence of a free and unfolded aptamer attached with FAM. The LOD for the sensor is 24.1 nM of ochratoxin A and it has a linear detection range of 25 to 200 nM. The technique is highly sensitive, selective, and specific. It has been tested in 1% beer containing buffer solution spiked with varying concentrations of ochratoxin A (Guo et al. 2011).

Similarly, graphene oxide NPs can be used to quench the fluorescence of FAM attached to an ochratoxin A-specific aptamer. The LOD for the sensor is 1.9 μM with a linear detection range from 2 to 35 μM. Ochratoxin A and its structural analogs adsorb onto the graphene oxide. The sensor has high selectivity that specifically responds to ochratoxin A without interference from other structure analogs and with only limited interference from ochratoxin B. The sensor has been tested for the presence of toxin in 1% red wine containing buffer solution spiked with different concentrations of ochratoxin A (Sheng et al. 2011).

A novel aptasensor for mycotoxin detection has been developed using multiplexed FRET, multicolor UCNPs, and graphene oxide. The fluorescent UCNPs act as donors and graphene oxide acts as the acceptor. The UCNPs were synthesized and functionalized and further immobilized with ochratoxin A-aptamers and fumonisin B1 (FB1)-aptamers. The aptamer-modified UCNP is brought in proximity to the graphene oxide surface. The strong upconversion fluorescence is quenched by the graphene oxide because of a good overlap between the fluorescence emission of multicolor UCNPs and the absorption spectrum of graphene oxide. However, in the presence of mycotoxins, the aptamers bind to their corresponding mycotoxins, resulting in changes to the conformation of aptamers. This causes the aptamer-modified UCNPs to be far from the graphene oxide surface. The aptasensor developed had LOD of 0.02 $ngmL^{-1}$ for ochratoxin A and 0.1 $ngmL^{-1}$ for FB1. The aptasensor was used for ochratoxin A and FB1 level detection in naturally contaminated maize samples (Wu et al. 2012).

Using QDs and aptamers, a simple fluorescent strip sensor was developed to detect ochratoxin A. The LOD of the sensor was 1.9 ng mL^{-1} and it could detect the toxin in 10 minutes. The nanosensor strips had high selectivity and could be used for samples with complex compositions (Wang et al. 2011).

DNA-scaffolded nanoclusters have been used for developing signal-on fluorescent biosensor platforms for the detection of ochratoxin A. The sensors make use of structure-switching of the anti-ochratoxin A aptamer and magnetic bead technology. The sensor has high sensitivity and can detect 2 pg/mL of the toxin with high specificity and has been validated in food samples such as wheat. The DNA-scaffolded silver NPs have a higher fluorescence

quantum yield and photostability than conventional sensors. The other advantages of these biosensors are their simple fabrication, easy operation, low-cost technology, rapid speed, and portability (Chen et al. 2014b).

An electrochemiluminescent biosensor using an aptamer as the recognition element and N-(4-aminobutyl)-N-ethylisoluminol as the signal generator has been used for ochratoxin A detection. The aptamer complementary DNA 1 sequence is immobilized onto the surface of a AuNP-modified gold electrode. Another aptamer for the signal producing compound sequence is generated, DNA 2, and is hybridized to DNA 1 and used as a probe. The presence of toxins in the sample causes the aptamer recognition of the target toxin and the dissociation of DNA 3 from DNA 1, resulting in decreased electrochemiluminescence. The LOD of the biosensor is as low as 0.007 ng mL^{-1} and it has been applied to measure the mycotoxins in wheat samples (Wang et al. 2010).

A screen-printed carbon electrode modified with polythionine, iridium oxide NPs, and an animated aptamer selective to toxins has been used to develop an aptasensor for ochratoxin A detection. Electrochemical impedance spectroscopy is used to measure the aptasensor assay. The label-free detection technique has LOD of 14 pM or 5.65 ng/kg. The biosensor performance has been validated in a white wine sample (Rivas et al. 2015).

