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Edited by Hussaini Anthony Makun

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Contributors

Nivia Coelho, Luciana Coelho, Diego Pessoa, Kenia Oliveira, Priscila Sousa, Licia Silva, Antonio Valero, Magdevis Yanet Rodríguez, Prof. Guiomar Denisse Posada-Izquierdo, Fernando Pérez-Rodríguez, Elena Carrasco, Rosa María García-Gimeno, Saulat Jahan, Yun-Hwa Hsieh, Aslı Uçar, Mustafa Volkan Yılmaz, Funda Pınar Çakıroğlu, Biljana Damjanović-Vratnica, Matilde Jimenez-Coello, Gerardo Dzib-Paredes, Jose Alberto Rosado-Aguilar, Karla Y. Acosta-Viana, Eugenia Guzman-Marin, Antonio Ortega-Pacheco, Zhenbo Xu, Brian Peters, Bing Li, Lin Li, Mark Shirtliff, Ntambwe Malangu, Kirkwood M. Land, Luisa W. W. Cheng

Notice

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Meet the editor

Hussaini Anthony Makun is currently working as Professor of Biochemistry and Director of Research in the Federal University of Technology, Minna. He completed his PhD in Biochemistry (Toxicology) from the same University. The researcher was a Postdoctoral Fellow (PDF) with Food Environment and Health Research Group of the University of Johannesburg (UJ) between 2008 and 2010. He is the Lead Researcher of the Food and Toxicology Research Group (FTRG), which conducts researches on mycotoxins, African medicinal plants and substances of abuse in Nigeria. He has won six national and international grants and is a member of the African Union Expert Committee on Contaminants in Food and the Joint FAO/WHO Expert Committee on Contaminants in Food (2012-2016). He has 57 publications in peer-reviewed journals, technical papers and books.

Contents

Preface

Food-borne diseases are the major causes of morbidity and mortality in the world. It is estimated that about 2.2 million people, a majority of whom are children living in developing countries, die yearly due to food and water contamination. Typhoid fever occurs in 16.6 million people and causes 600,000 deaths every year around the world. Even in the developed countries where food hygiene and laws are enforced, food-borne diseases are a major public health concern. For instance, in the United States, contaminated foods are responsible for nearly 76 million infections, 325,000 hospital cases and 5000 deaths every year. While in the developing countries, particularly Sub-Saharan Africa, the food safety issues are exacerbated by food shortage such that even visibly mouldy and spoiled foods are consumed. Food safety and consequently food security are therefore of immense importance to public health, international trade and world economy.

The book, which has 10 chapters, provides information on the incidence, health implications and effective prevention and control strategies of food-related diseases. Hence, the book will be of importance to undergraduate and postgraduate students, educators and researchers in the fields of life sciences, medicine, agriculture, food science and technology, trade and economics. Policy makers and regulatory officers in food industry can acquire substantial knowledge from the book, which will be useful in the course of their duties. The first chapter provides information on the factors that propagate common pathogens encountered in the food industry and proffers hygienic practices for their elimination from the food chain. While the second chapter reviews the risk factors enhancing microbial contamination in food service centres, the third chapter explains the risk factors associated with food poisoning in Africa as well as describing the short- and long-term public health impacts of food-borne diseases in the continent. The two subsequent chapters critically analyse the potential exposure and risk associated with metal contamination of food and current technologies in detecting botulinum neurotoxins in foods, respectively. Chapters 6, 7, 8 and 9 discuss the epidemiology, prevention and control of cholera, staphylococcal food poisoning and *Toxoplasma* gondii and prion diseases in the food value chain, respectively. The last chapter gives important information on effective techniques for extraction, as well as chemical composition and antimicrobial activity of herbal extracts use in preventing food-borne diseases.

It is our hope that this book will provide valuable information to researchers, policy makers and regulatory officers in the food industry and will be used as a specialized reference and text book. The contributions of all the authors are highly appreciated without which the book would have not been a reality. I also wish to express my sincere gratitude to my wife, Evelyn Pambelo Hussaini, for her encouragement during the course of the work. Finally, the authors and editor wish to express our appreciation to InTech for publishing the book.

Professor Hussaini Anthony Makun
Department of Biochemistry,
Federal University of Technology,
Minna, Nigeria

Chapter 1

Food Safety – Problems and Solutions

Aslı Uçar, Mustafa Volkan Yilmaz and Funda Pınar Çakıroğlu

Additional information is available at the end of the chapter

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Abstract

When certain disease-causing bacteria, viruses or parasite contaminate food, they can cause food-related diseases. Another word for such a bacterium, virus, or parasite is "pathogen". Since food-related diseases can be serious, or even fatal, it is important to know and practice safe food-handling behaviors to help reduce the risk of getting sick from contaminated food. According to the Codex Alimentarius Commission (CAC), "food safety is the assurance that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use". Foodborne diseases are widespread throughout the world. The process by which a foodborne disease spreads begins with the features of the disease, contaminating the food, which in turn threatens both individual and public health by means of the foods. Healthy, or what can be termed as safe food, is food that has not lost its nutritional value, that is clean, in physical, chemical and microbiological terms and that is not stale. The factors causing the contamination of the food may threaten the safe consumption of it and thereby make the foods harmful to human health. For this reason, it is necessary to utilize various resources to prevent the food from being contaminated in all stages of the food chain, from harvest to consumption. The aim of this chapter is to determine the factors affecting food safety and proffer effective intervention strategies against food-related diseases.

Keywords: Food safety, food hygiene, food handlers hygiene, kitchen and equipment hygiene, nutrition

1. Introduction

The diseases caused by food, or the foodborne diseases, are described as the illnesses with which people are infected by the foods they eat [1]. These diseases are a widespread public health issue and are expensive to treat [2]. Foodborne diseases result from the consumption of contaminated foods and products. Contamination of the food at any stage, from production to consumption, produces bacteria, viruses, parasites, chemical agents and toxins, which eventually cause the foodborne diseases [1].

These diseases are seen as a pervasive, permanent problem that can lead to morbidity and, occasionally, to mortality. Foodborne diseases are increasing worldwide, particularly in the developing countries, due to neglect of personal hygiene and food hygiene [3].

Foodborne illnesses pose a threat to international public health safety and economic development. With the increasing amount of trade, travel and immigration, the rate at which dangerous contaminants and pathogens pass through the borders has also risen. Every year, approximately 2.2 million people, a majority of whom are children living in developing countries, die as a result of food and water contamination [1]. Typhoid fever occurs in 16.6 million people and causes 600,000 deaths every year around the world. In the United States, contaminated foods are seen as being responsible for nearly 76 million infections, 325,000 hospital cases and 5000 deaths every year [4]. According to 2011 data from the Centers for Disease Control and Prevention (CDC), it was estimated that in the United States, one out of every six persons was infected with foodborne illness (48 million people) and that foodborne illnesses resulted in 128,000 hospital cases and 3000 deaths [5].

In 2013, FoodNet, a CDC-established program that tracks foodborne illnesses in the United States, found that foodborne illnesses were responsible for 19,056 infections, 4,200 hospital cases and 80 deaths. The incidence of bacteria responsible for causing diseases in every 100.000 people was determined to be 15.19 for *Salmonella*, 13.82 for *Campylobacter*, 4.82 for *Shigella*, 2.48 for *Cryptosporidium*, 1.15 for *STEC non-O157*, 0.51 for *Vibrio*, 0.36 for *Yersinia*, 0.26 for *Listeria* and 0.03 for *Cyclospora*. Incidences of *Cyclospora*, *Listeria* and *Vibrio* were found to be the highest among the elderly, aged 65 years and older, whereas for all of the other pathogens, the highest incident rates were found among children younger than 5 years of age [6].

The diseases caused by *Salmonella* and *Campylobacter*, the main agents responsible for foodborne infections, are dramatically increasing in some countries, including Denmark, Finland, Iceland, the Netherlands, Norway, Sweden, Switzerland and the United Kingdom. In 1999 and 2000, the number of reported foodborne disease cases was 84,340 and 77,515, respectively in Turkey. While *Salmonella* is the most frequently encountered disease agent, the actual figures on foodborne infections and toxins are not reflected, as it is not mandatory to report these diseases [7].

The world's growing population and the consumers' desire to be provided with a wider range of foods have resulted in a longer and more complex food chain. Today, foods reach consumers after being collected from fields, farms and factories and then pass onto many countries, traveling distances of thousands of kilometers. With this global food distribution, an infection that occurs at any point within the food chain has the potential of affecting any given population in the world. It is therefore essential, given the number of interactions taking place between the actors involved in the food chain and the long distances between them, that multisectorial and international collaboration take place. As no country can provide food safety on its own, safety measures need to be enhanced in many countries [8].

While experts on food safety and health have determined that millions of foodborne disease cases are reported every year, the actual numbers are clouded by uncertainty, as most cases go unreported. Furthermore, foodborne diseases are difficult to diagnose, since they have

various symptoms, including fatigue, chills, mild fever, vertigo, upset stomach, dehydration caused by diarrhea, severe cramps and, in some cases, even death. In many of the reported cases, foods prepared outside of the home are the primary cause of foodborne diseases, though it is not uncommon for home-made foods to also cause diseases [9]. Studies conducted on the distribution of foodborne diseases across the world have demonstrated that a majority of these diseases occur during the processing of the food in the preparation stage at home or at food production sites [10]. In fact, most foodborne diseases can be prevented if the regulations governing food safety were complied with, from production stages to consumption [11].

Improper heating of the food, such as undercooking, re-heating and waiting in the heat, or improper cooling of the food account for 44% of the foodborne illnesses. Inadequate preparation and improper cooking practices, such as those involving cross-contamination, insufficient processing, poor hygiene and the re-use of leftovers, are responsible for causing 14% of these diseases [7].

As indicated by these figures, foodborne diseases are widespread throughout the world. The process by which a foodborne disease spreads begins with the features of the disease contaminating the food, which in turn threaten both individual and public health by means of the foods. Healthy, or what can be termed as safe, food is food that has not lost its nutritional value, that is clean, in physical, chemical and microbiological terms and that is not stale. The factors causing the contamination of the food may threaten the safe consumption of it and thereby make the foods harmful to human health. For this reason, it is necessary to utilize various resources to prevent the food from being contaminated at all stages of the food chain, from harvest to consumption [12].

This study conducts an analysis of the factors responsible for jeopardizing food safety and food safety policies throughout the world.

2. The factors that affect food safety

Foods are the basic building blocks of living things, yet they may pose a threat and become harmful to human health in some situations [13]. Many people throughout the world become ill because of the food they eat. These diseases associated with food consumption are referred to as foodborne diseases, and they may result from dangerous microorganisms [14]. Foods can become harmful to human health or even fatal when combined with bacteria, mold, viruses, parasites and chemical toxins [13]. Therefore, it is absolutely necessary that consumers be provided with a safe food supply. The factors involved in the potential threat caused by foods are inappropriate agricultural practices, poor hygiene at any stage of the food chain, lack of preventive controls during processing and preparation of the food, incorrect use of the chemical materials, contaminated raw materials, food and water and inappropriate storage [15].

These issues were classified into three categories: **food hygiene**, **personal hygiene of food handlers** and **kitchen sanita**tion.

2.1. Food hygiene

Many factors serve to undermine food hygiene. The hygienic quality of the foods is negatively influenced by purchasing low-quality or stale foods, storing food in inappropriate conditions, cooking large amounts of food, more than is necessary, and letting it sit in inappropriate environments, storing raw and cooked foods together and preparing, cooking and storing food using incorrect methods [13]. If foods are contaminated at any stage, from production to consumption, the hygiene of the food is compromised, depending on the temperature, humidity and pH values of the environment it is stored in, and the food then becomes potentially harmful to human health. An infection or intoxication caused by the consumption of a contaminated food or drink is called food poisoning [16]. The causes of food poisoning are classified as microorganisms, parasites, chemicals, naturally created food toxins, naturally created fish toxins, metabolic disorders, allergic reactions and radioactive substances [17].

Salmonella, Campylobacter and *Enterohemorrhagic Escherichia coli* (EHEC) are foodborne pathogens that affect millions of people every year. Symptoms of food poisoning caused by these pathogens include fever, headache, upset stomach, vomiting, abdominal pain and diarrhea. Although food poisoning is mostly caused by bacteria, some parasites and viruses can also be factors. Parasites such as *Trichinella spiralis* and *Toxoplasma gondii* can remain alive by using the nutritional elements in the carrier. Viruses such as *Hepatitis A* can behave like parasites and infect people as well as the entire food chain [9,18].

Staphylococcus aureus, Clostridium perfringens, Salmonella, Streptococcus, Shigella, Clostridium botulinum, E. coli 0157:H7, Campylobacter and *Bacillus cereus* are the microorganisms that most frequently cause food poisoning [9,13,18,19].

S. aureus is a gram-positive coccal bacterium about 0.5–1.0 μm in diameter. The optimum growth temperature is 37° C. The normal ecological habitat of *S. aureus* is human body [16]. *S. aureus* can be cultured from multiple sites of the skin and mucosal surfaces of carriers; the primary reservoir of staphylococci is thought to be the nostrils of the nose. Spread of *S aureus* generally is through human-to-human contact. Carriage of *S. aureus* in the nose appears to play a key role in the epidemiology and pathogenesis of infection. In the general population, a mean carriage rate of 37.2% was found [20]. *S. aureus* is conveyed to the food by the person handling it. Persons with skin, nose or throat infections or inflammatory wounds pass this microorganism onto the food. The foods posing a particular risk for containing *Staphylococcus* include cooked meat, potato salad, desserts with milk, such as custard, and chicken, fish and other meat salads [9,13,21]. It causes food poisoning by releasing enterotoxins into food. After 3–6 hours of consuming contaminated food, symptoms begin. The most marked and severe symptoms are nausea and vomiting. The others are stomachache and diarrhea [16]. This bacterium cannot be eliminated by cooking. Therefore, it is necessary to keep foods refrigerated; the use of aprons and gloves by staff reduces skin-to-skin contact and, therefore, the staff should further follow the rules of hygiene and minimize physical contact with food [9,13,21]. For staphylococcal food poisoning, phage typing can be performed to determine whether the staphylococci recovered from the food were the source of infection. Depending on the type of infection present an appropriate specimen is obtained accordingly and sent to the laboratory for definitive identification using biochemical or enzyme-based tests. Furthermore, for

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differentiation on the species level, catalase, coagulase, DNAse, lipase and phosphatase tests are all done [19].

C. perfringens is a gram-positive, rod-shaped anaerobic, spore-forming pathogenic bacterium [19]. *C. perfringens* is very common in nature. Especially, it is found in the digestive system of humans as well as of animals and in soil [13]. This bacterium is the third most common cause of foodborne illnesses in the United Kingdom and United States. According to some estimates, this type of bacteria causes nearly a million illnesses each year. Poisoning occurs after contamination of foods. Cooking kills the growing *C. perfringens* cells that cause food poisoning, but not necessarily the spores that can grow into new cells. If cooked food is not promptly served or refrigerated, the spores can grow and produce new cells [22]. Because the spores of some strains are resistant to temperatures as high as 100°C for more than 1 h, their presence in foods may be unavoidable. Furthermore, the oxygen level may be sufficiently reduced during cooking to permit growth of the clostridia. Spores that survive cooking may germinate and grow rapidly in foods that are inadequately refrigerated after cooking [23]. It easily reproduces in meat that has been sitting at room temperature for a long time after being cooked, in undercooked and repeatedly re-heated meat and in meat served cold [13,19]. *C. perfringens* cells lose their viability when foods are frozen or held under prolonged refrigeration unless special precautions are taken. Such losses may make it difficult to establish *C. perfringens* as the specific cause of a food poisoning outbreak. It is recommended that samples that cannot be examined immediately be treated with buffered glycerin salt solution and stored or shipped frozen to the laboratory [23]. It can be detected in contaminated food [if not heated properly) and feces. Incubation time is between 6 and 24 hours after consuming of contaminated food [24]. Usual symptoms typically include abdominal cramping, diarrhea; vomiting and fever. Very rare, fatal cases of clostridial necrotizing enteritis (also known as pigbel) have been known to involve "Type C" strains of the organism, which produce a potently ulcerative β-toxin. Many cases of *C. perfringens* food poisoning likely remain subclinical, as antibodies to the toxin are common among the population. This has led to the conclusion that most of the population has experienced food poisoning due to *C. perfringens*. Despite its potential dangers, *C. perfringens* is used as the leavening agent in salt rising bread. The baking process is thought to reduce the bacterial contamination, precluding negative effects [25]. This microorganism can be prevented from harming the food by cooling the cooked food rapidly, saving leftover food in shallow containers and storing food in appropriate conditions [13,19].

Salmonella is a rod shaped bacteria and can live in a variety of habitats. Some strains of *Salmonella* live in water, soil, food plants and feces of contaminated humans. Generally the bacterium is able to access those places through cross-contamination of already-infected organisms or feces. When present in water, *Salmonella* can live for several weeks; in soil the bacteria can live up to several years, while in feces the bacteria may only survive a few days. The bacteria can survive in salt water and cooler environments, but a too hot of an environment may kill the bacteria [19,26]. Salmonellosis in humans is generally contracted through the consumption of contaminated food of animal origin (mainly eggs, meat, poultry and milk), although other foods, including green vegetables contaminated by manure, have been implicated in its transmission. Person-to-person transmission through the fecal–oral route can

also occur. Human cases also occur where individuals have contact with infected animals, including pets [27]. Usually, symptoms start 12–72 hours after ingestion of bacteria. It is usually characterized by acute onset of diarrhea, fever, abdominal cramps, nausea and vomiting. In most cases, the illness lasts for 4–7 days, and most people recover without treatment. But, *Salmonella* can cause more serious illness in older adults, infants and persons with chronic diseases [26]. *Salmonella* food poisoning has long been, and continues to be, an important global public health problem. In much of Europe and North America, *Salmonella* is mostly found in raw or undercooked chicken, meat, eggs and fish and in unpasteurized milk. It is very easy to control and can be killed by cooking foods at sufficiently high temperatures. To prevent *Salmonella* contamination, the food should be stored at appropriate temperatures, sanitation and hygiene rules should be followed and rodents and flies should be removed from the work environment [9,13,27].

Streptococcus is a gram-positive and nonmotile bacterium and the name refers to the bacterium's characteristics of grouping in chains that resemble a string of beads. The natural habitat of the bacteria are pharynx, rectum and skin [19]. Certain *Streptococcus* species are responsible for many cases of pink eye, meningitis, bacterial pneumonia, endocarditis, erysipelas and necrotizing fasciitis [18]. *Streptococcus* is found inside the human mouth, on hands and in nose secretions and can be conveyed to foods through contact with these secretions. For this reason, infected persons should not prepare food, and the food should be kept in appropriate conditions after being cooled rapidly [19,21].

Shigella is a nonmotile, gram-negative, facultative anaerobic, non–spore-forming, rod shaped bacterium. It is one of the leading bacterial causes of diarrhea worldwide [28]. *The primary host and natural reservoir known at this point for Shigella* is the human gastrointestinal tract. *Shigella* can survive in fecal-contaminated material but has a low survival rate without the optimal acidic environment in the intestinal tract [29,30]. They can easily multiply between 10 and 48 °C [16]. The optimum growth temperature for this bacterium is 37 °C [30]. There are two different mechanisms for pathogenicity. Firstly, bacterial number increases very fast in intestine and then settles into mucosal entry and colon. Because they may cause leakage of blood into the lumen, bloody diarrhea occurs. Secondly, the production of endogenous toxin, which is known as *Shiga toxin*, results in diarrhea [16]. *Shigella* infection typically occurs by ingestion (fecal–oral contamination); depending on age and condition of the host, fewer than 100 bacterial cells can be enough to cause an infection. Food prepared by the contaminated person may easily become contaminated with *Shigella* bacteria [30]. Symptoms of shigellosis include mild to severe diarrhea, bloody diarrhea, fever, dehydration, nausea, vomiting and stomach cramps. They usually appear between 1 and 7 days after contracting the bacteria [31]. The diagnosis of shigellosis is made by isolating the organism from diarrheal fecal sample cultures. It can infect the food in any environment where hygiene rules are not followed. The most important protective factor against *Shigella* is to follow proper personal hygiene rules [13,18,21]. Hand washing before handling food and thoroughly cooking all food before eating decrease the risk of getting shigellosis [32].

C botulinum is a gram-positive, rod-shaped, anaerobic, spore-forming, motile bacterium with the ability to produce the neurotoxin botulinum [33,34]. The natural habitats of the *C. botuli-*

num are soils and marine sediments throughout of the world. Since it is found in the soil, it may contaminate vegetables cultivated in or on the soil. It also colonizes the gastrointestinal tract of fishes, birds and mammals [35]. *C. botulinum* is not a very common bacterium, yet it is very dangerous when it does infect a person [9,18]. Foodborne botulism generally occurs 18 to 36 hours after exposure [range 6 hours to 8 days]. Initial symptoms can include nausea, vomiting, abdominal cramps or diarrhea. After the onset of neurological symptoms, constipation is typical. Dry mouth, blurred vision and diplopia are usually the earliest neurological symptoms. They are followed by dysphonia, dysarthria, dysphagia and peripheral muscle weakness. Symmetric descending paralysis is characteristic of botulism [35]. There are no fever and no loss of consciousness. The symptoms are not caused by the bacterium itself, but by the toxin produced by the bacterium. Incidence of botulism is low, but the mortality rate is high if prompt diagnosis and appropriate, immediate treatment (early administration of antitoxin and intensive respiratory care) are not given. The disease can be fatal in 5 to 10% of cases [36]. Because it is an anaerobic bacterium, it can only grow in the absence of oxygen. Foodborne botulism occurs when *C. botulinum* grows and produces toxins in food prior to consumption. The growth of the bacteria and the formation of toxin occur in products with low oxygen content and certain combinations of storage temperature and preservative parameters. Canned foods improperly prepared and heated and particularly low-acid foods, such as green beans, spinach, mushrooms, meat and vegetables, are very risky in regard to *C. botulinum* contamination [9,18]. Occasionally, commercially prepared foods are involved. Though spores of *C. botulinum* are heat resistant, the toxin produced by bacteria growing out of the spores under anaerobic conditions is destroyed by boiling (for example, at internal temperature >85°C for 5 minutes or longer). Therefore, ready-to-eat foods in low-oxygen packaging are more frequently involved in botulism [36]. Food inside cans that are deformed or warped should under no circumstances be consumed [9,18]. Prevention of foodborne botulism is based on good practice in food preparation, particularly preservation and hygiene. Botulism may be prevented by the inactivation of the bacterial spores in heat-sterilized or canned products or by inhibiting bacterial growth in other products. Commercial heat pasteurization may not be sufficient to kill all spores, and therefore the safety of these products must be based on preventing bacterial growth and toxin production. Refrigeration temperatures combined with salt content and/or acidic conditions will prevent the growth of the bacteria and formation of toxin [18,35,36].

E. coli is a gram-negative, facultative anaerobic, rod-shaped bacterium [18]. *E. Coli 0157:H7* is a very common bacterium found in the human intestines [9]. Provided resource availability and key abiotic conditions (availability of energy and nutrient sources, pH, moisture and temperature) are propitious, *E. coli* populations can survive and even grow in open environments such as soil, manure and water. There are also possibilities for migration between these habitats [37]. Whether food is prepared at home, in a restaurant or in a grocery store, unsafe handling and preparation can cause contamination. Common causes of food poisoning include failing to wash hands completely before preparing or eating food, using utensils, cutting boards or serving dishes that are not clean, causing cross-contamination, consuming dairy products or food containing mayonnaise that have been left out too long, consuming foods that have not been stored/cooked at the right temperature, especially meats and poultry, consuming raw seafood products, drinking unpasteurized milk and consuming raw produce

that has not been properly washed *[38].* Fecal contamination of water or foods is responsible for causing the infection [39]. It can be conveyed through raw or undercooked minced meat or unpasteurized milk. Infection by this bacterium can cause hemolytic–uremic syndrome, which can cause bloody colitis with severe abdominal pain, bloody diarrhea, nausea and vomiting, and the syndrome can lead to renal failure, brain damage, heart attack, paralysis and even death [9]. People with weakened immune systems, pregnant women, young children, and older adults are at increased risk for developing these complications [38]. Person-to-person contact is an important mode of transmission through the oral-fecal route. The duration of excretion of EHEC is about 1 week or less in adults, but can be longer in children. Visiting farms and other venues where the general public might come into direct contact with farm animals has also been identified as an important risk factor for EHEC infection [39]. To protect against contamination, the meat should be cooked very well and foods that include meat should be prepared in perfectly hygienic conditions [9]. The prevention of infection requires control measures at all stages of the food chain, from agricultural production on the farm to processing, manufacturing and preparation of foods in both commercial establishments and household kitchens [39].

Campylobacter is a gram-negative, microaerophilic, non-fermentative bacterium. It grows best in habitats with an oxygen level lower than 5%, and it is typically found in the intestinal tract of animals. They are able to move via flagella. The optimum growth temperature is 42–45 °C and they cannot proliferate in room temperature [16]. *Campylobacter* is one of the bacteria most frequently responsible for causing gastroenteritis. While its actual incidence is not known exactly, in high-income countries, its incidence ranges between 4.4 and 9.3 in every 1000 people each year *[27]*. The infection can pass through animal-sourced food, particularly those of flying bird species, domestic animals, such as cats and dogs, contaminated surface streams, unpasteurized milk and direct physical contact with infected animals. Infection from this bacterium leads to foodborne diseases usually through uncooked meat and other products and raw or unpasteurized milk. Contaminated water and ice are also sources of infection [39]. *Campylobacter jejuni* can be found in both fresh water and sea water and can live for 5 weeks in this environment. Wild birds, farm animals, farm areas and surfaces of still waters create an ideal ecological system for different types of *Campylobacter*. The presence of this microorganism in nature indicates that fecal contamination has occurred in that area. These microorganisms are unable to survive and reproduce anywhere except for in their hosts, and they die in sunlight. Contamination occurs in the environment and on other animals particularly through the droppings of wild birds, such as ducks, geese and seagulls [21]. The most frequent symptoms associated with this infection are Guillain-Barre syndrome (GBS), reactive arthritis (ReA) and irritable bowel syndrome (IBS) *[27].* To prevent its infection, it is necessary to take protective measures in all stages of the food chain, from production to consumption, and to apply these protective measures in both industrial and domestic environments [39].

B. cereus is a gram-positive, rod-shaped, soil-dwelling, facultative anaerobic bacterium. It can grow between 10 and50 °C, but the optimum growth temperature is 28–37 °C. It can be destroyed at the boiling temperature in 5–30 minutes [16]. *B. cereus* is present in dust and soil and can cause contamination in cereals, particularly those made of rice, in food that sits out

for a long time and in meat products [19,21]. The microbe is able to grow either in the presence or in the absence of oxygen. Its spores are sufficiently heat resistant to survive pasteurization treatment of milk and standard cooking temperatures reached in domestic kitchens. It cannot survive the high-temperature treatment used to process canned foods. The symptoms start after 30 minutes of ingestion. Firstly, nausea and vomiting can be seen and then diarrhetic syndrome generally starts [40]. In case of foodborne illness, the diagnosis of *B. cereus* can be confirmed by the isolation of more than 105 *B. cereus* organisms per gram from epidemiologically implicated food, but such testing is often not done because the illness is relatively harmless and usually self-limiting [41]. To prevent infection, foods should be washed thoroughly, not left at room temperature for a long time after being cooked and not left in an open container on the kitchen floor [19,21].

Giardia duodenalis, Cryptosporidium parvum, Cyclospora cayetanensis and *T. gondii* are the parasites that pose the biggest threat to food safety. *G. duodenalis*, previously called *Giardia lamblia*, and now commonly known as "intestinalis", is a microscopic parasite, which lives in human and animal intestines. It usually infects people through contaminated water and uncooked meat. The parasite attaches to the epithelium by a ventral adhesive disc and reproduces via binary fission. The most frequent symptoms associated with this parasite are diarrhea, abdominal cramps, gas and nausea. Giardia lamblia is difficult to detect, which often leads to a delay in diagnosis or misdiagnosis; several tests should be conducted over a 1-week period. Giardiasis is a global disease that infects nearly 33% of people in developing countries and 2% of adults and 6–8% of children in developed countries worldwide. It is especially important that proper hand hygiene be practiced for protection against this parasite [42].

C. parvum is a unicellular microscopic parasite protected by a shell. It is responsible for causing the disease cryptosporidiosis, also known as "Crypto", and it is the main cause of worldwide diseases originating from water and food. Generally, it infects people through soil, foods, water and infected animal parts that had contact with feces. Its most widespread symptoms are diarrhea, abdominal cramps, upset stomach and mild fever. Some cases, however, may not cause any symptoms. In order to protect against this parasite, proper personal hygiene should be performed, only clean water should be used for drinking and vegetables or fruits that have been fertilized with unprocessed fertilizer should not be consumed [42]. This parasite is transmitted by infected food handlers or processors' contact to the food or by foods produced with using animal feces as a fertilizer. When the parasite is involved, watery diarrhea is commonly seen as the main symptom. There is no known effective treatment method. [17].

C. cayetanensis is a unicellular microscopic parasite that causes cyclosporiasis. Although the incidence of cyclosporiasis is reported to be increasing in many countries, this organism is not very well known. In the incubation stage of the parasite, *C. cayetanensis oocysts* infect people through the consumption of water contaminated with this parasite or by eating anything that has come into contact with the feces of an animal with cyclosporiasis disease. The disease may cause symptoms such as diarrhea, loss of appetite, abdominal ventricosity, nausea and vomiting. To protect against infection from this parasite, it is necessary to perform proper personal hygiene and to wash or peel vegetables and fruits before eating them [43].

T. gondii is a unicellular microscopic parasite that causes toxoplasmosis disease, and it exists all over the world. It is among the top three causes of death from foodborne diseases worldwide. It can infect people through the consumption of uncooked meat or by drinking from fresh water sources, such as lakes or streams. This parasite can also infect the fetus through the mother. Moreover, as cats are major hosts for *T. gondii*, domestic cats should receive proper cleaning and hands must be washed after handling them. Symptoms associated with infection from this parasite include diarrhea, upset stomach, vomiting and abdominal pain [44]. In food-caused toxoplasmosis cases, *T. gondii* infects people through consumption of uncooked or undercooked meats. Especially, this parasite spreads faster by products that are contaminated with cat feces. With the higher consumption of raw meats, it shows increased prevalence in Europe and South Africa. *Toxoplasma* infections can be diagnosed by response of antibodies with serologic applications [17].

Infections caused by microorganisms are largely the result of the poor hygiene of the person responsible for preparing the food. These microorganisms can rapidly reproduce in temperatures outside the safe ranges specified by food safety regulations [1].

Cooked foods should not be left to sit in room temperature for longer than two hours. These foods should be cooled rapidly and kept refrigerated, preferably at a temperature under 5°C. Microorganisms can reproduce very rapidly at room temperature. Temperatures below 5°C and above 60°C cause the reproduction of microorganisms to slow down or stop. Table 1 presents the measures that should be followed to ensure food safety [1].

Step	**Danger**	**Measure**
Supply/Purchase	Contamination of raw foods	Purchase foods from reliable suppliers. Make sure that hygienic conditions are provided and maintained during supply and transportation.
	Contamination of ready-to-eat foods	Purchase foods from reliable suppliers. Purchase foods from companies that apply HACCP* system.
Storage	Contamination	Keep foods in wrapped or closed containers. Perform pest control.
	Reproduction of bacteria	Monitor the time and temperature of storage. Follow the FIFO** principle.
Preparation	Contamination resulting from personal hygiene	Wash hands before touching the food. Prevent cross-contamination by surfaces and containers. Separate cooked foods from raw foods. Use boiling water, especially if the food will not undergo additionally cooking.
	Reproduction of bacteria	Pay close attention to the amount of time foods remain at room temperature.

Step	Danger	Measure
Cooking	Survival of the pathogen	Make sure that the food is cooked well (the food in its entirety should have a temperature of 70°C)
Cooling and keeping at cool temperatures	Reproduction of the bacteria and spores which did not die in high temperature; toxin production	Make sure that the temperature of the food drops below 5°C as soon as possible when cooling it. Do not let foods remain at room temperature longer than two hours. Avoid storing too much food in the refrigerator or in the cool spaces in it. Beware of the thermal agitations in long-term cold storage.
	Contamination by various sources	Wrap the foods appropriately and prevent their direct or indirect contact with raw foods Make sure that the food containers are clean when storing the cooked foods.
Waiting in high temperature	Reproduction of the bacteria and spores which did not die in high temperature; toxin production	Keep temperature of the food above 60°C.
Re-heating	Survival of the bacteria	Re-heat the food properly.
Service	Reproduction of bacteria, production of spores, and toxins	Re-heat the food properly.
	Contamination	Do not touch the food with hands. Serve the food hot. Prevent contact between uncooked foods and unclean containers.
*HACCP: Hazard Analysis Critical Control Point		
**FIFO: First In First Out Method		

Table 1. Measures to Be Followed to Ensure the Provision of Food Safety [45]

2.2. Personal Hygiene of Food Handlers

The food processing stage is one of the most important stages in the food chain, and those responsible for performing the duties involved in this stage assume major responsibilities in the prevention of food poisoning cases [46,47]. The food processing staff should include healthy individuals who do not have any diseases, and they should undergo regular medical check-ups. In addition to being healthy, it is also important that the workers take particular care for their personal hygiene and execute proper food handling behavior. This is especially important because food handlers can cause cross-contamination between raw and cooked foods, and they may jeopardize food hygiene by improper preparation, cooking and storage of foods [47]. A study confirmed by the Food and Drug Administration (FDA) determined that

81 foodborne diseases were caused by foods contaminated via food processing workers [48]. It should be noted that food workers have the power to make a remarkable impact on public health. In reducing the foodborne diseases or food poisoning, the personal hygiene practices of workers at food production sites are a key factor [49]. It is well known that proper personal hygiene is the best way to mitigate the risks associated with contamination by most of the bacteria generally seen as being responsible for foodborne diseases (Table 2) [40].

Pathogen	Foods involved/sources	Prevention
Campylobacter	Unpasteurized dairy, poultry and meats, infected food handler	Cook all foods thoroughly. Use only pasteurized dairy products. Wash hands properly.
Hepatitis A	Water, ice, shellfish, salads, cold cuts, sandwiches, fruits, fruit juices, milk, milk products, vegetables, any food that will not undergo further heat treatment	Purchase shellfish from approved suppliers. Prevent cross–contamination through hands. Ensure food handlers practice proper hand washing, and prevent bare hand contact with food.
Norovirus	Raw fruit, raw vegetables, prepared salads, raw shellfish	Cook foods thoroughly. Wash hands. Use certified shellfish. No bare hand contact with food.
(Staph) *S.* aureus	Ready-to-eat foods, i.e., sandwiches, salads, ham and other meats, potato salads, custards, warmed-up foods; food handlers' infected cuts, throat, nose and acne	Practice proper hand washing and hygiene. Avoid cross-contamination. Keep bare hand contact with foods to a minimum. Prohibit workers who have cuts and lesions from handling the food. Cool foods rapidly.
Salmonella	Undercooked or raw meats, poultry & shell eggs, poultry and egg salads, egg custards and sauces, protein-containing foods, pets and infected food handlers	Avoid cross-contamination. Cool and refrigerate foods immediately. Cook meats / poultry thoroughly. Practice proper hand washing.
Shigella	Ready-to-eat foods associated with bare hand contact (salads, sandwiches, etc.), source: human feces and flies	Practice proper hand washing after using the bathroom. Use only approved water and foods Control flies. No bare hand Contact with food.

Table 2. Pathogens that threaten food hygiene, the foods they infect and preventive measures

In the Codex Alimentarius [50], the topics involving workers at food processing sites and food hygiene were classified under the following titles: **Health status, Illness and injuries, Personal cleanliness** and **Personal behavior**.

Persons who have any disease that may cause food infection or persons who are suspected to be hosts should not be allowed into the food processing site. Workers at food processing sites who have any symptoms associated with infections should be reported to the administration, and they must be examined by a physician [50]. Accordingly, the law that entered into force

in Turkey in 1996 includes the provision: "The staff to be hired to work in the processing of foods and food additives cannot be employed without first providing a medical report. Those who are determined to be carriers should immediately be treated. Those who do not obtain a clean bill of health after completing their treatment should definitely not be employed. The owner or administrator of the workplace is responsible for ensuring that the workplace is in compliance with all of these practices" [51]. Similarly, it is reported that in certain regions in the United States, workers at food processing sites are required to show a health card to their employers [52]]. In the WHO consultation report, routine medical and microbiological examinations of food handlers are not generally recommended, but if food handlers are suffering from an illness that includes symptoms such as jaundice, diarrhea, vomiting, fever, sore throat, skin rash or skin lesions, like boils or cuts, they should report this to their supervisor before starting work [53]. If workers have a sore throat or fever, open cuts or infected wounds, Norovirus, diarrhea, vomiting or jaundice or if they have had contact with someone who has *Salmonella typhi, E. coli 0157:H7, Hepatitis A* or *Shigella,* they should immediately be removed from the food production sites. The employer must take the necessary precautions within 24 hours and report these workers to the relevant institutions. Many studies have recommended that food production sites include health service units and that it was important that workers report their diseases [54–57].

The hygiene practices that should be performed by food processing workers include precise adherence to personal hygiene regulations and the wearing of special, protective attire such as bonnets and gloves to help secure their hygiene. It is important that these clothes be regularly cleaned and cared for [50]. Reports have shown that the lack of personal hygiene among workers at food processing sites was among one of the practices that contributed to food borne diseases and that proper hand washing was the most commonly neglected practice. The practice of improper hand washing may be an important factor in the spreading of foodborne diseases by cross-contamination. It was reported that of the staff working in food production sites, 60% did not wash their hands correctly, and of the foodborne diseases, 25 to 40% were linked to staff working in food processing and food services industries [58,59]. It was also reported that food processing and food service workers were the asymptomatic carriers of the pathogens which caused food poisoning, due to their failure to wash their hands properly after using the restroom [60]. Another study determined that the foods became infected due to improper cleaning of contaminated hands after using the restroom [61]. Aycicek [62] took samples from bare hands and hands with gloves during the food preparation stage (180 in total) and found that the bacteria load on bare hands was significantly higher than that of the gloved hands ($p < 0.05$). The most common bacteria found were *S. aureus* (126/180), *Bacillus spp.* (19/180) and *E. coli* (14/180).

In addition, many studies have reported that workers in the food processing industry did not show enough care in washing their hands properly when necessary and in using protective attire (e.g., gloves, bonnet) [63–66].

The situations when workers should wash their hands are summarized below.

- Before starting to prepare food

- Before touching unpacked foods and clean equipment
- Before serving the foods and touching the clean utensils and equipment
- Before changing tasks from raw meat to ready-to-consume foods in order to prevent cross-contamination during food preparation practices
- After touching any part of the body
- After using the restroom
- After coughing, sneezing or using a handkerchief
- After smoking
- After eating or drinking something
- After touching unclean equipment and tools
- After leaving the kitchen and before entering again
- After washing the dishes
- After touching the handle of the refrigerator, door or any other place commonly used by people during food preparation practices
- After touching working clothes
- Before and after entering areas where foods are kept and stored
- After handling cash
- Before wearing gloves and after taking them off, hands should be properly washed according to hygiene rules [67]

The steps for proper hygienic hand washing are: wash hands and wrists with soap under clean running water, being sure to rub between the fingers; use a nail brush to clean nails; rub arms and wrists with soap and water; soap and rub hands together for 10 to 15 seconds; dry hands with hot air or paper towel and use a paper towel to turn off the tap [63,68]. It is now known that hand-drying methods are as important as hand-washing methods in the prevention of contamination by microorganisms [69]. Studies have demonstrated that among all forms of drying methods, hand dryers posed the greatest threat of contamination. It was found that hand drying machines are less than an ideal alternative for drying hands after they have been washed, as these machines allow the bacteria to be able to spread as far as one meter away; therefore, the use of paper towels should be encouraged [70,71].

In general, humans are the primary source of food contamination, posing a risk to food safety as carriers. Along with the many extraneous factors, such as hands, clothes, accessories, hair and mustache, internally derived factors, such as the breath, spit and wounds, can be sources of contamination. It was reported that food processing workers were capable of spreading 10,000 to 100,000 microorganisms every minute [72,73]. Thus, workers should avoid certain behaviors at the production site, such as smoking, coughing, sneezing, chewing and eating. They also should not wear accessories, watches or hairpins [50].

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2.3. Kitchen sanitation

Another important issue in the provision of food safety is kitchen sanitation. A study conducted in child care centers in the states of North Carolina and South Carolina in the United States found that most kitchens were not in compliance with the FDA's 2009 Food Code [74]. In order to minimize the risk of foodborne illnesses in the production and processing of foods, it is necessary to minimize the risk of contamination in the placement, arrangement and decking of kitchen utensils, to set up the area so that it is equipped to perform maintenance, repair, cleaning and disinfection and to ensure that surfaces and materials in the kitchen are anti-toxic, that the kitchen has control mechanisms for temperature and humidity, if possible, and that effective measures are taken against pests [50].

2.3.1. Kitchen hygiene

Issues related to kitchen hygiene should be addressed prior to even completing the construction of the kitchen. The plan and interior design of the kitchen should be arranged in such a way as to facilitate proper hygiene practices (e.g., protection against cross-contamination) [50].

The kitchen should be constructed with durable materials that are easy to care for and clean. These materials should be free of any substances that can potentially render the food unsuitable for consumption, such as parasites, pathogenic microorganisms and toxins, or raw materials, food components and others substances used in the production of processed products that have been infected by foreign substances [75].

The surfaces should be designed in such a way as to not accumulate dirt, to prevent foreign substances from infecting foods and to not allow the creation of dense liquids or mold. Pests should also be prevented from entering the workplace. Drainages should be easy to clean and prevent pests such as rodents from entering and waste liquids from re-entering back into the kitchen environment [76].

There should be warnings written and hung on the walls of the workplace about the rules the staff should obey and the best hygiene practices to be performed. The staff should be provided with changing rooms that include a sufficient number of lockers to hold both work and civilian clothes. The staff should not keep any food in these lockers [75].

Ventilation systems should be capable of eliminating smoke, odors, soot and evaporation, keeping heat inside and preventing dust, dirt and pests from entering. Filters and other parts of the systems should be easily accessible for cleaning or changing. The kitchen should have natural or artificial lights that are equal to the natural light of the day, and the intensity and color of the lights should not impact the production or the quality of the foods in a negative way. There should be continuous control on humidity and temperature in the food storage sites [76].

To maintain a hygienic kitchen, the continuity of cleaning and disinfection procedures is as important as the layout plan of the kitchen. Therefore, a cleaning and disinfection plan should be developed for the kitchen, and all cleaning and disinfection practices should be done

according to this plan and recorded. The staff should be trained on the sanitation and disinfection of the kitchen [75].

2.4. Equipment hygiene

Equipment that comes into regular contact with foods should be made of material able to be cleaned and disinfected, resistant to corrosion and non-toxic. The equipment should be arranged in a way as to enable it and the area around it to be cleaned sufficiently. When it is necessary that chemicals be used to clean the equipment, the instructions governing the use of those chemicals should be followed. Calibration checks of the equipment and tools should be made regularly, and these checks should be recorded [76].

3. Food safety systems

Effective food control systems are needed to improve the applicability and control of food safety [77]. Currently, the HACCP, ISO 22000 and PAS 220 are the most commonly used internationally approved food safety systems.