An electrochemical biosensor platform containing three single-stranded DNA molecules, and the aptamer immobilized on an electrode surface, has been used for the ultrasensitive detection of ochratoxin A. The binding of a toxin to the aptamer changes the redox current of the methylene blue probe. AuNP-functionalized DNA has been used for signal enhancement, which has led to a detection of 30 pg/mL of toxin (Kuang et al. 2010).

A chemiluminescence immunoassay-based biosensor platform using functionalized MNPs has been used for ochratoxin A detection. Amine-functionalized MNPs are used to concentrate toxins and a horseradish peroxidase tagged antibody is used as a label. Amine-targeted inhibition is used for a non-specific extraction platform to decrease the background and improve signal-to-background ratio. A lab-built drop-type chemiluminescence biosensor platform with luminol–H_2O_2 reagent was used to detect the toxin in rice samples and the LOD was as low as 1.39 pg mL^{-1} (Kim and Lim 2015).

An electrochemiluminescence biosensor using $Ru(bpy)_3^{2+}$-doped silica NPs and molecularly imprinted polymer was used to detect ochratoxin A. The fabrication of a sensor with NPs led to the binding of the toxin to the sensor surface. The elution and rebinding and their reaction with $Ru(bpy)_3^{2+}$ immobilized on the electrode surface caused high-energy electron-transfer reactions and led to the emission of a electrochemiluminescence signal. The signal intensity decreased when the target molecule rebound to the molecularly imprinted polymer. The highly sensitive and selective sensor had LOD of 0.027 pg/mL and was successfully validated for toxin detection in corn (Wang et al. 2016).

12.3.2 Aflatoxins

A magnetic solid phase extraction technique with polydopamine-coated magnetic NPs was used for extracting and detecting aflatoxins from liquid samples. The magnetic NP-containing adsorbent phase was prepared from amine-terminated NPs and dopamine through in situ oxidative self-polymerization. Using this technique, the extraction yield was 59.3% for AF G_2 and 89.0% for AF B_1. On coupling with high performance liquid chromatography (HPLC) and mass spectrometry (MS), the technique had LOD of 0.0012 ng/mL for AF B_1, AF B_2, and AF G_1, and 0.0031 ng/mL for AF G_2 (McCullum et al. 2014).

Mesoporous carbon NPs functionalized with an antibody have been used to develop an electrochemical and immunoassay-based biosensor platform for the sensitive and rapid detection of aflatoxin B_1. Mesoporous carbon is initially functionalized with electroactive thionine molecules and is further conjugated with a polyclonal anti-AFB_1 antibody. In the biosensor, the electrostatic interaction occurs between negatively charged nafion film and a positively charged anti-AFB_1 antibody on the nanostructures; the signal is carried by thionine to the mesoporous carbon. The toxin reacts with the labeled anti-AFB_1 antibody present on the mesoporous carbon, based on specific antigen–antibody reaction, resulting in the dissociation of thionine–MSN nanostructures from the sensing interface. This decreases the signaling of the thionine molecules. The biosensor has a high sensitivity with LOD as low as 3.0 pg mL^{-1} and is found to be simple and low cost, and eliminates the sample separation and washing step (Lin et al. 2015).

QD beads have been used to develop biosensors for the ultrasensitive detection of aflatoxin B_1 in maize. The QDs were fabricated by encapsulating CdSe/ZnS to prepare highly luminescent labels. The QD-based immunechromatographic assay had LOD of 0.42 pg mL^{-1} for aflatoxin B_1 in a maize extract, which is an improvement over the conventional ELISA test. The performance of the biosensor was validated with an ELISA kit and LC–MS. The biosensor can be used for the rapid and cost-effective quantitative detection of mycotoxins in foods (Ren et al. 2014).

12.3.3 Bacterial Toxins

The pathogenicity of the foodborne pathogen *E. coli* O157:H7 is due to the production of Shiga-like toxins SLT-1 and SLT-2. An MNP-based assay platform has been developed to rapidly detect SLT-1. The MNP core consists of iron oxide and its surface is coated with a thin layer of alumina (Fe_3O_4@ Al_2O_3 MNPs) functionalized with pigeon ovalbumin that selectively binds to SLT-1B. The functionalized MNP effectively enriches SLT-1B from complex cell lysates. Gal-α(1→4)-Gal disaccharides are used to dissociate SLT-1 from the MNP. The released SLT-1 is identified by matrix-assisted laser

desorption/ionization MS. A 20 μL sample volume and 1 minute enrichment is used for SLT-1B detection. The nanosensor has been used for the effective and rapid detection of SLT-1 from complex cell lysates, ham, and juice samples (Kuo et al. 2015).

Core-shell-structured iron-gold magnetic NPs and a gold nanorod SERS probe have been used to develop sensors for the detection of staphylococcal enterotoxin B (SEB). Magnetic gold nanorod particles are functionalized with aptamers to recognize and bind SEB, whereas the gold nanorod particles are used as SERS probes. The binding constant between the toxin and aptasensor-nanoconjugate was $8.0 \times 107\ M^{-1}$. The LOD for the assay platform was found to be 224 aM, that is, 2697 SEB molecules in a 20 μL sample. The biosensor has been applied for SEB detection in artificially contaminated milk, blood, and urine (Temur et al. 2012).

12.4 Detection of Contaminants and Adulterants

Nanobiosensors can be used to detect contaminants and adulterants in food matrices. Some of the examples of biosensors developed using nanotechnology for the detection of food contaminants are discussed in this section (Table 12.2).

12.4.1 Melamine

AuNP-based biosensors have been used to detect the presence of melamine in pet foods and infant milk formulations. The AuNPs conjugated with cyanuric acid can selectively bind to melamine. If melamine is present in the food, it induces the aggregation of AuNPs, which results in a noticeable color change from red to blue. It is an analyte-concentration-dependent colorimetric measurement and can be used to detect 2.5 ppb of melamine adulteration in foods (Ai et al. 2009).

AuNP formation has also been used to detect melamine. The gold ions on reacting with a chemical reductant form AuNPs and the solution turns red. However, if melamine is present in a sample, it interacts with the reductant, preventing AuNP formation. Thus, there is no change in color indicating the presence of melamine in the sample (Cao et al. 2010).

The sensitive detection of melamine in foods is also possible with the use of nanoaptasensors. The aptamer for melamine was designed from an abasic-site-containing triplex molecular beacon (tMB). The triplex structure has high binding affinity for melamine. The tMBs were integrated with fluorescent silver nanoclusters. The signaling of DNA-templated silver nanoclusters acts as a turn-on for melamine detection. The interaction of melamine with tMB increases the fluorescence of a silver nanocluster attached to the