3.1. Hazard analysis and critical control points (HACCP)

HACCP was first used in the 1960s by the American Pillsbury company for the purpose of producing "zero defect" products for the US Army and NASA. Later, starting in the 1970s, it began to be used as a reference by the Food and Drug Administration (FDA) in official supervisions. It was adopted by the Codex Alimentarius Commission in 1992 and published as the HACCP international standard for the first time. Since then, the food industry and official authorities have been using it to protect against and control the risks of potential dangers that could threaten food safety [78].

Initially, HAACCP had three principles:

1. Identification and assessment of hazards associated with food products
2. Determination of critical control points to control identified hazards
3. Establishment of a system to monitor the critical control points

The HACCP, as it is applied today, has five starting steps and is governed by seven principles. The starting steps were created by Codex, and they should be completed prior to implementing the seven HACCP principles. The starting steps help to ensure that the HACCP system is implemented and managed in the most effective way possible [79].

The HAACP system is applicable for any company operating within the food chain, regardless of their size. In the implementation stage, the HACCP system should be supported by certain preliminary condition programs. A company interested in implementing this system should already be following the requirements of this preliminary condition program. Preliminary condition programs include national regulations, codes of practice or other food safety

prerequisites. In general, preliminary condition programs involve factories and equipment, staff training, cleaning and sanitation, maintenance chemical control, waste management, storage and transportation [78].

Five starting steps	The seven principles of the HACCP system
1. Assemble HACCP team	1. Conduct a hazard analysis
2. Describe the product	2. Determine Critical Control Points
3. Identify intended use	3. Establish critical limits for each CCP
4. Construct flow diagram	4. Establish a monitoring system for each CCP
5. Conduct on-site confirmation of flow diagram	5. Establish corrective actions
	6. Establish verification procedures
	7. Establish documentation and record keeping

Table 3. HACCP Implementation in 12 Steps

HACCP is an internationally accepted system and in most countries, it is required that companies within the food industry implement this system.

3.2. ISO 22000

In 2005, The International Organization for Standardization (ISO) published a standard for the Food Safety Management System known as ISO 22000. The ISO 22000 system is a combination of preliminary condition programs, HACCP principles and implementation steps defined by the Codex Alimentarius Commission and ISO 9001:2000 standard components. After it was defined, it began to be used in more than 50 countries within 2 years [80].

The basic approach of the ISO 22000 standard is to implement a preventive system that serves to protect consumers from foodborne diseases. This standard controls all the processes in the food chain, including infrastructure, staff and equipment. In business establishments, the Food Safety Management System implementations include production control, product control, equipment control, maintenance, general hygiene practices, staff and visitor hygiene, transportation, storage, product information, training, the selection and evaluation of suppliers, communication and other similar issues [81].

The main goal of this standard is to have a system in place that determines the unacceptable risks that may result from process errors and to secure product safety and consumer health. Food safety supervision over product, design, production and quality control determines and eliminates the potential dangers. The fundamental role of ISO 22000 is not only to provide food safety but also to improve the sensory and nutritional quality of food, and it also plays a primary role in the quality assurance of service practices in industrial production. Lastly, this standard helps to reduce operational losses by instituting a more effective use of resources to increase productivity, and thereby, directs the establishment to a system of total quality [80].

3.3. PAS 220 (publicly available specification)

This standard was created by the major global food producers in cooperation with the Confederation of Food and Drink Industries (CIAA) with the purpose of eliminating the weaknesses of the ISO 22000 food safety system standard. Nestle, Unilever, Danone and Kraft, the sector leaders generally known as "G4", collectively published the PAS 220 standard, which refines the preliminary conditions programs. The PAS 220 standard is applicable for all types of companies and was made available in 2008. It was intended that the PAS 220 standard be used together with the internationally accepted ISO 22000 standard [82].

The content and topics of PAS 220 elaborate on the 10 sub-titles in the ISO 22000 standard and adds 5 of its own, resulting in the following 15 items [82]:

- Structure and placement of buildings
- Placement of work site, buildings and their wings
- Supporting plants (air, water, energy)
- Supporting services, including wastes and sewage
- Adequacy of the equipment, cleaning and preventive care
- Management of purchased materials
- Measures against cross-contamination
- Cleaning and sanitation
- Pest control
- Staff hygiene and workers' lodgings
- Re-processing
- Product recall procedures
- Storage
- Informing consumers about products
- Food defense, biodefense and bioterrorism

4. Good agricultural practices

Today, increasing attention is focused upon the impact farming practices are having on the environment, and there is an increasing emphasis on more sustainable methods of crop production. Systems need to be adopted that are more sensitive to environmental issues, genetic diversity, wildlife and their habitats and in some cases the social structures of rural communities. Furthermore, consumers around the world are more sophisticated and critical

than in the past, demanding to know how and what has been used to produce their agriculturally derived products.

Good Agricultural Practices (GAP) are defined "practices that address environmental, economic and social sustainability for on-farm processes, and result in safe and quality food and non-food agricultural products" by the FAO. The aims of the GAP are as follows:

- Ensuring agricultural production harmless to environment, human and animal health
- Safety of natural resources,
- Ensuring traceability and sustainability in agriculture,
- Improving workers health and working conditions,
- Ensuring safety and quality of produce in the food chain.

General principles for GAP were first presented to the FAO Committee on Agriculture (COAG) in 2003 in the paper "Development of a Framework for Good Agricultural Practices" the annex of which broadly outlined farm-level GAP recommendations in 10 fields; which are "soil", "water", "crop and fodder production", "crop protection", "animal protection", "animal health and welfare", "harvest and on-farm processing and storage", "energy and waste management", "human welfare, health, and safety" and "wildlife and landscape".

Although there are some GAP used by different organizations to succeed different purposes and goals, GLOBALGAP (EUREPGAP) is the widespread certificate in agricultural produce worldwide. GLOBALGAP documents consists of; ISO 9001:2000 Quality Management System, ISO 14000 Environmental management system, OHSAS 18001 Work Health and Safety Management System and ISO 22000 Food Safety Management System principles [83].

5. Conclusion

Food safety ultimately deals with the consumption stage, where the existence and level of the dangers caused by foods are of chief concerns. The observance of rigorous control procedures throughout the course of the food chain is a fundamental necessity, given that risks to food safety can surface in any stage of the chain. Therefore, all parties involved in the food chain share the responsibility for ensuring food safety.

The design of a food safety system involves numerous factors. To begin with, minimum hygiene standards should be determined by laws and regulations, food producers must apply food safety measures and procedures and official bodies must supervise and inspect food industry companies to confirm that they are conducting their operations in a manner consistent with the regulations in force. Food poisoning cases that threaten public health globally occur as a result of the contamination of foods in any stage, from production to consumption. Although the factors jeopardizing food safety seem to be easy to control in theory, studies and current practices indicate that there is still a long way to go in practice.

Author details

Aslı Uçar*, Mustafa Volkan Yilmaz and Funda Pınar Çakıroğlu

*Address all correspondence to: aucar@ankara.edu.tr

Ankara University Faculty of Health Sciences, Department of Nutrition and Dietetics, Ankara, Turkey

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Chapter 2

Risk Factors Influencing Microbial Contamination in Food Service Centers

Antonio Valero, Magdevis-Yanet Rodríguez,
Guiomar Denisse Posada-Izquierdo, Fernando Pérez-Rodríguez,
Elena Carrasco and Rosa Maria García-Gimeno

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Abstract

An improvement of food service centers in recent years has been made based on the implementation of the principles of the Hazard Analysis and Critical Control Points (HACCP) system. Food safety preventive measures have been focused on training of handlers in hygiene practices and on improving the sanitary quality of meals. In Europe, an increasing trend in foodborne outbreaks has been attributed to catering businesses. This fact highlights that the impact of preventive measures in the past few years has not been sufficiently effective as expected. Special attention should be paid to food services destined to susceptible population, such as hospitals, long-term care facilities, or school canteens, because people could be more susceptible to become ill when exposed to foodborne agents. There are numerous relevant factors influencing microbial contamination of foods, according to the preparation method, hygienic sanitary conditions of catering facilities, or food handling, storage, and distribution. In the present chapter, a review of the most significant risk factors influencing microbial contamination of foods in food service centers are described with special focus on those establishments where susceptible population (i.e., children, elderly, immunocompromised people) is present. Besides, potential preventive measures to be considered in that establishments and correct implementation of food safety actions are given to provide useful recommendations to food handlers, food operators, and risk managers.

Keywords: Food safety, catering establishments, risk factors, handlers, microbial indicators

1. Introduction

Some individuals tend to eat out of the home, often at food service operations, such as cafeterias, canteens, fast food outlets, bars, and restaurants [1]. In recent years, the catering

sector has been experiencing an increase in technological innovation in correspondence with changes in consumer habits of the population, transformed by numerous factors and changing lifestyles, demographic trends, and so on, which have increased consumer preferences for healthy, safe, and convenient foods.

Food service or catering industry defines those businesses, institutions, and companies responsible for any meal prepared outside home. These industries include restaurants, school and hospital cafeterias, catering operations, and some other small- and big-scale establishments. A catering food establishment also means an approved food establishment that is serving or preparing food at a location other than its permitted location for a contracted food service event. During these operations, foods are often transported, distributed, handled, and consumed in a short-time framework (often less than 1 week).

The catering sector can be divided into three groups based on the population they serve, the way of working, the technology used, or the food types served (Table 1): commercial (residential and non-residential) catering sector, and non-commercial residential establishments.

Residential	Hotels, guest houses, holiday parks, farmhouses, public houses, bed, and breakfasts
Non-residential	Restaurants, cafes, fast-food outlets, wine bars, delicatessen and salads, bars, take-away outlets, schools catering, and burger vans
Non-commercial residential establishments	Hospitals, residential homes, prisons, and armed services

Table 1. Types of catering establishments

The Hazard Analysis and Critical Control Points (HACCPs) system has been recognized as a useful tool to prevent food contamination in food service establishments. Control measures are mainly focused on training of food handlers in hygiene practices to improve safety of served meals.

Unquestionably, consumption outside home is linked to a fact of modern life that causes some concern among health professionals and food authorities because of the potential adverse effects that may entail for human health. Food poisoning poses a serious problem for public health worldwide, and the most vulnerable population (children, elderly, pregnant, and sick) counts among the most affected.

Data from the World Health Organization assert that about 2.2 million people become daily ill in the world for more than 200 foodborne diseases and about two-thirds of the outbreaks that occur originate in their homes and in catering establishments. Given the higher volume of meals prepared in the later ones together with meals exposure to handling and environmental factor, the risk of becoming ill is expected to be considerably higher [2]. In Europe, an

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increasing trend in foodborne diseases outbreaks has been attributed to catering businesses [3]. This fact highlights that the impact of prevention measures in the past few years has not been sufficiently effective as expected. Important aspects such as the size of establishments and heterogeneity of foods served justify the creation of specific regulations on food safety management, to reduce the risks of foodborne illness.

Moreover, it seems to be necessary to strengthen food hygiene and compliance with HACCP system to prevent food outbreaks. These outbreaks are mainly caused by foodborne pathogens and norovirus. Special attention should be paid to food services destined to susceptible population, such as hospitals, long-term care facilities, or school canteens, because people could be more susceptible to become ill when exposed to foodborne agents. Besides this, the large number of meals served per day could have an impact on public health if prepared foods are contaminated.

In food service environments, various factors may be related to foodborne diseases. According to the Food and Drug Administration (FDA), these factors are the food served coming from unsafe sources, poor personal hygiene, inadequate cooking, improper holding temperatures, or utilization of contaminated equipment. On the other hand, the World Health Organization (WHO) [4] suggested that drinking water could be an important factor related to foodborne diseases. It was already demonstrated that training of food handlers and knowledge acquisition in hygienic food preparation, processing, and distribution of meals is crucial in the prevention of most types of foodborne diseases [5]. The use of normalized questionnaires on good manufacturing practices (GMPs) was achieved to evaluate training of food handlers. It was found that, after implemented, there is a substantial improvement in GMP [6–8]. However, it is recognized that these training activities should be repeated over time to minimize the reluctance of food handlers to apply the acquired knowledge [9].

Legislation in food hygiene at EU level prioritizes control measures to protect public health, making food operators responsible to assure product safety [10]. Implementation of HACCP system in food service operations may increase food safety management. However, given the complexity of the food chain and variety of menus and meals prepared, simplified and flexible self-control measures must be required in most cases to increase efficiency and homogeneity of implemented food safety management systems [7]. Evaluation of microbial risks is crucial to determine food safety of prepared meals [11].

In the present chapter, a review of the most significant risk factors influencing microbial contamination of foods in food service centers will be described with special focus on those establishments where susceptible population (i.e., children, elderly, immunocompromised people) is present. Besides, potential preventive measures to be considered in that establishments and correct implementation of food safety actions will be given to provide useful recommendations to food handlers, food operators, and risk managers.

2. Factors enhancing microbial foodborne outbreaks worldwide

The increase in the global burden of foodborne diseases constitutes a concern to governments and food operators today than a few years ago. Identification of emerging pathogens (or

environmentally adapted) causing life-threatening conditions, introduction of ethnic foods, environmental changes, food security aspects, migrant populations, the ease of worldwide shipment of fresh and frozen food, and the development of new food industries including aquaculture are some of the reasons for this concern. Over the past years, foodborne diseases caused by bacteria, viruses, parasites, and prions have been prioritized by governments of industrialized countries generating substantial media attention.

However, to monitor foodborne diseases, an effective surveillance system at the local, national, and international levels should be implemented. The appearance of multistate outbreaks (i.e., contamination in a commercial product occurring in one country and affecting persons in several other countries, or tourists being infected abroad and possibly transmitting the pathogen to others at home) contributes to the increase in the number of illnesses at international level. Currently, funding sources are not easily available for non-developed countries and regions where most of the information comes from passive reporting mechanisms [12]. In the industrialized countries, a need for funding sources allocated to the improvement of foodborne disease surveillance, and control is required.

Several factors (such as environmental, socioeconomic, chemical, physical, and biological) are influencing on such public concern.

Environmental factors such as weather influence the transport and dissemination of microbial hazards via rainfall and runoff and their survival and/or growth through according to temperature or humidity conditions [13]. It is shown that the increase in climate variability influences on current and future deficiencies in areas, such as watershed protection, infrastructure, and storm drainage systems, thus enhancing the risk of food contamination events. More knowledge is needed about transport processes and fate of microbial hazards to predict risks associated to weather variability. In this sense, application of existing technologies such as molecular fingerprinting to track contaminant sources could be expanded.

On the other hand, despite of the development of novel traceability systems and inspection controls, infectious diseases still remain a leading cause of global disease burden with high morbidity and mortality in non-developed countries. The emerging and re-emerging diseases have been a big impact at socioeconomic and public health levels. Their control requires continuing surveillance, research and training, better diagnostic facilities, and improved public health system. Food safety is of particular concern in a developing country context given the higher incidence of foodborne illnesses and their associated economic and social cost [14]. Furthermore, the economic and social changes associated with development (i.e., urbanization, changes in food production systems, and consumption patterns) could increase the risk of emerging foodborne illnesses. This recognizes the connections between disease and socioeconomic factors such as poverty and malnutrition and the wider economic, social, physical, and cultural environment in which people live [15]. Promotion of trade together with improvement of agricultural and animal practices at primary production will definitely help developing countries to better manage food safety. Improving food safety in international trade would also require numerous policy and technical interventions that include an effective market access; a better analysis of the costs and benefits of global trade rules for developing countries or integration of developing countries into the global economy [16].

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Besides environmental and socioeconomic factors, according to the Center for Disease Control and Prevention (CDC), the absence of control, a key elementary factor along the production chain, contributes to the extension of foodborne outbreaks, some of them affecting to food service centers:

- Producing and harvesting food: lack of quality assurance programs and unsafe agricultural practices.
- Processing foods: absence or undefined inspection systems at industrial facilities; poor processing preservation technologies (i.e., thermal treatments, addition of preservatives, and unsafe food formulations).
- Distributing and preparing foods: absence of food purchasing specifications, untrained food handlers, improper hand-washing procedures and facilities, and lack of food safety education programs for consumers.

3. Residential catering

Institutional food establishments have a key role to public health. Consumers that are generally more likely to suffer from foodborne diseases occupy them, such as children, elderly, sick, or immunocompromised individuals. Their physiological characteristics often require high degree of food safety in the meals served.

According to the European Federation of Contract Catering Organizations (FERCO), approximately 33% of firms or collective organizations currently have a contract with a Contract Catering company. Indeed, this is a sector that represents an alternative to meet the basic food needs of a group of very important people. Among institutional catering companies stand Eurest SA authorities; Serunión S.A; Sodexho S.A. Spain; and Aramark catering services, SL, among others, that allocate their production mainly to hospitals, nursing geriatric, dining, and study centers.

The fact of preparing and serving large volumes of food in a relatively short-time framework involves the use of new technologies for conservation and/or optimization of existing technologies, to ensure the hygienic and sanitary quality and shelf life of food.

Studies related to systems development, maintenance, and transportation of prepared meals cite the refrigerated cold chain, frozen cold chain, hot chain, or vacuum cooking [17]:

- In the refrigerated cold chain, food is abated once drawn up, from 65°C to 10°C in a time not exceeding 90 min, before being stored at 0–3°C. Afterward, food is regenerated until reaching the appropriate temperature before serving.
- In the frozen cold chain, food is placed in a blast chiller given the same procedures above and subsequent cold storage at –18° C until consumption, at which time must be regenerated to its proper serving temperature (65°C).

- In the hot chain, foods are maintained at temperatures above 65°C and should be consumed within a maximum of 12 h.
- In the vacuum cooking, the food is prepared at low temperatures and for a long time. The food is then packaged in a waterproof material, which is not affected by high temperatures. Before cooking, air is extracted and sealed. After cooking, the product is cooled quickly and is cooled, and then regenerated (65°C) before consumption.

Utilization of more traditional food preservation techniques is also common [17] such as industrial pasteurization or chemical preservation (salting, smoking, marinating, and pickling).

Lastly, other preservation technologies are used to reduce the amount of food handling in the kitchen, such as high hydrostatic pressure, the light pulses, dehydration, irradiation, and modified atmosphere preservation, among others [17–19].

4. Health and socioeconomic implications of microbial contamination and its effects on international trade

Provision of safe foods supports national economies, international trade, and consumer confidence, thus underpinning sustainable development. However, globalization and changes in consumers' habits to a more convenient and healthy foods led to increase the awareness of potential and/or emerging hazards for public health. This also triggered a growing consumer demand for a wider variety of foods, thus leading to a more complex food chain.

The consequence of the population growth is an intensification of agriculture and animal production to meet consumer demands. Food safety challenges should consider the potential effect of climate change because temperature changes can modify the risk profile of a given food commodity during the whole production chain [20]. These effects produce a greater degree of responsibility to food producers and handlers to ensure food safety. It should be highlighted that the spread of a localized outbreak can increase largely due to the globalized food chain and international trade. Examples include the contamination of infant formula with melamine in 2008, and the 2011 Enterohemorrhagic *Escherichia coli* outbreak in Germany linked to contaminated sprouts. This multistate outbreak was reported in eight countries in Europe and North America, leading to 53 deaths. Losses caused to farmers and food industries were quantified in US$ 1.3 billion, while emergency aid payments to 22 EU member states were around US$ 236 million [21].

According to data reported by USDA, foodborne illnesses are annually costing the economy more than $15.6 billion. Each year, more than 8.9 million Americans will be sickened by one of the 15 pathogens, with more than 5.4 million of those illnesses due to the stomach churning, but usually short-lived, Norovirus. In EU countries, 5196 foodborne outbreaks, such as waterborne outbreaks, were reported [22]. Governmental authorities should make food safety a public health priority, as they play key role in developing policies and regulatory frameworks. They also are in charge of establishing and implementing effective food safety systems

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that ensure that food producers and suppliers along the whole food chain operate responsibly and supply safe food to consumers.

Among the food commodities causing outbreaks, meat products are the most frequently reported, given the high consumption associated to these products. Vegetable salads are recognized as potential vehicles for enteric pathogens, as they are not subjected to any heat treatment before consumption. In Table 2, notified outbreaks in EU and the United States for produce commodities are reported. Scientific studies highlight the importance of an adequate training of food handlers and implementation of GMPs when elaborating vegetable salads in catering establishments.

Regarding cooked meat products, they are susceptible to be contaminated after heat treatment, during storage, and distribution. This is because they can be subjected to poor handling practices (i.e., slicing, packaging) during preparation in catering establishments. As an example, in Table 3, notified outbreaks in EU countries and the United States associated to consumption of meat products are reported. It is concluded that problems in kitchen design, inadequate handling and disinfection practices, and lack of knowledge on food safety by handlers are the main risk factors influencing microbial contamination.

Year	Vehicle	Microbial hazard involved	Place	Country	Number of cases	Reference
2012	Romaine lettuce	*E. coli* O157:H7	Retail outlets	The United States	58	[23]
2011	Bean sprouts	*E. coli* O104:H4	Multiple places	Multistate EU outbreak	3910	[24]
2011	Romaine lettuce	*E. coli* O157:H7	Retail outlets	The United States	60	[25]
2011	Fresh basil	*Shigella sonnei*	Not available	Norway	46	[26]
2010	Lettuce	*E. coli* enterotoxigénica and norovirus	Catering establishments	Denmark	260	[27]
2007	Shredded lettuce	*E. coli* O157	Processing industries	The Netherlands, Iceland	50	[28]
2007	Alfalfa sprouts	*Salmonella stanley*	Domestic homes	Sweden	51	[29]
2006	Shredded romaine lettuce	*E. coli* O145	Processing industries	The United States	26	[30]
2004	Salad lettuce	*Salmonella newport*	–	The United Kingdom	375	[31]
1994	Iceberg lettuce	*S. sonnei*	Domestic homes	The United Kingdom, Norway	–	[32]

Table 2. Notified foodborne outbreaks in Europe and the United States by the consumption of produce commodities

Year	Vehicle	Microbial hazard involved	Place	Country	Number of cases	Reference
2011	Turkey meat	*Salmonella* Heidelberg	–	The United States	77	[33]
2009	Cured RTE meat	*Salmonella* montevideo	–	The United States	272	[34]
2009	Cooked meat	*E. coli* O157	School canteens	Wales	150	[35]
2008	Not available	*Listeria monocytogenes*	Processing meat industry	Canada	22	[36]
2006	RTE pork meat	*Yersinia enterocolítica* O:9	–	Norway	11	[37]
2006	Fermented sausages	*E. coli* O103:H25	–	Norway	17	[38]
2005	Minced beef	*E. coli* O157:H7	–	France	69	[39]
2002	Turkey meat	*L. monocytogenes*	Processing industry	The United States	54	[40]
2001	Cooked meat	*E. coli*	Butchery	The United Kingdom	30	[41]
–	Minced beef	*E. coli* O157	–	–	732	[42]
2000	Ham	*L. monocytogenes*	–	New Zealand	28	[43]
2000	Turkey meat slices	*L. monocytogenes*	Processing industry	The United States	11	[44]

Table 3. Notified foodborne outbreaks in Europe and the United States by consumption of meat commodities

5. Microbial contaminants of prepared meals in catering establishments

During the whole production chain, there is constant exposure of food to microbial contamination. Therefore, a strict quality and safety food control should be promoted with a view to minimize the incidence of food poisoning.

Undoubtedly, for catering establishments, the HACCP system assesses the condition under which the product was elaborated, determines the main risk factors of food contamination, and manages effective measures to reduce contamination by pathogenic and spoilage microorganisms.

Microbial indicators are able to highlight deficiencies in the hygienic and sanitary food quality. Indeed, their presence at high levels leads to a reduction of shelf life and is probably related to the presence of pathogenic microorganisms.

According to the International Commission on Microbiological Specifications for Foods (ICMSF), microbial indicators do not offer a direct risk to human health. These groups are

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mainly aerobic mesophilic bacteria, lactic acid bacteria, total coliforms, fecal coliforms, enterococci, enterobacteriaceae, *Staphylococcus aureus* and *E. coli* [45]. In the next subsections, the most representative microbial indicators will be described. Besides, *Listeria monocytogenes* is included due to its relevance and presence in a wide range of food commodities as well as for the current EU regulation (No. 1441/2007) [46] where it is included as safety criteria for ready to eat foods.

5.1. Aerobic mesophilic microorganisms

Microbial species belonging to this group are quite heterogeneous and include all bacteria, fungi, and yeasts growing at aerobic conditions. The presence of aerobic mesophilic microorganisms in fresh foods demonstrates the effectiveness of sanitary procedures during processing, handling, and storage before [47].

Ready to eat foods (apart from fermented foods, cheeses, and dairy products) with significant concentration levels of aerobic mesophilic microorganisms should not be considered suitable for human consumption, even if microbial species are not pathogenic.

Generally, contamination occurs because of the use of contaminated raw materials or inefficient health treatments as well as inadequate conditions of storage time and temperature [48].

In general, high levels pose a greater risk of pathogen contamination. Several authors agree that the recommended concentrations for ready to eat foods should be less than 5.0 log cfu/g [49]. However, other guidelines for ready to eat foods such as those proposed by the Health Protection Agency (UK) [50] establish acceptable limits between 6 and 8 log cfu/g, depending on the food type.

5.2. Lactic acid bacteria

Lactic acid bacteria comprise a wide range of microorganisms with common morphological, metabolic, and physiological characteristics. Some of the most representative species are *Streptococcus* spp., *Pediococcus* spp., *Leuconostoc* spp., *Lactobacillus* spp., and *Lactococcus* spp. [51].

In the food industry, they have multiple uses as starter cultures in the manufacture of cheese, yogurt, and fermented meats. They are also recognized as natural antimicrobial agents against foodborne pathogens in biopreservation processes [52]. They represent the predominant group in fermented meat products reaching levels between 8 and 9 log cfu/g during the maturation processes. The most common species are *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Pediococcus acidilactici*, and *Pediococcus pentasaceus* [53].

Despite its protective function, they are able to produce end metabolites that lead to food spoilage and thus shortening its shelf life. Their final levels depend largely on the storage temperature and packaging methods [54]. Deterioration caused by the growth of lactic acid bacteria is shown by undesirable changes in smell, taste, color, and gas production. Some studies have found these changes in vacuum-packed meat products or modified atmosphere products [55].

5.3. Enterobacteriaceae

Enterobacteriaceae are considered as food quality indicators including *E. coli* being mainly related to fecal contamination. Generally, the presence of these microorganisms in foods is closely linked with the implementation of inadequate handling practices, inefficient cooking processes, cross-contamination, inadequate personal hygiene of food handlers, equipment and food-contact surfaces as well as inadequate holding time and temperature conditions [56].

Enterobacteriaceae species are Gram-negative bacteria, aerobic or facultative anaerobic, non-sporulated, mobile or immobile, and being able to ferment glucose and to reduce nitrate to nitrite. Some of the most representative species include *Salmonella enterica*, *Shigella* spp., *Yersinia* spp. (intestinal pathogens in humans), *Edwarsiella* spp., *Hafnia* spp., *Proteus* spp., *Morganella* spp., *Erwinia* spp., *E. coli*, *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., or *Klebsiella* spp. Most of them produce endotoxins and thermolabile and/or thermostable exotoxins. Some *E. coli* serotypes are producing verotoxins and shigatoxins, which are linked to a high rate of morbidity and mortality in humans [57].

Food commodities where Enterobacteriaceae can be found are processed meat products [58], nutritional formulas for infants [59], mixed salads, raw vegetables, and milk/dairy products, among others [60].

5.4. Total and fecal coliforms

Total and fecal coliforms are specific groups within the Enterobacteriaceae family, including species, such as *E. coli*, *Klebsiella* spp., *Enterobacter* spp., or *Citrobacter* spp. These are Gram-negative bacteria, aerobic or facultative anaerobes, non-sporulated, whose optimal growth temperature is around 35–40°C. These food quality indicators are taking part of the intestines of humans and warm-blooded animals and other organisms often located on the ground or plant.

The main difference between total and fecal coliforms is that the latter group ferments lactose at temperatures between 44 and 45°C. The group includes primarily *E. coli* (~90%) with certain *Klebsiella* and *Citrobacter* species. Coliforms are considered a reliable indicator of fecal contamination and are sometimes found in contaminated equipment and utensils, as well as in a wide variety of foods.

Contamination of ready to eat foods by coliforms is commonly attributed to environmental contamination, the use of inadequate hygiene practices, and/or insufficient control of the storage temperature. In the case of thermally treated food, the presence of coliforms is indicative of inadequate treatment or post-processing contamination as they are thermolabile microorganisms [47].

5.5. *Escherichia coli*

Enteropathogenic *E. coli* comprise different serotypes that can be present in contaminated foods. Most of them are able to produce Shiga-like toxins and/or other heat-labile or heat-stable

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toxins that can potentially cause diarrheagenic diseases in humans [61]. Besides, some serotypes of enterotoxigenic *E. coli* can also produce a cytotoxin to Vero cells (VTEC *E. coli*).

Normally, outbreaks caused by VTEC serotypes are of low prevalence (1.2 cases per 100,000 people in the EU) [39]; however, the high infectivity and severity of the disease increase the importance of performing novel research on this pathogen. It is reported that human outbreaks attributed to *E. coli* serotypes were mostly originated from catering services or restaurants [62].

Generally, *E. coli* can be present in animal origin foods (pork, beef, and poultry), water sources, or produce such as cabbage, lettuce, or spinach. They can enter the food chain through cross-contamination or recontamination phenomena [63] or through the irrigation with contaminated water, which may result in the internalization of certain *E. coli* serotypes in vegetables [64].

E. coli O157:H7 was the most studied serotype due to the severity of the illnesses caused and its low infective dose, around 100 cells [65]. However, other non-O157 serotypes have been associated to human infections through the ingestion of risk food products, such as fermented and minced meats or raw milk [66].

5.6. *Staphylococcus aureus*

S. aureus has been reported as a microbial indicator most likely associated to reduced water activity (a_w) foods, such as ready to eat cooked or cured meats [67, 68]. The presence of *S. aureus* is often associated to contamination of raw material, such as poultry carcasses or raw chicken samples [69] or cross-contamination events occurring because of mishandling during processing and storage [63].

Food poisoning is attributed to the ingestion of foods that contain thermotolerant Staphylococcal Enterotoxins (SEs) in doses around 20–100 ng [70]. The staphylococcal enterotoxin A (SEA) is the one most frequently reported. A wide range of environmental factors, such as pH, a_w, temperature, food type, and processing conditions, have been suggested to play an important role on SEs production. Generally, growth of *S. aureus* is necessary for SE production, although this phenomenon does not always accompany growth [67]. Indeed, some published studies consider hazardous *S. aureus* levels from 6 log cfu/g in contaminated foods for SE production [71].

5.7. *Listeria monocytogenes*

L. monocytogenes is a foodborne pathogen causing listeriosis, with high mortality rates between 20% and 30% [72]. It is mainly distributed in the field, soil, contaminated water sources, and decaying vegetation. It is also categorized as a psychrotrophic microorganism, being ubiquitous in food-processing environments. Consequently, *L. monocytogenes* is often found as a post-contamination pathogen in food products like sliced cooked meat products, smoked fish, cut vegetables, or ready-to-eat (RTE) products. Raw chicken, milk, and raw meat are frequently implicated in foodborne outbreaks [73]. The associated high mortality rates to pregnant women and their unborn child, neonates, elderly people, and immunocompromised people

makes that its level in food products should remain low. The Commission Regulation No. 1441/2007 *on microbiological criteria for foodstuffs* states that, for *L. monocytogenes*, in the food category *RTE foods able to support the growth of L. monocytogenes other than those intended for infants and for special medical purposes, two different microbiological criteria are proposed: (i) L. monocytogenes levels should not exceed 100 cfu/g throughout the shelf life of the product and (ii) absence in 25 g of the product before the food has left the immediate control of the food business operator*, who has produced it. Their application depends on the ability of the food operator to demonstrate that the targeted food is able or not to support the growth of *L. monocytogenes* up to the end of the shelf life. Also in the United States, the limit of 100 cfu/g for *L. monocytogenes* that does not support growth of the microorganism in foods is being considered [74].

6. Risk factors associated to microbiological contamination and foodborne outbreaks

Foods can become contaminated during growth and harvesting of raw materials, storage and transport to the industry, and processing into finished products. Recontamination can also occur during transport to retail outlets and before consumption at domestic homes and/or in catering establishments. Contamination vectors are mainly animals, surfaces, environment (air, water), and people in contact with foods (food handlers). Processing conditions, packaging materials, and equipment used can also be contamination sources. Survival of microorganisms on contaminated surfaces could lead to their multiplication at high levels, thus compromising food quality and safety [75].

Animals are important reservoirs of microorganisms, and slaughter of animals could introduce high concentration of microorganisms in food industries. Zoonotic pathogens are normally present on the skin and in the gastrointestinal and respiratory tracts. Pathogens carried on the hands are also a major source of contamination [76].

Airborne contamination represents a significant medium for the microbial transfer to food products. [77] Installation of proper air filters is recommended; otherwise, microorganisms can be present together with dust, debris, or insects.

Water sources are used in the food industry as an ingredient, a processing aid, and for cleaning. Therefore, not to increase both microbial and chemical contaminations, it is important to use decontaminated water (i.e., chlorinated and electrolyzed). Water used in hand-washing facilities can pose a potential risk because of the presence of condensations, leaking pipes, or aerosols. Microorganisms colonizing these surfaces can multiply rapidly if conditions are favorable. Thus, checking the microbiological quality of water is essential to guarantee food safety.

Food handlers can act as vectors for food contamination leading to the transmission of enteric and respiratory pathogens to food, e.g., through aerosol droplets from coughing near the processing line [78]. They can also favor cross-contamination through the skin if hand-washing is not properly done.

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Finally, pests, such as birds, insects, and rodents, are potentially a major contamination problem. Therefore, care must be taken to avoid their entrance into food factories. Facilities should be designed in such a way they cannot live and breed. To do so, appropriate disinsectization fumigation methods must be achieved.

The above factors when combined together may increase the risk of food contamination. According to CDC data, 1527 foodborne disease outbreaks, resulting in 29,444 cases of illness, 1184 hospitalizations, and 23 deaths were reported within 2009–2010. [79] Among the 790 outbreaks with a laboratory-confirmed illness, norovirus was the most commonly reported infection, accounting for 42% of outbreaks, followed by *Salmonella*, with 30% of outbreaks. Outbreaks caused by some pathogens were particularly severe. For example, *Listeria* outbreaks resulted in the highest proportion of persons hospitalized (82%), followed by *Clostridium botulinum* (67%). Among the 23 deaths, 22 were linked to bacteria (9 *Listeria*, 5 *Salmonella*, *E. coli* O157, 3 *Clostridium perfringens*, and 1 *Shigella*), and 1 was linked to norovirus. Regarding European data [22], in 2013, *Campylobacter* continued to be the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union (EU) and has been so since 2005 (214,779 cases) with an EU notification rate of 64.8 per 100,000 population. However, the high mortality rate associated to *L. monocytogenes* was confirmed since 191 deaths were reported in 2013, much higher than deaths associated to *Campylobacter* or *Salmonella* (59 or 56, respectively).

6.1. Risk factors affecting microbial safety of foods in catering establishments

6.1.1. Hygienic food handlers` practices

In production processes, storage, and distribution of prepared foods, the role of food handlers seems essential to ensure food safety, supported mainly on good hygienic practices and implementation of improved self-control measures. The food handler is defined as "anyone who by their work have direct contact with food during preparation, manufacture, processing, manufacturing, packaging, storage, transport, distribution, sale, supply and service."

Then existing laws applied to food handlers are cited as follows:

- Regulation (EC) 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs [10].
- Regulation (EC) 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with the legislation on feed and food law, animal health, and animal welfare [80].

In a food catering environment, hygiene procedures may be improved as part of food poisoning occurs as a result of the risk factors associated with food handling, related to poor hygiene, improper cooking procedures, cross-contamination, or improper storage of food [81].

Handlers sometimes act as vehicles for the spread of indicators or pathogens directly and indirectly through the hands to other food-contact surfaces and handlers. In the United States, the hygienic practices of food handlers are one of the five most important risk factors of food poisoning and about 89% of the outbreaks occur mainly by inadequate hygiene [78].

In certain circumstances, the hands may represent the most important vehicle of fecal and respiratory microorganisms [82]. It has been shown that microorganisms, such as *S. aureus*, *E. coli*, and *S. enterica.*, can survive on the hands if hygiene measures are not sufficiently appropriate. Several studies describe outbreaks of food poisoning associated with catering establishments [83]; these studies indicate that sanitary measures may be insufficient when dealing with consumer food safety.

Ayçiçek et al. [84] evaluated the sanitary measures of food handlers in a hospital central kitchen. They concluded that these measures were insufficient as significant counts of *S. aureus* and *E. coli* in both gloved hands and bare hands were presented when handling food. Specifically, *S. aureus* positive samples were obtained in 70% of the isolates. Other microorganisms, such as *Bacillus* spp. or *Staphylococcus* coagulase negative, were also isolated. Lues and Van Torden [85] attempted to relate the microbiological contamination found in the hands of food handlers and that presented in cloths and aprons. To do this, they visited several retail points in South Africa dedicated to the selling of RTE meat. In the study, it was found aerobic mesophilic counts (hands and aprons, respectively) in 98% and 8% of analyzed samples, total coliforms (40% and 26%), Enterobacteriaceae (44% and 16%), and *S. aureus* (88% and 40%). However, they did not find any significant correlation between the microbial counts, so potential cross-contamination could not be concluded. Besides, they considered that inadequate hygiene could be a potential risk factor in the microbiological contamination of food, as 32% of the hands analyzed presented high counts for total coliforms. Fecal coliforms also prevailed in more than half of the samples from the hands (55.6%) of food handlers of several school canteens in Brazil [8]. The lack of the annual medical examination (51.9%), lack of regular training for handlers (74.1%), and poor hygiene practices (100%) could have an influence of these results, as stated by other authors [86]. Other microbial agents, such as norovirus and hepatitis A virus in humans, can survive in the hands of food handlers when they do not follow good hygienic practices [2]. In this case, contamination is often associated with asymptomatic carriers and direct hand contact with contaminated food. At the same time, contamination of food-contact surfaces is also promoted.

While hand-washing is a quick and simple method, it is also considered by many authors as the most convenient and effective way to reduce foodborne pathogens [82]. If done correctly, it prevents the risk of cross-contamination and the presence of high microbial loads in foods not submitted to intense inactivation treatments.

The principles for hand-washing are universal, though effective reduction of microorganisms depends on the following considerations [82]:

- Origin and level of organic and microbial contamination.
- The use of water power.
- Washing time (15–30 s).
- Type of soap and amount used.
- Degree of exposure to the washing process of the fingers, palms, back, wrists, nails, and subungual region.

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- Frequency and intensity of rubbing fingers and palms during rinsing.

In the light of the importance of maintaining adequate hygiene habits and attitudes of food handlers to ensure food safety, various methods have been used according to legal regulations for guidance handlers' hygiene. The Theory of Planned Behavior (TPB) has been advocated by many researchers to predict determinants of food handler's behavior [87].

However, some studies warn of possible deficiencies in hygiene by food handlers. For example, Green et al. [88] conducted a study to identify factors related to the hygienic practices of food handlers. To do this, they performed an observational study that evaluated the hand-washing and use of gloves of 321 food handlers. The results showed that washing hands and wearing gloves were most frequent in food preparation activities than when handling soiled equipment and direct contact with body parts. They also found that the use of gloves resulted in a decrease in the frequency of washes hands. In Clayton and Griffith [89], the habits of 115 handlers in 29 establishments during food preparation together with their corresponding hygiene measures were analyzed. The results indicated that only 9% of handlers washed their hands after touching their hair or face and 25% washed their hands after handling of contaminated equipment. Lubran et al. [90] examined the frequency of contact of the hands of food handlers with objects and food ready for consumption before the sale, washing hands and changing gloves during food preparation, as well as hygienic measures used for the equipment, utensils, and food-contact surfaces. The study was conducted in nine retail stores of RTE food and found a high percentage (60–80%) of handlers that did not wash their hands when handling food while maintaining contact with other surfaces. Likewise, the hygiene of the hands of food handlers in 15 retail shops was evaluated in another study [91]. Out of the 29 food handlers examined, only 48.3% used soap to wash their hands properly and rubbed hands including washing between fingers, fingertips, and wrists. They also found that most handlers who washed their hands in less than 10 s (41.4%) had slightly higher levels of contamination of aerobic mesophilic bacteria and *S. aureus* than those food handlers who took a longer time for hand-washing. To this end, some studies [82] highlight the importance of rubbing hands and increase in the frequency of hand-washing to have higher efficiency to remove microbial load.

On the other hand, hand-drying is also a very important in the hand-washing stage. Some authors consider most critical is the last washing stage and needs to be implemented correctly to ensure proper hygiene, thus reducing the risk of cross-contamination [92]. According to the study conducted by Michaels et al. [93], effective hand-drying may reduce microbial population up to 90%. As result of inappropriate drying practices, residual moisture of hands, drying hands with sheets of cloth, or inefficient air dryers that lengthen the drying time are encountered [94]. It should be pointed out that rubbing hands during drying could promote skin contamination after washing. In an observational study by Clayton and Griffith [89], it was found that the effectiveness of hand-washing was affected by a high percentage of inappropriate drying practices (61%).

6.1.2. Availability of health resources

Obviously, the availability of resources and the functioning of health facilities (sink, hot water, soap, etc.) in the catering establishments constitute also another factor that may adversely affect the personal hygiene practices of food handlers [94]. Another study aimed at evaluating

the implementation of the HACCP system and knowledge of hygiene and food safety as well as food handlers' attitudes and practices in 20 establishments in Spain. [95] revealed that the difficulties encountered were improper maintenance of sinks and showers in 95% of the establishments visited, as well as the availability of hot water in bathrooms and changing rooms (50%). Likewise, another study evaluated 123 food retail outlets in the United States to investigate the knowledge and hygienic practices of food handlers, as well as the availability of resources for hand-washing [96]. The results indicated that the main constraints were linked to the absence of brushes to wash the nails (38%) and insufficient sinks (24%) concluding that only the fully equipped establishments (55%) had a properly trained supervisory staff.

6.1.3. Gloves

It is already demonstrated that hand-washing does not always guarantee complete removal of microorganisms. Thus, the use of gloves is necessary to prevent direct hand contact with food and food-contact surfaces as a measure of increasing food safety and minimizing risks of cross-contamination in the food industry. The most important issue is that sometimes the use of gloves can create a false sense of hygiene among handlers [97] and jeopardize the food safety.

It should be noted that the irregular change of gloves as well as their improper use could enhance cross-contamination. In the United States, this is attributed as one of the main reasons that favor the occurrence of food poisoning [98]. In another study [99], it was found that the use of the same pair of gloves for handling different foods increases the risk of transfer of pathogens. In this case, they found an increased risk of transfer of *L. monocytogenes* from contaminated raw chicken to cooked meat slices. In turn, if gloves are changed, this risk was minimized. Besides, not only the frequency of gloves changing but also their integrity is highly important to avoid cross-contamination. Some authors state that washing hands before and after the change of gloves is highly desirable to reduce this microbial contamination [81]. Even the process of changing gloves is fraught with hazards, because many glove materials cause excess moisture build-up, causing difficulty in disinfecting contamination from the nail region to spread all over the hand. However, gloves should be changed regularly because the risk of transmission of foodborne pathogens could be higher from dirty, unchanged gloves than from bare hands.