TABLE 12.2

Application of Nanobiosensors in Detecting Adulterants and Contaminants in Foods

Target/Analyte	Nanomaterial	LOD	References
Adulterants			
Melamine	AuNPs	2.5 ppb	Ai et al. 2009
Sudan I	Gold-labeled Abs	10 ng/g	Wang et al. 2013
Sudan I and Bisphenol A	ZnO/CNTs	BPA: 9.0 nmol L^{-1} Sudan I: 80 nmol L^{-1}	Najafi et al. 2014
Sudan I	ZnO/CNTs	0.003 μmol L^{-1}	Elyasi et al. 2013
Bisphenol A	Cd-doped ZnO QDs	13.1 ng mL^{-1}	Zhang et al. 2014
Bisphenol A	Multi-walled CNTs	9.0×10^{-10} M	Kim et al. 2013
Pesticides			
Chlortoluron	ZnO NPs	0.47 nM	Haddaoui and Raouafi 2015
Parathion methyl	AuNP and CdTe QDs	0.018 ng mL^{-1}	Yan et al. 2015
Parathion methyl	Zirconia macroporous polyaniline	2.28×10^{-10} mol L^{-1}	Wang et al. 2015
Parathion methyl	AuNP	0.08 ng mL^{-1}	Wu et al. 2014
Malathion	Copper oxide and CNT	0.1 ppb	Huo et al. 2014
Lindane	AgNP	5 nM	Kaur et al. 2015
Aldrin, dieldrin, lindane and α-endosulfan	Ag and AuNP SERS	10^{-8} M	Kubackova et al. 2015
Carbofuran and carbaryl	Cobalt(II) oxide-decorated reduced graphene oxide	Carbofuran: 4.2 μg/L Carbaryl: 7.5 μg/L	Wang et al. 2014
Atrazine	Single walled CNTs	0.001 ng mL^{-1}	Belkhammsa et al. 2016
Chlorpyrifos	Fe_3O_4 coated CNTs	6.3 pg/mL	Sun et al. 2015
Pyrethroids	Molecularly imprinted silica: QDs	1.2 μg/kg	Xiao et al. 2016
Pentachlorophenols	Gold nanoclusters/ Graphene	1.0×10^{-14} M	Luo et al. 2014
Thiram	copper ions with $NaYF_4$: Yb/Tm UCNPs	0.1 μM	Mei et al. 2016

tMB. The highly sensitive and label-free aptasensor platform will have potential use in detecting melamine from food samples (Wang et al. 2015).

12.4.2 Gluten

Apart from colorimetric fluorescence, assays have also been used to develop nano-based biosensors. An enhanced fluorescence-linked immunosorbent

assay (EFLISA) has been used to detect gluten in gluten-free-labeled foods. A nanostructured silver island film enhances fluorescence in rhodamine-labeled anti-gliadin antibodies. These have been used to detect gliadin in foods. Gliadin causes intestinal inflammation on consumption of gluten-containing foods in celiac disease patients (Staiano et al. 2009; Kuang et al. 2011).

12.4.3 Sudan I

Nanocolloidal gold-labeled Mab has been used for the rapid detection of Sudan I adulteration in foods. A simple semiquantitative dip strip was developed by coating a protein-Sudan red I conjugate on a nitro cellulose membrane strip in a defined test line. Sudan I detection led to the formation of an intense red color in the test line due to the complex formation between nanocolloidal gold-labeled Mab and Sudan red I. The visual LOD was 10 ng/g of Sudan red I in food samples such as tomato sauce and chili powder. The rapid strip test requires a minimal time of 10 minutes for Sudan I detection in food samples. It can be stored for 2 months at 4°C (Wang et al. 2013).

Adulterants such as bisphenol A (BPA) and Sudan I can be simultaneously detected using electrochemical nanosensors. The electrode surface containing carbon paste is coated with nanocomposites made of zinc oxide (ZnO)/CNTs and an ionic liquid, 1,3-dipropylimidazolium bromide. The nanosensor uses voltammetry for the detection of a mixture of adulterants. At a neutral pH, the mixture of Sudan I and BPA can be seen as two anodic well-separated peaks. Sudan I has an anodic peak of 0.47 V, while BPA forms an anodic peak at 0.70 V at pH 7. The LOD for BPA and Sudan I in samples is 9.0 nmol L^{-1} and 80 nmol L^{-1}, respectively. The electrochemical nanosensor can be used to detect a mixture of adulterants such as BPA and Sudan I (Najafi et al. 2014).