The pros and cons of using gloves are extensively explained [97]. However, it should be highlighted that a proper glove change must be done because their efficiency as physical barrier for microorganisms is limited over working time.

Besides this, physical properties of gloves (tensile strength, flexibility, resistance to puncture, and tears), material used (natural rubber gloves, vinyl, nitrile, polyurethane), and glove features (single use vs. multiuse, powder-free, allergenic reactions, etc.) should be primarily considered in the quality control system as they have a great impact on pathogens transmission from food handlers to prepared meals.

Gloves can be made of different types of material, according to their use and physical properties. Polyethylene copolymer gloves could be the least expensive of all glove types. Density materials are quite variable, and they usually have a loose fit. They are built for using in short-

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time periods, and some glove types contain antibacterial compounds. However, their use is quite limited in comparison to other gloves.

Vinyl gloves (PVC) can be used as an alternative to latex gloves being more resistant to heat damage. However, they are susceptible to being torn with snag on nail edges. They also have a limited use in food industries because of their short shelf life.

Nitrile gloves were also developed to replace latex gloves. They are less elastic but have a longer shelf life. They are more resistant to chemicals but sensitive to alcohol degradation. Although they are sensible to be torn, their garish color helps them to be distinguished within a food lot.

Natural rubber latex gloves are most commonly used because they are most cost-efficient and comfortable. They provide good tactile sensitivity and good dexterity. However, latex and chemical compounds added to the gloves can produce allergies and migration of particles to food, especially in the presence of bleach.

In summary, according to the intended use, convenience, and cost-effectiveness, glove material should be carefully chosen. However, it has been demonstrated that regardless of the gloves used, handling practices and gloves changing are critical steps that influence microbial transfer to foods.

6.1.4. Cross-contamination

As mentioned above, the cross-contamination is a major cause of food poisoning worldwide. Cross-contamination phenomena arise as a consequence of the application of inadequate hygiene practices, contact with contaminated equipment and utensils, by direct hand contact with foods prepared by the improper storage of food, bad processing food, by direct food contact with air or contaminated environments, and so on [94]. Recontamination routes and sources (e.g., raw materials, food contact surfaces, food handlers) were revised [100] demonstrating their relevance to foodborne disease outbreaks. These information sources should also be incorporated in Quantitative Microbiological Risk Assessments (QMRAs) to perform mitigation strategies and reduce foodborne disease [101].

Particularly, RTE foods are highly susceptible to be contaminated during handling. Some studies refer to the direct contact of the RTE food (e.g., meat) with food-contact surfaces and contaminated cutting utensils [58]. It is also emphasized its importance as a source of transmission of enteric pathogens, *L. monocytogenes*, or *S. aureus* [102].

6.1.5. Food storage

Temperature is the most important factor that governs microbial growth in food. Most microorganisms grow at temperatures between 5°C and 60°C (called danger zone), being the optimum growth temperature at 37°C. Thus, maintaining the cold chain and a correct heat treatment for hot foods are essential measures to maintain food safety, where food handlers play a key role throughout the production chain.

Table 4 presents the recommended temperature storage/transport and freezing of raw materials and finished products in catering establishments.

Maintaining food to inadequate time intervals/storage temperature often constitutes one of the most common risk factors for food poisoning. Previous studies indicate that in school canteens and other related catering establishments exposure to abuse refrigerated temperature for extended periods of time could lead to an increase of pathogens at hazardous levels for human health from preparation to the distribution thereof [103]. Other possible deficiencies are related to temperature control storage of raw and processed ready for consumption, lack of knowledge of food handlers about cooking and refrigeration temperatures suitable to prevent the growth and survival of microorganisms, inadequate cooling and warming food and preparing several hours before consumption [104], or joint cooling of raw and cooked foods [105].

Food product	**Temperatures**	
	Storage/ Transport	**Freezing**
Meat	≤7°C	≤–18°C
Meat wastes	≤3°C	≤–18°C
Broiler meat	≤4°C	≤–18°C
Minced and mechanically separated meat	≤2°C	≤–18°C
Meat preparations	≤4°C	≤–18°C
Ham, cooked meat, deli meats	0–5°C	≤–18°C
Prepared meals to be consumed within 24 h from preparation	≤8°C	
Prepared meals to be consumed after 24 h from preparation	≤4°C	
Prepared meals (frozen)	≤–18°C	
Hot meals	≥65°C	
Frozen fruits and vegetables	≤–18°C	

Table 4. Recommended storage temperatures of raw ingredients and prepared meals

6.1.6. Training of food handlers

Training of food handlers has been considered an important measure as a part of the HACCP systems, given that it helps to prevent most foodborne diseases. Although knowledge alone is not enough to change practices, food handlers with adequate knowledge can change their practices easier if they are closely supervised and supported by their onsite managers. In addition, guidance and supervision by their managers during work improve attitudes and practices [106].

For some time ago, questionnaires or "checklists" have represented an effective tool to evaluate the level of knowledge and skills on food hygiene and safety of food handlers [98]. Neverthe-

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less, further studies detailing more sophisticated methods to obtain a greater quantity and reliability of information to improve the training of food handlers are needed.

Additionally, novel strategies leading to more effective training methods have been performed. For example, some studies proposed to strengthen the training of less experienced food handlers and validate the knowledge of those more experienced in a period not exceeding 10 years [107]. In this sense, a better knowledge on food safety by food handlers ensures better performance and motivation [108]. In this sense, it is highly important to food handlers in the HACCP systems companies to correct their attitudes and behavior at work. Also, a periodic training is found as an effective way to raise awareness of food handlers [9].

Training of food handlers in food hygiene is a mandatory requirement for the food industry. At EU level, requirements on food safety and hygiene procedures are stated in Regulation (EC) 852/2004 [10].

Specifically, in its Annex II (Chapter XII), the food business operators must ensure the following:

- "Supervision and instruction or training of food handlers in food hygiene matters, according to their work."
- "That those who are in charge of the development and maintenance of the procedure referred to in paragraph 1 of Article 5 of this Regulation or the application of relevant guides have received adequate training in regard to the application of the HACCP principles."
- "Compliance with all requirements of national legislation concerning training programs for persons working in certain food sectors."

Recently, the European Federation of Food, Agriculture and Tourism (EFFAT) and the FERCO launched the project "Food hygiene training for all" [109]. The project is based on the development of a software tool for basic training of food handlers of the contract-catering sector across Europe. This tool is available online free of charge (www.contract-catering-guide.org/food-hygiene-training-for-all) and aims at obtaining a better qualification of workers in catering establishments and also offers the opportunity for training staff in those companies that do not have sufficient resources to invest in training.

6.1.7. Intervention strategies against microbial foodborne outbreaks

The burden reduction of foodborne diseases is a major goal of societies. The strategies developed by countries to achieve this goal are numerous and very different depending on issues, such as political and socioeconomic status, actual or emerging pathogens, resources, trade (import/exports), temporal limitations, and inter-regional cooperation.

Woteki and Kineman [110] described different approaches to reducing foodborne illness and grouped them into four categories: (i) population surveillance and better outbreak detection, (ii) prevention-based regulatory approaches, (iii) information and education, and (iv) risk-based system.

Early identification of foodborne outbreaks and the implicated organism should be directed to controlling the outbreak, stopping exposure, and perhaps more importantly, preventing future outbreaks [111]. Also, a rapid and coordinated response is needed among state officials and federal agencies. Some authors [111, 112] pointed out that surveillance based on molecular analysis of foodborne pathogens involved in outbreaks and sporadic cases together with the creation of a platform to share this information would allow for anticipation of potential future episodes. In this sense, Fisher et al. [113] reported the creation in the EU of platforms where data and information on potential outbreaks of foodborne pathogens are available and can be disseminated rapidly to those who need to know; the Enter-net is a surveillance network database of bacterial enteric pathogens, while Salm-gene is a molecular typing network. In the United States, similar platforms are available [110], i.e., FoodNet, a system of disease surveillance that provides information on the incidence of foodborne illness, and Pulse-net, a common name for National Molecular Subtyping Network for Foodborne Disease Surveillance.

Regulatory agencies allow food business operators to set up performance standards in the industry through the well-known HAZARD plan. The HAZARD plan should be designed upon the analysis of the likely hazards in the food and the strategies put in place to eliminate them or to reduce them to acceptable levels [114]. This system has shown to have a very positive and crucial impact on food safety, thus on public health, as has been recognized by key organizations like the World Health Organization [115]. Special attention deserves establishments, which deliver meals to a large number of people, and even more, in those centers where there is an important proportion of consumers with a weak or impaired immune system like hospitals or nurseries. Unfortunately, in the past years it has been reported some cases where the HACCP plan was not fully implemented. This is the case of the study by Kokkinakis et al. [116], who reported that only two out of the seven major hospitals interviewed in Crete (Greece) had implemented the HACCP plan during the period of 2004–2009. These authors identified 14 crucial elements for HACCP implementation in hospitals. Shih and Wang [117], in their study on factors influencing HACCP implementation in 23 public hospitals in Taiwan, revealed that the most important concern perceived by managers was related to economic issues, i.e., "getting funds from the hospital" and "difficulty of allocation funds for facility improvement." In addition, it was shown that more support, HACCP training, and coordination with other hospitals were necessary to avoid staff reluctance to implement the HACCP plan. Shih and Wang [117] also pointed out that kitchen design and flow charts of food production are the first two issues to consider before the HACCP implementation. The lack of financial support and poor HACCP training were also reported by Garayoa et al. [95] in their survey directed to staff from 20 contract catering companies throughout Spain.

In the food industry, emerging and existing technologies should be assessed in terms of food safety [118]. However, a new concept of food safety arisen in the early 2000s, with Regulation 178/2002, *laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety*. In this document, risk analysis was introduced as the pillar on which food law should be based on the aim to achieve the general objective of a high level of protection of human health and life. This risk-based approach would enable the setting of national and international targets for disease reduction as well as provide the basis for such reduction efforts [110, 119].

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Education and information of consumers are highly relevant to prevent outbreaks, and above all, sporadic cases. The World Health Organization launched in 2001 an educational campaign called "Five keys to Safer Foods," where five brief and clear messages were given to food handlers: (i) keep clean, (2) separate raw and cooked, (3) cook thoroughly, (4) keep food at safe temperatures, and (5) use safe water and raw materials (WHO, 2006). Other campaigns like Fight bac™ [120] or Thermy™ [121] aimed at getting consumers informed about hygienic food handling practices the former, and the use of thermometers in the cooking of food products the latter. Other most recent campaigns like "The chicken challenge" clearly show short messages with the objective of cutting *Campylobacter* food poisoning in half by the end of 2015 [122].

The reduction of foodborne illness incidence is a challenge for governments, which should manage the different strategies to lower the risk posed by food hazards up to acceptable levels. Current knowledge and tools on risk assessment allow for science-based decision-making.

7. Conclusions and recommendations

By following a systematic approach in assessing risks from production to serving food safety managers will better define the control measures to be adopted in catering settings to prevent foodborne infections. GMPs and HACCP principles should be followed together with special training of food handlers. Although microbiological quality of prepared meals is often satisfactory, special care should be taken regarding indicator microorganisms or prevalence of pathogens such as *L. monocytogenes*. Control of time and temperature along the food chain might prevent microbial growth until risk levels. Other measures such us excluding key high-risk foods to the most susceptible population (i.e., children, elderly, immunocompromised people) would also be advisable.

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Author details

Antonio Valero*, Magdevis-Yanet Rodríguez, Guiomar Denisse Posada-Izquierdo, Fernando Pérez-Rodríguez, Elena Carrasco and Rosa Maria García-Gimeno

*Address all correspondence to: avalero@uco.es

Department of Food Science and Technology, University of Cordoba, Cordoba, Spain

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Chapter 3

Risk Factors and Outcomes of Food Poisoning in Africa

Ntambwe Malangu

Additional information is available at the end of the chapter

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Abstract

Food poisoning is one of the common health problems in most African countries. This review was conducted to describe the situation in Africa with regard to specific risk factors and outcomes of food poisoning in the African setting based on published literature.

It is noted that food poisoning in Africa involves the staple foods used throughout the continent, but due to lack of surveillance systems, the actual burden of food poisoning is unknown. While the general mechanisms of food poisoning apply to the African settings, inherent issues specific to the African context include macro-environmental issues such as infrastructural problems, inadequate policy environment and lack of regulatory food control systems, leading to unsafe foods being sold in the streets. In addition, individual sociodemographic factors, including the age, gender, health status and lack of food safety knowledge among the population and food handlers and preparers, as well as the country's contextual factors such as unsafe harvesting practices, are all associated with the occurrence of food poisoning in Africa.

Also due to a lack of surveillance systems and population-based studies, the outcomes of food poisoning are not adequately documented. It is clear that food poisoning is responsible for a significant number of health facility visits and hospitalisations as well as deaths. Moreover, several long-term effects such as cancers and other conditions affecting children, pregnant women and the elderly have been also reported.

To conclude, food poisoning is one of the major causes of morbidity in Africa. Several risk factors influence its occurrence. It is recommended that the control systems that are lacking should be instituted and that public education should be conducted so that hygienic and safe food handling practices can be implemented. Moreover, the preservation of the sources of food, namely the fauna and flora ecosystems, as well as environmental media, namely air, soil and water, should be considered not only as a necessity but as a shared responsibility that each human being should accept every day in the way he or she lives, make decisions and eat.

Keywords: Food poisoning, risk factors, Africa

1. Introduction

1.1. Definition and mechanisms of food poisoning

Food poisoning is a term applied to an acute intestinal disease acquired by the consumption of food or drinks (e.g. juice, water, wine). The toxic agents responsible for food poisoning include the following: microorganisms that occur naturally in humans, animals and/or the environment; parasites, in particular intestinal worms and others that can be transmitted through contaminated food and water; contaminants, adulterants and poisoning agents that can occur in food through normal modes of contamination or by deliberate addition; naturally occurring toxins that occur in food naturally or produce toxins inside the food through normal biological processes; agro-chemical and veterinary drug residues as a result of their purposeful use; spores and prions such as the bovine spongiform encephalopathy or its human variant responsible for the Creutzfeldt-Jakob disease; persistent organic pollutants that accumulate in soil, plants, animals and the human body; and heavy metals such as lead, mercury, cadmium and others; and various allergens [1–5].

1.2. How does food poisoning occur?

Food poisoning is often confused with food allergy and food adverse effects, which are, respectively, an immune-mediated reaction and a clinically abnormal response, attributed to an exposure to a food or food additive [1, 6]. Food poisoning results from exposures to toxic agents present in the food that may lead to harmful effects based on the reaction of the body to these agents or the food itself. Exposures may happen through ingestion, contact or transdermal, or inhalation. The resulting effects of the exposure may be localized, or generalized; they may also be topical or systemic [7, 8].

At the core, the adverse functional or morphological changes observed upon clinical or histopathological examinations are almost invariably a consequence of biochemical lesions [9]. This means that, in general, toxicity arises from interaction of the offending toxic element or its derivatives with molecular sites of the host system that leads to the derangement of the biochemical processes involved in the normal function and regulation of the cells, tissues, organs and systems of the body. Hence, it is the overloading of the biochemical processes, because of the simple presence or the excessive quantities of the offending toxic agent, beyond the capacity of the host systems to adapt and restore to these processes at their normal level that leads ultimately to cell, tissue, or organ injury or the elucidation of the toxic effects as signs and symptoms [10–12].

The most common microorganisms involved in food poisoning are bacteria such as *Bacillus cereus*, *Staphylococcus aureus*, *Clostridium botulinum*, *Vibrio cholerae*, *Escherichia coli* and *Shigella* species and others that produce toxins that cause foodborne intoxications. *Listeria monocytogenes* can grow at temperatures below 5°C and so can multiply in refrigerated food but can be eradicated by thorough cooking and by pasteurization. Other microorganisms such as *Norovirus* and *Rotavirus* as well as parasites such as *Giardia* spp. and *Cyclosporidium* spp. can also be involved [13]. The main reason why microorganisms predominate in causing food

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poisoning is that they have the ability to grow in the foods and to dwell there even when reduced to spores, particularly bacteria. Moreover, even when bacteria do not grow in the food itself, they may be carried by the foods that are notoriously known to be involved in outbreaks of dysentery [14, 15]. It is important to note that most foods carry microorganisms, but some foods are more prone in being potential carriers of food poisoning microorganisms; these foods are principally raw meat, poultry, milk, seafood and raw vegetables [16, 17].

The microorganisms inducing food poisoning are often associated with the gut of humans, animals and birds. *Clostridium perfringens* forms part of the normal gut flora and is widely present in food and the environment. Its spores can survive heating to standard cooking temperatures particularly when food is kept warm for prolonged periods after cooking; its effects are mediated by an enterotoxin produced by the bacterium after ingestion. It should be noted that any food contaminated with faeces has the potential to cause food poisoning as well as contact with ill animals [18, 19]. It should be noted that *Campylobacter* spp. is the commonest reported food poisoning caused by bacteria worldwide. It typically causes fever, diarrhoea and abdominal cramps. The infection is often contracted by eating undercooked poultry, unpasteurized milk, untreated water and food that has been contaminated. Two species account for the majority of infections: *Campylobacter jejuni* and *Campylobacter coli* [20, 22].

Raw meat is often a direct food poisoning hazard as it contains microbes; it is also an indirect factor of food poisoning through cross-contamination of cooked meats, other foods and water. It has been established that raw meat can contain *Salmonella* that may affect food handlers or they may transfer to other foods. It is also known that *Salmonella* may grow in meat products kept at room temperatures; products such as meat pies, sausages, cured meats (ham, bacon and tongue) and sandwiches are prone to this effect as they are normally allowed to stand at room temperatures during or before being processed or cooked [23–25]. Besides meat, water, a major component of most foods and drinks, is often contaminated as explained above or it may be polluted from its sources and thus carry with it substances that may cause food poisoning [26–28].

Furthermore, substances released in foods by insects, bacteria or other living microorganisms, hence called "toxins", may also cause food poisoning. This is well documented in the case of mushroom toxins and ciguatera fish poisoning. With regard to fish, the toxin is produced by a marine alga *Gambierdiscus toxicus*. Ciguatera fish poisoning occurs when toxins created by dinoflagellate microorganisms are passed up the marine food chain and bioaccumulate in large fish such as barracuda, grouper, red snapper, kingfish, coral trout and others [29, 30]. It is important to note that these toxins are not destroyed through cooking, smoking or freezing, they are odourless and tasteless and do not alter the appearance of the fish.

Another type of fish poisoning is scombroid poisoning, which results from improper handling and refrigeration of fish containing high levels of naturally occurring histidine. Contamination with bacteria will convert the amino acid histidine into histamine, causing symptoms similar to allergic reactions, which occur very rapidly; the symptoms include headache, abdominal cramps, diarrhoea, itching, flushed face, dry mouth, heart palpitations and difficulty breathing. Scombroid poisoning affects fish from the Scombridae family, such as tuna, mackerel, skipjack and bonito. It can also affect other species, such as herring, bluefish and sardines [31–33].

The other type of fish poisoning is shellfish poisoning that is associated with species found in temperate and tropical areas, such as oysters, clams, cockles, mussels, scallops, crabs and lobsters. These fishes ingest toxins produced by dinoflagellates and produce diarrheic shellfish poisoning, neurotoxic shellfish poisoning or even paralytic shellfish poisoning, which is characterised by the numbness of face arms and legs, loss of coordination and dizziness. In severe cases, respiratory failure and paralysis may occur, leading to death; sometimes, amnesic shellfish poisoning occur, which produces seizures, muscle weakness or paralysis and disorientation. Permanent short-term memory loss has been observed and, in severe cases, can lead to coma or death [30, 33].

Finally, puffer (Fugu) fish poisoning is caused by a tetrodotoxin accumulating mainly in the liver, intestines and ovaries of puffer fish, ocean sunfish, globe fish and porcupine fish. Symptoms of poisoning include profuse sweating, salivation, headache, hypothermia, heart palpitation as well as neurological symptoms, such as numbness, loss of coordination, tremors and paralysis [29].

Chemicals such as pesticides, fungicides, preservative agents, food additives, colorants and taste-enhancing or altering chemicals may also cause food poisoning [34]. Some pesticides such as lindane and thiosulfan have been covertly and illegally used to harvest fish from rivers in some countries. Similarly, carbofuran was used to harvest birds for the bush meat trade in some countries in East Africa. The poisoned fish and bush meat are sold to unsuspecting customers who may be subsequently poisoned [35–38].

It should be noted that some of these chemicals found in foodstuffs in small doses but taken more frequently can lead to chronic poisoning, whereas the others when ingested in high doses may result in acute food poisoning. This occurs when recently treated and unwashed fruits or vegetables that are contaminated with pesticides are eaten [39, 40].

The above remarks suggest that in most cases, the exact mechanism of toxicity is not known, but the time between the exposure and the resulting toxic effects determines whether an exposure is acute or chronic. In an acute poisoning incident, the effects occur almost immediately after an exposure or conventionally within 24 hours. When the effects of toxicity appear or become apparent after 1 day to several years later from the actual date of first intake of the offending agent, this is referred to as "chronic poisoning" [41].

Furthermore, the exposures may be deliberate or accidental. Deliberate exposures occur when the victims expose themselves to toxic agents with the intention to harm themselves or to seek attention from the people close to them [41]. This could happen when a person decide not to preheat or warm the food before eating with the very intent of falling sick to get the attention or sympathy of their loved ones or avoid performing certain acts. This kind of exposure results in suicide or para-suicide depending on whether the victim had or did not have actual intention to die. Other instances are simply bizarre like the old practice of adding tablets of lead to wines, despite knowing the dangers of acute lead poisoning [42–45].

On the other hand, food has been used to intoxicate other people. This deliberate exposure whereby the manipulation of food or drink by a third party, whether adulteration, contamination or addition of high doses of a chemical, drug or other substances in order to harm the

intended victim is usually done for criminal or emotional purposes [46]. Motivations for criminal poisoning include the desire to rob, to punish someone, to benefit financially, get political power, to end the miserable life of a loved one, to eliminate an enemy or to simply to terrorise [46–50].

For instance, it is well accepted that the Emperor Claude was poisoned by his wife, who gave him a meal of poisonous mushrooms of *Amanita phalloides*, which contain amanitin polypeptides, so that upon his death, his son, Nero could become the king. Amygdalin, a glycoside yielding hydrocyanic acid (HCN) through hydrolysis, is present in the family of Rosacea species such as in certain seeds of apples, cherries, peaches and plums. HCN is a chemical that inhibits the action of the enzyme cytochrome oxidase and prevents the uptake of oxygen by cells. As little as 0.06 g can cause death in humans; hence, it has been reportedly used as an assassination weapon in the famous killing of the Russian monk, Grigori Yefimovich Rasputin [48, 51].

Some would resort to criminal deliberate intoxication in order to get attention as in the case of Munchausen syndrome by proxy [52, 53]. Many authors have reported on drug-facilitated sexual assaults where a variety of medicines and drugs of abuse have been added in drinks or foods/meals of unsuspecting victims (Gilfillan, 1965; Douglas et al., 1992; Uges, 2001; Weber et al., 2009) [54–56]. The author is also aware of cases where food poisoning has been perpetrated with the intent to kill. In one dramatic case, a woman crushed a glass bottle and added the fine particles in the porridge and gave it to the three children of a widowed man she wanted to marry. The two kids younger than 5 years old died due to severe intestinal haemorrhages; the girl who was 7 years old survived as she did not eat much as she sensed that something was not right with the porridge.

Accidental food poisoning occurs when the victims unwittingly ingest the offending food or drink with no intent or expectation of harm [57]. This occurs due to negligence, lack of knowledge or simply as a random event. Sometimes it might be due to a confusion of pills looking as candies or edible legumes with their look-alike toxic species. Such a case happened in South Africa, where the author attended to children aged 4 to 8 years old who had ingested colourful multivitamin sugar-coated tablets and colourful chlorpromazine tablets that found at municipal dumpsite. They had mistaken them for candies and ate or swallowed them; two of the seven children died.

Similarly, there was an outbreak in Cambodia where adult villagers confused the edible *Melientha suavis* with the non-edible and toxic *Urobotrya siamensis*, both plants from the Opiliaceae family [58]. In other settings, mass poisoning incidents have occurred through eating of birds carrying a toxic substance from plants. This happened in Algeria more than 50 years ago. Edmond Sargent, one of the leading African toxicologists, proved through experimental study that the poisonous birds or pigeons of Algeria had been carriers of cotinine, a poisonous alkaloid that is not harmful to the birds but to the humans who ate these birds [59, 60].

Other famous cases include the mass intoxication of Greek soldiers by eating honey from *Rhododendron ponticum* and *Rhododendron flavum*. The honey grown from these plants had high doses of diterpene grayanotoxins. In other settings, people have been intoxicated from teas brewed from plant parts or after consuming leaves, flowers or seeds from toxic plants

(Dickstein and Kunkel, 1980; Bain, 1985; Joskow et al., 2006) [61–63]. Accidental food poisoning incidents have been reported after people consumed recently sprayed maize that was obtained illegally. The author knows of an incident in South Africa where 10 family members where brought at the hospital after consuming such maize. Of the 5 children younger than 7 years who ate this food, 2 died.

2. Risk factors for food poisoning

With regard to food poisoning, several factors play a role in its occurrence. These factors include the individual factors such as their age, gender, socioeconomic status, their residence, health status and others; other factors include the contextual country and place of residence together with the characteristics of their macro- and micro-environment as explained below.

In the macro-environment, the chemicals and biological agents are found in environmental media such as water, soil, air and food itself. Moreover, there are interactions between these media with regard to food poisoning. For instances, crops grown in soils that are polluted by heavy metals and other chemicals as well as those sprayed with toxic pesticides may contain high levels of these chemicals that may lead to acute or chronic poisoning based on the circumstances of consumption [64, 65].

Several contextual determinants affect the occurrence of food poisoning; these include the accessibility, the availability, the affordability of controlled chemicals and drugs that may be used for instance in para- or suicide, criminal food poisoning or harvesting of fish or hunting. Other associated determinants are the seriousness of the rule of law and the enforcement of food control regulations. Sadly, in African settings, even controlled chemicals such as pesticides and pharmaceuticals can be found and brought from the streets in most cities. And as discussed further, foods vended in the streets are one of the major risk factors for food poisoning [66–69].

Foods and drinks form part of the micro-environment as they are found in the house where people live and at other settings, such as workplaces and places of entertainment. The presence of foods in the household means that accessibility and availability are guaranteed. Hence, if the storage and means of preparations are inadequate, the foodstuffs or drinks may be subjected to spoilage, contamination or chemical degradation and fermentation that would render them prone to cause food poisoning. This observation is equally true for restaurants and other food-serving venues [69–71].

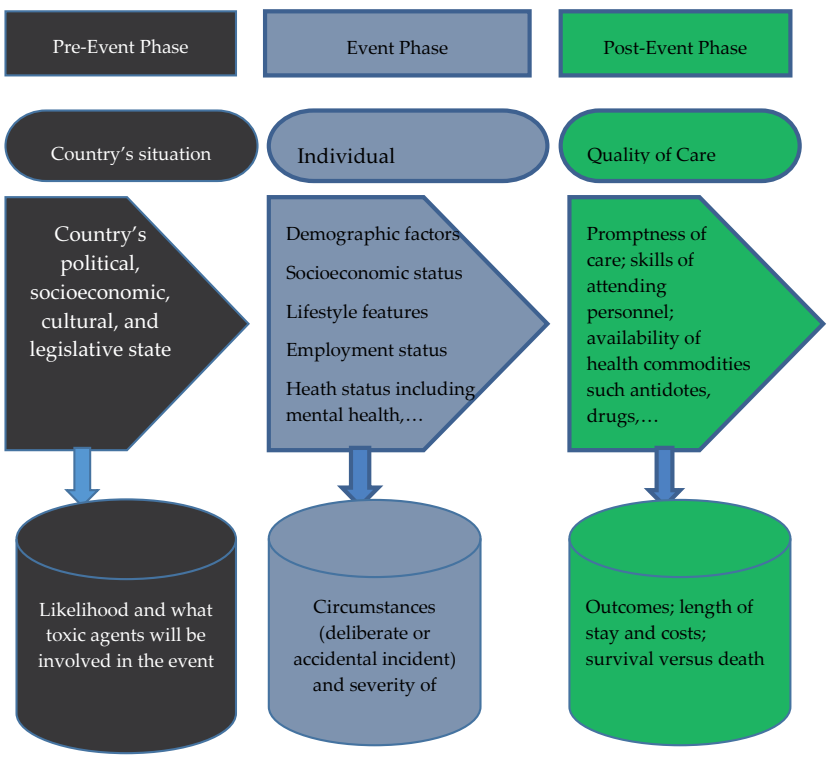
If any of these areas in the micro-environment have food likely to cause poisoning, the only determinant that will trigger the occurrence of food poisoning will be the individual circumstances including how susceptible the person is to the toxic agents in the offending food, the freedom of choice they have, which is linked to their economic status and the amount of offending food consumed [72–74].

Furthermore as explained by Haddon [75], several factors are involved in the three phases of a poisoning event. These phases are the pre-event, the event and the post-event. It is noted that

factors in the pre-event phase contribute to the likelihood or the possibility that a poisoning event may occur; the factors mediating in the event phase affect the likelihood that a poisoning event or an injury will actually occur and how severe it would be. Finally, the factors in the post-event phase influence the outcomes or consequences of the poisoning event once it has occurred.

In the case of acute food poisoning, the factors in the 'pre-event phase' include the whole political and regulatory frameworks that ensure that crops and foods standards of quality and safety are respected by producers, importers and retailers in such a way that consumers can have access to foods that are safe and nutritious [76, 77].

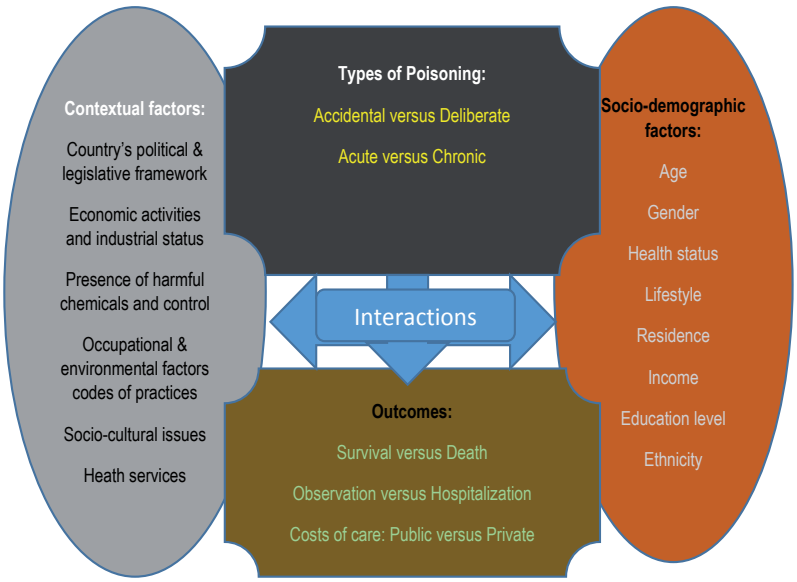
In the event phase, the sociodemographic, socioeconomic and psychosocial factors of the potential victims of poisoning determine whether they will consume foods that will lead to a poisoning event. These include their age, gender, socioeconomic status, how hungry they are and the type and state of foods available to them. While, in the post-event phase, the factors in this phase include the promptness and quality of care that the victims of food poisoning will receive in line with the existing health care system [78].



Source: Malangu (2011). Acute poisoning in three African countries: Botswana, South Africa and Uganda. Available at: http://hdl.handle.net/10386/674.

Figure 1. Phases of a poisoning incident.

The above phases of poisoning event are intertwined in their interactions; the country' context, the individual characteristics and the status of offending foodstuffs combine to determine whether a food poisoning occurs or not. Whether the country is experiencing food shortage or there is food for everyone depends on the economic situation and on the distribution of income across various segments of the populations [79–81]. As depicted below, the country's context and the characteristics of an individual interact to produce the type of poisoning as well as the outcomes of the incident.



Source: Malangu, N. (2014). Contribution of plants and traditional medicines to the disparities and similarities in acute poisoning incidents in Botswana, South Africa and Uganda. African Journal of Tradition Complement Altern Medicine 11(2): 425–438.

Figure 2. Risk factors involved in the occurrence of a poisoning incident.

With regard to sociodemographic factors of individuals, a comparative study of acute poisoning in three countries reported that females were more significantly affected by food poisoning than men in Botswana and Uganda, as well as in South Africa although the difference was not statistically significant. With regard to age, food poisoning occurred in ≤10% of cases and affected more young adults aged 20 to 30 years in South Africa; more adults aged over 30 years in Uganda; while in Botswana food poisoning affected equally children younger than 12 years old and adults over 30 years [78].

The above findings suggest that the contextual circumstances of individuals in their respective countries affected the onset of food poisoning. It also suggests that all strata of populations are susceptible to food poisoning particularly to staphylococcal food poisoning. However, the severity of symptoms may vary depending on the amount of the offending agent consumed in the food and the general health status of affected individuals. Hence, the young and elderly,

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because of their weak immune status or defence mechanisms, are more likely to develop more serious symptoms [82–84].

Moreover, although noroviruses have been reported as a leading cause of sporadic cases and outbreaks of acute gastroenteritis across all age groups, a systematic review estimated that 12% of all cases of sporadic acute gastroenteritis caused by *Norovirus* occur in children [85].

With regard to socioeconomic and regulatory factors, in the African region, poverty is considered as the underlying cause of consumption of unsafe foodstuffs that are likely to cause food poisoning because it exacerbates food safety problems through unsanitary living conditions in rapidly growing urban centres, lack of access to clean water, unhygienic transportation and storage of foods and low education levels among consumers and food handlers, together with a false sense of invulnerability as people eat unsafe foods with the belief that nothing will happen to them [86–89]. The poor environmental conditions, particularly unsafe sanitation systems, exacerbate the situation as explained above.

Interestingly, most countries do not have appropriate policies and legislation to deal with street vending [90]. Even when national legislation and regulations exist, there is a limited capacity and capabilities to control the quality of both locally produced and imported foodstuffs [91]. It has been documented that several street food vending facilities often do not meet proper hygiene standards, in large part because of weak regulatory systems, inadequate food safety laws, lack of financial resources to invest in safer equipment and lack of education for food handlers [92]. Because street food is frequently cooked well in advance of consumption, it is prone to contamination from exposure to dust, flies, bacteria and their spores. The handling and processing of foods by the preparers who may be carrying germs open opportunities for contamination of food if adequate precautions are not implemented [93, 94].

Yet because of inadequate coordination between surveillance, food laboratories and food inspection services, there is a disorganised sampling and quality control of foodstuffs. Furthermore, the emphasis is on sampling for enforcement purposes and often there is no systematic monitoring for food contaminants as well as no surveillance systems capable of identifying common agents of foodborne diseases [95, 96].

Moreover, food safety control laboratories generally do not exist in some countries; and when they do exist, they do not function as a result of lack of human, material and financial resources. In addition, most of the public health laboratories in the African region lack the capacity to test for chemical contaminants and naturally occurring toxins [97, 98]. There is a lack of skilled inspectors who have relevant competencies and means to do their work properly [97, 99, 100].

With regard to environmental factors, it is also established that they contribute to the occurrence of food poisoning. As explained above, food may serve as a carrier of either the microorganisms (bacteria, parasite or virus) or chemicals that ultimately elicit the symptoms of food poisoning. Several well-known examples of food poisoning in which the environment has a strong influence include the case of *Salmonella* spp. and heavy metals poisoning from drinking water. Contamination of surface and ground waters by pesticides is a clear example of the influence of poor environmental management resulting in poisoning [34, 40, 102]. Other examples are *S. aureus*, a bacterium that causes food poisoning, and

Source: Mwamakamba, L., Mensah, P., Takyiwa, K., et al. (2012). Developing and maintaining national food safety control systems: Experiences from the WHO African region. African Journal of Food, Agriculture, Nutrition and Development 12(4): 6291–6304. – African Scholarly Science Trust.

Figure 3. Status of food policy formulation in Africa.

S. aureus, a bacterium that is commonly found in the environment (soil, water and air) and also found in the nose and on the skin of humans. It is established that food handlers carrying enterotoxin-producing *S. aureus* in their noses or on their hands are regarded as the main source of food contamination via direct contact or through respiratory secretions when proper hygiene is not maintained [103].

This situation is not unique to Africa. A survey of food from retail markets and dairy farms in Turkey was performed between 2007 and 2008. Enterotoxigenic *S. aureus* was found in at least 10% of samples of meat and unpasteurised milk but in less than 10% of samples of dairy and bakery products [104]. While in an Italian survey performed between 2003 and 2005, also less than 10% of samples of dairy products and meat products were tested positive for *S. aureus*

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[105]. In Japan, a retail survey performed between 2002 and 2003 found 17.6% of raw chicken were tested positive for *S. aureus* [106].

Another case involves *Vibrio* species that grow naturally in marine environments worldwide and are able to survive and replicate in contaminated bodies of water particularly those with increased salinity. The *V. cholerae* "O1 and O139" produce cholera toxin, which is responsible for the onset of cholera. Asymptomatically, infected humans can also be an important reservoir for this organism in areas where *V. cholerae* disease is endemic. It has been reported that water contaminated with human faeces or sewage is the main route of infection, but some cases have resulted from the consumption of fish and other seafood products [107, 108].

In the case of seafood, it is known that some of them are susceptible to surface or tissue contamination originating from the marine environment due to polluted waters from broken sewage, overboard sewage discharge and sewage run-off after heavy rains or flooding. Moreover, during various processing and preparation activities, contamination of seafood by pathogens with a human reservoir can occur. Other contributing factors may include inappropriate storage and transportation at inappropriate temperatures and cross-contamination through contact with contaminated seafood or seawater [109].

In the case of agricultural products, several reports have been written about fungal toxins, namely mycotoxins such as aflatoxins, which pose a serious challenge as they contaminate various agricultural commodities either before harvest or under post-harvest conditions [110, 111]. Generally, tropical conditions such as high temperatures and moisture, unseasonal rains during harvest and flash floods are favourable to fungal growth and production of mycotoxins. Additionally, poor harvesting practices, improper storage and less than optimal conditions during transport and storage at market places can also contribute to fungal growth and increase the risk of mycotoxin production. Hence, chronic poisoning due to foodstuffs containing aflatoxins has been reported [112–115].

Similarly, food processing factors play a role in the onset of food poisoning mediated by meat. For instance, the level of bacterial contamination associated with live animals can be amplified through slaughtering plant operations. During transportation, the animals infected with *Salmonella* do shed it and thus contaminate other animals. The level of contamination is believed to increase during the containment of the animals in holding pens before slaughter [116]. After slaughter, the subsequent dressing of meats increases the spread of *Salmonella* on meat surfaces and by the time the meat is in retail outlets, contamination levels may have increased by up to 20% [17].

Street vending of foods is also a major risk factor for food poisoning. The major concern with street foods is their safety, mainly because vending is done in places that may have poor sanitation and inappropriate utensils. Street foods in some African countries have been tested for various microorganisms including faecal coliforms, *E. coli*, *S. aureus*, *Salmonella* spp. and *B. cereus*. It is noted that *E. coli* and *S. aureus* were recovered in a significant proportion of the food, water, hand and surface swabs tested in Harare. Samples of fufu and other staple foods tested in Accra yielded positive results and counts for *E. coli* and *S. aureus*. One study reported that of 511 street food items examined in Accra, up to 69% contained bacteria. In the same

study, *Shigella sonnei* was isolated from macaroni, *Salmonella arizonae* from meat-based soup and *E. coli* from macaroni, tomato stew and rice. In a separate study, it was observed that over 26% of street food samples analysed in Nigeria contained *B. cereus*, whereas 16% contained *S. aureus* [117–120].

In North Africa

The following discussion illustrates the issues about mycotoxins. These substances are ingested orally but they may also be inhaled and may be transdermally absorbed. Of the more than 400 mycotoxins identified in the world, ochratoxin A (OTA) is one of the most prevalent. It is a ubiquitous mycotoxin produced by several fungal species belonging to the genera *Aspergillus* such as *Aspergillus ochraceus* and *Penicillium* such as *Penicillium verrucosum*, is responsible for chronic food poisoning in North Africa [120, 121]. OTA contaminates several agricultural products such as wheat, barley, rice and sorghum, cereal-derived products, dried fruits, spices, beer and wine. Additionally, OTA was found in high amounts in animal feeds. Because of its long elimination half-life (about 35 days in serum), as a consequence of its binding to plasma proteins, its enterohepatic circulation and its reabsorption from urine, OTA is the most detected mycotoxin in human blood. Its human exposure has been clearly demonstrated by its detection in human blood and urine (Zimmerli and Dick, 1995; Zaied et al, 2011) [122–124]. Its adverse effects in a variety of animal species have been described, such as teratogenicity, immunotoxicity, genotoxicity and mutagenicity [125, 126]. Its most important toxic effect in humans is nephrotoxicity; it was implicated in the human "Balkan endemic nephropathy" which is similar to the Tunisian chronic interstitial nephropathy of unknown aetiology in many aspects [127, 128].

In Tunisia, a clear correlation has been established between the consumption of OTA-contaminated food and nephropathy; it has been speculated that this is due to the favourable climatic, geographic, social and economic conditions that Tunisia provides for the growth and survival of this toxigenic fungi [127–129]. Similarly, studies from Morocco have detected that of 60 samples of grains of cereals sold in Morocco, 40% to 55% of samples of corn, wheat and barley were contaminated by OTA. In a follow-up study by the same authors, 10% of samples of corn flour sold in Rabat exceeded the maximum recommended limits for aflatoxins [130, 131].

In East Africa

The following discussion illustrates the role of food handlers in the onset of food poisoning. In Ethiopia, the prevalence of intestinal parasites among food handlers was found to be 29.1% to 63% [132]. This high prevalence has been attributed to poor personal hygienic practices and poor environmental sanitation. Of concern is that in one study, 6.5% of food handlers working in the kitchens had been suffering from diarrhoea at the time of the survey. And the microorganisms associated with the diarrhoea were identified as *Entamoeba histolytica*, *Giardia lamblia* and larva of *Strongyloides stercoralis*. It is important to note that food handlers can directly transmit *Giardia* to consumers if they do not exercise appropriate hygiene practices [131, 133].

In Central Africa

The following discussion illustrates how food poisoning can result from the way the food is harvested. The use of plant-based poisons to harvest fish and bush meat has been in Africa

since centuries. Plants such as *Tephrosia* sp. and *Mundulea sericea* were used throughout tropical Africa; but in recent years, because of their ease of accessibility, availability and affordability, synthetic chemicals are used to harvest fish in the Democratic Republic of the Congo and Cameroon and to kill birds and animals even vultures in several African countries [134–137].

In southern Cameroon, some respondents in a survey claimed that eating fish harvested with the use of synthetic chemicals induced vomiting and stomach pains, and in some cases even death (reference). It is also reported there that a massive killing of cane rats (*Thryonomys* spp.) has been performed using pesticides. These rats were subsequently eaten as bushmeat. A major incident of human deaths due to eating fish contaminated with pesticides occurred around Lake Victoria and ultimately prompted authorities to take action against this fishing method [136–140].

In West Africa

The influence of lack of knowledge and relevant equipment for safe handling of food processing activities as well as cultural ethos in the onset of food poisoning is plausible as shown in the following reports. In Nigeria, it has been reported that the unhygienic states of some restaurants have contributed to the onset of foodborne diseases. It is known that in some parts of Nigeria, food poisoning is believed to be associated with evil spirit, malice or curses. Moreover, an urban adult Nigerian eat food from street vendors regularly because it is easily available, affordable and usually fresh [141–144].

However, street-vended foods pose some risks due to lack of basic infrastructure and services, such as potable water and reliable electricity, as well as poor knowledge of basic food safety measures. In addition, there are other issues such as poor drainage systems, unsanitary waste disposal systems, presence of flies and apparent lack of facilities for food protection [118, 120, 145, 146]. Inappropriate personal hygiene practices such as the use of unwashed fingers to feel foodstuffs or sometimes to dish out augur a high potential for contamination in the handling and preparation of foods. Moreover, an investigation about food poisoning among three families in Kano due to yam flour consumption showed that the use of certain unsafe preservatives for the processing of yam flour was responsible for the incident [142–144, 147]. Furthermore, the lack of regulatory monitoring of foodstuffs results in unsafe foodstuffs being sold in the market. For instance, a recent survey of heavy metal levels in commonly consumed canned and non-canned beverages available in the Nigerian market indicated a high prevalence of beverages with levels of heavy metals that exceed the maximum contaminant levels (MCLs) for these metals [148–150].

In Southern Africa

The influence of food processing factors in the occurrence of food poisoning is illustrated as follows. Botswana is one of the leading beef cattle–rearing countries in Africa. Although as a semi-desert, the country is fairly free from most tropical diseases that are associated with heavy rainfall and high humidity, foodborne diseases which have been reported in Botswana to be mainly associated with poor handling as well as lack of awareness with regard to safety of food [151]. It has been observed that the meat exported from the country undergoes extensive scrutiny by both local and international experts under the supervision of the Botswana Meat Commission and the European Commission (EEC); yet *Salmonella* spp. has been isolated in local abattoirs, which process meat for local consumption [151, 152].

In Mozambique, cassava is one of the main staple foods in some areas where it is harvested from August to October each year. Bitter cassava, which is high in cyanogenic glucoside content, is mostly produced and harvested. Hence, indigenous knowledge has taught communities that sun drying preceded or not by fermentation is the process that helps to lower the cyanogenic glucosides [153]. Sometimes this is not done. Consequently poisoning may result from this mishap. In 1981, the first epidemic of Konzo was reported in Mozambique; over 1,100 cases occurred in northern Nampula Province. This condition, Konzo, is characterised by the sudden onset of irreversible spastic paraparesis. Epidemiological findings showed that this was associated with prolonged high dietary cyanogenic glucoside consumption and a diet deficient in sulphur amino acids. Laboratory findings confirmed that people in the affected areas had high serum thiocyanate concentrations [154–157]. Sadly, Konzo is reportedly still spreading to new areas in Mozambique; it is being diagnosed outside major agricultural crises, with persistent cases and smaller epidemics. Affected communities continue to suffer cyanide intoxication at the time of the cassava harvest [158–160].

3. Outcomes of food poisoning

3.1. Short-term outcomes

Like any other health condition, food poisoning can be measured in terms of morbidity and mortality. However, due to the lack of population-based data, it is not possible to establish exactly the mortality rate due to food poisoning; rather, it is the case fatality rate (CFR) that is calculated from the number of people who have been affected after eating the offending food. The morbidity due to food poisoning can be measured by its incidence and prevalence.

Usually, the prevalence of acute food poisoning in patients attending health facilities has been determined as well as the factors associated with such episodes. In several African countries, the prevalence of acute food poisoning in patients seen at Emergency Departments of hospitals ranges from 0.5 to 11.5% [72, 73, 78, 161, 162]. As for chronic food poisoning, it is even more difficult to estimate its prevalence because of the diagnostic limitations [67, 163]. However, as explained below, the prevalence of health conditions resulting from chronic food poisoning can also be estimated.

In general, morbidity and mortality are higher in elderly individuals due to their increased susceptibility, age-associated comorbidities such as decreased immunity, decreased production of gastric acid and intestinal motility, malnutrition, lack of exercise. It has been reported that elderly persons are more likely to die from food poisoning events implicating toxin-releasing bacteria [7, 14].

It is estimated that over 3 million cases of acute food poisoning and 20,000 deaths occur annually due to exposure to pesticides in foods [164]. The following observations illustrate the extent of the problem in some African countries. It has been reported that more than 200,000 people die of food poisoning annually in Nigeria from foodborne pathogens [23, 165].

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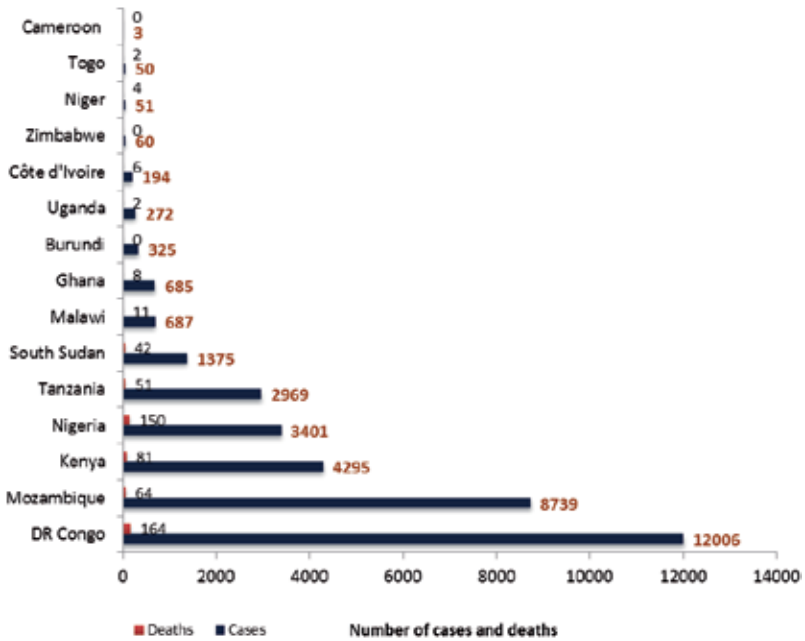
In the case of cholera, several outbreaks have been reported in African countries. In refugee camps, because of unsanitary conditions, environmental contaminants and improper food handling, outbreaks of foodborne diseases are common. Notable examples include the July 1994 outbreak, in which 14,000 deaths were recorded in refugee camps in Goma, Democratic Republic of the Congo. Another outbreak in April 1997 led to a total of 1,521 deaths among 90,000 Rwandan refugees residing in temporary camps in the Democratic Republic of the Congo, yielding a CFR of 1.69% [166, 167].

In Angola, an outbreak of cholera in February 2006 was reported in several provinces including the capital city, Luanda. In total, 26,979 cases were identified and 1,085 deaths were registered or a CFR of 4% [168]. In South Africa, an outbreak of cholera from November 2008 to April 2009 was resulted in over 12,000 suspected cases, 1,144 laboratory-confirmed cholera cases and 57 deaths, a CFR of 0.5% [169]. Previously, a breakdown in the drinking water pipeline system in a South African suburb caused an outbreak of typhoid, causing illness in nearly 4,000 persons as well as several deaths [170].

Recent data show that cholera is ever present in African countries. As shown in the Figure 4, the most affected countries are the Democratic Republic of the Congo, Mozambique, Kenya, Nigeria and Tanzania. The most recent report suggests that the cumulative number of cases of cholera in Tanzania was 4,835 cases that resulted in 68 deaths, a CFR of 1.4% [171].



Source: WHO/AFRO (2015). Epidemic alert and response. Available online at: www.afro.who.int/en/clusters-a-programmes/dpc/epidemic-a-pandemic-alert-and-response/outbreak-news/4764-outbreak-bulletin-vol-5-issue-5-31-october-2015.html.

Figure 4. Cholera cases and deaths in African countries, January to September 2015.

With regard to mycotoxins, the outbreak of acute aflatoxicosis in Kenya in 2004 was one of the most severe food poisoning events involving contaminated maize; it has affected a total of 317 people resulting in a massive CFR of 39% [113, 172].

In Madagascar, acute food poisoning associated with consumption of fish or other marine animals contaminated by ciguatera is common from October to May each year. In 2013, a total of 116 cases and 1 death (CFR: 0.8%) were reported [173].

Finally with regard to incidence of food poisoning, the following Table 1 illustrates the situation in several African countries.

Country	Annual incidence per 1,000 inhabitants		
	Minimum	Mean	Maximum
Ethiopia	1.22	5.36	10.73
Zimbabwe	26.83	33.75	40.78
Cuba	11.45	19.00	28.82
Brazil	22.12	26.88	32.18
Mexico	66.00	79.20	95.00
Thailand	39.64	48.45	58.56
India	3.26	5.44	8.41
Malaysia	12.02	20.72	32.29
Tunisia	32.11	36.89	41.67
Egypt	8.39	10.11	12.15

Source: World Health Organization (2002). World Health Statistics 2002. WHO, Geneva.

Table 1. Incidence of foodborne salmonellosis in some countries.

The mean incidence of foodborne salmonellosis ranges from 5.36 to 79.20 per 1,000 people [174]. This suggests that the morbidity due to this type of food poisoning is important in the African continent. As will be discussed further, this requires interventions particularly because long-term effects may also result from food poisoning.

3.2. Long-term outcomes

3.2.1. Chronic poisoning

There are some evidence built from data of the last 50 years that chronic poisoning due to dietary aflatoxins, heavy metals, pesticides and other organic compounds has resulted in people being affected by several conditions. Mycotoxins are toxic and carcinogenic metabolites produced by fungi that colonize food crops; aflatoxins, the most prevalent mycotoxins have been linked to liver cancer and have also been implicated in child growth impairment and

acute intoxication as reported above. On the other hand, fumonisins have been linked to oesophageal cancer and neural tube defects; ochratoxin A has been associated with renal diseases as described above [175–178].

There is evidence that the above conditions that affect some African populations may have resulted from exposures to the above chemicals through food and other means. In the case of aflatoxin, its presence has been detected in human breast milk of women from Sub-Saharan Africa and Arab countries [110, 111, 130, 179–182]. Similarly, the presence of endocrine disruptors, who are consumed through polluted waters and contaminated fishes and bush meat, has been confirmed also from analyses of breast milk, umbilical cords samples of women and children from several African countries [183–186].

With regard to heavy metals, their implication in chronic poisoning through the food chain has been demonstrated and explained above. The health consequences are also multiple; in the case of mercury, its neurotoxicity has been noted since the Mina Mata incident in Japan [187]. To date, it is now established that it causes cognitive deficits at low exposure levels and severe psychiatric and neurologic effects at very high levels of exposure. Its toxic effects on the kidney, cardiovascular and immune systems are well known as well as its developmental neurotoxicity in case of in utero exposure [188–191].

Consumption of fish contaminated with mercury is one of potential sources of human exposure besides mining and the use of mercury-containing cosmetics used for skin lightening in Africa [192, 193].

Another heavy metal, lead, it is a naturally occurring heavy metal whose exposure is associated with several adverse effects across different population subgroups. From several studies, the effects of lead on neurodevelopment and, in particular, its negative effects on intelligence quotient and behaviour have been documented [190, 191, 194, 195]. More recently, lead has been associated with other adverse health impacts, such as increased risks of attention deficit hyperactivity disorder in children and cardiovascular mortality in adults [196, 197]. Although foodstuffs have not been reported as major sources of exposures to lead, lead was found in contaminated dust and soils that growing children may consume or inhale as well as in contaminated ceramics, crayons, pencils and piped water [188, 198].

With regard to pesticide exposure among African populations, it is a public health concern because of their uncontrolled use coupled to weak regulatory enforcement [175, 199]. In addition, there is a lack of competencies to use them appropriately due to lack of training and access to relevant safety information and personal protective equipment [200]. Pesticides are used for pest control in several environments, such as in the home and in various forms of agriculture. It is not surprising that pesticides used in agriculture have been detected in rivers and lakes that are located around farming communities [201, 202].

Human exposure to pesticides used in agriculture is reportedly higher among farmworkers and their families. In a study conducted in Kenya, it was reported that breast milk samples taken from non-farmer urban Kenyan mothers up to 4 weeks postpartum had detectable levels of nine organochlorine pesticides including dichlorodiphenyltrichloroethane (DDT) (and its metabolite dihydrodiphenylethane (DDE), dieldrin and lindane. Similarly, DDT was also

detected in breast milk of mothers residing in some areas of Kwazulu, South Africa and Ghana [203]. Collectively, these studies suggest both historical and ongoing pesticide exposures in individuals through agriculture and dietary sources [77, 184, 202, 203].

3.2.2. Sequelae

Several sequelae have been described in populations exposed to specific toxic agents. For African populations that have been exposed to several classes of pesticides through agriculture, the environment and foods, it is expected that they may experience similar adverse effects. Well-established sequelae include neurotoxicity, endocrine-disrupting adverse effects and birth defects documented agricultural communities [204–207].

With regard to heavy metals, it is estimated that each year 9,129 to 119,176 additional cases of bladder cancer, 11,844 to 121,442 of lung cancer and 10,729 to 110,015 of skin cancer worldwide are attributable to inorganic arsenic in food [208].

Other sequelae include stunted growth in children or being chronically underweight, susceptibility to infectious diseases and hepatocellular carcinoma (HCC) or liver cancer. This cancer is the third leading cause of cancer deaths worldwide, with roughly 550,000–600,000 new HCC cases globally each year [209]. Aflatoxin exposure in food is a significant risk factor for HCC [175, 210].

4. Public health interventions

Food poisoning is a health condition that is multifactorial; it therefore requires a multi-sectoral approach in addressing it. The interventions should be targeted to address the risk factors discussed above. For food poisoning, the youth, from teenagers to young adults, particularly those with any psychiatric or psychological disorder, those with history of child abuse and those who are addicted to any substance, constitute a high-risk group for deliberate self-poisoning, whereas the children, the elderly and pregnant women constitute a high-risk group for accidental events with a high fatality potential [211, 212]. Thus, the two public health approaches, namely the individual-centred approach or 'high-risk strategy', and the population-based approach or 'population-based strategy' need to be implemented synchronically to address food poisoning [213].

In order to reduce the prevalence and incidence of food poisoning as well as its related mortality, multiple approaches ought to be implemented. Based on the population-based strategy, these include legislative measures, public educational programmes and establishment of poison or toxicological information centres and services and the establishment of surveillance systems [78, 214, 215]. Reports from several other settings have shown that legislative measures that restricted access to toxic agents resulted in a decrease in mortality associated with the targeted agents [212, 215–217].

In addition, good agricultural, manufacturing or processing practices can help in preventing and addressing food poisoning. For instance, in the case of aflatoxins, the risk of contamination

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of food and feed in Africa is increased by environmental and agronomic factors. Environmental conditions especially high humidity and temperature favour fungal proliferation as well as drought conditions. In the same vein, some inappropriate agricultural practices such as extended field drying and leaving the harvested crop in the field prior to storage facilitate fungal infection and insect infestation [218, 219].

For the above reasons, agronomic management practices that can reduce the risk of aflatoxin development such as the use of resistant varieties, crop rotation, well-timed planting, weed control, pest control especially control of insect pests and avoiding drought and nutritional stress through fertilization and irrigation should be implemented. Similarly, measures to control aflatoxins through the use of appropriate pesticides and the implementation of effective post-harvest initiatives, such as rapid and proper drying, proper transportation, adequate packaging and insect control during storage, should also be considered [220, 221].

Other practical approaches include the reduction of the frequency of consumption of 'high risk' foods (especially maize and groundnut) by consuming a more varied diet and diverting aflatoxin-contaminated foods to animals using clay-locking systems [222]. Of even more programmatic interest is the health education of the public and millers who should avoid blending visibly contaminated products to reduce the overall risk of spreading mycotoxins [218, 223].

Similarly, in order to reduce pesticides related poisoning from the food chain, several measures can also be contemplated such as encouraging the use of less hazardous and cost-effective pesticides currently available; establishing a national programme to monitor applicators' exposure and use of personal protective equipment; ensuring the respect of the correct time lag of crops that have been sprayed; and ensure proper disposal of obsolete, unused or unwanted pesticides and wastes [224].

Based on high-risk strategy, managerial decisions at facilities' level can be implemented such as assessment of the health status of food handlers, the audit of food-serving facilities based on the Hazard Analysis Critical Control Point (HACCP) approach. This approach is well known for its use in identifying points where controls could be applied to prevent or eliminate these microbiological hazards or reduce them to acceptable levels [225].

The implementation of HACCP has been reported in some settings on the African continent but it is not widely used particularly in food outlets frequented by the majority of people or businesses [226–228]. This failure is reflective of the general lack of enforcement of regulatory and legislative texts relating to foodstuffs in Africa [168, 214]. This is an area where advocacy is required from the civil society and other community voices to ensure that authorities are called upon to enforce existing legislation [96, 229].

Some authors have suggested the ' four Cs' namely cleaning, cooking, chilling and avoiding cross contamination as the backbone for basic hygiene, food preparation and storage. This suggestion is supported by findings from surveys that reported that at least three-quarters of the public had never heard of the most common causes of food poisoning and believe they are

unlikely to get food poisoning from food cooked at home; yet the majority of them did not store raw meat properly and/ or wash their hands after handling raw meat or fish [230–233].

With regard to epidemiological surveillance, it is necessary to assess the magnitude of the food poisoning problem and its major risk factors in defined contexts. Based on the findings, evidence-based interventions could then be designed and implemented [90]. Together with the establishment of a food poisoning surveillance system, national or regional toxicovigilance centres should be funded so that they can collect, aggregate, analyse, and report on food and other causes of acute and chronic poisoning with specific focus on deliberate poisoning. These centres should serve both the public and clinicians with relevant information for the management of poisoning events [78].

With regard to academic training, there is a need to create awareness on the issues of food production, processing, distribution, handling and cooking among all people studying towards a degree in any field of science, particularly for those in agricultural and health sciences. Public education and awareness campaigns aimed at the whole population are required so that risks about various types, sources and consequences of food poisoning are communicated regularly to influence behaviour change [234–238]. Public education programs should also strive to increase the population's awareness of the general risks of poisoning at home. This includes designing messages dealing with appropriate storage of foods and other products in the home, and explaining what to do in case of a suspected poisoning incident.

5. Concluding remarks

Food poisoning is a significant cause of both short-term and long-term morbidity; it is equally a cause of observed and insidious morbidity. While short-term consequences of food poisoning include hospitalisations, out-of-pocket expenses, loss of productivity and deaths; its long-term consequences include dreadful diseases, such as cancers, birth defects and more indirect deaths.

For this reason, the contribution of the human element in the occurrence of food poisoning is worrying, particularly because of the existence of unsafe harvesting, uncaring handling, unhygienic food-serving facilities, unhealthy processing practices and criminal use of foods. Yet all these issues and practices can be addressed with available technologies, systems and evidence-based practices within the national and international regulatory frameworks.

Given the reliance of the human race on foods for its survival and the fact that every food that humans eat comes ultimately from the natural environment; the implementation of hygienic and safe food handling practices and the preservation of all fauna and flora ecosystems as well as environmental media, namely air, soil and water, is not only a necessity but a shared responsibility that each human being should shoulder every day in the way he or she lives, make decisions and eat.

Author details

Ntambwe Malangu*

Address all correspondence to: gustavmalangu@gmail.com

School of Public Health, Sefako Makgatho Health Sciences University, Pretoria, South Africa

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Chapter 4

Potential Exposure and Risk Associated with Metal Contamination in Foods

Luciana M. Coelho, Diego R. Pessoa, Kênia M. Oliveira, Priscila A.R. de Sousa, Lícia A. da Silva and Nívia M.M. Coelho

Additional information is available at the end of the chapter

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Abstract

Humans require several trace elements as components of the diet. Some of these elements are required in extremely small quantities (only micrograms per day). On the other hand, in higher concentrations, some elements may also have deleterious, even lethal, effects. Metals such as arsenic, chromium (Cr), lead (Pb), and mercury (Hg) are naturally occurring chemical compounds. The contamination of food with these metals occurs mainly through human activities, such as farming and industry, or from contamination during food processing and storage. People can be exposed to these metals by ingesting contaminated food or water, and their accumulation in the body can lead to harmful effects over time. The main objective of this chapter is to provide a literature review on the various types of foodborne poisoning caused by the contamination of food with arsenic, Cr, Pb, and Hg and on food safety issues associated with the presence of these metals in food. Research findings from various studies carried out to examine the relationship between metal exposure and the adverse health effects of metals are addressed.

Keywords: Diseases, chemical contamination, metals, food

1. Introduction

Foods can be contaminated with harmful chemicals and microorganisms, which can cause illness in humans. Chemical contaminants can be classified according to the source of contamination and the mechanism by which they enter the food product. In the case of metal residues in food, contamination mostly occurs during food processing and storage [1].

In human nutrition, metals are well recognized by public health agencies, nutritionists, and researchers from various areas of knowledge. Humans require several metals as components

of their diet. Some of the metals are required in extremely small quantities, while some, such as arsenic, chromium (Cr), lead (Pb), and mercury (Hg), in certain amounts can adversely affect the nervous system, kidneys, and other vital organs of the body, which can be life threatening in extreme cases.

In general, sources of contamination are contaminated food and beverages and packaging [1, 2].

Metals can often be inadvertently and unintentionally introduced into food products. If these contaminants are not detected, they can become a major safety hazard for consumers. Metals such as arsenic, Cr, Pb, and Hg exist as naturally occurring chemical compounds. These metals are of particular concern in food because of their toxicity, especially in the case of long-term (chronic) intake, because they can accumulate in the body and cause organ damage particularly in susceptible groups, such as children [2].

Arsenic may be present as a contaminant in many foods, such as grains, fruits, and vegetables, where the metal is present because it is absorbed in the plant through the soil and water, and also trace amounts of arsenic can enter the food chain through the application of agricultural chemicals like fertilizers, which may contain arsenic. While most crops do not readily take up much arsenic from the ground, rice is different because it takes up arsenic from soil and water more readily than other grains. Also, arsenic exposure occurs through the consumption of aquatic food, especially shellfish and animals that feed from the bottom of the sea [3].

Cr exposure occurs mainly through the diet. Food crops that are polluted through contaminated soil or water may contain high concentrations of this metal.

Pb is a toxic substance present in the environment in small amounts, and everyone is exposed to some Pb from daily actions such as inhaling dust, eating food, or drinking water. Tobacco smoking and the use of leaded petrol in vehicles are reported to be major sources of Pb exposure, although the Pb content in petrol has dramatically declined over recent decades, thereby reducing environmental exposure [3].

Hg exposure can occur through dental fillings that contain Hg compounds, occupational exposure, and herbal medicines. However, to date no studies have been able to show an association between amalgam fillings and ill health. Most dietary exposure is in the form of inorganic Hg. However, some fish may bioaccumulate the more toxic organic form, methylmercury, in significant quantities. Thus, diets rich in fish can be a cause of organic Hg exposure [3].

Thus, these metallic contaminants have been evaluated by international authorities, and safe reference values have been established. The maximum concentrations of these contaminants allowed by legislation are often well below toxicological tolerance levels, because such levels can often be reasonably achieved by using good food manufacturing practices. Even so, food contaminant testing is needed to assure the safety and quality of food products. Chemical analysis can be very useful in the food industry, with the development of new techniques to accurately and precisely quantify metals present in low concentrations in foods. These data can be applied in the area of toxicology to prevent diseases through prior diagnosis.

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2. Arsenic

2.1. Chemistry of arsenic

Arsenic is a chemical element found in several oxidation states (+III, +V, 0, and -III) and various inorganic and organic forms. Arsenic rarely occurs as a pure element. The most common ores of arsenic are arsenopyrite (FeAsS) and arsenic sulfur compounds [orpiment (As_2S_3) and realgar (As_4S_4)]. The inorganic forms are considered to be of greater toxicity, and the ascending order of toxicity is elemental arsenic < arsenobetaine < methylated forms < arsenate < arsenite < arsine [4]. As(III) and As(V) are apparently of comparable bioavailability but differ in terms of their biochemistry. Preferentially, As(III) binds thiol groups, whereas As(V) does not. So, the oxidation state of arsenic can affect its toxicity [5, 6].

2.2. Occurrence in the environment

Arsenic compounds are used in glass and semiconductor production, as a preservative for wood, and as a feed additive to increase weight gain for poultry and swine [7]. Historically, arsenic compounds were used in agriculture as insecticides or herbicides. Due to its widespread use, it can contaminate the environment. Environmental pollution by arsenic occurs as a result of anthropogenic activities and natural phenomena such as volcanic eruptions and soil erosion [8].

Arsenic can be present as a contaminant in environmental compartments, such as water, soil, and plants, and ultimately can seriously affect the human health through exposures to these contaminated compartments. Inorganic arsenic species are the most important chemical forms of arsenic in natural waters [9].

2.3. Dietary sources of arsenic

Arsenic can be found in fish, shellfish, meat, poultry, dairy products, and cereals. However, in fish and shellfish, organic chemical species are found, and thus the arsenic is less toxic. Marine organisms tend to accumulate more arsenic than those living in freshwater or terrestrial environments, which typically have lower arsenic concentrations of around 0.25 mg kg^{-1} [10–12].

China has established the acceptable level of arsenic in rice as 200 ng g^{-1}, while the Codex Alimentarius Committee on Contaminants in Food considers levels of 200 and 300 ng g^{-1} in polished and raw rice, respectively, to be safe [13]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reports that the provisional tolerable daily intake (PTDI) of inorganic arsenic is 0.002 mg kg^{-1} body weight, equivalent to 0.12 mg day^{-1} for an adult of 60 kg [14]. However, if contamination with arsenic trioxide occurs, it should be noted that the minimum lethal dose of this compound is 70–180 mg in humans [15]. In general, high levels of arsenic are found in rice and the concentration can vary from 10 to 510 μg kg^{-1}, when rice is irrigated with contaminated water, contributing to the daily intake of arsenic [16].

Due to the toxicity and the many diseases resulting from the ingestion of arsenic, the concentrations of this metalloid and its species in different types of food need to be investigated. Numerous studies on arsenic levels in food have been conducted, and the results have been published in journals, newspapers, and other media. Figure 1 shows the number of publications per year in the Web of Science on the contamination of food with arsenic, demonstrating the interest of the scientific community.

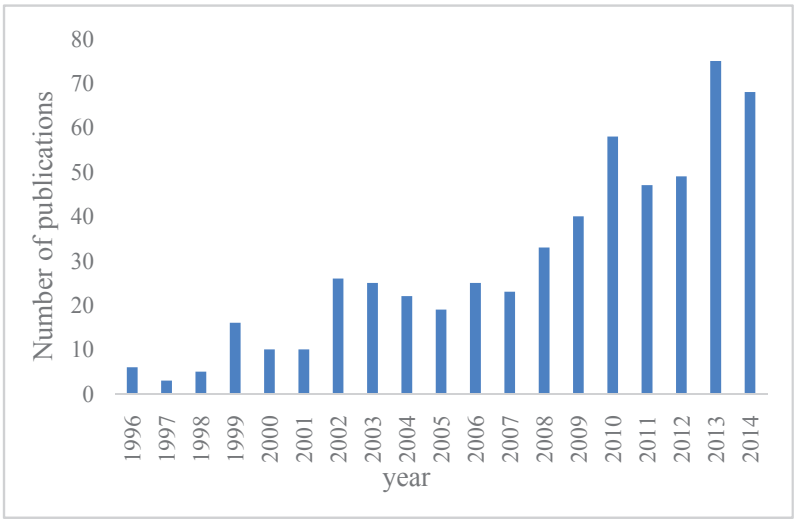

Figure 1. Publications on food contamination with arsenic.

The concentration of arsenic that is safe to human health is under discussion by organizations such as the World Health Organization (WHO), the Food and Agriculture Organization (FAO), the US Food and Drug Administration (FDA), the Food Standards Agency (FSA), and the European Food Safety Authority (EFSA) [17].

The Codex has adopted 0.2 mg kg^{-1} as the maximum level of arsenic in rice. This committee has the task of establishing international food safety and quality standards for consumers worldwide, which are widely used as a basis for national legislation.

To date, the European Union (EU) has not set maximum levels for arsenic in food. However, for water intended for human consumption, the value is 10 mg L^{-1} for total arsenic, with no distinction between the various chemical species of arsenic [18, 19]. Thus, extensive research should be conducted, aimed at more in-depth studies and attaining greater consistency in the data for the creation of relevant legislation regarding arsenic in foods [20–23].

2.4. Routes of entry into plants, animals, and humans

The most important route of exposure to arsenic is the ingestion of foods and beverages, which in most cases are contaminated from the use of irrigation water with a high concentration of the element. In general, the water is contaminated by dissolved minerals and contains different

forms of arsenic [24]. Exposure to arsenic occurs via the oral route (ingestion), inhalation, dermal contact, and the parenteral route [25–27].

Arsenic occurs in both inorganic and organic forms, which exhibit large differences in their metabolism and toxicity. The high toxicity of arsenic is well known because arsenic compounds are readily absorbed by either inhalation or ingestion, the extent of absorption being dependent upon the solubility of the compound.

2.5. Metabolism or transformation in the living system

There is evidence that arsenic has a physiological role related to methionine metabolism [28]. However, the site of action of arsenic remains unknown. Studies with rats fed adequate amino acid–based diets have shown that arsenic deprivation had little effect on growth in rats. However, in rats fed suboptimal methionine, arsenic deprivation resulted in a significant reduction in body weight. It was shown that arsenic deprivation reduces the hepatic concentration of *S*-adenosylmethionine, indicating that arsenic maintains the metabolic pool of *S*-adenosylmethionine [28].

In addition, arsenic status affects DNA methylation in animal and cell culture models, resulting in an apparent hypomethylation of DNA [29]. This process is associated with an increased incidence of cancer. So, there is an amount of dietary arsenic that is harmful or beneficial to humans [30, 31].

2.6. Biological functions

In body, arsenic is present as arsenite and arsenate. Arsenic species interact strongly with sulfhydryl groups of organic molecules. It affects several enzymes, causing damage in several cell systems. Because of their similar properties, arsenate can substitute for phosphate and other phosphate intermediates in several biochemical reactions. At the cellular level, arsenate depletes adenosine triphosphate (ATP) in human erythrocytes, interrupting the production of energy [31, 32].

Although some research has indicated that arsenic is an essential nutrient for rats, chickens, and pigs, however, no studies have been published in the literature to determine the nutritional importance of arsenic in humans [32].

2.7. Mechanisms of toxicity of arsenic

The toxicity of arsenic is highly influenced by its oxidation state and solubility, as well as many other factors [33]. As(III) binds to thiol or sulfhydryl groups on proteins and can inactivate over 200 enzymes. As(V) can replace phosphate, which is involved in many biochemical pathways [34].

Mechanism by which arsenic exerts its toxic effect is due its ability to interact with sulfhydryl groups of proteins and enzymes and to substitute phosphorous in various biochemical reactions [34].

In humans, As(III) is methylated to two major metabolites via a non-enzymatic process to monomethylarsonic acid (MMA), which is further methylated enzymatically to dimethyl arsenic acid (DMA) before excretion in the urine [34].

Various hypotheses have been proposed to explain the carcinogenicity of inorganic arsenic. Nevertheless, the molecular mechanisms by which this arsenic induces cancer are still poorly understood [35, 36].

2.8. Incidence of (acute and chronic) toxicity

Arsenic can cause numerous human health effects. Several epidemiological studies have reported a strong association between arsenic exposure and increased risks of both carcinogenic and systemic health effects [25]. The severity of adverse health effects is related to the chemical form of arsenic and is also time and dose dependent [26, 37, 38]. Among the notable effects and diseases are skin lesions, neurotoxicity, cardiovascular diseases, abnormal glucose metabolism, diabetes, peripheral vascular diseases, coronary heart diseases, myocardial infarction, stroke, gangrene, kidney failure and liver failure, cancer of the internal organs, particularly the bladder and lung, skin pigmentation, keratoses, and skin cancer [24, 26, 34–37].

2.9. Comparative analysis of analytical techniques

Several techniques have been used for the detection of arsenic in foods. Among the combined techniques are the use of chromatography or inductively coupled plasma coupled with mass spectrometry (MS) [39], mass spectrometry–desorption electrospray ionization (MS-DESI) spectrometry, inductively coupled plasma–optical emission spectrometry (ICP-OES), and hydride generation–atomic absorption spectrometry (HG-AAS) [39, 40], which generally allow the chemical speciation of arsenic and other techniques such as capillary electrophoresis coupled to inductively coupled plasma and mass spectrometry (CE-ICP-MS) [41]. However, these techniques are expensive. In this regard, an interesting approach to determining arsenic species with detection by electrothermal atomic absorption spectrometry after cloud point extraction (ETAAS/CPE) was developed by Baig *et al.* [42] and Costa *et al.* [43].

3. Chromium

3.1. Chemistry of chromium

Cr is a naturally occurring element present in the earth's crust and is found in two oxidation states, namely hexavalent chromium (Cr(VI)) and trivalent chromium (Cr(III)). Hexavalent chromium (Cr(VI)) compounds are, in general, more toxic than Cr(III) compounds. Cr rarely occurs as a pure element. The most common ore of Cr is ferrochromite [44, 45].

3.2. Occurrence in the environment

Cr is an element available in the environment, found mostly in minerals, rocks, plants, soil, water, dust, and volcanic gases. Cr can be present as a contaminant in the environment from

various natural and anthropogenic sources [46]. Cr released into the environment as anthropogenic activity occurs mainly from metallurgical and chemical industries such as tannery facilities, chromate production, stainless steel welding, and ferrochrome and chrome pigment production.

The health hazard associated with exposure to Cr depends on its oxidation state, ranging from the low toxicity of the Cr(III) form to the high toxicity of the Cr(VI) form [47]. Cr(III) is an essential trace mineral present in trace amounts in some foods, such as meat, whole grains, oleaginous plants, and legumes.

3.3. Dietary sources of chromium

Cr(III) is considered to be essential to mammals for the maintenance of glucose, protein, and lipid metabolism, whereas Cr(VI) is detrimental to human health even at relatively low concentration levels, because it can be involved in the pathogenesis of some diseases such as liver, kidney, lung, and gastrointestinal cancers [48–50]. Cr(III) is a stable and biologically active state of Cr, and it is found in many types of foods, including egg yolk, whole grains, cereals, coffee, nuts, green beans, broccoli, meat, beer yeast, and drinks produced from grapes. Cr is also available in many dietary supplements and is responsible for the proper functioning of the metabolism of carbohydrates and lipids. Table 1 shows the amounts of Cr that can be found in some foods.

Food	Amounts of chromium (μg 100 g^{-1})
Broccoli	11–22[b]
Dry garlic	60[a]
Mashed potato	1.5[a]
Whole wheat bread	4.4[a]
Champagne	1.1–3.6[c]
Red wine	0.7–9.0[c]
White wine	0.7–4.4[c]
Green grapes	0.3–2.1[c]
Red grapes	0.2–6.5[c]
Apple	0.8[a]
Grape juice	4.0[b]
Orange juice	1.0[a]

[a]Adapted source by NHI [51].

[b]Adapted source by Oliveira and Machine [52].

[c]Adapted source by Cabrera-Vique *et al*. [53].

Table 1. Quantity of chromium in some foods.

3.4. Routes of entry into plants, animals, and humans

Cr-containing compounds have been a major concern because of Cr release into the environment and the high risk of Cr-induced diseases in industrial workers occupationally exposed to Cr(VI) [54]. The route of human exposure to Cr is through skin and mainly through inhalation, and the lung is the target organ [55–57]. Non-occupational exposure occurs through ingestion of Cr- containing food and water. Cr content in foods varies greatly and depends on the processing and preparation of foods.

3.5. Metabolism or transformation in the living system

The main path for Cr(III) to get into the organism is through the digestive system. The mechanism of Cr intestinal absorption is not yet fully known, but it is known that Cr(VI) compounds are absorbed better than Cr(III) compounds. Absorbed Cr circulates in blood bound to the β-globulin plasma fraction and is transported to tissues bound to transferrin or other complexes at the physiological concentration [58]. Cr from blood is relatively quickly absorbed by bones, accumulating also in the spleen, liver, and kidneys. Cr is excreted especially by the urinary system.

3.6. Biological functions

Cr plays an important role in carbohydrate, lipid, and glucose metabolism [58–60]. Studies show evidence that Cr acts as a cofactor for insulin, and therefore, Cr activity in the organism is parallel to insulin functions. It is assumed that the activity of Cr is mediated by the anabolic action of insulin. Cr supplementation intensifies amino acid uptake by tissues such as these the binding of Cr to nucleic acids is stronger than in other metal ions [60]. Cr(III) seems to be involved in the structure and expression of genetic information in animals. Also, Cr protects RNA from heat denaturation and, among other functions, promotes the growth of the animals.

3.7. Mechanisms of toxicity of chromium

The toxicity of Cr compounds depends on its oxidation state and solubility [61–63]. Cr(VI) compounds are more toxic than Cr(III) compounds most likely due to the ease with which Cr(VI) can pass through cell membranes and its subsequent intracellular reduction to reactive intermediates [64–66]. As Cr(III) is poorly absorbed by any route, the reduction of Cr(VI) is considered as being a detoxification process. If Cr(VI) is reduced to Cr(III) extracellularlly, then Cr(III) is not readily transported into cells, and so toxicity is not observed. Under physiological conditions, Cr(VI) can be reduced Cr(III) by hydrogen peroxide (H_2O_2), glutathione (GSH) reductase, ascorbic acid, and GSH [66, 67].

3.8. Incidence of (acute and chronic) toxicity

Cr is of particular interest because its toxicity is highly dependent upon its chemical forms and concentration. Cr(VI) shows high toxicity and is related to clinical cases such as nasal irritation

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and ulceration, hypersensitivity reactions, and dermatitis through contact. The lethal dose is between 50 and 100 mg kg^{-1}, which is much lower than that of Cr(III), with a lethal dose between 1900 and 3300 mg kg^{-1} (both cases tested by oral ingestion in rats). Furthermore, Cr(VI) is classified as carcinogenic because it penetrates the cell membranes of living organisms [59]. Exposure to Cr(VI) can occur mostly through inhalation, skin contact, and ingestion. Cr(VI) inhalation, for example, besides causing severe irritation of the respiration system, is also carcinogenic. Although the WHO has established a limit for human consumption of 0.005 mg kg^{-1} body weight per day, no scientific studies have proved that Cr ingestion can cause disease. The potential effects of Cr(VI) vary mainly with the species, the amount absorbed into the bloodstream, and the route and duration of exposure [68–70]. Thus, Cr(VI) is found in most lists of high-toxicity elements for which strict control procedures apply. The difficulty in establishing a recommended dietary allowance (RDA) for Cr is mainly due to the limitations related to estimating the ingestion levels of this mineral, which range from the absence of data on the amount of Cr present in foods, due to analytical difficulties given the trace concentrations, to environmental contamination problems [70].

The ingestion of Cr(VI) is detrimental to human health even at relatively low concentration levels because it may be involved in the pathogenesis of some diseases, such as liver, kidney, lung, and gastrointestinal cancers. Following studies, many authors have suggested that chromium picolinate can cause DNA damage [60, 61], but there is no confirmation of carcinogenesis in animals [62]. There are reports of toxicity after supplementation, but the results of other investigations did not indicate hepatic alterations [64–70]. Based on this impasse, the US Agency of Toxic Substance and Disease Registration concluded that there is no conclusive evidence that supplementation causes liver damage, although it does have proven deleterious effects on the kidneys [67].

3.9. Comparative analysis of analytical techniques

The determination of Cr can be carried out through sensitive techniques that are able to quantify a few micrograms of this element. One of these techniques is graphite furnace–atomic absorption (GF-AAS), consolidated after 1981, and is able to detect Cr concentrations of around 0.2 μg kg^{-1} in food. More sensitive techniques developed later, such as ICP-MS and ICP-OES [71], are used for the determination of Cr. These techniques quantify only total Cr without promoting speciation, and separation techniques, such as chromatography, are required.

The official method for the analysis of Cr in food samples is ICP-OES using nitric acid and hydrogen peroxide to oxidize organic materials in food samples [71]. This technique has high sensitivity (of the order of 1 ng L^{-1}). However, this equipment is sophisticated and expensive with high operational costs. An option that combines higher sensitivity and lower cost is GF-AAS technique. This technique has important advantages such as a reduced amount of sample and high sensitivity, and the analysis can be carried out with minimal or no sample preparation [71].

4. Lead

4.1. Chemistry of lead

Pb is a heavy metal that has malleability, low melting point, low electrical conductivity, and high corrosion resistance. These properties allow its widespread use in the manufacture of blades and pipes of high flexibility and resistance in welds and coatings in the automotive industry; protective plates against ionizing radiation (e.g. X-rays); alloys; coating cables; and paints, dyes, and plastic additives [72]. Usually, inorganic Pb compounds are found as Pb(II) and rarely found as a pure element. Its most common mineral is galena or lead sulfide (PbS). The solubility of Pb compounds is enhanced at lower pH, suggesting that the increased mobility of the Pb is found in ecosystems under stress acidification [73].

4.2. Occurrence in the environment

Pb is a metal that occurs naturally, making up only about 0.0013% of the earth's crust. However, most Pb concentrations that are found in the environment are the result of human activities such as burning of fossil fuels and mining [74]. Pb can be found in the atmosphere in particulate form, being deposited in water systems, interfering with the characteristics of the water. In other cases, this metal may be found complexed with natural organic compounds [75]. In contact with the ground, Pb can remain for a long time and in various forms (such as insoluble and soluble complexes and colloids) and absorbed by plants, accumulating in the edible parts, causing contamination in humans and animals [74–78].

4.3. Dietary sources of lead

The WHO and the Expert Committee on Food Additives—"JECFA" initially established a provisional tolerable weekly intake (PTWI) for lead of 50 μg kg^{-1} body weight for adults. However, after assessing the risk to health, the JECFA later reduced this value to 25 μg kg^{-1} body weight, equivalent to 3.5 μg kg^{-1} body weight per day (equal to 1.75 mg $week^{-1}$ or 1750 μg $week^{-1}$ for a person weighing 70 kg) [20, 21].

The Expert Committee noted, however, that some foods with high levels of Pb remain commercially available [22]. A reference value for Pb of 0.01 mg L^{-1} in drinking water was established by the WHO. The concentrations in drinking water are typically below 5 μg L^{-1}, although higher concentrations (above 100 mg L^{-1}) have been reported. The EPA regulations establish limits in the form of maximum contaminant levels (MCLs), and the value for Pb in drinking water is 0.015 mg L^{-1}, even though the EPA has also established a goal for zero Pb in this regard [23].

4.4. Routes of entry into plants, animals, and humans

The main routes of human exposure to Pb are by ingestion (food, water, and soil), inhalation, and skin [79]. The compounds of tetra-alkyl Pb (Pb tetra acetate, etc.), for example, are rapidly absorbed through the lungs, gastrointestinal tract, and also the skin. Usually, a high level of

metal enters the body through the ingestion of contaminated cereals and vegetables [80]. Once absorbed, inside the body Pb is distributed by the blood reaching the soft tissue and then is deposited in the bones and other hard body parts. It is slowly excreted in urine and feces [79].