Similarly, electrochemical nanosensors have been developed with Pt/CNTs nanocomposite ionic liquid instead of ZnO/CNTs coated carbon paste electrodes. The nanosensor uses a number of methods such as cyclic voltammetry, electrochemical impedance spectroscopy, and square-wavevoltammetry (SWV). Due to the catalytic activity of the sensor, Sudan I gets oxidized. This results in an increase in the oxidation peak current and a reduction in the oxidation peak potential. The electron transfer coefficient, diffusion coefficient, and charge transfer resistance of Sudan I on oxidization at the electrode surface is measured. The LOD for Sudan I by the nanosensor was found to be 0.003 μmol L^{-1}. The biosensor is highly specific for Sudan I detection in food samples (Elyasi et al. 2013).

12.4.4 Bisphenol A

BPA has also been detected using QD-based nanosensors. Cd-doped ZnO QDs were made hydrophilic by functionalizing them with

poly(amidoamine) (PAMAM) dendrimers. These QDs were conjugated with BPA antibodies by covalent linkage. The QD-BPA Ab conjugates were used in a competitive fluorescence-linked immunoassay (FLISA). This biosensing platform had a linear working range of 20.8–330.3 ng mL^{-1} and LOD of 13.1 ng mL^{-1} for BPA. The rapid and sensitive FLISA QD-coupled sensor has been validated for detecting BPA in aqueous samples (Zhang et al. 2014).

The nanocomposites of multi-walled CNTs doped with titania-nafion have been used to modify a glassy carbon (GC) electrode to detect BPA. This modified electrode has higher catalytic activity to BPA in comparison to a GC electrode. Hence, it showed a better voltammetric response for BPA. Adding a cationic surfactant such as cetyltrimethylammonium bromide (CTAB) to the sample increased BPA accumulation due to the hydrophobic interaction between CTAB and BPA. The electrochemical nanosensor had a linear response ($r^2 = 0.999$) of 1.0×10^{-8} M to 5.0×10^{-6} M and an LOD of 9.0×10^{-10} M. The nanosensor was highly sensitive and specific and has been applied for BPA detection in packaged food samples (Kim et al. 2013).

12.5 Detection of Pesticides

12.5.1 Chlortoluron

Disposable and highly sensitive nanosensors using ZnO NPs have been used for the detection of chlortoluron herbicide. The ability of the herbicide to inhibit tyrosinase activity has been taken advantage of in designing the sensor. The biosensing platform consists of tyrosinase-modified screen-printed carbon electrodes and is nanostructured by ZnO NPs for the electrochemical function. In the presence of the target analyte, the tyrosinase activity decreases. The biosensor has LOD of 0.47 nM for pesticide detection (Haddaoui and Raouafi 2015).

12.5.2 Parathion-methyl

AuNPs and fluorescent QDs have been used for organophosphorus pesticide detection. The ratiometric fluorescent QDs are made of two differently colored CdTe QDs. The red emissive QDs within the silica sphere are used as a reference signal, and the green emissive QDs on the silica surface are the response signals. By the inner-filter effect, AuNPs can quench the fluorescence of QDs. Protamine turns on the fluorescence by its electrostatic attraction with AuNPs. Trypsin hydrolyzes protamine, preventing fluorescence. Pesticide parathion-methyl can inhibit the trypsin activity. Thus, pesticide

concentration is proportional to fluorescence emission. The QD-based biosensor has LOD of 0.018 ng mL^{-1} for parathion-methyl detection (Yan et al. 2015).

Zirconia/macroporous polyaniline has been used to develop an electrochemical sensor for the detection of methyl-parathion. Zirconia has a high affinity to the phosphate group and the macroporous polyaniline has high catalytic activity and good conductivity, making them both good biosensors with an LOD of 2.28×10^{-10} mol L^{-1}. The electrochemical sensing platforms with simple, sensitive, selective, and fast analysis have been successfully applied to detect methyl-parathion in apples and cabbages (Wang et al. 2015).