4.5. Metabolism or transformation in the living system

In the human body, Pb is not metabolized, but it forms complexes with macromolecules. Pb forms complexes (sulfur groups, –SH) through covalent bonds, causing the intoxication of humans [81]. Pb can disturb the metabolic functions in two ways: (1) it accumulates, thereby disrupting the function of vital organs and glands such as the heart, brain, kidney, bone, liver, so on and (2) it moves the vital nutritional minerals from their original location, hindering their biological function [82].

4.6. Biological functions

Pb is a toxic metal that would not have known beneficial effects to the body, and its accumulation over time in the bodies of animals and humans can cause severe illness [83].

4.7. Mechanisms of toxicity of lead

One of the main reasons by which Pb exerts toxic effect is its ability to substitute diverse cations (calcium, zinc, and magnesium) in their binding sites. Pb has a greater affinity than calcium and zinc ions to protein-binding site because of its larger ionic radius and greater electronegativity. For example, Pb interacts with oxygen and sulfur binds to sulfhydryl and amide groups of enzymes, altering their configuration and diminishing their activities, and competes with calcium in skeletal tissue and to interact with proteins [84].

In the blood, Pb is distributed to the remaining tissues, where it accumulates; the amount of metal accumulated depends mainly on the vasculature and metabolic characteristics of each tissue [85]. The half-life of Pb is 35 days in the blood and is about 2 years in the brain, and it can last for decades in bone.

Many investigators have demonstrated that Pb affects biomolecules and hence physiological systems. For example, calmodulin is a protein found primarily in the brain and heart. The binding of calcium ions of this protein allows the binding of this protein to cyclic nucleotide phosphodiesterase and adenylate cyclase with subsequent activation. Thus, this protein modulates the levels of AMP and cyclic GMP [86]. Pb is a more potent activator than calcium for calmodulin. According Kern, Pb modifies several signaling cascades and proteins that participate in the vesicular cycle [87]. The alterations caused by the abnormal protein operation in second messenger systems and exocytic processes greatly contribute to Pb neurotoxicity [87].

Pb affects various cellular organelles, for example, mitochondria and endoplasmic reticulum, in different ways. In the mitochondria Pb affects energy metabolism, while in the endoplasmic reticulum Pb increases the cytoplasmic concentration of calcium with a consequent reduction in ion concentration inside this organelle. Many signaling pathways that are within the endoplasmic reticulum are calcium dependent; because the amount is not appropriate, various processes are impaired. Besides, Pb binds to Ape1 nuclease, whose function is to detect and

repair DNA damage, inhibiting its operation and allowing the accumulation of mutagenic damages [85–88].

4.8. Incidence of (acute and chronic) toxicity

Pb is one of the most common environmental contaminants. This element has no known physiological function in the organism, and its damaging effects can affect almost every organ and system in the body [89]. The main way in which Pb enters the body is through the respiratory route (occupational exposure), followed by the digestive route. Organic Pb compounds can penetrate in the body through skin contact and are rapidly absorbed [90, 91].

Exposure to Pb can result in a wide variety of biological effects, depending on the exposure level and duration. The major diseases related to Pb contamination are shown in Table 2. Pb is toxic to various organs and systems, and its effects may vary from enzyme inhibition and anemia to diseases of the nervous, immune, reproductive, and cardiovascular systems, impaired kidney function, and even death.

Studies have suggested an association between Pb exposure and lung cancer and, to a lesser extent, stomach cancer [90]. Pb is hypothesized to be a carcinogen and to enhance the genotoxic effects of other agents. Renal tumors developed in mice that had received high doses of certain Pb compounds and various other animal studies have shown increases in the yield or genotoxicity of known carcinogens. The US Environmental Protection Agency has determined that Pb is a probable human carcinogen [89–91].

Effects on health	Site of the body affected	Adverse effects
Neurological	Central nervous system, peripheral, and autonomic	Acute and chronic encephalopathy; peripheral neuropathy
Hematological	Blood	Anemia
Endocrine	Bone tissue and serum	Damage to the kidneys and development of cells, teeth, and bones. Possible damage to the thyroid.
Growth	Bone	Reduced growth
Reproductive	Male and female reproductive systems	Reduced fertility, high probability of miscarriages
Carcinogenic	Kidneys and cells—genomic DNA	Carcinogenic to animals and epigenetic involvement in the expression of the modified gene
Cardiovascular	Cardiovascular system	Likely increase in blood pressure, cardiac lesions, and abnormal electrocardiograms
Gastrointestinal	Gastrointestinal tract	Colic
Hepatic	Liver	Reduced functional capacity of the cytochrome P-450 to metabolize drugs

Table 2. Main health effects related to lead contamination [89–91].

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4.9. Comparative analysis of analytical techniques

The determination of traces of Pb in various food samples is of great importance because Pb is recognized as a cumulative poison in humans and other animals [92]. The determination of Pb requires procedures that are sufficiently sensitive for detection at the pg L^{-1} level. Traditionally, GF-AAS has been applied in such cases, but the direct determination of Pb in complex matrices is usually difficult owing to matrix interference and separation procedures often being required before the sample analysis [93]. The ICP-MS technique is favored because of its low detection limits [93], although many researchers prefer AAS owing to its simpler and less expensive instrumentation. Lead hydride generation and its application to spectrometry analysis have been reviewed by Madrid and Cámara [94]. HG-AAS is a well-developed technique that can be used for the determination of volatile hydride-forming elements such as arsenic, selenium, antimony, and others at trace levels [95]. The advantages of HG-AAS over other atomic absorption spectrometric techniques such as the flame and graphite furnace methods are increase in atomization efficiency and higher selectivity because the analyte is removed from the matrix as a volatile compound and detection limits at the pg L^{-1} level or lower for the elements cited above. Considering these advantages, this technique could be applied for the determination of Pb, and it is possible to include this element in multi-element analysis schemes involving hydride generation.

The generation of Pb hydride was described by Carrijo et al. [96]. In this study, a flow injection–hydride generation–atomic absorption spectrometry (FI-HG-AAS) system was used for Pb determination. The main characteristics of the flow injection system, that is, high sampling rate and good accuracy, precision, and sensitivity, are maintained.

5. Mercury

5.1. Chemistry of mercury

Hg is a metal found in various chemical forms, which can be divided into the following categories: elemental or metallic Hg, inorganic Hg, mainly in the form of mercuric salts ($HgCl_2$ and HgS), and mercuric (Hg_2Cl_2) and organic Hg, for example, methylmercury and ethylmercury [68, 72].

Metallic mercury (Hg) is in the liquid state at room temperature and easily volatilizes into the atmosphere forming Hg vapors. Hg is a metal with widespread use, especially the production of scientific precision instruments, electrical industry, dentistry (production amalgams), the production of certain types of toys, mining, metal smelting, among others [97].

5.2. Occurrence in the environment

Hg is a metal found naturally in the earth's crust, occurring in air, soil, and water [98]. It rarely occurs free in nature and is found mainly in cinnabar ore (HgS). It can be found in metal form, as salts of Hg or organic Hg compounds. Once released, Hg remains in the environment among the circulating air, water, sediment, soil, and biota, which assumes various chemical forms.

Most emission to air occurs in the form of elemental Hg, which is very stable and can remain in the atmosphere for months or even years, enabling transport over long distances around the globe [98]. Most of the Hg released by human activities in air is by combustion of fossil fuels, mining, smelting, and combustion waste [99].

The Hg vapor in the atmosphere can be deposited or is converted into the soluble form, returning to the earth's surface in rainwater. From there, two important chemical changes may occur. The metal can be cast again and return the Hg vapor in the atmosphere or may be "methylated" by the microorganisms present in the water sediments, turning into methylmercury [98]. Furthermore, the Hg can also be released directly in the soil or in water by the application of agricultural fertilizer and disposal of industrial waste water [100].

Atmospheric emissions are the major source of environmental contamination, followed by water pollution and soil contamination, when there is improper disposal of effluents and waste [98].

5.3. Dietary sources of mercury

Usually, Hg contamination occurs by the presence of this metal in water, soil, air, or food, mostly in the form of methylmercury [100, 101]. The most important source of exposure through diet for the general population is the consumption of fish and other marine organisms. Hg is concentrated in the tissue of fish, becoming increasingly potent in predatory fish and mammals that feed on small fish. The larger carnivorous fish have higher concentrations than smaller ones [99]. The average daily intake of methylmercury (mainly from fish) that can cause demonstrable effects on the health of sensitive individuals is 300 mg day^{-1} or 4.3 μg Hg day^{-1} kg body $weight^{-1}$ [102].

Industrial products can also be contaminated by Hg during the processing steps. Studies have shown the contamination of Hg in breast milk (4–15 μg kg^{-1}) [103], in tea (6 ng g^{-1}) [104], and in products for infant feeding (0.50 μg kg^{-1}) [105].

5.4. Routes of entry into plants, animals, and humans

The absorption of Hg by humans and animals can be by pulmonary route (inhalation), as well as by gastrointestinal or cutaneous route. In the case of pulmonary route, Hg after inhalation and the presence in the lung is distributed throughout the body, accumulating in various parts of body [106–108]. Soluble compounds are absorbed by mucous membranes following vapor inhalation and by the skin and the sebaceous glands. In the body, organic and inorganic Hg binds GSH [108]. It acts as inactivator because it readily binds to thiol groups of cellular enzymes and disrupts its function by inactivating the metabolism. The non-absorbed Hg is excreted in feces, and absorbed Hg forms are excreted via saliva and skin.

5.5. Metabolism or transformation in the living system

Hg and its organic compounds in low concentrations cause damage to human health. Their concentrations in surface and ground waters are below 0.5 mg L^{-1}. However, aquatic micro-

organisms convert the organic Hg into inorganic Hg compounds, which accumulate in the food chain. Methylmercury is the most relevant toxicant [109]. The gastrointestinal tract is the second way (after airway) through which Hg, already now in its organic form, enters the human body through the consumption of fish, shellfish, and other aquatic organisms.

5.6. Biological functions

Hg has no biological role. All Hg compounds are extremely toxic, particularly methylmercury [109].

5.7. Mechanisms of toxicity of mercury

The mechanism of toxicity of Hg is based on its chemical activity and biological features. The main mechanism of toxicity of Hg compounds involves their reactivity with sulfhydryl groups. Once in the cell, both Hg^{2+} and MeHg form covalent bonds with cysteine residues of proteins and deplete cellular antioxidants [110].

5.8. Incidence of (acute and chronic) toxicity

Metallic Hg and its organic compounds in very low concentrations cause damage to human health (such as neurotoxic, immunotoxic, and teratogenic properties) and can have high persistence and a high bioconcentration factor (BCF), accumulating in animals, fish, and the environment. Hg poisoning levels and the main symptoms and diseases related to acute and chronic poisoning by Hg are shown in Tables 3 and 4, respectively [111].

24-hour urine	
0.00–0.01mg	Non-toxic
0.02–0.09 mg	Danger of poisoning
0.10–0.80 mg	Chronic intoxication
Above 1.00 mg	Acute intoxication
Above 2.00 mg	Subacute poisoning

Table 3. Mercury poisoning levels.

Acute intoxication	**Chronic intoxication**
Dark gray appearance in the mouth and pharynx	Digestive disorders
Severe pain	Nervous disorders
Vomiting (may even be bloody)	Cachexia
Bleeding gums	Stomatitis
Metallic taste in the mouth	Salivation

Acute intoxication	Chronic intoxication
Burning in the digestive tract	Bad breath
Severe or bloody diarrhea	Loss of appetite
Inflammation of the mouth (stomatitis)	Anemia
Tooth decay and/or loose teeth	Hypertension
Glossitis	Loosening of the teeth
Swelling of the gum mucosa	Central nervous system disorders
Kidney nephrosis	Mild kidney disorders
Serious liver problems	Possibility of chromosomal alteration
Can even cause sudden death (1 or 2 days)	–

Table 4. Main symptoms and diseases related to acute and chronic poisoning by mercury.

5.9. Comparative analysis of analytical techniques

The determination of Hg in food samples is critical to assess the degree of human exposure, and thus reliable analytical techniques with high sensitivity are required. However, in most situations, the determination of Hg species is not an easy task due to low concentrations in the samples and the characteristic volatility [112, 113]. The volatility of Hg requires special consideration when treating the sample. Food sample preparation using a microwave oven has been widely employed [114].

In the case of the quantification of methylmercury in fish samples and seafood, depending on the nature of the sample and the technique used, an additional pre-concentration step is required. Hg determination has been performed using cold vapor coupled to atomic absorption spectrometry (CV-AAS), cold vapor coupled to atomic fluorescence spectrometry (CV-AFS), inductively coupled plasma optical emission spectrometry (CV-ICP-OES), and inductively coupled plasma mass spectrometry (CV-ICP-MS). Hyphenated techniques involving gas or liquid chromatography separations with detection by element-specific detectors such as ICP-MS and atomic absorption/emission are the most commonly reported [114].

6. Conclusions

The information gathered herein highlights the risks associated with arsenic, Cr, Pb, and Hg contamination in foods. Therefore, measures should be taken to reduce exposure of the general population to these contaminants to minimize the risk of adverse health effects. The development of simple strategies suitable for obtaining quantitative information regarding some species of great interest should be encouraged.

In the food industry, analytical chemistry plays an important role, contributing new analytical procedures and instrumentation. Methods for the determination and monitoring of metals are

still scarce. However, there are inherent difficulties associated with the types of samples involved. There are various types of food samples and a great variation in their compositions. This hinders the application of analytical techniques for the fast and accurate monitoring of metals in real samples. More sophisticated techniques are of interest in some fields of application, but these techniques have not yet reached the food industry. Thus, chemists need to direct their attention toward these trends with the aim of narrowing the gap between science and the food industry. These studies require an interdisciplinary approach to cover the various aspects involved and could achieve important advances in toxicology, chemistry, and food science.

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Author details

Luciana M. Coelho[1], Diego R. Pessoa[2], Kênia M. Oliveira[2], Priscila A.R. de Sousa[1], Lícia A. da Silva[1] and Nívia M.M. Coelho[2*]

*Address all correspondence to: nmmcoelho@ufu.br

1 Department of Chemistry, Federal University of Goiás, Catalão, GO, Brazil

2 Institute of Chemistry, Federal University of Uberlândia, Uberlândia, MG, Brazil

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Chapter 5

Technologies for Detecting Botulinum Neurotoxins in Biological and Environmental Matrices

Luisa W. Cheng, Kirkwood M. Land, Christina Tam, David L. Brandon and Larry H. Stanker

Additional information is available at the end of the chapter

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Abstract

Biomonitoring of food and environmental matrices is critical for the rapid and sensitive diagnosis, treatment, and prevention of diseases caused by toxins. The U.S. Centers for Disease Control and Prevention (CDC) has noted that toxins from bacteria, fungi, algae, and plants present an ongoing public health threat, especially since some of these toxins could compromise security of the food supply. Botulinum neurotoxins (BoNTs), produced by *Clostridium* spp., are among those bacterial toxins that pose life-threatening danger to humans. BoNTs inhibit the release of acetylcholine at peripheral cholinergic nerve terminals and cause flaccid paralysis. BoNTs are grouped in seven serotypes and many subtypes within these groups. Rapid and accurate identification of these toxins in contaminated food as well as in environmental matrices can help direct treatment. Herein, we discuss current methods to detect BoNTs with a focus on how these technologies have been used to identify toxins in various food and environmental matrices. We also discuss the emergence of new serotypes and subtypes of BoNTs and the increasing number of cases of botulism in wildlife. Finally, we consider how environmental changes impact food safety for humans and present new challenges for detection technology.

Keywords: Botulism, Toxins, Food matrix, Environmental detection, Foodborne illness

1. Introduction

The U.S. Centers for Disease Control (CDC) have summarized the risks that biological toxins pose to human health [1]. Bacteria, fungi, parasites, and plants all produce toxins in the environment that can impact food safety. Furthermore, changes in the environment have caused emergence of new problems associated with toxins. One example is the production of toxins by *Clostridium botulinum*. This pathogen, which is a gram-positive, anaerobic spore-

forming bacterium, produces botulinum neurotoxins (BoNTs). Humans are susceptible to the effects of these poisons, which are among the most toxic molecules known [1]. The parenteral lethal dosage for humans is 0.1–1 ng/kg, and the oral dose is 1 μg/kg. A single gram of BoNT released into the environment and subsequently inhaled could kill more than one million people [2, 3]. BoNTs exert their biological effects by blocking acetylcholine release by neurons. To date, BoNTs have been divided into seven serotypes, denoted as A through G, of which A, B, E, and F are known to be toxic to humans [4–6]. However, all of the botulinum serotypes are possibly toxic to people. In addition to the principal serotypes, at least 40 additional subtypes have been described based on differences in both primary peptide sequence and three-dimensional structure [4–6]. In this discussion, we review the basic biology of BoNTs and current methods to detect these molecules in biological and environmental matrices.

C. botulinum isolates are categorized into different groups [5, 6]. Members of Group I are referred to as "proteolytic" and produce toxin types A, B, or F. They are widely distributed in the environment and often found in various raw foods. BoNTs can cause symptoms at levels as low as 5 ng. Although the onset of symptoms typically takes 12–36 hours, the time course depends on the amount of toxin ingested [2, 3]. It can take much longer for symptoms to manifest. Initial symptoms include diarrhea and vomiting followed by neurological effects that include blurred vision, weakness, and difficulty in swallowing, talking, and breathing. Unless diagnosed early, mortality rates can be as high as 40% [1–4]. Timely response and current treatments have reduced mortality to less than 10%. The most common foods involved in outbreaks are improperly preserved meat or fish products, but a range of other foods have been implicated, such as cheeses (including vegetables preserved in oil and cheese). Because botulinum toxins are not heat stable, they can be inactivated at cooking temperatures.

Strains in Group II are classified as "non-proteolytic" [5, 6]. These *C. botulinum* strains synthesize neurotoxin B, E, or F. These bacteria can grow at temperatures <3°C are ubiquitous in the environment. Moreover, one can find Type E strains in aquatic habitats [1–4]. It is not known whether these strains can synthesize neurotoxins in refrigerated processed foods without visible spoilage. The endospores of strains in this group are not as resistant to heat as those strains in Group I. Neurotoxins synthesized by strains in Group II toxins have shown to be less potent than those of Group I; at least 0.1 μg of neurotoxin is required to cause symptoms of botulism. However, their other biological properties are similar. Foods involved in outbreaks of Group II botulism include cold-smoked fish and other preserved fish products.

Group III botulinum produces toxins of serotype C or D and is associated with avian and non-human mammalian botulism [5, 6]. Whole genome sequencing analysis indicates that strains of physiological group III are probably more related to *Clostridium haemolyticum* and *Clostridium novyi* than to *C. botulinum* serotypes belonging to Groups I and II. Group IV is rare and has not been well characterized. However, it does synthesize neurotoxin serotype G.

Bacteriophages contain the neurotoxin genes of *C. botulinum* serotypes C and D [5, 6]. The BoNT prophage replicates in the bacterium as a large plasmid, and strains containing the phage can become toxigenic via either type C or type D phages. The distinction between types C and D is not clear because chimeric sequences have been isolated from the environment. These toxin genes have been identified in avian isolates. They contain sections from both BoNT/C

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and BoNT/D genes and are referred to as type C/D [5, 6]. The chimeric toxin is more pathogenic to avians than either serotype C or D individually. In *C. botulinum* serotypes C and D, there is a small amount of a binary toxin, denoted as the C2 toxin. The genes encoding the C2 toxin have been localized to a plasmid. Structurally and functionally, the C2 toxin contains a translocation domain and an ADP-ribosylating domain that has been shown to target cellular actin. The occurrence of other chimeric botulinum toxin genes has yet to be determined [5, 6].

At the amino acid sequence level, BoNT serotypes can differ from one other by 34–64% [5, 6]. Significant genetic variation within each serotype has also been observed. In fact, 32 toxin subtypes with amino acid sequence differences of 2.6–32% have been identified thus far, and more will likely be identified in the future [5, 6]. This serotype and subtype diversity confound direct antibody and molecular-based assay designs. It is rare that one probe can bind to all serotypes. In *C. botulinum*, the neurotoxins are first synthesized as a large holotoxin (approximately 150 kDa). They are then processed by a trypsin-like protease in *C. botulinum* yielding two polypeptides (one approximately 100 kDa and the other approximately 50 kDa) that are still bound by a single disulfide [2, 3]. The neurotoxin structure mimics other known A–B dimeric toxins found in other bacterial pathogens. The ~100 kDa fragment is called the heavy chain (HC) and aids the binding of the neurotoxin to host cell receptors and its translocation from vesicles to the cytoplasm [2, 3]. The ~50 kDa fragment, called the light chain (LC), contains the enzymatically active domain of the neurotoxin. Recombinantly, expressed LC is routinely used for activity-based neurotoxin assays. Antibodies specific for the HC and LC are used for immunoassays for detecting neurotoxins as well as for neutralization.

2. Important factors to consider when developing toxin detection assays

The development of a robust assay for the detection of any pathogen or biological product of a pathogen (such as a toxin) requires consideration of several factors: sensitivity, specificity, matrix effects, and biological activity [8–10]. Each of these factors is briefly discussed below in the context of assay methods for *C. botulinum* toxins.

3. The mouse bioassay

In the laboratory, a rodent bioassay is considered the "gold standard" method for detecting BoNTs [8–10]. Despite much effort to replace the use of animals, it is still the most sensitive and reliable assay to model all aspects of BoNT intoxication: binding, translocation, enzymatic activity, and pathology. Alternatives to the mouse bioassay have been developed (discussed below) with shorter assay times and equal or greater sensitivity.

The mouse assay quantitatively determines the amount of BoNT required to kill all mice in a test group. This measurement is expressed as a minimal lethal dose (MLD). Although protocols may vary, mice are usually injected intraperitoneally with 0.5 mL of BoNT sample in a dilution series and then monitored over several days for signs of intoxication and death

[11, 12]. If enough sample is available for an assay, the specific neurotoxin can be identified using neutralization with antibodies specific to each of the neurotoxin serotypes (A–G). The toxin serotype is therefore revealed based on which antibody confers protection from death. The mouse bioassay is highly sensitive and useful for detection of different neurotoxins in different matrices. However, despite its versatility, the mouse assay has limitations that include: long assay times and the use of animals requiring specialized animal facilities, substantial costs, trained staff, and consideration of ethical issues, especially when death is used as an endpoint. There is also substantial variation in results observed among different research laboratories [8–12].

Alternative "refined" animal assays that do not use death as an endpoint, such as the mouse phrenic nerve hemi-diaphragm assay, have been evaluated [13, 14]. Despite being more sensitive and rapid compared to the use of whole animals, these assays usually require the use of specific equipment and personnel with specialized training. Furthermore, these alternative animal assays are not feasible with larger samples and those containing a complex matrix. However, a recent study described an *in vivo* assay using a toe-spread reflex model. This method was used to detect neurotoxins in simple buffer solutions, samples containing serum, and milk [15]. This new assay provides results more quickly than standard mouse bioassays. Whether these results can be translated into a user-friendly, deployable kit has yet to be determined.

4. DNA and other nucleic acid–based methods

Numerous nucleic acid methods have been developed for detecting clostridial DNA in biological and environmental matrices. The polymerase chain reaction (PCR) to identify the presence of *C. botulinum* DNA was originally used to detect the presence of bacterial spores in samples [16]. The method is capable of detecting the presence of as few as 100 spores per reaction mixture for serotypes A, E, and F and only 10 spores per reaction mixture for serotype B.

Multiplex PCR methods have also been developed to analyze unknowns for a battery of different targets such as different pathogens and/or associated gene products of these pathogens. Multiplexed assays employ different combinations or sets of PCR primers, each one specific for a gene of interest, to amplify multiple targets in one PCR tube. One such multiplex method could possibly discriminate among BoNT serotypes A, B, E, and F. As previously described, Peck et al. [16] developed a culture enrichment method that, when coupled with multiplex PCR, could identify strains of *C. botulinum* that were non-proteolytic (such as BoNT serotypes B, E, and F). This method was robust and rapid enough for use with food samples contaminated with *C. botulinum*.

Real-time PCR (RT-PCR) or quantitative PCR (qPCR) is also useful in studies of gene expression, specifically differential expression of genes under various environmental conditions or comparative studies of different organisms. For detection of clostridial DNA, RT-PCR methods examine expression of the NTNH (non-toxic, non-hemagglutinin) and numerous other genes in *C. botulinum* serotypes A, B, E, and F [18]. Pentaplex methods have been developed to

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simultaneously identify and discriminate among larger numbers of different serotypes, using a wider array of different genes [19]. This technology may prove to be efficient and cost-effective.

The GeneDisc Cycler is an apparatus to perform RT-PCR applications using the GeneDisc system. The GeneDisc is a disposable plastic reaction tray that is the size of a compact disc. This method has been designed for simultaneously testing for the BoNT/A, BoNT/B, BoNT/E, and BoNT/F genes. In 2011, Fach et al. evaluated the GeneDisc Cycler equipment with neurotoxin-producing clostridia and non-BoNT-producing bacteria isolated from various clinical, food, and environmental samples [20]. Results obtained using this "macroarray" were also compared to the mouse bioassay. The toxin genes were detected in all clostridial serotypes A, B, E, and F as well as in toxigenic *Clostridium baratii* Type F and toxigenic *Clostridium butyricum* Type E. No cross-reactivity was observed with bacteria not toxigenic to humans as well as *C. botulinum* Types C, D, and G. An evaluation of the GeneDisc array was performed in four European laboratories with BoNT-producing clostridia and 10 different samples that included food matrices and clinical isolates [20]. Results demonstrated the technology to be specific and reliable in the identification of *C. botulinum* cells containing genes encoding neurotoxins A, B, E, and F. Furthermore, contaminated food and fecal samples were successfully tested. This assay is highly sensitive, capable of detecting as low as 5–50 genome copies in each PCR assay. The GeneDisc Cycler can also be used for monitoring neurotoxin-producing clostridia in food samples, clinical samples, and environmental matrices. A similar study was carried out examining a focused microarray for detection of genes-encoding BoNTs [21].

Recently, Kolesnikov et al. [22] described a new method called "proteolytic PCR" in which PCR is used to assay the proteolytic activity of botulinum toxin. This technology starts with DNA–protein complexes attached to a solid phase. Proteolytic cleavage releases DNA into solution. The DNA can then serve as a template for PCR. This study described its use to detect botulinum toxin and tetanus toxin proteolytic activity [22].

5. Immunological and antibody-based assays

Enzyme-linked immunosorbent assay (ELISA) is a common assay used to detect BoNTs. This method utilizes anti-BoNT capture and detector antibodies arranged in a "sandwich" format. The detection formats are most commonly luminescent- or colorimetric-based. Prior generations of BoNT immunoassays were approximately 10 times less sensitive than the mouse bioassay described in the previous section. Although not as sensitive, ELISA methods are relatively fast, inexpensive, and simple to perform. They are also less subject to inhibitory matrix effects. An amplified ELISA for detecting toxins in food matrices has also been described [23]. Toxins for serotypes A, B, E, and F could be detected in liquids, solid food, and semisolid food. Assay performance was evaluated in a range of food matrices, such as broccoli, orange juice, bottled water, cola soft drinks, vanilla extract, oregano, potato salad, apple juice, meats, and dairy foods. The assay sensitivity varied for each botulinum serotype. The tests readily detected 2 ng/mL of serotypes A, B, E, and F in various foods tested. Recently, traditionally

formatted, very sensitive sandwich ELISAs used high affinity mAbs against BoNT/A and BoNT/B to detect BoNT/A as low as 5 and 25 pg/mL in buffer and in a milk matrix, respectively; and BoNT/B at 100 fg/mL and 39 pg/mL in buffer and milk matrix, respectively [24–26].

These mAbs were used in an electrochemiluminesence (ECL) immunosorbent assay using the Sector 2400 Imager (Meso Scale Discovery [MSD], Rockville, MD, USA) instrument [27, 28]. Detection sensitivities for BoNT/A in this system were similar to traditional ELISAs in buffer but were markedly improved in liquid food matrices because of the reduced background signal. The higher sensitivity and reduced time required for these new immunosorbent methods make them potential alternatives to the mouse bioassay. Sharma et al. recently developed another ECL assay for simultaneous detection of several biothreat agents (including clostridial neurotoxins) in milk products, with limit of detection (LOD) of 40 pg/mL for BoNT/A complex [28]. The ECL assay was also successfully applied to screen *C. botulinum* serotype A outbreak strains. The study also showed that this sensitive ECL assay is rapid (it can be completed in less than 6 hours). The ECL assay also has potential for using as an *in vitro* screening method, complementing or replacing other immunoassays.

Cheng and Stanker [27] evaluated the performance of antitoxin mAbs using the same electrochemiluminescence immunoassay platform (Sector 2400 Imager, MSD). The ELISA and ECL methods were observed to be more sensitive than the mouse bioassay. In fact, the ECL assay was able to outperform ELISA in terms of detection sensitivity—including food matrices spiked with BoNT/A and in some food matrices spiked with BoNT/B. The ELISA and ECL methods are fast and simple alternatives to the mouse bioassay and can be used for detecting BoNTs in food matrices and serum samples.

One example of mAb development using a recombinant immunogen was the work of Liu et al. [29], who expressed the recombinant H(C) subunit of BoNT type A (rAH(C)). Two out of 56 mAbs were selected to establish a highly sensitive sandwich chemiluminescence enzyme immunoassay (CLEIA) with LOD for both rAH(C) and BoNT/A of 0.45 pg/mL. This CLEIA can be used to detect BoNT/A in matrices, such as milk and beef extract. This method is 20–40-fold more sensitive than the mouse bioassay and takes only 3 hours to complete, making it a useful method to detect and quantify BoNT/A.

The multiplex technology discussed above to detect nucleic acid has also been applied to the development of methods to analyze multiple epitopes on a single antigen and multiple targets in a single sample. This approach uses multiple mAbs as well as polyclonal antibodies to reduce false-positive and false-negative results. A commercialized system, Luminex xMAP technology, utilizes microsphere beads conjugated with antibodies. It employs paramagnetic beads instead of non-magnetic polystyrene beads and is very useful for the analysis of food matrices. The antibody-bead complexes detect multiple epitopes in a single sample. This technology was used to detect abrin, ricin, BoNTs, and staphylococcal enterotoxins in spiked food samples [30].

Zhang et al. [31] developed ELISA-based protein antibody microarrays to simultaneously detect six serotypes of BoNTs. Using numerous different food and other matrices, the microarray is capable of detecting BoNT serotypes A through F. Using engineered, high-affinity

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antibodies, these serotypes were detected to similar levels in various matrices and were comparable to detection in buffer.

Accurate and sensitive detection of contaminated food and other biological samples in the environment is critical. Brunt et al. [32] have developed an affinity column-based assay for detecting neurotoxin in food matrices—specifically serotypes A, B, E, and F. The detection limit for BoNT/A was reported as 0.5 ng, which is two-fold more sensitive than lateral flow methods (also see Section 6) [32]. For serotypes B, E, and F, the minimum detection limit ranged from 5 to 50 ng. Although not as sensitive as ELISA or mouse bioassay, rapid immunochromatographic methods generally require only 15–30 minutes to complete. They do not require enrichment steps and are amenable to use in the field.

Koh et al. have presented a new technology called SpinDx [33]. This method utilizes a centrifugal microfluidic platform to detect BoNTs based on a sedimentation immunoassay. A reagent mixture is prepared, consisting of capture beads conjugated with target-specific antibodies and fluorescent detection antibodies. The reagents are mixed with the sample and forced through a channel containing dense medium, a process that washes the sample and removes interfering substances. The beads that collect at the end of the channel are queried to determine the amount of antigen bound. SpinDx was used to quantify BoNTs with sensitivity that surpassed the mouse assay.

6. Lateral flow methods

The development of lateral flow methods for detecting toxins has also led to the commercial availability of numerous kits for sensitive and rapid testing. Lateral flow methods employ capture antibodies that are "printed" on nitrocellulose membranes in a process akin to inkjet printing technology. Detection antibodies are labeled with visible materials, such as colloidal gold or colored latex beads. The sample is added to a reagent pad containing labeled toxin-binding detection antibodies and is wicked across the membrane. Toxin is retained by the capture antibody, which also concentrates the labeled detection antibody. A positive reaction is revealed as a colorimetric change and is presented as a line on the device. In general, lateral flow methods are qualitative and simply determine the presence or absence of neurotoxin. Sharma et al. [34] compared several commercial lateral flow devices for their capacities to detect toxin in food samples. They were able to detect BoNT/A and BoNT/B as low as 10 ng/mL and BoNT/E at 20 ng/mL in various liquids, such as milk, soft drinks, and fruit juices. Ching et al. [35] used the same mAbs described in the ELISA section above [24–26] in lateral flow devices to achieve sensitivities of 0.5 and 1 ng/mL for BoNT/A in buffer and milk, respectively. Although simple lateral flow tests have lower sensitivities compared to other methods, they produce rapid results and are most useful for the rapid screening of samples suspected of frequent contamination at relatively high level of BoNT. They have many applications and are ideal for field use by non-technical personnel. Self-contained and not necessarily requiring additional reagents or equipment, they can be easily interpreted in the field.

An innovative approach for toxin detection has recently been developed that combines antibodies with the amplification power of PCR, immuno-PCR (I-PCR) [36]. In I-PCR, template DNA is conjugated to the antibody, replacing a secondary antibody conjugated to the detection enzyme. Upon binding of toxin by the antibody, the presence of toxin is revealed using PCR. Chao et al. [36] described an I-PCR method for detection of BoNT/A with femtogram (10^{-15} g) sensitivity. These investigators compared competitive and sandwich ELISA to the I-PCR method. The I-PCR method was 10^3–10^5 times more sensitive with LODs for the ELISA methods of about 50 fg. The use of I-PCR for highly sensitive detection of BoNT in food matrices or other biological and environmental backgrounds has yet to be reported (as of late 2015).

7. Mass spectrometry-based methods to detect toxins

Mass spectrometry (MS) has been used as a method to dissect components of botulinum toxin complexes [37–39]. The MS-based method, called ENDOPEP-MS, uses antibodies to concentrate and extract BoNT from test samples [38]. The concentrated toxins are then subjected to an endopeptidase activity–based assay to generate target cleavage products. Finally, MS is used to identify these products. This approach has been successful in identifying BoNT serotypes A, B, E, and F in various food and clinical matrices with greater sensitivity than the mouse bioassay.

Morineaux et al. [40] recently described a MS method that employs immunocapture enrichment by antibodies specific for BoNT/A-L chains. The enriched analyte is then analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) on a triple quadrupole mass spectrometer (QqQ) in multiple reaction monitoring (MRM) mode. Peptides from BoNT LC specific to the subtypes BoNT/A1–A3 and BoNT/A5–A8 could be identified. BoNT/A subtypes were correctly identified in culture supernatants, water, and orange juice samples with a LOD of 20–150 mouse lethal doses (LDs), but there was a lower sensitivity in serum samples.

Kalb et al. [41] have described the development of a quantitative enzymatic method for the detection of four BoNT serotypes using matrix-assisted laser desorption/ionization—time of flight (MALDI-TOF) MS. Factors that might affect the linearity and dynamic range for detection of BoNT cleavage products were carefully examined, including the concentration of the substrate and internal standard, the length of time for the cleavage reaction, and the components present in the reaction solution. Longer incubation time produced more sensitive results but was not capable of determining higher toxin concentrations, whereas a shorter incubation time was less sensitive. To address these limitations, a novel two-step analysis was developed [41]. By combining the results from a two-stage quantification, four or five orders of magnitude in dynamic range are observed for detection of BoNT serotypes A, B, D, and F. To minimize the number of cleavage reactions and analytical samples, the assay can be multiplexed using mixtures of different neurotoxin substrates. Numerous different research groups (including Kalb et al. [42], Björnstad et al. [43], and Hines et al. [37]) have used MS to dissect the components of the BoNT/G complex, revealing BoNT/G as well as other toxin protein components, namely NTNH, HA-17, HA-33, and HA-70. Overall, the use of MS can provide rapid and definitive results.

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8. Enzymatic assays to detect toxins

Rapidly distinguishing between the presence of active versus inactive toxin is critical for effective medical intervention in toxicoses. As BoNTs are zinc metalloproteases, knowledge of the human targets for these enzymes has enabled development of enzyme-substrate assays. Activity assays have been developed using a wide variety of detection systems. Toxin samples can be treated with recombinant versions of host–target substrates (such as SNAP-25), and the cleavage products can be detected using immunoblotting. Alternatively, fluorogenic peptide substrates emit a signal when cleaved. One such system uses a peptide ("SNAPtide") with reverse-phase HPLC and a fluorescence detector to detect as low as 5 pg/mL of BoNT/A in skim milk [45]. Other peptide substrates (VAMPtide and SYNTAXtide) have been used for detection of their cognate BoNTs [46]. The levels of substrate cleavage correlate well with toxin activity.

9. Cell-based assays

Cell-based assays measure BoNT receptor binding, translocation, and enzymatic activity and can be *in vitro* alternatives to the mouse bioassay. Several neuronal and non-neuronal cell lines have been analyzed for use in neurotoxin assays. These include the following: BE(2)-M17 cells, chick embryo neuronal cells, neuroblastoma cells, and rat spinal cord cells [47–50]. In general, the endpoint of cell-based assays for BoNT/A is the proteolytic cleavage of its intracellular substrate, the vesicle-trafficking SNARE protein called SNAP-25. Recently, Hubbard et al. [51, 52] described the functional analysis of numerous different biological neurotoxins, including BoNTs, in networked cultures of stem cell–derived central nervous system neurons. The investigators demonstrate synaptic activity in cultured neurons of humans and rodents, suggesting that these could serve as comparable methods to animal studies. Hong et al. [53] have also developed a similar assay using a motor neuron-like continuous cell line. Pathe-Neuschäfer-Rube et al. [54] developed a N-terminal tagged luciferase-expressing neuronal cell line. Luciferase is released from these transfected cells during depolarization, which is blocked by botulinum toxin. Cell-based methods may prove to be equally sensitive, or better, than animal studies and may provide a new alternative for *in vivo* experiments. For example, for the first time, the U.S. Food and Drug Administration approved a cell-based assay developed by the biotechnology company Allergan, Inc. (Irvine, CA, USA) for its use as an alternative to the mouse bioassay. However, the details of the Allergan assay have not been published.

10. New antibody and biosensor technologies

Diamant et al. [55] have used an interesting approach for generating antibodies that have higher specificity against serotypes A, B, and E, and possess neutralizing capabilities. Mice were immunized with a "trivalent mixture" of recombinant fragments of neurotoxins A, B,

and E. The method generated numerous different monoclonal antibodies against each serotype. Most of the monoclonal antibodies had higher ELISA titers compared to polyclonal antibodies and had specificities with five orders of magnitude greater specificity. These antibodies also protected against neurotoxin dosages of 10–50 LD_{50}. They also observed a neutralizing synergy when serotype-specific monoclonal antibodies were combined into an oligoclonal mixture.

Detection methods can also utilize highly sensitive antibodies to enrich or enhance sample preparation as well as amplify the signal. For example, an assay with a large immunosorbent surface area (ALISSA) [56, 57] utilizes an antibody to concentrate the neurotoxin onto the surface of a large bead. The "captured" toxin molecules are then used in an enzyme assay. Using food matrices, the LOD for ALISSA was observed as low as 50 fg/mL. This is far more sensitive than the mouse bioassay, immunoassay, or enzyme assay and suggests that it may be useful for detecting food contamination. Marconi et al. [58, 59] have also described the use of surface plasmon resonance (SPR) to examine synaptic vesicle capture by antibodies against BoNT substrates, such as SNAP25 and VAMP2. SPR could be used with cultured neurons in 96-well plates incubated with either BoNT/A or BoNT/B and may be an alternative to animal studies. Further development of label-free and optical biosensors for detecting botulinum toxin [61, 62] will provide additional technologies with possible impact on food safety.

11. Challenges for botulinum neurotoxin detection: new serotypes in the environment

Kull et al. [62] described the isolation of a novel *C. botulinum* strain associated with an outbreak of botulism in Germany. Genotyping of the isolate and subsequent comparison of its neurotoxin gene sequences with database sequences revealed it as a novel BoNT/A serotype. This novel isolate has been called BoNT/A8, and its neurotoxin gene is located within a HA-orfX+ locus. Unique among all other BoNT/A subtypes known so far, an arginine insertion was identified in the HC domain of the HC. Both the full-length neurotoxin and the recombinant LC of BoNT/A8 had lower endopeptidase activity compared to BoNT/A1. Reduced ganglioside binding and lower enzymatic activity may both contribute to lower biological activity of BoNT/A8 as determined using the phrenic nerve hemi-diaphragm assay. Nevertheless, the novel BoNT/A8 subtype caused severe botulism in a 63-year-old male. These findings reiterate that subtyping of BoNT is highly relevant to food safety, epidemiology, and clinical diagnostic and therapeutic practices. Hill et al. [63] carried out a detailed genetic analysis of *bont* genes and confirmed their location on chromosomes, phagemids, and plasmids, as well as variations among different genes. Close examination of sequences confirmed that horizontal gene transfer, site-specific insertions, and recombination events have contributed to the observed variation among different neurotoxins. Understanding the details of toxin gene sequences, protein sequences, and their function can pave the way for the development of novel therapeutics and tailor-made antitoxins. Ongoing development of diagnostics for new and emerging toxins is critical to food safety and human health.

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12. Botulinum neurotoxin detection in the environment: role of climate change and algal blooms in avian botulism and the challenges of environmental matrices

Increased global temperature has been associated with increased algal blooms. The role of these algal blooms in disease is unclear. However, recently, a connection between algal blooms and botulism has been explored. Avian botulism is a disease that often occurs on a yearly cycle and results from the ingestion of neurotoxins by birds. This disease has become increasingly common in the U.S. Great Lakes [64], as have blooms of the green alga *Cladophora*, which can serve as a potential habitat for *C. botulinum*. The interactions between *Cladophora* and *C. botulinum* are unclear due to the complex food web associated with this disease. Investigators in several recently published studies [64–66] reported a high number of botulism cases in shoreline birds in Lake Michigan. This increased incidence was correlated with increasingly large accumulations of *Cladophora* in the water. Sadowsky et al. [65] examined algal mats that were collected from Lakes Michigan, Ontario, and Erie in 2011–2012 and then compared them with algal populations in sand and water. They found that 96% of *Cladophora* mats collected from the shorelines in 2012 contained *C. botulinum* Type E. Among the algae samples containing detectable *C. botulinum*, the large number of detected *C. botulinum* type E cells indicated that *Cladophora* mats are principal sources of this pathogen. Mouse toxin and antitoxin bioassays further confirmed the toxin in collected samples as serotype E. Further examination of *Cladophora*-associated *C. botulinum* may lead to a model system to study algal–clostridial interactions and result in lower bird mortality.

In a follow-up study using PCR, Sadowsky et al. [66] reported that algae mats from different shores of the Great Lakes contained the serotype E gene. Also, *C. botulinum* was found to be present in amounts of up to 15,000 cells per gram of dried algae, based on quantitation of gene copies encoding serotype E. Moreover, genes for serotypes A and B, which are associated with human diseases, were detected in several of the algal samples. Using mouse toxin assays and subsequent neutralization assays, it was confirmed that *Cladophora*-associated *C. botulinum* was serotype E. One might consider that with increased incidence of extreme drought and other environmental changes, algal blooms may happen more often in water-restricted areas, and *C. botulinum* growth may pose a threat to humans if toxin is produced in algal mats. Developing sensitive detection methods for toxins within algal matrices is urgent, as is monitoring other matrices that could provide an environment for botulinum toxin production. The increased avian botulism associated with increased algal blooms highlights the need to develop new technologies for detection of toxin in the environment, or a re-evaluation of current methods and their use in environmental matrices.

Vidal et al. [67] examined numerous environmental factors that influence the prevalence of the unusual mosaic BoNT serotype C/D. Between 1978 and 2008, 13 avian botulism outbreaks were observed, killing 20,000 birds. A significant association was found between the number of dead birds recorded in each botulism outbreak and the mean temperature in July (with average temperatures being higher than 26°C). The presence of *C. botulinum* type C/D in wetland sediments was detected by qPCR. Furthermore, low concentrations of chloride ions

and high organic matter content were correlated with the presence of *C. botulinum*. The digestive tracts of dead birds found during botulism outbreaks were also analyzed; *C. botulinum* was present in almost 40% of the studied samples. Recently, Le Maréchal et al. [68] examined livers from dead birds suspected of having botulism and showed that this organ can serve as a reliable matrix for RT-PCR confirmation of disease. This finding may provide wildlife investigators with a faster method to confirm avian deaths due to botulism.

The presence of *C. botulinum* was detected in aquatic invertebrates and flesh-eating invertebrates collected around bird carcasses. Moreover, the presence of *C. botulinum* bacteria in the adult fly stage of some invertebrates raises the question of whether flies can transport *C. botulinum* from one carcass to another. The same investigators examined whether adult blowflies could play a significant role in botulism outbreaks by carrying *C. botulinum* between carcasses. A field experiment and subsequent laboratory tests determined that blowflies could transport *C. botulinum* Type C/D between carcasses [69]. These results confirm that adult flesh-eating flies could play a role in avian botulism outbreaks. An environmental monitoring protocol for botulinum-carrying flies has not yet been established. It is a matter for future research to determine whether these or other insects could serve as mechanical vectors for botulinum isolates that pose greater threats to humans than the avian isolates.

Probably, one of the greatest challenges is determining which environmental matrices should be collected and analyzed, and which ones would provide the most definitive information about potential threats to humans and animals. For instance, Anza et al. [70] examined the role of eutrophication and avian botulism outbreaks in wetlands receiving effluents from urban wastewater treatment plants. Numerous different avian pathogens, including clostridial pathogens, were present in wastewater and could pose a threat to birds living in wastewater wetlands. Methods to detect BoNTs in environmental matrices could be adapted from previous studies of food and clinical samples or may require new technologies. Future studies in this area are clearly warranted.

13. Future technologies to detect botulinum neurotoxins

The discussion herein has presented a general overview of methods currently being used to detect BoNTs. Many current methods to detect BoNTs in food and environmental matrices have been adapted from the clinical laboratory. New possibilities to consider, to name a few, could exploit the tools of nanotechnology, mHealth, and the use of mobile devices, the capability of miniaturization for even more sensitive and rapid detection of BoNTs. The application and practical use of these technologies might be valuable advancements to current methods to detect BoNTs.

14. Conclusions and recommendations

To maintain a safe food supply and to detect toxins in an ever-changing environment, an ongoing, concerted effort in assay development and validation is essential for human health

and safety. Some areas for investigators to consider include the development of new antibodies and binding molecules specific to BoNT serotype F as well as new hybrid serotypes. The impact of different types of neurotoxin accessory proteins on the detection of BoNTs should also be examined. Furthermore, the impact of food processing conditions on the stability and bioavailability BoNTs is an area in need of further study. The development of new bioassays based on non-mammalian systems and cell cultures should also be supported as well as the advancement of new portable and field-deployable testing methods, including those based on miniaturization of current bench top instruments. These are only a few recommendations, but their development and use should help to further ensure food safety and animal and human health.

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Author details

Luisa W. Cheng[1*], Kirkwood M. Land[2], Christina Tam[1], David L. Brandon[1] and Larry H. Stanker[1]

*Address all correspondence to: luisa.cheng@ars.usda.gov

1 Foodborne Toxin Detection and Prevention Research Unit, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, CA, USA

2 Department of Biological Sciences, University of the Pacific, Stockton, CA, USA

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Chapter 6

Cholera – Epidemiology, Prevention and Control

Saulat Jahan

Additional information is available at the end of the chapter

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Abstract

Cholera is an important public health problem, causing substantial morbidity and mortality especially in the developing countries. It is an indicator of socioeconomic problems and is a global threat to public health. Worldwide, approximately 3–5 million cholera cases and 100,000–120,000 deaths due to cholera occur annually. Cholera is transmitted by drinking water or eating food, which is contaminated with the *Vibrio cholerae*. Approximately 5–10% of persons suffer from severe cholera and if untreated, 50% of severe cases are fatal. The frequency, severity, and duration of cholera infection vary and keep on changing in different parts of the world. Environmental factors such as climate variability, temperature, and rainfall play an important role in cholera transmission. Population density, urbanization, and overcrowding also influence cholera transmission. It is also closely associated with the social and behavioral aspects of individuals as well as communities. Each year, many cholera outbreaks are reported from different regions of the world. These outbreaks have negative impact on social and economic conditions of the affected countries. An integrated, multisectoral program, designed on evidence-based interventions, is required to prevent and control cholera.

Keywords: Cholera, foodborne diseases, outbreaks, public health, *Vibrio cholerae*

1. Introduction

Cholera is an acute diarrheal illness, caused by the toxigenic strains of the bacterium *Vibrio cholerae* serogroups O1 or O139 [1, 2]. It is one of the important public health problems in Asia and Africa and causes substantial morbidity and mortality [3]. Since centuries, cholera has been a subject of interest for epidemiologists. The studies regarding cholera helped in the development of new epidemiological methods which led to the understanding of not only cholera transmission but also helped in the development of the science of infectious disease epidemiology [4]. The purpose of this chapter is to discuss this important infectious disease, i.e., its historical aspects, clinical features, epidemiology, and the outbreaks caused by cholera.

Moreover, the preventive measures for cholera and methods of control of cholera outbreaks will also be discussed.

1.1. Historical aspects of cholera

For centuries, cholera remained one of the most horrific diseases [5]. It was first described by Hippocrates in the fifth century BC. Traditionally, the Ganges Delta region in Asia is considered the home of cholera. It is believed that cholera spread throughout the world from this region. Several epidemics occurred in Asia during the fifteenth and eighteenth centuries. Seven major pandemics of cholera have occurred since 1817 [3, 6].

Historians believe that the impact of cholera epidemics on the cultural evolution of Western Europe, was far reaching and it altered the social matrix of European culture. During the nineteenth century cholera was not only considered a terrifying disease, but was also a challenge to national identity and national economy [7].

John Snow performed pioneer work on cholera in London in the 1800s. He established an association between cholera transmission and contaminated water [4]. He discovered the method of prevention and control of cholera by tracing its source back to drinking water. Due to his work on the Soho cholera outbreak in 1854, John Snow has become a legend in epidemiology [5]. Later, in 1883, Robert Koch described the causative agent for cholera as a curved bacillus, *V. cholerae* [3].

In 1892, a major cholera outbreak occurred in Germany, killing 10,000 people. It was found to be caused by a defect in the design in the German waste removal system [7]. Seven cholera pandemics occurred during the nineteenth and twentieth centuries. The seventh pandemic began in Indonesia in 1961, reached West Africa in 1970 and the Americas in 1991 [3, 6].

In the United States of America, the risk of cholera is very low. However, in 2005, the floods caused by Hurricane Katrina, created the fear of a cholera epidemic for the first time in a century. The US Centers for Disease Control and Surveillance had 11 confirmed cases of people becoming ill from *Vibrio* sp. infection. However, only one of those cases had *V. cholerae*, which was not from the two epidemic-causing serogroups, i.e., O1 and O139 [5].

1.2. General burden of cholera on human population

Cholera is an indicator of a lack of social development and is a global threat to public health. With rapidly increasing population in developing world, the populations living in unsanitary conditions are increasing and the re-emergence of cholera has also been noted [8].

Every year, approximately 3–5 million cholera cases occur, worldwide. An estimated 100,000–120,000 deaths due to cholera occur every year. The number of cholera cases reported to the World Health Organization (WHO) continues to rise. In 2011, a total of 589,854 cholera cases including 7816 cholera deaths were reported from 58 countries, with a case-fatality rate of 1.3% [8, 9]. A total of 838,315 cases belonging to the period 2004–2008 had been notified to the WHO, as compared to 676,651 cases notified from 2000 to 2004 [1]. In 2006, 52 countries reported 236,896 cholera cases including 6311 deaths with a case fatality rate of 2.7% [10].

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The above-mentioned numbers are an underestimation of the real picture as the true number of cholera cases is thought to be much higher. Many cases may not be reported due to limitations in health-care systems and the surveillance systems. There are inconsistencies in cholera case definitions and there is a lack of standard vocabulary. Notified cases are often diagnosed on clinical grounds, leading to a number of undiagnosed and unreported cases. Some countries notify only laboratory-confirmed cases. It is a challenge in the estimation of disease burden due to the dearth of microbiology laboratories capable of detecting *V. cholerae* O1 and O139 in the countries where the disease is prevalent. Fear of sanctions regarding travel and trade may also result in underreporting of cholera cases [10, 11].

2. Causative agent

The causative agent of cholera, *V. cholerae*, is a waterborne and foodborne gram-negative bacterium. *Vibrio cholerae* can cause global pandemics which makes it unique among the diarrheal pathogens [12]. The serotype O1 or O139 colonizes and multiplies within the human small intestine [13]. There are two biotypes of *V. cholerae* O1: Classical and El Tor. Each of these biotypes has two serotypes: Inaba and Ogawa. Both these biotypes cause similar symptoms, however, El Tor biotype mostly causes asymptomatic infection or causes a mild illness [9]. Until 1992, only *V. cholerae* serogroup O1 was responsible for causing epidemics. However, in 1992, a previously unrecognized serogroup of *V. cholerae*, labeled as O139, was found to be responsible for large outbreaks of cholera in India and Bangladesh [3]. Multiple antibiotic-resistant strains of *V. cholerae* have also emerged and the severity of the disease appears to be intensifying [1].

2.1. Mode of transmission

Cholera is transmitted by drinking water or eating food contaminated with the *V. cholerae*. Fecal contamination of water or foods may result in large epidemics. The disease may also be transmitted through eating contaminated raw or undercooked shellfish [9]. Before the late 1970s, it was believed that person-to-person transmission of cholera is the main mode of transmission. Now, *V. cholerae* is found in riverine, estuarine, and coastal waters throughout both temperate and tropical regions of the world. It is recognized as a component of coastal and estuarine microbial ecosystems [14].

2.2. Incubation period

The disease has a short incubation period of 18 hours to 5 days, and it can spread rapidly through a population [12].

3. Clinical features of cholera

The infection may be mild or asymptomatic in most of the cases, but sometimes it can be severe. Approximately 5–10% of persons suffer from severe cholera. In severe cases, the clinical

features include profuse watery diarrhea, vomiting, increased heart rate, loss of skin elasticity, dry mucous membranes, low blood pressure, feeling of thirst, muscle cramps, metabolic acidosis, and restlessness or irritability. These patients can develop acute renal failure, severe electrolyte imbalances, and coma, leading to hypovolemic shock and death [3, 15]. If untreated, 50% of severe cases are fatal, while proper treatment and fluid replacement reduce mortality to less than 1% [3, 12].

3.1. Diagnosis

The confirmatory test for cholera is done by culture of a stool specimen or rectal swab. For transport of specimen, Cary Blair media is the most appropriate, and for isolation and identification of the organism, the selective thiosulfate–citrate–bile salts agar (TCBS) is the medium of choice. Commercially available rapid test kits should not be used for routine diagnosis as they cannot determine the subtypes and are not able to isolate the antimicrobial susceptibility. However, they are useful during epidemics [16].

3.2. Treatment

Rehydration by oral rehydration salts and, if required, intravenous fluids and electrolytes, is the cornerstone of cholera treatment. In addition, antibiotics may be needed for severe cases. Commonly used antibiotics include tetracycline, doxycycline, furazolidone, erythromycin, or ciprofloxacin [17].

4. Epidemiology of cholera

Cholera is said to be a 'forgotten disease' which mainly affects 'forgotten people' of the world. It comes into limelight when an extensive cholera outbreak occurs, although some neglected populations continually suffer from the recurrent episodes of cholera [10]. Cholera is the main cause of epidemic diarrhea in the developing countries. For the last four decades, there is an ongoing global pandemic in Asia, Africa, and Latin America [9].

4.1. Person distribution

In endemic regions, the majority of cases occur among children less than 5 years of age and in reproductive-age women [3]. However, various studies conducted in different parts of the world have reported varying age distribution for cholera patients. A study conducted in Pakistan during the period 2000–2001 found the mean age of patients infected with *V. cholerae* O139 as 40 years as compared to those infected with *V. cholerae* O1 strains where the mean age was 23 years [18]. Tamang et al (2005) conducted a study in a teaching hospital in Nepal between May 1, 2004 and October 31, 2004. They investigated 148 stool samples from patients with acute diarrhea and found out that 46 cases (31%) were positive for *V. cholerae* serogroup O1, biotype El Tor, serotype Ogawa. In their study, younger age group of less than 30 years was mostly affected. Males and females were equally affected [19]. During a cholera

outbreak in southwestern Nigeria from November 20, 2005 to January 1, 2006, a total of 115 cases and 11 deaths were reported. Overall case fatality rate was 9.6% with a case fatality rate in males of 11.9% as compared to 7.1% in females. During this outbreak, the age group of 15 years and above was the most affected comprising of 68.3% cases and 90.9% deaths [20]. A study conducted in southeast of Iran examined 3,178 patients with watery diarrhea, referred to a teaching hospital over a period of 4 years. A total of 362 (11.4%) samples contained *V. cholerae* strains; 336 (92.8%) were *V. cholerae* O1 Ogawa strain. A majority of the cases (270) were referred from rural areas. In this study, the patients ranged in age from 1 to 65 years. Approximately one-fourth (26%) were children under the age of 5 years [21]. In Mexico, the distribution of cholera cases by gender was reported to be similar. Individuals aged 25–44 years had the greatest proportion of cases while individuals aged over 65 years had the highest rates of infection. The authors state that this distribution differs from the distribution of cholera cases in endemic regions. The authors interpret this difference because of the occupational exposure and eating habits of the older individuals [3]. Most of the studies have mentioned a pattern of disparities between richer and poorer people with a higher incidence of cholera in lower socioeconomic groups [22]. Malnourished children and people with low immunity, such as HIV cases, have a higher mortality risk from cholera infection as compared to the normal population [8].

4.2. Place distribution

The frequency, severity, and duration of cholera infections vary and keep on changing in different parts of the world [13]. Cholera is endemic in Africa, south and southeast Asia [23]. In contrast, cholera is almost eradicated from most of the developed countries [5]. Incidence is as low as 0–5 cases per year in the United States [9].

In some countries such as Bangladesh and India, cholera infections occur every year [13]. Cholera reached West Africa and Ghana during the seventh pandemic [24]. In East Africa, cholera epidemic was first reported in 1836 but no cases were reported across Africa between 1870 and 1970. Cholera returned to Africa in 1970 as a result of seventh cholera pandemic. In 1974, cholera cases were reported for the first time in Tanzania, and then reported each year since 1977 [12]. In 1970, the first cholera case was reported in Ghana, after which cholera has been endemic in Ghana. Between 1999 and 2005, Africa accounted for about 90% of the cholera cases and 96% of the cholera-related deaths worldwide [24]. In contrast, other regions such as parts of South America have historically had only sporadic epidemics [13].

During 2013, a total of 56,329 cases were reported from Africa, which shows a decrease of 52% as compared to 2012 when 117,570 cases were reported. Africa accounted for 43.6% of the total cases in 2013 as compared to 93–98% of the total cases during the period 2001–2009. In contrast, 11,576 cases were reported from Asia, representing an increase of 57% as compared to 2012 when 7367 cases were reported from Asia [11].

In 2013, a total of 26 countries reported deaths due to cholera; 17 of these countries belonged to Africa. The case fatality rate was <1% in 4 countries, 1–5% in 17 countries, and >5% in 5 countries. In 2013, a majority (65%) of the deaths were reported from the African continent. A total of 1366 deaths were reported with a case fatality rate of 2.43% [11].

Various studies have reported differences in incidence of cholera in rural and urban areas. Sepúlveda et al (2006) reported high incidence of cholera in rural and suburban areas due to lack of basic sanitation infrastructure [3]. Other studies have reported cholera predominance in the urban areas, due to overcrowding and unsanitary living conditions. In urban communities in Ghana, intermittent water supply along with indiscriminate sanitation practices increases the risk of cholera. Urban slum areas are also at high risk of cholera infection as a lack of safe drinking water, poor sanitation, high population density, and crowding are common features in these areas; all of which are the risk factors for cholera [12, 24].

Bompangue et al. (2008) explored geographic proximity of the cholera cases [25]. The number of cases was found to be statistically significantly higher in the presence of a lake, a main road, or a harbor. Proximity to surface water, high population density, and low educational status were also identified as the predictors of cholera in an endemic area of Bangladesh. Poverty, urbanization, and proximity to coastal areas were important geographic predictors of cholera in Mexico. Proximity and density of refuse dumps also play a significant role in cholera transmission [6]. Epidemiological studies have demonstrated an inverse relationship between diarrhea and access to tube well water, and positive associations with canal water compared with river or pond water [13].

4.3. Time distribution

Vibrio cholerae are found in seas, rivers, and ponds of coastal areas of the tropical countries. It is postulated that environmental factors such as climate variability, temperature, and salinity play an important role in cholera transmission. Cholera is also associated with rainfall patterns. Recurrence of epidemic cholera has also been related to climatic factors [6, 13, 14].

Even in endemic areas, the magnitude of cholera incidence varies from year to year. In rural Bangladesh, there is a premonsoon epidemic almost every year. Cholera is rare during the winter as the temperature is low and there is little rainfall. Gradually, the temperature gets hotter till the monsoon arrives. The incidence of cholera is low in the actual monsoon period as compared to premonsoon period. This lower incidence in the monsoon period is explained by the dilution effect reducing the amount of bacteria in the aquatic environment. However, postmonsoon epidemics are much larger than the premonsoon epidemics [22].

A regular seasonal cycle for cholera outbreaks related to the different strains: classical, El Tor and O139, is described for Bangladesh. El Tor is reported to be most incident from September to November. Some studies have reported two annual El Tor cholera peaks; smaller peak in April followed by a larger outbreak from September to December. *Vibrio cholerae* O139 has a similar seasonal pattern [13].

In Pakistan, the incidence of cholera increases from November to January and from April to May, while in India, cholera cases peak in April, May, and June. In South America, cholera peaks in summer months, January to February and in the rainy season. In eastern African countries such as Kenya, Somalia, Uganda, and Tanzania, cholera outbreaks occur following summer rainfall. From 1979 to 1983, two cholera peaks were reported from Tanzania; from October to December; and from March to May. Both of these peaks coincide with increased

rainfall. In southern Tanzania, the highest incidence of cholera is reported in June and July, while during 2002 the northern parts of southern Africa reported maximum cholera cases from the last week of January to mid-March. In Mozambique, peak of cholera cases occurs from December to May [13].

Cholera outbreaks occur more commonly closer to the equator than at higher latitudes and do not follow a clear seasonal pattern near the equator. Although annual peaks are evident, it is difficult to determine distinct seasonal patterns in cholera outbreaks across regions. However, grouping countries by latitude range, rather than region, makes these seasonal peaks more obvious. Countries near the equator have higher and more constant temperatures, and have a greater incidence of cholera outbreaks [13].

The seasonality of cholera outbreaks may also be explained by secondary transmission. Several studies find that the severity of secondary transmission is associated with local environmental variables, predominantly water sources for household consumption. People who use contaminated surface water for drinking, cooking, and bathing are more likely to contract cholera than those who do not [13].

4.4. Social and behavioral epidemiology of cholera

Cholera transmission is closely associated with the social and behavioral aspects of individuals as well as communities. There are many demographic factors which predispose an individual or community to cholera infection. An increase in population density can result in overburdening existing water supply and sanitation systems. Thus, population density, urbanization, and overcrowding influence intensity of the cholera outbreak [24].

With increasing world population, urbanization is expected to increase. Most of this urban growth will occur in the developing countries. Because of economic issues, most of urban growth in the developing countries is the expansion of squatter settlements. These settlements comprise of households that lack access to safe drinking water and sanitation facilities. These conditions are of particular concern in the spread of infectious diseases which have been associated with conditions prevalent in urban squatter settlements [12].

There are obvious socioeconomic disparities in the occurrence of cholera. Even in the developing countries, the incidence of cholera is higher in socially deprived communities as compared to economically privileged class [13]. Among vulnerable groups living in areas at high risk for cholera, the case fatality rate is high. The secondary cases of cholera occur through fecal–oral transmission, and are related to poor hygiene, poor water, and sanitation environment [6, 22]. The water and sanitation environment as well as proper hygiene and educational level are associated with socioeconomic status of individuals as well as communities. Individuals with low socioeconomic status lack financial resources to install an appropriate sanitary system or obtain cleaner water sources. It is reported that cholera is more common in poorer households without safe water and proper sanitation system as compared to those having appropriate water and sanitation systems [22].

Surface water is contaminated with human excreta and sewage at place where rivers pass through overcrowded cities. In some African countries such as Ghana with multiethnic

population, the traditional laws for protection of water bodies are no longer obeyed. Thus, waste dumps are a common sight at the banks of surface water bodies in urban communities. These urban inhabitants have to use such polluted water at the times of water shortage. The urban slums and squatter settlers have lack of accessibility and affordability to safe drinking water and sanitation. Sometimes, public utility providers are not bound legally to serve the urban slums because of technical and service regulations. In addition, many urban slums are located at low lying areas leading to higher vulnerability to floods, making them more prone to cholera infection. Once the disease is introduced in a population, these demographic and social variables further aggravate the situation [6, 24].

Rapid urbanization can lead to overcrowding, unprotected water sources, and improper disposal of solid waste, liquid waste, and human feces. All these conditions make the environment conducive for cholera transmission. In urban communities, cholera outbreaks have been attributed to poor waste management and sanitation systems [24].

The behavioral aspects of community regarding cholera are important because if cholera is recognized as a serious and life threatening condition by the community, it will be more receptive to community health education program. Human behavior is also important in the acceptance of vaccine program [2].

A humanitarian crisis in a country or region can result in cholera outbreaks. The risk of cholera epidemics also increases after large-scale natural disasters. Some areas of Democratic Republic of Congo have been the site of large scale disasters including invasion and occupation by foreign forces, civil war, population displacements, and earthquakes. These areas have also shown high incidence of cholera [26].

Outbreaks of cholera have negative impact on social and economic conditions of the country as it leads to widespread fear and travel sanctions. Moreover, within the cholera-affected countries, resources are diverted to the care of cholera patients. Certain countries such as Peru and Mexico suffered economically as a result of cholera outbreaks, because of a decrease in tourism and decreased exports by other countries [3].

5. Cholera outbreaks

Cholera outbreaks have always proved challenge for the resource limited health-care systems of countries that usually suffer from these outbreaks. During 2014, cholera outbreaks were reported from South Sudan while in 2013 Mexico reported cholera outbreak [27]. Since October 2010, there is an ongoing outbreak in Haiti and the Dominican Republic. During 2013, the cholera cases during this outbreak accounted for 47.3% of all reported cases [11]. In 2013, outbreaks were also reported from other Central African countries such as Angola, Burundi, and Congo [11]. Namibia reported 1557 cholera cases including 17 deaths while Congo reported 1624 cases and 221 deaths in an outbreak which started in November 2012 [11]. In Cuba, an outbreak started in July 2012, and a total of 181 cholera cases were reported in 2013 [11].

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6. Prevention and control

6.1. Cholera prevention

For the prevention and control of cholera, it is important to understand the factors that are responsible for initiating and sustaining cholera infection in a community [28]. Measures for the prevention of cholera include provision of clean water and proper sanitation to the cholera-endemic communities.

6.1.1. Health education

Health education regarding personal hygiene and food hygiene should be provided to these communities. Media, community leaders, and religious leaders should participate in health education and social mobilization campaigns [29].

CDC has listed five basic cholera prevention messages. These include drinking and using safe water; washing hands with soap and water; using latrines or proper sanitation methods; proper cooking of food, covering it, and eating it hot; proper cleaning up of places used for bathing and washing clothes [30].

6.1.2. Vaccines

Currently, there are two oral cholera vaccines available: Dukoral and Shanchol. Dukoral is a killed whole cell vaccine including V. cholerae O1 serogroup and recombinant B subunit of cholera toxin. It can be given to children ≥2 years and to adults. For children 2–5 years of age, three doses, 1–6 weeks apart, are given orally, and booster dose is given after 6 months. For adults and children ≥6 years, 2 doses, 1–6 weeks apart, are given orally while booster dose is given after 2 years. The earliest onset of protection is 7 days after the second dose, and the protection at 6 months is 85–90% [31]. Shanchol is a killed bivalent (O1 and O139 serogroups) whole-cell vaccine suspension. It can be given to ≥1 year of age; 2 doses, 2 weeks apart, are given orally. The earliest onset of protection is 7–10 days after the second dose, and there is 65% protection for at least 5 years [31].

In cholera-endemic regions and those at high risk for outbreaks, cholera vaccines should be used along with other prevention and control strategies. In these regions, high risk population may be targeted for vaccination. The high risk groups include preschool and school-aged children, pregnant women, and HIV-infected individuals [23].

6.2. Cholera control

Cholera-endemic areas should prioritize cholera control measures [23]. Countries facing complex emergencies and displacement of internally displaced people (IDP) on a large scale or refugees to places where the provision of safe water and proper sanitation is compromised, and they are vulnerable to cholera outbreaks. In such situations, it is critical to depend on surveillance data to watch for an outbreak and to implement appropriate intervention

measures [32]. Thus, strengthening of surveillance system and early warning system is vital in places at high risk of cholera outbreak [29].

The main strategies for cholera control include appropriate and prompt management of cholera cases; strengthening laboratories; training and capacity building of health-care workers; and availability of adequate medical supplies for management [3, 29]. In addition, access to safe water, proper sanitation, appropriate waste management; personal hygiene and food hygiene practices; improved communication and public information are needed for the control of cholera outbreaks.

6.2.1. Cholera vaccines

Oral Cholera Vaccine should always be used as an additional public health tool in complex emergencies and should not replace usually recommended control measures such as improved water supplies, adequate sanitation, and health education. Once a cholera outbreak has started, the vaccine is not recommended as it takes time to provide protection and is also not cost-effective [33]. Reyburn et al (2011) estimated that an organized mass vaccination campaign could prevent 34,900 (40%) cholera cases and 1695 deaths (40%) in Zimbabwe. However, the cost of the vaccines was an important barrier along with other logistic issues [34].

A well-organized, multisectoral approach is required to control cholera outbreaks. The effectiveness of public health interventions depends on an efficient surveillance system. There must be frequent and timely information-sharing at local as well as global level [11]. The administration of cholera vaccines may be considered for high risk population in high risk areas. Funds and resources should be provided to the deserving countries to improve cholera prevention and preparedness activities.

6.2.2. International travel and trade

Currently, there is no obligation of cholera vaccination for international travel. It is learned with experience that quarantine and restrictions on travel and trade are not very effective in controlling the spread of cholera. However, the travelers should be provided information regarding signs, symptoms, and prevention of cholera. The neighboring countries of cholera-affected areas should be advised to enhance their surveillance system for early detection and prompt response if any outbreak occurs [11].

Author details

Saulat Jahan

Address all correspondence to: saulatjahan@hotmail.com

Research and Information Unit, Public Health Administration, Qassim, Ministry of Health, Kingdom of Saudi Arabia

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Chapter 7

Staphylococcal Food Poisoning and Novel Perspectives in Food Safety

Zhenbo Xu, Brian M. Peters, Bing Li, Lin Li and Mark E. Shirtliff

Additional information is available at the end of the chapter

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Abstract

Ingestion of food is the major way for human beings to obtain nutrient substances for basic living, and therefore, the quality and safety of food is a major concern. Foodborne illness includes any illness resulting from the consumption of contaminated food that contains pathogenic bacteria, viruses, or chemical or natural toxins. Consequently, food safety is considered to be a globally expanding issue and thus a leading topic in public health, no longer limited to foodborne illnesses but extended to nearly every safety issue regarding "farm to table" food approaches. Bacterial foodborne infections occur by ingestion of food contaminated with growth of pathogenic bacteria, toxin production, and continuous bacterial growth in intestines. In the past decade, a large number of cases or reports have been available on food containing unhealthy, harmful, or toxic substances (other than food poisoning outbreaks) worldwide. Foodborne microorganisms, primarily associated with pathogenic bacteria and toxic substances produced in food, have presented major challenges for food safety. As a global foodborne pathogen, *Staphylococcus aureus* is typically capable of causing a large number of infections, including food poisoning. Thus, this chapter aims to review several factors contributing to the rise of staphylococci as a growing concern for the food safety industry, including the growth of *S. aureus*, production and regulation of staphylococcal enterotoxins, the viable putative but nonculturable (VPNC) state, and antimicrobial resistance of *Staphylococcus* caused by the indiscriminate use of antibiotics in both clinical and veterinary settings.

Keywords: *Staphylococcus aureus*, Enterotoxins, Food poisoning, Biofilm

1. Introduction

Foodborne illness, also known as foodborne infection, foodborne diseases, or food poisoning, is defined as any illness resulting from the consumption of contaminated food that contains pathogenic bacteria, viruses, or chemical or natural toxins (such as poisonous mushrooms) [1,

2]. Remaining as the leading concern in public health and food safety globally, the annual occurrence of foodborne illness has been reported to be 47.8 million foodborne cases (with 128,000 hospitalizations and 3,000 deaths) in the United States, 750,000 cases (with 113,000 hospitalizations and 460 deaths) in France, and 5.4 million cases (with 18,000 hospitalizations and 120 deaths) in Australia [3–5]. Foodborne infections and diseases are caused by a large variety of pathogens that contaminate food and related products, accounting for the major source of all foodborne illnesses, with an estimate of 14 million illnesses, 60,000 hospitalizations, and 1,800 deaths per year [2]. Therefore, bacterial foodborne infections occur when ingested food is contaminated with pathogenic bacteria, toxins produced (even if the growth of host bacteria is terminated), and continuous bacterial growth in intestines (for setting up an infection that causes illness).

1.1. Staphylococci

As a group of Gram-positive, facultative aerobic, frequently unencapsulated, osmotolerant microorganisms, staphylococci are carried, mostly transiently, by approximately 50% of healthy adults on the skin and anterior nares and widespread in untreated water, raw milk, and sewage. Staphylococci are responsible for various tissue infections and a multitude of diseases [6–9]. Up to date, more than 30 distinct types of staphylococci strains have been verified to be infectious for humans, and the associated illnesses and diseases range from mild to severe, from no treatment required to even potentially fatal [6, 7, 10]. Most of these infections are caused by *Staphylococcus aureus*, which has been regarded as a leading human and zoonotic pathogen implicated in both clinical medicine and food safety and typically is capable of causing a large number of infections, including skin infections and sometimes pneumonia, endocarditis, osteomyelitis, gastroenteritis, scalded skin syndrome, and toxic shock syndrome (TSS) [6]. Aside from this bacterium, coagulase-negative staphylococci (CoNS) are regarded as a frequent cause of nosocomial infection and bacteremia, especially in patients with indwelling medical devices [12, 13]. CoNS have also become the most frequently isolated pathogens in intravascular catheter-related infections (CRI), accounting for an estimated 28% of all nosocomial bloodstream infections [14].

1.2. Staphylococcal Food Poisoning (SFP)

Staphylococcal food poisoning (SFP) is a noncontagious gastrointestinal illness caused by the ingestion of food contaminated with preformed staphylococcal enterotoxins (SEs), with low fatality and symptoms commonly including nausea, retching, vomiting, stomach cramps, exhaustion, and diarrhea [1, 15]. The onset of SFP symptoms commonly occurs between 0.5 and 6 h, and the illness typically lasts for 1 day (up to 3 days), with rapid recovery [1]. Serving as one of the most economically important foodborne diseases, SFP is currently a major issue for global public health programs worldwide [16, 17]. In the United States, *Staphylococcus* has been among the top 5 pathogens responsible for foodborne illnesses. From 1983 to 1997, the annual number of SFP cases had been estimated to be 185,000, with 1,750 hospitalizations and 2 deaths, totaling a cost of 1.5 billion dollars [1, 2]. Based on the recent surveillance in 2011, SFP was found to be account for 241,148 foodborne illnesses, 1,064 hospitalizations, and 6

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deaths per year [1, 18]. In Europe, SFP ranks as the fourth most common causative agent of foodborne illness, with 926 SFP outbreaks reported in 15 European Union (EU) countries from 1993 to 1998 [2, 19]. In Japan, according to the Ministry of Health and Welfare of Japan, for a period of 20 years (1980–1999), a total of 2,525 outbreaks of SFP were reported, which involved 59,964 persons, resulting in 3 deaths [17]. In addition, an extensive SFP outbreak occurred in Japan in 2000, with a large number of patients (13,420 cases) ingested with dairy products contaminated with SEs [20]. In China, for a majority of regions, *S. aureus* was recovered from more than 15% of food samples, and in occasional outbreak cases, the identification rate approached 90% [2, 8, 21]. However, due to the lack of comprehensive surveillance and investigation and the prevalence and occurrence of SFP that varied considerably among different regions and areas in China, this discrepancy may be explained by different local eating habits and food product usage. In addition, staphylococcal strain-dependent differences may also contribute to the variation.

Food commonly involved in staphylococcal intoxication include protein food (even salty) such as ham, raw or processed meat, puddings, pastries tuna, chicken, sandwich fillings, cream fillings, potato and meat salads, custards, raw bilk milk, milk products (especially unpasteurized milk), cheese products, and creamed potatoes [22, 23]. In China, raw meat, milk and dairy products, frozen products, and cooked food have been found as major food types contaminated by *S. aureus*, taking up 38%, 20%, 16%, and 14%, respectively [2, 8, 21]. In Europe, meat and related products have been the common food vehicles for SFP. In Japan, SFP frequently occurs in rice balls and Japanese-style desserts [24]. Food made by hand requiring no further cooking or handled frequently during preparation are major targets for *Staphylococcus* contamination and at highest risk for the production of bacterial toxins, which eventually cause SFP. Although *S. aureus* contamination can be readily avoided by heat treatment of food, its ability to grow in a wide range of temperatures (7–48.5°C), pH [4.2–9.3] and sodium chloride concentrations (up to 15% NaCl) facilitates the contamination and transmission of this organism to various types of food [16, 17, 25, 26].

2. Staphylococcal Enterotoxins (SEs)

Some *S. aureus* strains are capable of producing SEs, and several CoNS species have also been reported to produce SEs, including *Staphylococcus epidermis* and *Staphylococcus haemolyticus*, among others [27–29]. Classified into members of the pyrogenic toxin superantigen family [30], SEs are a series of extracellular single-chain proteins primarily produced in food or culture media and secreted by some *Staphylococcus* strains, and the causative agent of SFP, which after ingestion may cause intoxication exhibited by vomiting (emetic action on the abdominal viscera) and diarrhea (epithelium of the intestinal tract and inhibition of water absorption in intestine), even collapse in severe cases [15]. In most SFP cases, a single enterotoxigenic staphylococcal strain isolated from the contaminated food is suspected to be the responsible strain [31]. However, from a number of SFP outbreaks with symptoms similar to gastrointestinal syndromes mediated by SEs, only nonenterotoxigenic staphylococci have been isolated, which may be explained by the outgrown enterotoxigenic staphylococci by the nonentero-

toxigenic ones. Containing low α-helix and high β-pleated sheet content, SEs have similar and flexible structure, with a low molecular weight between 24,000 and 30,000 Da [32]. One of the characteristic features of SEs is their heat stability (withstanding heating to 121°C for 10 min), as SEs are commonly produced ranging from 10°C to 50°C, with the optimum at 30°C to 40°C [24, 26, 33–35]. Consequently, when *Staphylococcus* strains grow in food under appropriate conditions and produce SEs undetectable by taste or smell, SEs remain active even when bacteria itself have been eliminated by heating. As concluded, effective ways include the prevention of food contamination from staphylococci, staphylococci organisms from growing, and SEs from being produced under either heating or freezing. SEs are also highly hydrophilic, with pH ranges from 4.8 to 9.0 (optimum between 5.3 and 7.0) and water activity (aw) ranges from 0.87 to 0.99 (optimum at 0.90) [24, 36, 37]. Being well studied and documented, SEs are found to possess a number of biological properties, including superantigenicity (induction of T-cell mitogenicity and human interferon), emetic activity, and pyrogenicity [38]. In addition to gastrointestinal symptoms in SFP as aforementioned, SEs have also been implicated in other diseases such as atopic eczema [39–41], rheumatoid arthritis [41–43], and urticaria [41, 44]. With binding of functional SEs (not presursor) to both the α-helical regions of the major histocompatibility complex (MHC) class II molecules outside the peptide binding groove of the antigen-presenting cells (APC) and the variable region (Vβ) on the T-cell receptor (TCR), a bridge between T cells and APC is formed, leading to nonspecific activation and proliferation of a large number of T cells [41, 45, 46] resulting in robust inflammatory cytokine release.

As classified according to the distinct immunological entities, until recently, a total of 25 types of SEs (A–V and X, with 3 subtypes for C) have been identified. The finding of SEs has been in accordance with the development of identification methodologies. In early studies, animal testing experiments had been commonly used for the observation of SE activity, requiring monkey, feline, or cavy models [47–49]. After feeding with contaminated food, animals exhibited abnormal behavior or gross morphology changes, and the SEs contained in food were determined by the number of vomiting events, the time until the first vomiting event, and behavioral changes [47]. These types of animal feeding experiments had been commonly used for the characterization of emetic activities of SEs, thus determining the roles for emetic activities of SEs during SFP. However, such methodologies were also significantly limited by low sensitivity and specificity, poor reproducibility, high expense and laboratory operation, diversity in animals, and incapability of quantification and accurate identification [47]. Therefore, the identification of individual single type of SEs was reported by the availability of serological assays. Based on the specific reaction between antigen and antibody, identification of SEs via specific antibody [50] was first reported in the 1930s. Ouchterlony double immunodiffusion, also known as agar gel immunodiffusion, had become applicable in 1948 [51], and the first serological study on distinct SEs was conducted in 1958 [52]. From 1960s to 1970s, a number of SEs with emetic activities were identified and reported by the employment of serological methodologies including Ouchterlony double immunodiffusion, radial immunodiffusion, and enzyme-linked immunosorbent assays (ELISA). With the development of molecular biotechnology such as PCR and even genome sequencing in the 1990s and 2000s, a large number of newly identified SEs (G–V and X) as well as variants (for instance, 17 distinct variants of *sel-x*) had been discovered on enterotoxin gene cluster (*egc*), staphylococcal

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pathogenicity islands (SaPIs), mobile genetic elements (MGEs), and even the bacterial genome (*sel-p* on genome of N315).

2.1. Classical SEs

Although investigation on the observation and detection of SEs from animal feeding experiments could be dated back from 1930s, the immunological characteristics were not clarified until a number of serological studies had been conducted in the 1950s and 1960s. These studies revealed 6 types (A, B, C_1, C_2, D, and E) of SEs that were characterized and further referred to as classic SEs (including C_3 reported in 1984). Antigenicity of SEs was validated when the antisera prepared from rabbits infected with SEs was demonstrated to protect cats [52], and later from a further serological study, the origins of two heat-resistant types of SEs had been verified [52, 53]. These extracellular immunologically distinct SEs responsible for the clinical manifestation of SFP were first referred to as type F (food poisoning) and type E (mostly produced by strains of "enteritis" origin) and later designated as A and B for a better sequential numbering system, with only type A commonly associated with food poisoning (A-1963). Sharing a similar basic three-dimensional structure, the 5 serological groups of SEs (A–E) exhibit nucleotide sequence identity of 50% to 85%, with types A, D, and E categorized into one group (52–83% amino acid identity) and types B and Cs falling into a separate, more closely related group (62–64% amino acid identity) [53, 54]. As origins and sources, types B and C are important causes of nonmenstrual-associated TSS, and types A and D are common causes of SFP with types B and C to a lesser extent [49].

2.1.1. Staphylococcal Enterotoxin A (sea or SEA)

SEA was first identified in 1959 from *S. aureus* strain FRI-196E [52, 55] and then named as type A [56], which has been considered to be the most commonly detected SE associated with food poisoning, with its minimal toxic dose for humans ranging from 20 to 100 ng [24]. Also, SEA has been verified to be responsible for a number of SFP outbreaks, including an extensive outbreak caused by ingestion of dairy products in Osaka, Japan, in 2000 [24, 56, 57]. After identification by serological methodology, the production of SEA in different media or condition had become a major concern. The maximal level of SEA production was found to occur during exponential phase. In semisolid BHI agar (pH 5.3], the production of SEA was acquired, and trace amounts of SEA and SEB were obtained with cellophane sac culture [58, 59]. As food samples were concerned, SEA production was detected in a number of meat samples (raw beef and pork, cooked beef and pork, and canned ham). Better growth of *S. aureus* and production of SEA were detected in cooked meat than raw meat despite no significant difference obtained, and such diversity may be explained by the bacterial competition between anaerobic and aerobic conditions (with the latter preferable for *S. aureus*) [50, 58, 59]. In milk, SEA production was also found to be associated with staphylococcal growth [60]. In fermented sausage, SEA production had been detected aerobically at pH 5.1 (with an inoculum of 4×10^7 cells/g sausage) but not anaerobically at pH 5.7 [61]. For co-growth food microorganisms, inhibition was observed to be more common than stimulation, including inhibition of both staphylococcal growth and SE formation with no apparent

effect on growth [62]. However, such influence of other microorganisms on *Staphylococcus* was affected markedly by environmental conditions, and discrepancy on inhibition had been also noticed between plate test and meat slurries [58, 59]. Despite acquisition of appropriate staphylococcal growth in both pure culture and in the presence of other food microorganisms, production of SEA was only formed in pure culture [58, 59]. Although SEB was reported to be produced in much larger quantities and more diverse among strains than SEA, SEA had been implicated in a larger number of food poisoning cases [63]. Resembling a primary metabolite (with SEB as a secondary metabolite), SEA is secreted by the bacterium during the exponential phase of growth, with various of factors affecting its production, including salt concentration (NaCl, $NaNO_2$, and $NaNO_3$ showing no influence), surfactants (increase in SEA secretion), pH (optimal ranging from 6.5 to 7.0), and antimicrobial agents (inhibition by chloramphenicol or 2,4-dinitrophenol, with streptomycin or penicillin G exhibiting no influence), which may explain the higher frequency and incidence of SEA in food poisoning [63–66]. In addition, temperature and inoculum size play important roles in SEA production. As temperature was concerned, SEA production was detected under a broad range of temperatures from 10°C to 50°C (in BHI broth) but not at low temperatures (such as 8°C or 10°C as reported) [24]. SEA was detected in the exponential phase from 15°C to 37°C, and its production increased with the elevation of temperature. Also, SEA was detected in the stationary or death phase at 10°C despite acquisition of the lowest SEA concentration at this temperature. Similar to SEE, SEA contains 2 MHC-II binding sites (Zn^{2+} dependent) and thus possesses strong superantigenicity for T-cell activation [67, 68]. Carried by a polymorphic family of lysogenic phages [69, 70], the gene encoding SEA has a length of 771 bp, and its translational product is SEA precursor of 257 amino acids. With 24-residue N-terminal hydrophobic leader sequence further processed, the mature form of SEA was composed of 233 amino acids [38, 53, 68–74]. Unlike other classic SEs (*seb*, *sec*, and *sed*), expression of *sea* had been found to be independent of *agr* regulation [54, 75, 76].