Functionalized AuNPs have been used in designing a molecular imprinting and electrochemical enzymeless sensor for methyl-parathion detection. Methyl-parathion is embedded in the imprinting sites by the molecular self-assembly of *p*-aminothiophenol and electro-polymerization with AuNP. The effectiveness of imprinting sites and the conductive performance is improved by AuNPs decorated with CNT nanocomposites. The nanosensor with LOD of 0.08 ng mL^{-1} has been applied for the detection of methyl-parathion in apples and vegetables (Wu et al. 2014).

12.5.3 Malathion

Copper oxide nanowires and single-walled CNTs have been used to design an electrochemical sensor for organophosphorus pesticides detection. The biosensor is highly stable and has a high affinity for malathion with LOD of 0.1 ppb. It also has good selectivity against other commonly used pesticides. It has been applied for malathion detection in spiked liquid garlic samples (Huo et al. 2014).

12.5.4 Lindane

A silver NP-based electrochemical sensor has been used for the detection of lindane. The silver NP was coated in polyaniline-nanocrystalline zeolite. The sensor has high electro-catalytic activity, stability, sensitivity, and selectivity and can detect lindane at nanomolar concentrations. It has LOD of 5 nM for lindane detection (Kaur et al. 2015).

12.5.5 Organochlorine Pesticides

A silver and gold NP SERS has been used for the detection of the organochlorine pesticides aldrin, dieldrin, lindane, and α-endosulfan. The metallic NPs were functionalized with alkyl dithiols. This enhanced the affinity of the pesticides with the sensors. The high sensitivity of SERS using nanosensors for the detection of the organochlorine pesticides had LOD of 10^{-8} M (Kubackova et al. 2015).

Cobalt(II) oxide-decorated reduced graphene oxide has been used to develop sensors for the detection of carbofuran and carbaryl. Two well-defined and separate voltammetric peaks were obtained with the sensors for carbofuran and carbaryl pesticides. The sensor had LOD of 4.2 μg/L for carbofuran and 7.5 μg/L for carbaryl and was successfully used for the detection of these pesticides in fruit and vegetable samples (Wang et al. 2014).

12.5.6 Atrazine

CNTs have been used in developing an immunosensor platform for atrazine detection. A network of single-walled CNTs acts as the conductor channel and constitutes the CNTs' field-effect transistors (FETs). Further, anti-atrazine antibodies are adsorbed on the CNT surface. The CNTs are coated with Tween 20 to prevent the non-specific binding of bacteria or proteins. In the presence of atrazine, immunoreactions occur and the CNTFETs detect the pesticide. The nanosensors could be used as label-free platforms to detect atrazine as they have LOD of 0.001 ng mL^{-1}. They have high sensitivity and can be used for detecting atrazine rapidly at low concentrations in water samples (Belkhammsa et al. 2016).

12.5.7 Chlorpyrifos

A biocompatible quinone-rich polydopamine nanospheres-modified glass carbon electrode has been developed as the sensor platform for the detection of chlorpyrifos. The sensor uses multi-horseradish peroxidase flake-like Fe_3O_4 coated in CNT nanocomposites as the signal label. The flake-like Fe_3O_4 coated CNTs (CNTs@f-Fe_3O_4) were synthesized with the coprecipitation of Fe^{3+} and Fe^{2+} on polydopamine-modified CNTs using ethylene glycol. The CNTs were used as the carrier of the multi-enzyme label due to the high loading of the secondary antibody (Ab_2) and horseradish peroxidase (HRP) and also the peroxidase-mimic activity of Fe_3O_4. The electrochemical immunoassay-based nanosensor had LOD of 6.3 pg/mL for chlorpyrifos detection. The sensor has high sensitivity, specificity, reproducibility, and stability for the fast analysis of the chlorpyrifos pesticide (Sun et al. 2015).

12.5.8 Pyrethroids

Molecularly imprinted silica layers appended to QDs (MIP-QDs) with customized selective artificial recognition sites have been designed for the detection of pyrethroids. The selective fluorescence quenching properties of MIP-QDs toward cypermethrin (CYP) are due to strong interactions between these molecules. An ELISA-like method based on the MIP-QDs was established under optimal conditions. The fluorescence quenching observed from this method showed a linear relationship with CYP concentration over the range of 0.05–60.0 mg/kg with a correlation coefficient of 0.9838.