2.1.2. Staphylococcal Enterotoxin B (seb and SEB)

Being the first identified (from *S. aureus* strain FRI-243) and the most studied SE, SEB was initially named as type E and subsequently designated as type B. As the most potent SE and requiring much lower quantities for toxic effect than synthetic chemicals, SEB are capable of causing multiorgan system failure and death at low concentration. As an exotoxin secreted by *S. aureus*, production of SEB had been reported from diverse clonal complexes, including CC8 (the most common), CC20, and CC59 [32, 77–79]. As a superantigen capable of cross-linking APCs and T cells to form a ternary complex between MHC-II and TCR at specific Vβ chain, SEB had been well studied as a causative agent for food poisoning, TSS, atopic dermatitis (common colonization of *S. aureus* and frequent occurrence of SEB-specific antibody from patients with AD), and respiratory diseases (asthma and nasal polyps) [32, 77, 80, 81]. As a well-characterized protein, SEB had been found to be extremely stable (retaining its activity even in acidic environment), water soluble, heat stable (among the most heat-stable proteins, with intact protein under 78°C to 80°C for 30 min), broadly pH tolerant [4–10], and resistant to proteolytic digestion (such as pepsin, trypsin, and papain) [47, 82, 83]. Nevertheless, SEB formation and production were influenced by a number of factors, including inhibition of SEB

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formation in BHI broth by medium filtrates (such as K_2HPO_4, KCl, $CoCl_2$, NaF, acriflavine, phenethyl alcohol, streptomycin sulfate, chloramphenicol, spermine phosphate, spermidine phosphate, and Tween-80) [82], decrease of SEB production by either temperature depletion (without affecting staphylococcal growth) or curing salt concentration elevation (more rapid reduction of SEB production than staphylococcal growth) [84], catabolite repression [85], and minerals (double SEB production was obtained when magnesium and potassium are under appropriate concentration) [34, 86, 87]. Generally, maximal SEB production occurs in postexponential growth. From early studies in the 1950s and 1960s, SEB was considered to be irrelevant to food poisoning [50, 88]. Located in either chromosome (strain FRI-243, FRI-277, or S6) or plasmid (strain DU-4916), *seb* is 705 bp in length, and the mature SEB consists of 239 amino acid residues sharing nucleotide and amino acid sequence homology with sec_1 and streptococcal pyrogenic exotoxin A [89–91]. Regulated by the staphylococcal two-component system, accessory gene regulatory (*agr*), the region between 59 and 93 nucleotides upstream of the transcription ignition site was found to be essential for transcription and expression [92–94]. *seb* had been commonly found in toxin-mediated foodborne and clinical *S. aureus* strains, and recently, *seb* (by PCR and Western blotting) was detected from 5% of 300 clinical *Staphylococcus* strains [95].

2.1.3. Staphylococcal Enterotoxin C (sec and SEC)

According to the new numbering system agreed on the American Society for Microbiology (ASM) meeting in 1962, the first verification of SEC was then reported in 1965, with its toxicity and specificity also confirmed [96]. In this study, the enterotoxins from *S. aureus* strains FRI-137 and FRI-361 were both discovered to react with a specific antibody; thus, strain FRI-137 (ATCC 19095) was selected as the prototype of SEC [96]. However, 2 years later, enterotoxins from strain FRI-137 and FRI-361 were purified as distinct enterotoxins [96, 98] and consequently labeled as SEC_1 (strain FRI-137) and SEC_2 (strain FRI-361). In 1984, the third enterotoxin C (SEC_3) was discovered from a *S. aureus* strain FRI-913 from prawn in England, which were serologically and chemically similar to SEC_1 and SEC_2 but identical by isoelectric focusing, radioimmunoassay (RIA), and N-terminal analysis [99, 100]. Despite cross-reactivity with same antibody, each of the 3 SEC had antibodies that reacted with minor determinants [99]. Located on chromosome (SaPIs), *sec* is composed of 801 bp and encodes a precursor protein of 267 amino acids, with the mature toxin of 239 amino acids [101, 102]. Aside from 3 types of classic SECs, additional *sec* variants (such as *sec*-bovine from SaPIbov) possessing >95% deduced amino acid homology among them had been also reported [103–107]. As SEC_1, SEC_2, and SEC_3 are all emetic enterotoxins with equal toxicity to that of SEA and SEB in both oral administration and intravenously [96, 99], SEC have been responsible for numerous SFP outbreaks (mostly caused by milk) [108]. Maximal production of SEC occurs during postexponential growth. SEC-positive strains of *S. aureus* are commonly associated with bovine, ovine, and caprine dairy products [109]. Yet interestingly, SEC expression has been noted to be reduced in cheeses [110]. From a recent study, milk environment was found to dramatically change the expression profiles of enterotoxin genes despite no influence on staphylococcal growth. In particular, SEC production was substantially reduced in milk compared to the

laboratory medium on the protein level, which may be explained by the down-regulation of the *agr* system [111].

2.1.4. Staphylococcal Enterotoxin D (sed and SED)

In 1967, SED was first reported from *S. aureus* strain FRI-293 (which also produced SEC; thus, strain FRI-494 was selected as the prototype strain, also known as ATCC 23235) and its emetic activity in cats, and specific neutralization of biological activity by antisera had been verified [112]. The production of SED alone and in combination with SEA was considered to play a key role in food poisoning (ranking second in frequency after SEA) and recognized as one of the most commonly recovered enterotoxins in SFP outbreaks [112, 113]. Encoding a toxin of 228 amino acids, *sed* is located on a 27.6-kb penicillinase plasmid pIB485 [114]. SED was found to be partially activated by *agr* via RNA III-mediated reduction of Rot (repressor of toxin) during postexponential growth phase, as independent formation from *agr* was found under high concentration. As a consequence of *agr* regulation via quorum sensing, during growth in BHI broth, a modest postexponential induction ratio (<10-fold) was obtained as *sed* reached maximal production during transition from exponential to stationary phase of growth [113, 115]. With the existence of a consensus -10 sequence, a less conserved -35 sequence, and a TG dinucleotide motif, the presence of 52-bp sequence (from -34 to +18) and transcription from +1 to +18 were important for promoter function and *agr* regulation [116]. Aside from regulation by the *agr* system, NaCl stress was capable of decreasing *sed* expression, although no significant effect was further verified. However, regulation under NaCl stress may be highly strain specific variable [117]. As food samples were concerned, in cheese manufacturing (with starter culture including 10^3 CFU/ml of milk), *sed* expression was not induced even when inoculated at 10^6 CFU/ml (equal to 10^8 CFU/g of cheese), presenting a low level of expression and a prolonged pattern that was similar to SEA [113, 118]. In different ham products, when *S. aureus* was inoculated for optimal growth in cultivation broth for 7 days, continuous *sed* expression was observed throughout the entire incubation period for both boiled and smoked ham [115]. However, much less production of *sed* (9 times less) was detected in the latter. For Serrano ham, SED was only detected after 5 days of incubation (*sed* expression still too low to determine), similar to which a second increase had been obtained for boiled and smoked ham after the same time span of incubation [115].

2.1.5. Staphylococcal Enterotoxin E (see and SEE)

In 1971, SEE was reported from a food poisoning *S. aureus* strain FRI-326, which produced distinct SEE having no immunoreactivity with specific antibodies to other SEs [119]. Its toxicity (in rhesus monkeys), specificity, and neutralization with specific antibody were also validated [119]. Located on the phage, *see* is composed of 771 bp and encodes a precursor with a molecular weight of 29,358 Da, which was further processed to a mature extracellular form with a molecular weight of 26,425 Da [120]. Containing a single polypeptide chain, SEE consists of 259 amino acid residues (no free sulfhydryl groups found), with serine and threonine as the NH_2- and COH-terminal amino acids, respectively [121]. Under extreme acidic (pH 2) and basic (pH 12) conditions as well as heating, the toxicity (emetic activity)

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and antigenicity (serological activity) were found to be destroyed, which is likely due to conformational change [121].

2.2. Staphylococcal enterotoxin-like toxins

Before the 1990s, a total of 7 types of classic SEs (*sea*, *seb*, *sec*$_1$, *sec*$_2$, *sec*$_3$, *sed*, and *see*) had been known as causative agents of SFP in humans due to emetic activity. However, starting from the discovery of *seh* in 1994 (aside from discovery of *sef* on 1981), a large variety of novel SE or SE-related toxins (as well as variants) had been reported (G–V and X) based on genetic homology with classical SEs. In 2004, the International Nomenclature Committee for Staphylococcal Superantigens has proposed that only staphylococcal superantigens inducing emesis after oral administration in a primate model should be designated as SEs, whereas other related toxins lacking either emetic properties in a primate model or verification of emetic activity should be otherwise designated as staphylococcal enterotoxin-like toxin type X [122, 123].

2.2.1. Staphylococcal Enterotoxin-Like Toxin Type F (sel-f and SEl-F)

In 1981, Bergdoll et al. had noticed an enterotoxin-like protein recovered from 93.8% [61/65] *S. aureus* strains sampled from patients with TSS, representing the first evidence of *sel-f* [124]. With its purification and preparation of specific antibody, *sel-f* was also recovered from 11.5% [3/26] of laboratory *S. aureus* strains, compared with only 4.6% [4/87] from other sources, which suggested the association between SEl-F and TSS [124]. However, from an investigation on the spread of a TSS strain, a temporal association of antibodies to SEl-F with cessation of recurrences of TSS was found, indicating that its production may not either reach clinically significant levels during infection or is insufficient to cause TSS [125]. Generally, studies and reports on SEl-F have been rarely available.

2.2.2. Staphylococcal Enterotoxin-Like Toxin Type G (sel-g and SEl-G)

In 1998, SEl-G and SEl-I (from *S. aureus* strains FRI-572 and FRI-445, respectively) had been identified and characterized, including verification on emesis (eliciting emetic response in rhesus monkeys) and superantigenicity (proliferation of T cells) [126]. *sel-g* consists of 777 nucleotides and encodes a precursor protein of 258 amino acids, which has typical bacterial signal sequences and is then cleaved to form mature toxin with 233 amino acids and with a molecular weight of 27,043 Da [127, 128]. SEl-G showed higher homology to SpeA, SEB, SEC, and SSA (38–42% amino acid identity) and exhibited similar epitopes with SEC_1 [126].

2.2.3. Staphylococcal Enterotoxin-Like Toxin Type H (sel-h and SEl-H)

In 1994, the first discovery of *sel-h* from *S. aureus* strain D4508 was reported, with its nucleotide and amino acid sequences identified [129]. One year later, SEl-H was identified and purified from *S. aureus* strain FRI-569, which elicited an emetic response in monkeys and was found to be antigenically distinct from other existent SEs [51]. SEl-H shares about 35% amino acid identity with SEA, SED, and SEE [130]. As a superantigen homologous to SEA subfamily, SEl-H displays unique MHC-II binding properties. As a potent T-cell mitogen, SEl-H was capable

of activating large amounts of T cells by cross-linking APC and T cells via Vα domain (Vα10, TRAV27) of TCR (with no TCR Vβ-specific expansion) by direct interaction between SEl-H and TCR Vα domain [131, 132]. With emetic activity, *sel-h* was commonly detected alone or together with *sea* [133] and responsible for a number of SFP outbreaks. In 1996, an outbreak was caused by cheese and *S. aureus* strains isolated from cheese were found to produce SEl-H [133]. From the SFP outbreak caused by reconstituted milk in Japan, SEl-H was also detected along with SEA. In a survey on 146 *S. aureus* strains isolated from humans, cows, and bovine in Japan, 7 and 4 strains were found to harbor *sea*$^+$*sel-h*$^+$ and *sel-h* alone, respectively [57, 133]. In December 2003, a suspected SFP outbreak involving 8 persons (3 adults and 5 children) with symptoms of vomiting, stomach cramps, and diarrhea shortly after lunch was caused by contaminated mashed potato, and *S. aureus* strains contained in raw bovine milk for preparation of mashed potato were found to produce sufficient SEl-H for food poisoning [134]. SEl-H production was influenced by a variety of factors, including aeration and pH conditions. Higher production level of SEl-H was acquired under aerobic incubation or pH controlled at 7.0, with decrease in SEl-H production under anaerobic condition or slight change of pH (such as 6.5 or 7.5) [135].

2.2.4. Staphylococcal Enterotoxin-Like Toxin Type I (sel-i and SEl-I)

As aforementioned, SEl-I were identified together with SEl-G in 1998 [126]. Unlike SEl-G, SEl-I was more similar to SEA, SED, and SEE (26–28% amino acid identity). *sel-i* consists of 729 nucleotides and encodes a precursor protein of 242 amino acids, which contains typical bacterial signal sequences and is further cleaved to form mature SEl-I of predicted 218 amino acids with a molecular weight of 24,928 Da [127, 128]. Although separated by DNA related to other SEs, linkage of *sel-g* and *sel-i* was discovered, and this enterotoxin gene cluster was designated as *egc*, with *sel-g* located 2002 bp downstream of *sel-i* [127]. In southern France, carriage of *sel-g*$^+$*sel-i*$^+$ and *sec*$^+$*sel-g*$^+$*sel-i*$^+$ was detected from 41.9% and 24.5% of 155 *S. aureus* strains isolated from various food samples [128]. In Taiwan, 14.5% [8/55] *S. aureus* strains of human origin and 9.4% [13/139] strains isolated from frozen food, Chinese sausage, and meal boxes were found to harbor *sel-g*, *sel-h*, and/or *sel-i*, suggesting a minor role that such SEs play in SFP outbreaks [136]. However, a discrepancy between the presence of *sel-g* and *sel-i* and the production of enough quantities of SEG and SEI was also noticed [128]. In 2004, 10.1% [11/109] wild *Staphylococcus* spp. stains were found to contain SEs and *egc*, and the *egc* from strain AB-8802 present variants of *sel-g* and *sel-i* (*sel-gv* and *sel-iv*) [137].

2.2.5. Staphylococcal Enterotoxin-Like Toxin Type J (sel-j and SEl-J)

In 1998, *sel-j* was first found to be located on the plasmid pIB485 encoding *sed*, which was separated from *sed* by 895 bp of intergenic region containing a perfect inverted repeat (with each arm of the repeat having a length of 21 bp) [138]. Most of *sel-j* was detected on *sed*-encoding plasmid, suggesting the coexistence of these 2 SEs and their relative contribution to the food poisoning symptomology [138]. With transcription in opposite directions, both *sel-j* and *sed* were capable of expression in *S. aureus* strains, with *sed* only under the transcrip-

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tional control of *agr* [138]. Containing 269 amino acid residues, sequence of SEl-J showed substantial similarity to the SE family of *sea*, *sed*, and *see*.

2.2.6. Staphylococcal Enterotoxin-Like Toxin Type K (sel-k and SEl-K)

Despite observation of the *sel-k* gene on SaPI1 (in 1998) and *egc* from *S. aureus* strains A900322 (in 2001), the first designation of *sel-k* from *S. aureus* TSS isolates MN NJ was reported in 2001, with its identification on SaPI3 together with *seb* [139, 140]. Possessing biochemical and biological properties similar to classic SEs, including superantigenicity (Vβ-specific T-cell activation), pyrogenicity, emesis, and lethality in primates, SEl-K was secreted by clinical *S. aureus* strains, with a molecular weight of 26,000 Da and a pI between 7.0 and 7.5 [48, 140]. An increase in the secretion of SEl-K was obtained when coexpressed with SEB (K-2014). However, regardless of the variation in SEl-K secretion amount *in vitro*, similar levels of SEl-K accumulation were found *in vivo* [141]. SEl-K was commonly detected in clinical isolates (more than half) and almost all USA300 strains. In addition, a genetic variation of *sel-k* was discovered, with 6 variants found among 20 clinical isolates [141].

2.2.7. Staphylococcal Enterotoxin-Like Toxin Type L (sel-l and SEl-L)

First noticed on *egc* from *S. aureus* strain A900322 [142], *sel-l* was identified on pathogenicity island SaPIbov (15,891 bp) from a bovine mastitis *S. aureus* isolate RF122 (*sel-l*) in 2001, with a molecular weight of 26,000 Da and an isoelectric point of 8.5 [105]. Lacking emetic activity, SEl-L was found to exhibit a number of biological properties similar to other SEs, including superantigenicity, pyrogenicity, enhancement of endotoxin shock, and lethality in rabbits when administered via subcutaneous mini-osmotic pumps, but the protein lacked emetic activity [105].

2.2.8. Staphylococcal Enterotoxin-Like Toxin Type M (sel-m and SEl-M)

In 2001, *sel-m* was reported to be located on the *egc* (enterotoxigenic gene cluster) together with *sel-g*, *sel-I*, *sel-k*, and *sel-l*, and SEl-M was found to exhibit superantigenicity activity with specific Vβ pattern [142]. However, the emetic activity has not been elucidated yet. Most clinical *S. aureus* strains harboring *egc* were found to carry such SEs regardless of the diseases, suggesting the potential derivation of SEs and the putative cluster of SE genes from *egc*.

2.2.9. Staphylococcal Enterotoxin-Like Toxin Type N (sel-n and SEl-N)

From the *egc* reported in 2001, *sel-n* was also found to be located between *sel-i* and *sel-g* [142]. A study was conducted on the cloning and expression of *sel-m* and *sel-n* from *S. aureus* strain FRI-1230, demonstrating that SEl-M and SEl-N were capable of stimulating T cells and inhibiting K562-ADM and B16 cells with an equivalent level to that of SEC_2 [143]. Although superantigenicity had been verified, the emetic activity of SEl-N is still unclear [144].

2.2.10. Staphylococcal Enterotoxin-Like Toxin Type O (sel-o and SEl-O)

In 2001, *sel-o* was identified from the *egc* cluster, on which other 4 SEs and 2 pseudogenes were also located, including *sel-i*, *sel-j*, *sel-m*, *sel-n*, *Ψent1*, and *Ψent2* [142]. However, the biological and biochemical properties of *sel-o* remains unclear despite validation of its superantigenicity [144].

2.2.11. Staphylococcal Enterotoxin-Like Toxin Type P (sel-p and SEl-P)

In 2001, *sel-p* (previously called *sep*) was first discovered from the genome of MRSA N315, and its biological properties were fully characterized in 2005 (with *sel-p* from *S. aureus* strain Sagal isolated from an SE unidentified food poisoning outbreak in Japan), including superantigenicity (induction of a substantial proliferative response and production of cytokines) and emetic activity (at relatively high dose as 50–150 μg/animal) [123, 145]. According to this study, SEl-P was detected in 60% of the 30 *sel-p*-positive *S. aureus* isolates, and all 10 strains harboring *seb* and *sel-p* produced SEB but not SEl-P, suggesting that inactivation of the *sel-p* locus associates with a particular SE genetic constitution [123]. Most recently, colonization with *sel-p*-positive MRSA increased the risk of bacteremia, which indicated *sel-p* as a predictive virulence factor for invasive disease [146].

2.2.12. Staphylococcal Enterotoxin-Like Toxin Type Q (sel-q and SEl-Q)

In 2002, a member of the new subfamily (group V), *sel-q* (from *S. aureus* strain MN NJ) was identified and located directly 5 of *sel-k*, with a molecular weight of 26,000 Da and isoelectric point between 7.5 and 8.0 [147]. Despite a lack of emetic activity (incapability in neither lethality in rabbits nor emetic activity in monkeys), *sel-q* had been found to possess superantigenicity, pyrogenicity, and ability to enhance endotoxin shock.

2.2.13. Staphylococcal Enterotoxin-Like Toxin Type R (sel-r and SEl-R)

In 2003, *sel-r* was recovered and identified from 4 *S. aureus* strains (Fukuoka 5, Fukuoka 6, Fukuoka 7, and Fukuoka 8) isolated from patients with nausea, vomiting, and diarrhea from a food poisoning outbreak occurred at a lunch-box shop in Fukuoka prefecture of Japan in September 1997 [148]. Located on 2 types of plasmid, pBI485 (and pBI485-like plasmids, encoding *sed* and *sel-j* as well) and pK0311 (pF5, pF6, and pF7), *sel-r* was found to most closely related to *sel-g* [148]. Investigation on the biological properties of SEl-R revealed its superantigenicity (T-cell stimulation activity via MHC-II) and emetic activity (induction of a reaction in animals within 5 h at 100 μg/kg) [148–150]. SEl-R production was also verified in seropositive *S. aureus* strains [148, 149]. A survey was conducted on the SEl-R production from 24 *sed*-positive *S. aureus* isolates, and *sel-r* expression was detectable from 22 isolates despite carriage of variant *sed* gene for seven strains lacking SED production [151].

2.2.14. Staphylococcal Enterotoxin-Like Toxin Type S (sel-s and SEl-S)

Two novel SE-like genes, *sel-s* and *sel-t*, had been reported on the plasmid pF5, where *sel-j* and *sel-r* were located. SEl-S (rSES) was characterized for biological properties, including super-

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antigenicity (specific stimulation of human T cells via MHC-II APC) and emetic activity (induction of emetic reactions in monkeys) [150].

2.2.15. Staphylococcal Enterotoxin-Like Toxin Type T (sel-t and SEl-T)

As aforementioned, a first identification of *sel-t* was reported on plasmid pF5 harbored by *S. aureus* strain Fukuoka 5 from SFP. Similar to SEl-S, SEl-T was found to exhibit both superantigenicity and emetic activity (induction of a delayed reaction after 24 h or 5 days postadministration). Data from the emetic study on SEs involved in the SFP outbreak in Fukuoka in 1997 combined with emesis studies in house musk shrews (similar as in the monkeys) suggest that SEl-R and SEl-S were validated to be the causative toxins of vomiting [150].

2.2.16. Staphylococcal Enterotoxin-Like Toxin Type U (sel-u and SEl-U)

From sequencing of 24 *S. aureus* strains harboring *egc*, *sel-u* was identified on 4 of the tested strains [152]. SEl-U was found to result from sequence divergence in the *Ψent1* and *Ψent2* pseudogenes, as *sel-u* was located between *sel-iv* and *sel-n* in *egc* of strain 382F (AY158703) with replacement of the *Ψent1* and *Ψent2* between *sel-iv* and *sel-n* in *egc* of strain Mu50 (AP003363) [144, 152]. A variant *sel-u*, designated as *sel-u2*, was recovered from an atypical *egc* locus and generated by a limited deletion in the pseudogenes *Ψent1* and *Ψent2*, which contained superantigenicity for activation of T-cell families Vβ-13.2 and Vβ-14 [144].

2.2.17. Staphylococcal Enterotoxin-Like Toxin Type V (sel-v and SEl-V)

In a broad surveillance on *egc* from 666 clinical *S. aureus* isolates, 63% [421/666] strains were positive for *egc* locus [144]. The archetypal *egc* harboring 5 SEs and 2 pseudogenes was found in 409 strains, and a novel SE-like toxin, designated as *sel-v*, was discovered from an atypical *egc* locus from *S. aureus* strain A900624 [144]. SEl-V was generated by recombination between *sel-m* and *sel-i*, and its superantigenicity for activation of T-cell families Vβ-6, Vβ-18 and Vβ-21 has also been validated.

2.2.18. Staphylococcal Enterotoxin-Like Toxin Type X (sel-x and SEl-X)

In 2011, *sel-x* was discovered from the core genome of 95% of phylogenetically diverse *S. aureus* strains with human and animal origins, including 17 distinct allelic variants (*sel-x*1 to *sel-x*14, *sel-xov*, *sel-xbov*1, and *sel-xbov*2). Acquisition of *sel-x* includes the horizontal transfer by a *S. aureus* progenitor, allelic diversification by point mutation, and assortative recombination, which explains the high genetic diversity of *sel-x*. With a unique predicted structure, SEl-X was well characterized by biological activities, including superantigenicity (activation of Vβ-specific T cells), pyrogenicity, and endotoxin enhancement. It is also noteworthy that SEl-X produced by strain USA300 (CA-MRSA) had been found to be responsible for the lethality in a rabbit model, which suggested a novel virulence determinant of CA-MRSA disease pathogenesis.

2.3. Pathogenicity Islands (PAIs) and *S. aureus* PIs (SaPIs)

2.3.1. PAIs

In 1986, before the first report of PAIs by Hacker et al. in Werner Goebel's group of Germany in 1994, two large segments had been found to be capable of deletion and thus enable the host bacterial to produce hemolysin and loss of P. fimbriae [139, 153, 154]. Considered to be foreign DNA segments integrated into the bacterial genome, such segments existed within pathogenic isolates (cause of virulence) but not on highly genetically similar nonpathogenic strains [153]. As a subclass of genomic islands, PAIs are defined as a group of gene clusters encoding bacterial virulence on a large DNA segment (ranging from 20 to l00 kb) located on the bacterial chromosome [139, 154–156]. PAIs are acquired by microorganisms via horizontal gene transfer via transduction, conjugation, and transformation. Acquisition of PAIs may rapidly and radically alter the genome of a bacterium, consequently strengthening or reducing its fitness within the host [154, 157]. Pathogens are capable of harboring one or more PAIs associated with one or more virulence genes. PAIs are capable of encoding genetic products, including secretory proteins (such as type III secretion system), cell surface proteins (such as erythrocytolysin, fimbriae, and heme binding factors), signal transduction systems, and regulation systems [139, 155–157].

As distinct DNA regions are present in the genome of pathogenic bacteria and absent in nonpathogenic strains (despite same or close species), typical PAIs are composed of mobility genes (such as integrases) commonly located at the beginning of the island and close to the tRNA locus or the respective attachment site. A number of virulence genes (V1–V4) are frequently interspersed with other mobility elements including insertion sequence (IS) elements (Isc, complete insertion element) or remnants of IS elements (ISd, defective insertion element) [153, 155, 156]. Commonly flanked by direct repeats (DR) and IS elements, PAIs are often genetically unstable and comprise some potential mobile components, such as IS elements, integrase, transposase, and plasmid replication initiation sites. As DNA sequences ranging from 16 to 20 bp (with maximum of 130 bp) with sequence repetition, DR plays a critical role in insertion and deletion (as recognition sites), such as integration of bacteriophages. Although combination of IS elements may be capable of mediating transfer of large DNA fragments, insertion mediated by IS also leads to inactivation of genes as well [153, 154, 156]. Consequently, PAIs are capable of deletion with distinct frequencies and loss of virulence traits encoded by PAIs are reported to occur at higher frequency than that encoded by mutation. PAIs are commonly inserted in the backbone genome of the host strain, typically located to specific sites such as tRNA loci or adjacent to tRNA genes, or sites associated with plasmid and phage integration, due to highly conserved genes encoding tRNAs among various bacterial species [153, 154].

PAIs differ from host chromosomes in GC content and codon usage, which may account for the discovery of novel PAIs and maintenance of the divergent nucleotide composition from the horizontally acquired DNA. Based on significant differences with respect to bacterial virulence, GC content, and codon usage, a hypothesis was proposed that such characteristics

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may be bestowed from DNA segments on the plasmid and phage; thus, acquisition of PAIs and the emergence of new pathogenic organisms can be correlated [153, 156].

2.3.2. SaPIs

As mobile pathogenicity islands with length ranging from 14 to 17 kb and carriage of genes for superantigen toxins and other virulence factors, SaPIs have been responsible for the TSS and other superantigen-related diseases, especially SE-like toxins. Located in specific loci of the chromosome and induced by bacteriophages, SaPIs are capable of incorporating small infective phage-like particles via a program of excision-replication-packaging. Containing most of the staphylococcal toxins and virulence factors, SaPIs facilitate the horizontal acquisition of MGEs and, thus, play an important role in the evolution of *Staphylococcus* [139, 154, 155].

2.3.2.1. SaPI families

Aside from SCC*mec* (introduction in detail as below), a large number of toxin genes have been found in SaPIs, including SE-like toxins and TSST. Several types of SaPIs have been identified. SaPI1 was found to be inserted in an *attC* site close to the *tyrB* gene and flanked by the region of *tst* gene, with a length of 15,233 bp [139]. The characteristic features of SaPI1 include mobility and instability, whereas SaPI2 was identified as a second locus. Transduction between SaPI1 and SaPI2 by via helper phage was demonstrated, and stable integration of these 2 SaPIs without phages had also been verified [139, 155, 156]. Inserted at the 3 end of the GMP synthase gene, SaPIbov was identified in a bovine isolate of *S. aureus* by PAI related to SaPI1, with a length of 15,891 bp and carriage of *sec*, *sel-l*, and *tst*. SaPI3 was identified to contain *sel-k* and *sel-q* [157]. SaPIs with similar structure between SaPI3 and SaPI1 had also been reported.

2.3.2.2. νSa families

Up to date, 7 conservative PAI types had been discovered in *S. aureus*, namely, νSa1 (including SaPI1 and SaPI3), νSa2 (SaPIbov), νSa3, νSa4 (including SaPI2), νSaα, νSaβ, and νSaγ [139, 155–157]. νSa1 to νSa4 were found to contain integrase genes as putative elements of genetic mobility. Derivation of *int* and *att* sites from phage genome was pointed out, as both were found in SaPIs. On the contrary, νSaα and νSaβ harbor transposase genes, which may be derived form transposons. Comprising SaPI1 and SaPI3, the νSa1 locus of CA-MRSA also carries a large number of genes encoding enterotoxins and TSST. Similarly, the loci of both νSa2 from CA-MRSA and SaPIbov were found to contain enterotoxins and TSST. Capable of high-frequency deletion and formation of an episomal circular DNA, νSa3 was identified in CA-MRSA MW2 and Mu50, and one type of νSa3 harbors novel allelic forms of *sec* and *sel-l*. With a lower frequency of excision than that of νSa3, the νSa4 family contains several allelic forms of a genomic island, and type I νSa4 carries *sec* and *sel-l* [157]. Despite the presence of both νSaα and νSaβ in all sequenced *S. aureus* genomes, the size and number of ORFs in νSaα, as well as the size and gene composition of νSaβ, were found to be highly variable, with neither SaPIs spontaneously excised from the chromosome [145]. The composition of νSaα includes 11 allelic forms of *set* genes (encoding exotoxins), *lukDE* genes (encoding leukotoxins), and lipoprotein gene clusters. However, all varieties of νSaβ contain a gene cluster for serine

proteins and superantigen genes absent in CA-MRSA but present in HA-MRSA strains. Adjacent to short DRs, the locus *etd* PAI contains exfoliative toxins *etd* and *edin-B* (encoding exfoliative toxins), IS element, and restriction/modification system [158].

2.3.2.3. Development and evolution of pathogenicity

The mechanisms of horizontal gene transfer in prokaryotic cells include transduction, conjugation, and transformation, among which phage transduction has been the primary transmission drivers of genes among different species and thus plays an important role in the formation of PAI [159]. The formation of PAIs may include 5 stages [160] as follows: (i) acquisition of virulence gene via horizontal gene transfer regulated by an operon and derived from "gene pool" of varied environment; (ii) integration of foreign genes (commonly derived from various complex genes of different donors) into the bacterial chromosome or plasmid via site-specific recombination or other mechanisms, following similar rules to complete integration and shaping obvious structure of genetic island; (iii) evolvement of MGEs into PAI via restructure, gene elimination, and acquisition of other genetic materials, during which the gene components associated with mobility may be inactivation or lost, such as origin of plasmid replication, self-transmissible plasmid tra and phage int; (iv) induced expression of foreign genes under temperate environment; and (v) acquisition or elimination of genetic information constantly via a serious of recombination, insertion or elimination, by which PAI may retrieve MGE and obtain the ability of excision and transfer the whole PAI from chromosome to another recipient bacterium.

Evolution of the bacterial genome may significantly influence its pathogenicity, mainly including point mutations, recombination, and horizontal gene transfer. Despite slow evolution due to relatively low frequency of point mutation, the horizontal gene transfer of large genetic segments (such as PAIs and SaPIs) undoubtedly speeds up the exchange of bacterial genes (as "quantum leap" in short time), leading to the consequent appearance and spread of various novel mutations or variants [161, 162].

3. Novel perspectives of *Staphylococcus* associated with food safety

3.1. Antimicrobial resistance mediated by MGEs

Antibiotics, as compounds or substances that kill or inhibit the growth of microorganisms, have been regarded as one of the greatest contributions to medicine and humanity and used to treat a wide range of infectious diseases caused by bacteria for both animals and human beings [5, 165]. Abuse of existing antibiotics contributes to the spread of antibiotic resistance and poses a predicament for the treatment of several bacterial infections, including therapy for individuals with food poisoning. In China, as one of the currently worst areas for antibiotics abuse, the annual prescription of antibiotics, including both clinical and veterinary treatment, is approaching 140 grams per person and has been roughly estimated to be 10 times higher per capita than that in United Kingdom [6, 7, 163, 164]. From a retrospective study conducted on 1,739 *Staphylococcus* isolates from a hospital in Guangzhou, China, from 2001 to 2010,

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antimicrobial resistance of tested drugs (exclusively for teicoplanin and vancomycin) was commonly observed among the isolates examined, with high resistance rates for β-lactamases (94.0% and 73.7% for penicillin and oxacillin, respectively) and resistance percentages for cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, trimethoprim-sulfamethoxazole, and tetracycline ranging from 83.9% to 19.4% [165]. As a consequence, antibiotic resistance in microorganisms still remains one of the core concerns in global public health, with methicillin-resistant staphylococci (MRS) strains representing one important group, commonly considered as "super bugs" [7]. Since their first discovery in 1961, MRS (including MRSA and MRCNS) have become among the most prevalent pathogens causing nosocomial infections throughout the world [13, 16, 17, 19, 166]. With the first report of an MRSA-mediated gastrointestinal illness outbreak [174], MRS strains have been considered a major contributor to both health-care-associated and foodborne illnesses. MRS had been identified from contamination of various food samples, such as milk, pork, chicken, veal, beef, turkey, and lamb meat [168–170] as well as in food production animals, such as cattle, chickens, pigs, and cows and are closely connected with the newly discovered MRSA designated as livestock-associated MRSA (LA-MRSA) [171–173]. Carriage of MRS strains in a wide variety of food and food production livestock may not be limited to only food hazard but also poses a significant occupational risk for the industrial staff, such as handlers, asymptomatic carriers, and uncolonized individuals. MRS strains show resistance to nearly all β-lactam antibiotics and commonly multiple other drugs due to the *mecA* and other resistance genes carried by an MGE designated as staphylococcal cassette chromosome *mec* (SCC*mec*). Additionally, the role of integrons as a mobile genetic mechanism in horizontal transfer of antibiotic resistance has also been well established [174–177].

3.1.1. SCCmec

As one of the major foodborne infectious pathogens, *S. aureus* (in particular, MRSA) has been considered to be a potential "super bug," posing a challenge to hospital infection control and a threat to global food safety. Due to the carriage of the *mecA* gene encoding a novel specific penicillin-binding protein (PBP2a), which exhibits a decreased binding affinity to antibiotics, MRSA presents resistance to virtually all β-lactam antibiotics [178]. Evolution from methicillin-susceptible *S. aureus* (MSSA) to MRSA occurred with the acquisition of a genomic island, the staphylococcal cassette chromosome (SCC*mec*). Harboring *mec*A and a large number of functional and regulatory genes, SCC*mec* is an MGE present in *Staphylococcus* species. With accurate excision and integration mediated by site-specific recombinase genes *ccrA* and *ccrB*, SCC*mec* is capable of integration into the bacterial chromosome, leading to the rapid spread of antimicrobial resistance (to β-lactam or other antibiotics) among staphylococcal strains.

MRSA was found shortly after the common use of methicillin, which was first licensed to treat penicillin-resistant *S. aureus* infections in Britain in 1959 [179], resulting in outbreaks of MRSA occurring worldwide. In the 1980s, an extraordinary large chromosomal DNA segment greater than 30 kb carrying *mec* was found to contain no allelic equivalence in MSSA strains by direct chromosome analysis of MRSA strains; this region was designated as *mec* DNA [180–183]. In 1987, the sequence of *mecA* gene cloned from a Japanese MRSA strain was determined [184,

185]. In consideration of the threat caused by this pathogen, the characteristics of MRSA were studied and SCC*mec* was found to be an MGE in MRSA. Additionally, the types of SCC*mec* were found to be genetically diverse. In 1999, the cloning and determination of the structure of the entire *mec* DNA sequence from a Japanese *S. aureus* strain N315 (first isolated in 1982) was reported [186]. Based on the structure, it was reported that *mec* DNA was a novel genomic element designated as staphylococcal cassette chromosome *mec* (SCC*mec*) driven by two site-specific recombinase genes referred to as cassette chromosome recombinases A (*ccrA*) and cassette chromosome recombinases B (*ccrB*) [178]. This was the first time that SCC*mec* was defined, and *ccrA* and *ccrB* were proposed as a novel set of recombinases, defining a new family of staphylococcal genomic elements. After the discovery of SCC*mec*, various types of SCC*mec* were continuously found by scientists around the world. In 2001, the identification of two additional types of SCC*mec* was isolated in other countries of the world and designated as type II SCC*mec* (found in N315) [187]. The two additional types of SCC*mec* were type I found in NCTC10442, which is the first MRSA isolate in England in 1961, and type III found in 85/2082 isolated in New Zealand in 1985. In 2002, a novel type of SCC*mec* designated as type IV was identified from CA-MRSA strains [188]. According to the foundation of type IV SCC*mec*, a new type of SCC*mec* designated as VI, which was originally mistaken as type IV SCC*mec*, was explored [189, 190]. In 2004, type V SCC*mec* was found in the chromosome of a CA-MRSA strain (WIS [WBG8318]) isolated in Australia [191]. Since 2008, novel types of SCC*mec* were found globally. Types VII and VIII SCC*mec* were identified in MRSA strains JCSC6082 (a Swedish isolate) [192] and C10682 (a Canadian isolate) [193], respectively. Novel types of SCC*mec* designated as types IX and X were identified in MRSA strains JCSC6943 and JCSC6945, respectively [194]. The latest type of SCC*mec* to be classified was designated as type XI; it was discovered in the MRSA strain LGA251 genome with a divergent *mec*A homologue (*mec*ALGA251) [195].

3.1.1.1. Structure and types

As a major member of the SCC family and a carrier for gene exchange in staphylococci strains, SCC*mec* is located near the replication origin of the *Staphylococcus* chromosome and inserted at *attB* site located at the 3 end of a novel ORF with unclear function (*orfX*). Typical SCC*mec* comprise 3 basic genetic elements: (i) *Ccr* complex, composed of two site-specific recombinase genes (*ccrA* and *ccrB*), and surrounding ORFs. Via site-specific recombination mediated by *ccrA* and *ccrB*, multiple antibiotic resistance and heavy metal resistance genes are capable of insertion into SCC*mec*. SCC*mec* is further integrated to the staphylococcal chromosome by accurate excision and integration, leading to adaption of the bacterial host to different environments and pressure of antibiotic selection. According to the different types of *ccrA* and *ccrB*, *Ccr* complex was classified into 8 allotypes: type 1 for *ccrA1* and *ccrB1*, type 2 for *ccrA2* and *ccrB2*, type 3 for *ccrA3* and *ccrB3*, type 4 for *ccrA4* and *ccrB4*, type 5 for *ccrC1*, type 6 for *ccrA5* and *ccrB3*, type 7 for *ccrA1* and *ccrB6*, and type 8 for *ccrA1* and *ccrB3*. (ii) *Mec* complex harboring *mecA* and related regulatory genes. Based on the regulatory genes located upstream and downstream of *mecA* and the difference of insertion sequences, *mec* complex was classified to five classes: class A carried the integrated *mecI-mecR1-mecA-IS431* structure, class B carried devoid the *IS1272-ΔmecR1-mecA-IS431* structure that contains integration insertion sequence,

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class C carried the *IS431-mecA-ΔmecR1-IS431* structure that contains two copies of insertion sequence *IS431*, class D carried the *IS431-mecA-ΔmecR* structure, and class E carried the *blaZ-mecALGA251-mecR1LGA251-mecILGA251* structure. Class C *Mec* complex was divided into two different class designations: class C1 (two *IS431*s were arranged in the same direction) and class C2 (two *IS431*s were arranged in the opposite direction) by the inserted direction of *IS431*. (iii) A junkyard region (J region) is located between *Ccr* complex and *Mec* complex. According to its location in SCC*mec*, J region was classified into J1 (also known as L-C region), J2 (also known as C-M region), and J3 (also known as M-R region) region, located at the upstream of *ccr* gene complex and downstream of *Ccr* complex and the upstream of *Mec* complex and downstream of *Mec* complex, respectively.

Up to date, SCC*mec* elements are classified into types I to XI based on the nature of *Ccr* and *Mec* complex and are further classified into different subtypes in accordance with diverse J region. Types and subtypes of SCC*mec* are described in detail as follows: (i) Type I SCC*mec* was first discovered and had a long history dating back to the 2000s. The characteristic MRSA strain carrying type I SCC*mec* was identified, designated as NCTC10442, which was recovered in the United Kingdom in 1961, representing the first MRSA strain [187]. Type I SCC*mec*, carrying class B *Mec* complex and type 1 *Ccr* complex, carried a *pls* regulator in J1 region. A subtype within type I SCC*mec* was designated as IA, containing a plasmid pUB110 located in J3 region [202]. According to the nomenclature proposed in 2006 [271], type I SCC*mec* was designated as 1B.1.1 and its subtype IA was designated as 1B.1.2. (ii) The characteristic MRSA strain carrying type II SCC*mec* had been identified and designated as N315, which was first isolated in 1982 and discovered in 1999 [178, 186, 196, 197]. Type II SCC*mec* harbored class A *Mec* complex and type 2 *Ccr* complex. In J3 region, an integrated copy of staphylococcal plasmid pUB110 was found and a *kdp* regulator was found in J1 region. A number of subtypes were designated as IIA, IIB, IIC, IID, IIE, and IIb and a variant in type II SCC*mec* in consideration with the difference of J1 and J3 regions. According to the nomenclature reported in 2006 [196], type II SCC*mec* was named 2A.1.1 and type IIb was designated as 2A.2. IIA, IIB, IIC, IID, and IIE were designated as 2A.3.1, 2A.3.2, 2A.3.3, 2A.3.4, and 2A.3.5, respectively. The variant of type II was designated as 2A.1.2. (iii) A Zelanian isolate designated as 85/2082 first isolated in 1985 was found carrying type III SCC*mec*, which was first discovered in 2001 together with type I SCC*mec* [196] and was known as the representative MRSA strain of type III SCC*mec* until now. Type III SCC*mec* carries class A *Mec* complex, type 3 *Ccr* complex, and an integrated copy of plasmid pT181 encoding tetracycline and mercury resistance in J3 region. Regarding the difference within the J3 region, there were several subtypes in type III SCC*mec* designated as IIIA and IIIB and two variants designated as IIIC and IIID. According to the 2006 nomenclature [196], type III SCC*mec* was designated as 3A.1.1, IIIA was designated as 3A.1.2, and IIIB was designated as 3A.1.3. (iv) The two commonly characteristic MRSA strains carrying type IV SCC*mec*, which was first discovered in 2002, were designated as CA05 (JCSC1986) and 8/6-3p (JCSC1978) [188]. Type IV SCC*mec* was found to have a unique combination of class B *Mec* complex and type 2 *Ccr* complex, and transposon Tn4001 was found in J3 region of type IV SCC*mec*. Diversity in subtypes of type IV SCC*mec* was obtained, including IVa, IVb, IVc, IVd, IVE, IVF, IVA, IVg, IVh, IVi, IVj, and IV1. Based on the 2006 nomenclature [196], IVa, IVb, IVc, and IVd were designated as 2B.1.1, 2B.2.1, 2B.3.1, and 2B.4, respectively. IVE was named 2B.