Good recovery (82.7%–92.4%) and a relative standard deviation of less than 10.1% were obtained from fish samples spiked with three levels of CYP. This method also demonstrated a low detection limit of 1.2 μg/kg and has been successfully employed to detect residual CYP in fish samples (Xiao et al. 2016).

12.5.9 Pentachlorophenol

An electrochemiluminescence sensor based on an Au nanoclusters/graphene hybrid has been used for the fast and sensitive detection of pentachlorophenol. It uses $S_2O_8^{2-}$ as a coreactant. Graphene facilitates both Au^- and SO_4^- production, and a high yield of the excited Au NCs (Au^{*+}) is produced due to the presence of Au^- and SO_4^-. The interaction between pentachlorophenol and the Au^{*+} results in a sensitive change in the electrochemiluminescence intensity. Other compounds that cannot be oxidized by the Au^{*+} show little interference in the detection of pentachlorophenol. The sensor has a sensitivity of 1.0×10^{-14} M concentration and has been tested in water samples (Luo et al. 2014).

12.5.10 Thiram

Copper ions with $NaYF_4$:Yb/Tm UCNPs have been used for the detection of thiram. Copper ions conjugated with $NaYF_4$:Yb/Tm UCNPs were fixed onto filter paper to form test paper. Further, in the presence of thiram, the blue luminescence on the paper strip is quenched through luminescence resonance energy transfer. These variations are monitored by the smartphone camera, and then the blue channel intensities of obtained colored images are calculated to quantify the amounts of thiram through a self-written Android program installed on the smartphone. The device offers a reliable and accurate LOD of 0.1 μM for thiram detection. Thus, by integrating upconversion nanosensors with smartphone digital imaging, a paper-based portable platform can be developed for rapid pesticide detection (Mei et al. 2016).

12.6 Nanosensors in Food Packaging Materials

12.6.1 Detection of Gases

Nanosensors are used to detect excess moisture and oxygen, as these are known to cause food spoilage. For example, a visual method to detect oxygen in modified atmosphere packaging (MAP) food has been developed using photoactivated indicator ink based on nanosized TiO_2 or SnO_2 particles and a redox-active dye (methylene blue). Even trace quantities of

oxygen cause color change in the detector. Similarly, the carbon dioxide content in MAPs by analyzing luminescent dyes was standardized by fluorophore-encapsulated polymer nanobeads at a detection range of 0.8–100% (Duncan 2011).

12.6.2 Time, Temperature, and Humidity Indicators

Triangular silver nanoplates are used as colorimetric indicators to detect time-temperature changes. They use the thermodynamic instability and strong in-plane dipole resonance mode in the visible radiation spectrum of Ag nanoplates. With storage time, the sharp corners of the nanoplate become rounded, causing the color to change from cyan to blue. The rate of color change is also temperature-dependent (Mihindukulasuriya and Lim 2014). Similarly, moisture content has been detected in packaged foods by using copper NPs. The NPs are coated with carbon and dispersed in a tensile film. Moisture content causes the swelling of the polymer matrix and thus inter-NP separation. This causes the sensor to reflect or absorb different colors of light and thus detect changes in humidity (Duncan 2011).

12.7 Conclusion

Thus, nanotechnology has increased the sensitivity and specificity of biosensing in detecting foodborne pathogens and biological and chemical contaminants. Application of nanotechnology has also improved the analytical methods used to determine the quality and shelf life of foods. Nanomaterials have been used in transducers for signal amplification and target recognition. This has led to the development of biosensors with specific target identification, rapid recognition, increased selectivity and sensitivity, portability, and reduced analysis time for ensuring safe foods for consumers.

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