3.3, IVF was designated as 2B.2.2, and IVA was designated as 2B.N.2. IVg, IVh, IVi, IVj, and IVk were designated as 2B.5.1, 2B.6.1, 2B.7.1, and 2B.8.1, and IV1 was designated as 2B.new.1. (v) The CA-MRSA strain WIS (JCSC3624) isolated in Australia was the characteristic MRSA strain carrying type V SCC*mec* [191], which carried class C2 *Mec* complex and type 5 *Ccr* complex. No subtype had been found so far within the group of type V SCC*mec*. According to the nomenclature proposed in 2006 [196], type V SCC*mec* was designated as 5C.1. (vi) In 2001, type VI SCC*mec* was first identified from a pediatric MRSA clone named HDE288, which was first reported in 1992 [189, 190]. Type VI SCC*mec* carries a class B *Mec* complex and type 4 *Ccr* complex. Until now, no subtype of type VI SCC*mec* has been found, which was designated as 4B according to the 2006 nomenclature [196]. (vii) CA-MRSA strain designated as JCSC6082 (p5747/2002) was isolated in 2002 [198] and identified to carry a type VII SCC*mec* in 2008 [192]. Type VII SCC*mec* carries a class C1 *Mec* complex that was different from class C2 *Mec* complex carried by type V SCC*mec* and type 5 *Ccr* complex. There was no subtype of type VII SCC*mec* reported. According to the nomenclature put forward in 2006 [196], type VII SCC*mec* was designated as 5C1. (viii) Type VIII SCC*mec* was first identified from a Canadian MRSA strain designated as C10682 isolated in 2003 [193]. It harbored a novel combination of class A *Mec* complex and type 4 *Ccr* complex. No subtype of type VIII SCC*mec* has been found currently. According to the nomenclature proposed in 2006 [196], type VIII SCC*mec* was designated as 4A. (ix) Type IX SCC*mec* was first reported to be identified in a MRSA strain designated as JCSC6943 isolated from a Thailand participant [194]. It was found carrying class C2 *Mec* complex and type 1 *Ccr* complex. No subtype of type IX SCC*mec* was found so far. According to the nomenclature put forward in 2006 [196], type IX SCC*mec* was designated as 1C2. (x) Together with type IX SCC*mec*, type X SCC*mec* carried class C1 *Mec* complex and novel type 7 *Ccr* complex was identified in a Canadian MRSA strain designated as JCSC6945 [194]. So far, no subtype of type X SCC*mec* has been found. According to the 2006 nomenclature [196], type X SCC*mec* was designated as 7C1. (xi) Type XI SCC*mec* isolated from MRSA strain LGA254 in southwest England of 2007 was a novel type different from other SCC*mec* with carriage of distinct class E *Mec* complex and type 8 *Ccr* complex. No subtype was found in type XI SCC*mec*, and according to the 2006 nomenclature, it was designated as 8E [195, 196, 199].

A thorough understanding of the molecular epidemiology and evolution of MRSA may aid in the further identification, control, prevention, and therapy of *Staphylococcus*-mediated human diseases, necessitating SCC*mec* typing as an essential tool for discrimination of different types and subtypes. Currently, there are several SCC*mec* typing methods available for the global evolutionary study of MRSA, with multiplex PCR as the major and widely used methodology. Multiplex PCR was first developed in 1988 and put in use to distinguish different types and subtypes of SCC*mec* in 2002 [198, 200]. In various multiplex PCR strategies, specificity of primer design has been the major concern determining the application of SCC*mec* typing. The multiplex PCR assay described in 2006 [201] was applicable for unique and specific typing of types and subtypes I, II, III, IVa, IVb, IVc, IVd, and V, respectively. After years of validation, this multiplex PCR strategy had been demonstrated to be a rapid, simple, and feasible method for SCC*mec* typing and serves as a useful tool for further prevention and control of *Staphylococcus*-mediated infections by clinicians and epidemiologists. However, with emergence of

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novel SCC*mec* (11 types and various subtypes to date), inclusive and novel SCC*mec* typing methodologies are desperately required.

3.1.1.2. Prevalence and occurrence

As different types and subtypes of SCC*mec* have been verified to influence the multidrug resistance and the antimicrobial MIC of β-lactam, a thorough understanding of the prevalence of SCC*mec* may aid in the further identification, control, prevention, and therapy of *Staphylococcus*-mediated human diseases. Consequently, surveillance of SCC*mec* has been performed globally in past decades. As the first identified type, type I SCC*mec* was nonpredominant in the 1970s, which was reported in a limited number of areas, including Brazil, Iran, Japan, Philippines, Spain, Switzerland, and the United States [202–210]. Type II SCC*mec* had been commonly found in Japan, Korea (occasionally in China), and the United States [206, 207, 211–215] and occasionally detected in Algeria, Brazil, China, Iran, Turkey, and Thailand [205, 216–221]. Type III SCC*mec* has been most frequently found among HA-MRSA and remains the predominant type in many countries or areas including Asia (China, Hong Kong, Iran, Malaysia, Singapore, Taiwan, and Thailand), Europe (Poland, Portugal, and Turkey), and South America (Brazil) [205, 208–214, 217, 220, 222–231]. Types IV and V have been implicated as CA-MRSA-associated SCC*mec*. A large number of variants (subtypes) have been reported within type IV, which is also the predominant type in Algeria, Brazil, Denmark, Korea, New Zealand, Portugal, Philippines, Sweden, Switzerland, Spain, and the United States [202–204, 209, 219, 221, 231–238]. Other types of SCC*mec* are rarely detected and reported [166]. According to our preliminary studies, from 2001 to 2006 in Guangzhou, analysis of the distribution of SCC*mec* type in 262 *Staphylococcus* strains demonstrated that the classic nosocomial SCC*mec* type (I–III) dominated among the tested strains, and none of the tested strain carried type IV or V. For MRSA strains, 3 and 198 strains belonged to SCC*mec* types II and III, respectively, with 8 strains untypeable. For MRCNS strains, 9, 24, and 12 strains were classified as SCC*mec* types I, II, and III respectively, with 8 strains untypeable. From a retrospective study conducted on 1,739 *Staphylococcus* isolates from a local hospital in Guangzhou from 2001 to 2010, SCC*mec* typing was performed on 263 randomly selected MRSA strains. Type III SCC*mec* was most frequently observed with an identification rate of 94.7% [249/263], with type II detected in 4 isolates (one individual isolate in 2001, 2002, 2005, and 2008, respectively) and 10 untypeable MRSA strains were recorded [165]. However, diversity in SCC*mec* types had been obtained from SCC*mec* surveillance of MRSA from another medical setting in Guangzhou from 2009 to 2012, as types I, II, III, IIIA, IV, V, and VI SCC*mec* carriage were found to be 17.6%, 56.8%, 6.2%, 10.7%, 4.1%, and 2.1%, respectively.

3.1.2. Other resistance determinants in Staphylococcus

Aside from SCC*mec*, the role of integrons as a mobile genetic mechanism in the horizontal transfer of antimicrobial genes or determinants among microorganisms has been recently well characterized, established, and documented, which may contribute to the broad distribution and spread of antibiotic resistance and ultimate emergence and unleashing of "super bugs" [174–177]. A complete and functional integron platform comprises three elements: (i) the

integrase gene (*intI*) encoding an integrase, (ii) a proximal primary recombination site *attI*, and (iii) a promoter gene (Pc) functionally demonstrated for all integrons [240]. Several classes of integrons have been identified and distinguished by differences and divergence in the *intI* sequences, and integron classes 1 to 3 are so-called multiresistant integron (RIs) with a capability of acquiring identical gene cassettes [173]. Class 4 integron is considered to be a distinct type of integron and termed super integron (SI), which was found on the small chromosome of *Vibrio cholerae* and known to be an integral component of various γ-proteobacterial genomes [17, 241, 242]. As a direct result of the linkage to Tn402-like transposons and associated with Tn3 transposon family (Tn21 or Tn1696), the class 1 integron platform has been the most ubiquitous among microbes and remains the focus of numerous studies, with a large variety of clinical Gram-negative organisms and a few Gram-positive bacteria reported to harbor this integron class [243–245]. The first observation of class 1 integron within *Staphylococcus* spp. was reported in 2004, with species including *Staphylococcus lentus*, *Staphylococcus nepalensis*, and *Staphylococcus xylosus* [246]. In Guangzhou, class 1 integrons were commonly found in MRSA strains (31.6%, 83/263) during 2001 to 2010, with decreasing identification rates observed [6, 7, 165, 166, 247]. From 2001 to 2004 in Guangzhou, the detection rate of class 1 integron for MRSA and MRCNS was 51.7% [46/89] and 56.6% [30/53], respectively [6, 13, 165, 247, 248]. From 2007 to 2010, class 1 integron was found in MRS isolates based on a series of studies of systematic integron investigation in hundreds of staphylococci strains from 2001 to 2006 [165, 247, 248]. Nevertheless, only 38.3% [46/120] of MRSA isolates carried class 1 integron. Undoubtedly, the commonly detected integron-based antimicrobial resistance mechanisms have contributed to the evolution of the resistance of MRSA and may further lead to dissemination of new waves of "super bugs." Class 2 integron has an organization similar to that of class 1 but is associated with the Tn7 transposon family [174, 249]. Class 3 integron contains a comparable structure to that of class 2 integron and up to date has only been found in a limited number of microorganisms, including *Pseudomonas*, *Alcaligenes*, *Serratia marcescens*, and *Klebsiella pneumoniae* [249–252]. Class 4 integron harbors hundreds of gene cassettes encoding adaptations that extend beyond antibiotic resistance and pathogenicity [253]. The remaining classes of integrons may also contain antibiotic resistance gene cassettes, but knowledge of their worldwide prevalence remains limited [240, 254]. As a genetic element existing in 9% of bacteria and representatives from a broad range of phyla and environments, integrons play a core role in antibiotic resistance among clinical organisms and contribute to the evolution and adaption of bacteria.

3.1.3. Mobility and evolution of MGEs in staphylococci

As a commonly found MGE with an antibiotic resistance gene (*mecA*) and site-specific recombinase genes (*ccrA* and *ccrB*), SCC*mec* has been classified into 11 types, various subtypes, and variants and plays a core role in antibiotic resistance, molecular epidemiology, and evolution of staphylococci. Through recognition of recombination sites (*attB*, *attSCC*, *attI1*, *attC*, secondary sites, etc.) and via this site-specific recombination event, MGEs are capable of capturing foreign genes. The mobility of MGEs is defined as being associated with mobile DNA elements (transposons or plasmids) and antibiotic resistance genes in addition to having a small array size and substantial heterogeneity in recombination sites [187, 229] From

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Southern hybridization analysis in preliminary studies, 58 staphylococci isolates were found to harbor one copy of class 1 integron on the chromosomal instead of plasmid DNA compared with their frequent location on plasmids for facilitation of conjugative-mediated transfer [13]. As natural capture systems and assembly platforms, MGEs in *Staphylococcus* (SCC*mec* or integrons system) allow bacteria to incorporate foreign genes and convert them to functional proteins by ensuring the correct expression. Despite affinity for self-transposition, integron systems are commonly associated with the transposons and conjugative plasmids serving as vehicles for the intra- and interspecies transmission of genetic material as well as gene cassettes capable of mobilizing to other integrons or to secondary sites in the bacterial genome [255]. This event has been regarded as a key mechanism in the dissemination and spread of resistance genes responsible for the swift spread of resistance genes and the rapid evolution of resistance to a wide range of unrelated antibiotics among diverse bacteria [251, 256]. Any ORF existing in the environmental "gene pool" is conceivably capable of being structured into the bacterial genome through the recombination platforms, and MGEs consequently have the potentially limitless capacity to exchange and stockpile functional genes, which enables rapid adaptation to selective pressure and may ultimately endow additional fitness and advantage to the bacterial host. In addition, a vast number of MGEs (such as conjugative plasmids, transposons, insertion sequences, and even entire chromosome) and the captured genes comprise the vast reservoirs of integrons and lead to the longstanding concept of a single massive "gene pool" that is available and temporally shared among bacteria [73]. The common observation of MGEs in microorganisms from the general environment and its enormous sequence diversity detected from such microbes, as well as various products unrelated to antibiotic resistance, strongly suggests that MGEs are ancient genomic structural elements and have played a general role in evolution and adaptation for a considerable period of time [43].

As a genomic island (G island) and MGE demarcated by a pair of DRs and inverted repeats, SCC*mec* has a set of site-specific recombinase genes (*ccrA* and *ccrB*) required for its movement and is inserted at the 3 end of *orfX* and located adjacent to the replication origin [220]. In the chromosome of staphylococci, SCC*mec* may have evolved from a primordial mobile element SCC, into which the *mec* complex was inserted. However, the function of the putative SCC*mec* may not be limited as the conveyer of antimicrobial resistance (mediated by *mec* complex) alone, and this MGE may serve as a vehicle for the exchange of useful genes for the better survival for staphylococci in various environments. In addition, SCC*mec* is a general genetic information exchange system of staphylococci with *ccrA* and *ccrB* involved in the recombination events (integration and excision), which plays a significant role in the evolution of *Staphylococcus*. MGEs serve as the reservoir for various genes and possess the function of interspecies genetic exchange. It is interesting to speculate whether multiple MGEs carried by staphylococci would speed up the rate of gene exchange or genome evolution, although these hypotheses require further investigation. From previous surveys, the influence of carriage of multiple MGEs on antimicrobial resistance had been investigated in MRSA. The presence of multiple MGEs was found to be strongly correlated with antimicrobial resistance, including erythromycin, gentamicin, tetracycline, and trimethoprim-sulfamethoxazole, which further limits the therapeutic options for deep-seated *Staphylococcus* infection and diseases. For treatment of complicated *Staphylococcus* infections, gentamicin is commonly prescribed by

many clinicians in combination with vancomycin due to enhanced efficacy based on synergistic antibacterial activity [257]. For penicillin-allergic patients, erythromycin has been frequently used. As the first choice for suspected CA-MRSA cutaneous infections, trimethoprim-sulfamethoxazole has also been commonly used in combination with rifampin for MRSA in carriers despite the high recurrence (up to 50%) and frequent emergent resistance of this organism.

Up to date, the most known functional genes carried by MGES are found to encode resistance to the oldest groups of antibiotics (such as tetracycline, streptomycin, and spectinomycin) that have been discontinued in clinical settings for decades but still available in veterinary practice. Although the indiscriminate use of these older antibiotics is no longer occurring in the clinical setting, their use in veterinary medicine may contribute to a novel and significant concern in food safety. Abuse of antibiotics leads to the emergence of antibiotic resistance and poses a predicament for the future treatment of bacterial infection, with MGEs undoubtedly facilitating the rapid spread and dissemination of a vast number of resistance genes among microorganisms.

3.1.4. Livestock-Associated MRSA (LA-MRSA)

As a common pathogen for both clinical medicine and food safety, MRSA was first reported as hospital associated before the 1990s and thus designated as HA-MRSA. Since the 1990s, CA-MRSA strains have increasingly been reported among groups of patients with no apparent connection to hospitals. It is noteworthy that a large number of such CA-MRSA-infected patients or carriers were pediatric associated. Aside from HA-MRSA and CA-MRSA, LA-MRSA has been recently documented and is known to be more persistent in food products from swine and cattle [258], which is also responsible for pneumonia, endocarditis, and necrotizing fasciitis by LA-MRSA carriers [259]. Nowadays, LA-MRSA acts as an increasing risk for public health and a challenge to livestock farming and related food products. LA-MRSA was mostly found among animals (particularly pigs) and humans with frequent contact to livestock farming or livestock food products [260–262]. After the first isolation of MRSA from livestock (cows with mastitis) [263], a extremely limited number of reports were focused on LA-MRSA. However, after an initial LA-MRSA case occurred in humans, described in 2005 [262], LA-MRSA have been the focus of numerous recent studies. In 2007, a transmission of MRSA (ST1, spa-type t127) between cows and humans was reported, verifying the transmission between animals and humans [264]. Afterwards, different types of LA-MRSA have been continuously discovered globally, and the prevalence and occurrence of LA-MRSA vary significantly in different areas. In Europe and America, the majority of LA-MRSA strains belong to sequence type (ST) 398, whereas ST9 is frequently discovered in Asia [265–268]. Both livestock and humans are potential carriers of LA-MRSA, and individuals working in animal clinics and livestock production environments with direct contact or exposed to MRSA-positive animals or ingestion of the MRSA-positive livestock food products have an increased risk of becoming MRSA carriers [269, 270]. A high risk of animal to human transmission of ST398 was found to result from direct association between animal and/or human MRSA

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carriages in the farm setting [261, 269, 271–273] despite much lower occurrence of transmission between humans by LA-MRSA and that of HA-MRSA [274–276].

As a clone of typical LA-MRSA, ST398-LA-MRSA has been responsible for serious infections and outbreaks worldwide [277, 278]. Containing various spa-types, ST398-LA-MRSA strains are mostly found to carry type IV or V SCC*mec*, which are nontypeable by standard PFGE using *SmaI* digestion due to protection from digestion by the presence of a restriction/methylation system [271, 279, 280]. According to the virulent properties of ST398 strains, most animal-associated ST398-LA-MRSA strains lack the major virulence factors in staphylococci, such as Panton-Valentine leukocidin (PVL), TSS toxin 1, and exfoliative toxins [281]. However, various resistance genes commonly present in staphylococci of human and animal origins are also recovered in ST398-LA-MRSA strains, including the β-lactamase gene cluster *blaZ-blaI-blaR*, the tetracycline resistance genes *tetM* and *tetK*, the macrolide-lincosamide-streptogramin B (MLSB) resistance genes *ermA*, *ermB*, and *ermC*, the lincosamide resistance gene *lnuA*, and arrays *aacA-aphD* or *aadD* for resistance to gentamicin-tobramycin-kanamycin or kanamycin-neomycin, respectively [281–285]. In addition, novel resistance genes were also discovered in ST398-LA-MRSA strains, such as *dfrK* (trimethoprim resistance), ABC transporter genes *vgaC* and *vgaE* (pleuromutilin-lincosamide-eptogramin A resistance), and *apmA* (apramycin resistance) [286].

Emergence, spread, and dissemination of ST398-LA-MRSA from animals, as well as its transmission between humans and animals, strongly suggest that the antimicrobial resistance caused by veterinary antibiotic abuse poses a hazard to both humans and animals regarding food safety challenges associated with animal origins.

3.2. Viable Putative but Nonculturable (VPNC)

In nature, bacteria exist in various states such as normal growth state, dead state, dormant state, and VPNC state, which was first reported in 1986 [287]. Differing significantly from the "starvation survival" state, VPNC state [previously known as viable but nonculturable (VBNC)] is a specific state under which bacteria remain alive but fail to form a colony on routine bacteriological media that normally support their growth. Consequently, routine bacteriological detection methodology fails to detect the VPNC bacteria. However, given the right conditions, bacteria in the VPNC state remain active and can "resuscitate" to the normal state. Hence, the VPNC food spoilage or pathogenic bacteria are considered to be a stealth source of contamination, posing a significant concern for traditional surveillance and control methodologies of foodborne pathogens.

3.2.1. Induction and resuscitation

Entering into the VPNC state is considered to be a survival mechanism for nonsporulation bacteria under a number of harsh environmental conditions, which is described in detail as follows: [1] Nutrient starvation [288]. Without essential nutrients, bacterial growth and metabolism may be terminated and thus enter the death-like status. Nutrient starvation, such as the absence of carbon source or nitrogen source, which is an extreme condition for the

growth of bacteria, can induce the VPNC state. [2] Extreme temperature [289]. The appropriate temperature for typical bacterial growth ranges from 20°C to 37°C, and termination of growth usually occurs under extremely high or low temperature. The temperature of 4°C or -20°C, at which bacteria stop growth and metabolism, is frequently used for induction of VPNC state. The combination of nutrient starvation and low temperature has also been widely applied as an induction condition. [3] pH value [290]. Most microorganisms grow in neutral and slightly acidic or alkaline pH conditions. Strong acidity or alkalinity may lead to bacterial death-like states, which has been occasionally used to induce the VPNC state. [4] Salinity [291]. As an extreme condition for bacterial growth, high salinity has been found to enable the entering of VPNC state. [5] Osmotic stress [291]. Extremely high osmotic stress was reported to be applied for VPNC state induction. [6] Oxygen availability [292, 293]. In an aerobic environment, anaerobic bacteria would enter into the VPNC state and the absence of oxygen would induce the VPNC state of aerobic bacteria and vice versa. [7] Existence of heavy metals [294, 295]. [8] Common food preservatives (cryopreservation, vacuum preservation, etc.). Currently, numerous bacteria are reported to have the ability to enter into VPNC state, such as *Salmonella* spp. [287, 296, 297], *Enterococcus* spp. [298–300], *Vibrio* spp. [301–309], *Campylobacter* spp. [310], *Pseudomonas* spp. [307, 311–313], *Shigella* spp. [314, 315], *Lactobacillus* spp. [316, 318], *Escherichia coli* [313, 316], and *Staphylococcus* spp. [318–324]. Furthermore, it has been well established and documented that bacteria in VPNC state can resuscitate and regain culturability when provided with appropriate conditions [308, 325, 326]. A variety of processes, including elevation of temperature gradually or directly [308], heat shock treating [325], adding nutrients [326], and adding organic matter (Tween-20, Tween-80, catalase, sodium pyruvate, etc.) were found to be applicable for resuscitation from the VPNC to normal state. The resuscitated bacteria are comparatively similar to their exponential-phase bacterial counterparts.

Currently, only 2 species of *Staphylococcus*, *S. aureus*, and *S. epidermidis*, were capable of entry into VPNC state [318–324]. In 2009, formation of VPNC *S. aureus* by radiation was reported for the first time, representing the first evidence of *Staphylococcus* cells entering the VPNC state [318]. One year later, induction of VPNC state by starvation of the *Staphylococcus* cells at low temperature (4°C) was also obtained [319]. Resuscitation of *S. aureus* strain under VPNC state was induced by temperature upshift (from 4°C to 22°C) or rich medium supplemented with sodium pyruvate [319, 320]. The prevention of resuscitation was observed by deficiencies in catalase or superoxide dismutase, indicating the relation of VPNC formation of *S. aureus* to oxidative stress [319, 320], constituting the initial studies on the mechanism of the formation and resuscitation of *S. aureus* in VPNC state. In addition, *S. aureus* cells in biofilm were found to enter into a VPNC state under antibiotic pressure (vancomycin or quinupristin/dalfopristin) [320, 321], suggesting that central venous catheter (CVC) or medical implant-associated biofilms may be potential reservoirs for *S. aureus* and *S. epidermidis* in the VPNC state [323]. Thus, both biofilm formation and VPNC induction may augment clinical challenges associated with antibacterial treatment options. *S. epidermidis* biofilms were reported to enter into the VPNC state when grown in excess glucose presumably due to accumulation of acidic compounds as the degradation products of glucose metabolism. This process was counteracted by high extracellular levels of calcium and magnesium added to the culture medium allowing

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modulation of the proportions of VPNC bacteria within *S. epidermidis* biofilms [324]. Although the induction and resuscitation of *Staphylococcus* cells in VPNC state has been verified, relatively little is known with respect to inducing and resuscitating condition, necessitating further investigation into this fascinating bacterial survival strategy.

3.2.2. Characteristics and mechanisms

Remaining metabolically or physiologically active, bacteria in VPNC state maintain cell integrity but exhibit dwarfing, which contribute to protect against a wide variety of stressors. The maintenance of metabolic activity and continuous gene expression under VPNC state [327, 328] indicates that potentially ingested bacteria may still be capable of causing foodborne illnesses. Such microorganisms also possess the capacity to regain culturability *in vivo* [329], exhibiting high ATP level, membrane potential [298], and retained plasmids, presenting higher autolytic capability than exponentially growing cells. The outer membrane protein profile also alters with entry into VPNC state [330]. Due to the diversity of VPNC bacteria, various characteristics among different species of microorganisms are being discovered worldwide. Regarding the mechanism of the VPNC state, the up- or down-regulation of genes and proteins associated with VPNC status compared to the exponential phase and the resuscitated status is considered to be potential factors for entering and exiting of VPNC state. However, it is currently unclear as to which genes are essential for these processes.

As for the pathogenicity of *Staphylococcus* cells under VPNC state, the viable cell numbers and gene expression had been found to remain constant in VPNC state by examination of epi-fluorescence microscopy, flow cytometry, and reverse transcription-PCR (RT-PCR) [320, 321]. This finding implied that *S. aureus* cells are likely still pathogenic in VPNC state and thus pose a significant concern on its threat to food safety.

3.2.3. Detection and identification

VPNC pathogenic bacteria are considered to be a threat to public health and food safety due to incapability of detection by the "gold standard" methodology for identification of food-associated microorganisms. Hence, the development, evaluation, validation, and further application of rapid and accurate detection methodology for VPNC bacteria are considered to be the leading concerns for the surveillance of bacterial cells in VPNC state as well as further understanding of the mechanisms on their survival and persistence in the extreme environment. The conventional detection method for VPNC bacteria was the combination of acridine orange direct count (AODC) (for total bacterial cell number counting), bright-field microscopy with nalidixic acid (for metabolically active cell number counting), and plate counting (for determination of culturability). The occurrence of entry of bacterial cells into VPNC state was validated and confirmed when colony counts were totally depleted on culture plates (with no observed colonies), which was designated as nonculturable, whereas the total bacterial and metabolically active cells still remained countable. Despite the limited application of nalidixic acid on Gram-negative microorganisms, the novel LIVE/DEAD Bacterial Viability Kit with requirement on differential fluorescence was employed for the detection of both Gram-positive and Gram-negative bacteria [331]. In consideration of the carcinogenesis and expense of

fluorescence substances, the development of molecular assays, such as random amplified polymorphism DNA and RT-PCR [328], was recently applied to identify bacterial cells in the VPNC state. As *Staphylococcus* species were concerned, an immunosensing system using impedance spectroscopy measurements was recently developed and applied for rapid verification and quantification of *S. aureus* cells in the VPNC state [322], with high sensitivity and specificity obtained.

In conclusion, foodborne pathogens, especially *S. aureus* strains, which contain various virulence genes, are capable of forming VPNC state and resuscitating into active and pathogenic state under specific conditions, posing a significant threat to food safety. The "farm to table" process includes food ingredients, processing, transportation, and storage, which involves a large variety of conditions. A number of such conditions (such as low temperature during refrigeration) may be sufficient for entry into the VPNC state, complicating the use of routine diagnostics by resulting in high "false-negative" rates of pathogen detection. However, once resuscitation occurs under proper conditions, foodborne pathogens remain active and virulent, which thus are highly likely to cause food poisoning outbreaks.

4. Concluding remarks

Ingestion of food is the major (although, not only) way for human beings to obtain nutrient substances for basic living; therefore, the quality and safety of food have recently become a major concern. Considered to be an expanding global problem and leading topic in public health, food safety is no longer limited to foodborne illnesses but has been extended to all safety issues associated with "farm to table" food approaches. In the past decade, a large number of worldwide cases or reports have been available regarding food containing unhealthy, harmful, or toxic substances (other than food poisoning outbreaks). Foodborne microorganisms, previously limited to pathogenic bacteria and toxic substances produced in food, have played a critical role in food safety. However, now due to diversity in the genus and species of microbes, variety of mechanisms on the regulation of growth and survival, and complexity of ecosystem involving polymicrobial interaction and environmental factors, a number of novel microbial issues associated with food safety have been recently acknowledged. Microorganisms may very well be capable of surviving the journey from farm to table via various evasion mechanisms at various food processing stages, including source (antimicrobial resistance caused by the use of drugs in veterinary medicine or livestock feed), processing (formation of biofilm and further survival of bacterial elimination), storage (formation of VPNC state and "false-negative" detection), and even after cooking (production of heat-stable toxins that remain active despite elimination of host bacteria). The contributions of aforementioned and novel evasion mechanisms with respect to food safety undoubtedly require further investigation in vitro and in vivo for improved diagnostic and decontamination procedures.

Author details

Zhenbo Xu[1,2*], Brian M. Peters[3], Bing Li[1], Lin Li[1] and Mark E. Shirtliff[2]

*Address all correspondence to: zhenbo.xu@hotmail.com

1 College of Light Industry and Food Sciences, South China University of Technology, Guangzhou, China

2 Department of Microbial Pathogenesis, School of Dentistry, University of Maryland, Baltimore, MD, USA

3 Department of Clinical Pharmacy, College of Pharmacy, University of Tennessee Health Sciences Center, Memphis, TN, USA

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Chapter 8

Toxoplasma gondii in Meat for Human Consumption – A Brief Review of the Most Described Strategies for Its Detection and Quantification

G.F. Dzib Paredes, A. Ortega-Pacheco, J.A. Rosado-Aguilar, K.Y. Acosta-Viana, E. Guzmán -Marín and M. Jiménez-Coello

Additional information is available at the end of the chapter

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Abstract

Toxoplasmosis is a parasitic zoonotic disease widely distributed worldwide and is caused by the intracellular parasite *Toxoplasma gondii*. The definitive host of *T. gondii* is the domestic cat and the entire cat family, in which the sexual stages of the parasite develop. *T. gondii* can also infect a wide range of intermediate hosts, affecting most warm-blooded animals including humans. In humans, toxoplasmosis is usually asymptomatic in healthy individuals, but can develop lymphadenopathy and nonspecific symptomatology or even be fatal in infants with congenital toxoplasmosis and in immunocompromised patients. Transmission to humans is mainly through food, especially by eating undercooked meat or meat contaminated with tissue cysts. This has led to various public health organizations worldwide monitoring programs on *T. gondii* in animals intended for human consumption, especially in meat samples. One of the techniques employed in the laboratory is that based on the polymerase chain reaction and some of its variants, which have proven to be valuable tools for the detection of *T. gondii* in tissues for human consumption and many other types of biological samples. The development of different strategies for the molecular detection of *T. gondii* has led to the identification and quantification methodologies varying widely among laboratories. Therefore, this chapter reviews the main methods of extraction, purification, detection and quantification of *T. gondii* DNA in tissue samples from different species destined for human consumption.

Keywords: *T. gondii*, meat, DNA quantification, parasite load, zoonosis

1. Introduction

Toxoplasmosis is a zoonotic disease widely distributed throughout the world and is caused by the intracellular parasite *Toxoplasma gondii*. The definitive host of the parasite is the domestic cat and the entire cat family, in which the parasite reproduces sexually. *T. gondii* can also infect a wide range of intermediate hosts, affecting most warm-blooded animals [1].

In humans, toxoplasmosis is usually asymptomatic in healthy individuals, but can develop lymphadenopathy and nonspecific symptomatology or even be fatal in infants with congenital toxoplasmosis (CT) and in immunocompromised patients (such as people with AIDS), in persons with problems in bone marrow and recipient patients of transplanted organs) [2].

Toxoplasmosis transmission to humans is mainly through food and from exposure to different stages of *T. gondii,* particularly by ingestion of sporulated oocysts found in the environment and contaminated water and food, or by ingestion of tissue cysts or tachyzoites contained in meat and products derived from the meat of various animals, [1,3,4].

The consumption of undercooked, raw or cured meat is a major mode of transmission of *Toxoplasmosis* to humans, especially in cases of CT, which has been attributed to 30% to 60% of cases of infection during pregnancy [5]. This has led to various public health organizations worldwide monitoring programs introduced on *T. gondii* in animals intended for human consumption, mainly in meat samples [6].

2. Occurrence of toxoplasmosis

T. gondii is a parasite that is widely distributed around the world, showing a higher incidence in tropical areas and a decrease when the latitude increases [7]. The estimated prevalence in the human population varies widely in different geographical areas, between different cities within the same city and between different ethnic groups even when they live in the same area [6,8]. In humans, toxoplasmosis is considered the third leading cause of death among food-borne diseases [7].

As well in humans, *T. gondii* is widely distributed worldwide. Parasite presence has been described in several animal species (wild, companion and production animals). However, food-producing animals may represent a real risk for transmission of the disease to humans, either directly or through farming [9]. Several serological assays have been performed in free-ranging chickens, because the information provided is usually used as an index of environmental contamination with oocysts [10]. Prevalence rates in these animals in Central and South America ranges from 40 to 60% [11],and other reports from India describe prevalence rates from 20 to 40% [12,13]; in Egypt there are rates reported around 40% [10,14] and in China, 30.36% [15]. Pigs and sheep are also commonly infected. Pigs are very susceptible to become experimentally infected and show high prevalence rates in some regions of the United States, as well in warm and tropical countries [16–21]. Prevalence rates of *T. gondii* in sheep are also widely distributed. For example in the United States, there has been described prevalence rate

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in range of 27 to 73 %; Uruguay, Argentina, Chile, Mexico and the United Kingdom showed prevalence rates of around 30%, but there are some countries with higher rates such as Ivory Coast, France and Turkey, where prevalence rates of 68, 89 and 95.7%, respectively, have been reported [22]. Cattle and buffaloes are not considered as probably sources of significant infection [22] as cows are relatively resistant to experimental infection [24].

In general, the occurrence infection by *T. gondii* in animals varies depending on weather conditions, geographical area, type of species, either productive or wild, as well as the age of the animals, the type of farming system (extensive, semi-extensive, intensive), the management and the existence of health programs [22,25,26]. Also, there have been described many factors that have an important impact on foodborne transmission of *T. gondii* to humans, such as the type of management and production of livestock, hygienic standards of slaughterhouses, food processing and technology, the density of cats or wild felines in the area and climate change, which may influence the sporulation of oocysts in the environment (i.e., temperature, humidity, wind) [6].

3. Clinical signs in animals and humans

3.1. Toxoplasmosis in humans

The clinical spectrum of the disease varies widely and depends primarily on the immune status of the host and *Toxoplasma* lineage to which a person was exposed. In humans, toxoplasmosis may manifest basically in five ways: asymptomatic, acute infection, congenital, ocular and the immunocompromised patient. In immunocompetent persons toxoplasmosis is usually asymptomatic or develops mild symptoms that go unnoticed in 80–90% of cases [27]. The usual clinical manifestations, trend to be laterocervical lymphadenopathy which is often treated, less frequently supraclavicular nodes, occipital, inguinal, mediastinal lymph node or any other chain are affected; patients with lymphadenopathy also have other symptoms such as fever, malaise, arthralgia, asthenia, night sweats, sore throat or maculopapular rash, no itching, which usually spares the palms and soles. The clinical picture of acute toxoplasmosis is usually benign and self-limiting; in <1 month, progression to the severe form is rare in immunocompetent hosts [27–30]. In immunocompromised subjects, acute toxoplasmosis usually occurs widely where the main sites of spread are the central nervous system, eyes, heart, liver and lungs, with preference in the tissues where the immune response is limited, causing injuries. In immunocompromised patients, such as AIDS patients with very low CD4 counts, patients under immunosuppression to prevent or treat transplant rejection and fetuses, a reactivation of an earlier infection, rather than a newly acquired one, is common. In these individuals the parasite can induce besides encephalitis and retinochoroiditis, carditis, pneumonia, and meningitis, among other manifestations [31]. Ocular toxoplasmosis is produced in most cases by breaking tissue cysts that are congenitally acquired; however, it can occur in acquired infections too. It manifests as uveitis or retinochoroiditis with exudate and decreased vision; the lesion can be observed by fundus evaluation. The ocular presentation is commonly characterized by necrotic lesions destroying retinal architecture, sometimes engaging and

choroid (retinochoroiditis) [28,30]. CT is the most significant complication of active infection. When the infection is acquired during pregnancy, either by reactivation of latent-phase bradyzoites or by a primary infection, the parasites migrate to the placenta and from there to the fetus. The severity of the disease depends on the stage of pregnancy when the parasites cross the placenta. At the beginning of pregnancy, fetal infection is rare, but when it happens, it triggers severe injury or death of the fetus. As the pregnancy progresses, congenital infection is most common, but the damage tends to be lower [29,32]. When the disease occurs in utero, there may be consequences observed at birth. Most congenital infections are asymptomatic, although in some cases the child is born with acute illness, where cases of hydrocephalus, eye damage and visceral can be found. In other cases, the disease develops after birth or become dormant for a long time [29,32].

3.2. Toxoplasmosis in animals

Natural infection in non-pregnant animals usually elapses without symptoms, but primary infection during pregnancy can cause embryonic death, abortion, birth weak or clinically normal but infected animals. Globally, *T. gondii* is the cause of 11–14% of the abortions that occur in sheep and goats [33]. In cattle, by contrast, *T. gondii* infection is not considered a common cause of abortion and presented asymptomatic. *T. gondii* in dogs is considered an opportunistic pathogen and infection is usually subclinical, but under certain conditions clinical signs are present, predominantly respiratory and neuromuscular manifestations (Dubey, 2010). Cats even as definitive hosts usually enrolled asymptomatic infection, even during removal of oocysts; however, sometimes clinical signs are present, primarily associated with respiratory type interstitial pneumonia, dyspnea, lethargy and anorexia, ocular signs (uveitis, retinochoroiditis) or neuromuscular signs [34]. In cats it has also been described as intrauterine infection; thus, infected animals generally have more severe signs: encephalitis, hepatitis, ascites, respiratory signs and perinatal death or weaning [35]. In the pigs, the disease usually attends in subclinical and can be seen in some cases of weak animals born or stillborn; in adult animals weight loss, anorexia and fever have been observed, which usually disappear by the third week after infection regardless of variant of *T. gondii*. Pigs are considered an important source of infection for humans because of the high parasite loads encountered in their tissues compared to other productive animals [22,35].

4. Economic impact of toxoplasmosis

The real economic impact of toxoplasmosis is difficult to estimate, because in most immunocompetent individuals, the infection goes unnoticed or has claimed clinical presentation to other diseases [22,29]. However, it is estimated that the economic impact should be very high due to the loss of one or more days of work in mild cases, treatment and care needs, sick children, especially those with mental retardation and blindness, loss of quality lifestyle and the costs of hospitalization in severe cases and the cost of monitoring pregnant women and treatment during pregnancy who are *T. gondii* positive [26,36,37].

In United States,, despite the low incidence, the economic impact of CT is high due to the severity of the infection, associated complications, treatment and social costs. CT costs have been estimated as $ 1.26 million per case and were mainly attributed to drug costs, annual losses of productivity, special education and health care costs [38]. On the other hand, in the United States, some 3,000 babies are born every year with CT and the annual cost of treating this disease is between US$ 31 and US$ 40 million [39]. The total economic impact of CT just in the United States has been estimated as over $ 7.7 billion per year, which makes it the second most important infection to humans after foodborne salmonellosis [40]. In the United Kingdom, the annual economic impact is estimated $ 12 million [26].

In most productive animals, toxoplasmosis occurs asymptomatically; however, in animal production, toxoplasmosis is considered as an economically important disease of livestock, especially sheep and goats, where it can cause early embryonic death and resorption, fetal death and mummification, abortion, stillbirth and neonatal death [41].

Regarding economic impact of infection with *T. gondii* in productive species, just in the United Kingdom, economic losses were estimated to be between 15 and 20%, from scanning to sale, but on some farms losses can reach over 30% [42]. This is often a result of a disease outbreak of *T. gondii* abortion. Other authors had mentioned that cost of *Toxoplasma* in the UK flock was estimated to be £12 million [43]. These estimates included loss of production, cost of treatment, control and monitoring but did not include the costs associated with human health. As a matter of fact, the economic losses caused by *T. gondii* infection in sheep are difficult to estimate because the disease occurs sporadically. Moreover, only a small number of the lambs aborted are subjected to diagnosis [43]. In addition, the material sent for diagnosis, besides being potentially inadequate, might also be examined erroneously and, finally, serological testing lacks specificity [45].

In pigs, the infection with *T. gondii* also use to be asymptomatic, but in some cases, the active infection or parasitemia reactivation could impact over reproductive parameters (abortions or weak stillborns), mainly when is present in concomitance with other viral agents such as parvovirus [46] or circovirus.

5. Molecular biology techniques for *T. gondii* detection

The diagnosis of toxoplasmosis is usually based on the detection of antibodies by ELISA serology, agglutination assays or other methods such as Western blot immuno- or Sabin-Feldman [47] staining. However, there are times when serological or with any of the aforementioned detection methods are not possible; in these cases the techniques of molecular biology have been helpful in the diagnosis of *T. gondii*, especially the PCR technique, which has proved to be a valuable tool [2,48].

Currently different strategies for molecular diagnosis of *T. gondii* have been developed; this has meant that various methodologies are reported (both for the extraction of nucleic acids and for the detection of different amplification targets) and brings about a large variation

between results from different laboratories [49]. So the aim of this chapter is to review the main methods of extraction, purification, detection and quantification of DNA from *T. gondii* in different species intended for human consumption.

5.1. DNA extraction methods

The extraction and purification of nucleic acids is the first stage of most molecular biological studies; extraction methods allow to obtain nucleic acids from various sources and then perform specific analysis by polymerase chain reaction (PCR) or its variants [49]. The quality and purity of the nucleic acids are two of the most important elements of such analysis since contaminants can interfere by inhibiting the amplification process in which PCR [50] rests. This makes clear that the importance of the process of sample preparation and DNA extraction methodology used, to have significant impact on the sensitivity of the test [50,51].

To extract the nucleic acids of the biological material, samples must be homogenized, causing cell lysis, proteins be removed by incubation with a protease and finally nucleic acids should be separated from other cellular components [50]. The ideal lysis procedure, which usually consists of a balancing techniques, must be strong enough to break the complex starting material (a fabric, for example), but gentle enough to preserve the nucleic acids. The lysis process is generally performed by physical or chemical processes, which break the bonds between the cells to facilitate interaction with lysis solutions that help release the genetic material [50, 51]. Among the conventional methods for extracting genetic material from T. *gondii* in tissue include the following procedures previous to DNA extraction.

5.1.1. Mechanical homogenization with liquid nitrogen

This process involves macerating the sample with liquid nitrogen using a mortar to obtain a fine powder. The nitrogen immediately can freeze the sample to prevent crystals formation, thus avoiding the breakdown of cell structures and the start of DNA degradation process by the action of DNase [51].

5.1.2. Chemical homogenization

In the chemical homogenization, the samples are maintained in solution at high temperatures in the presence of proteases, detergents and chaotropic agents to break the bonds between the cells or can even pierce the cell membrane. In fibrous tissue it is recommended that you cut into small pieces to facilitate their decomposition. Before starting this type of homogenization it is necessary to have information about the right amount of tissue; for rapid and complete homogenization, it is necessary to ensure the recovery of DNA and prevent degradation [51].

5.1.3. Pepsin digestion

Pepsin digestion is a method developed by Jacos et al. in 1960 and is modified by Dubey (1998) for retrieving from *T. gondii* from 50 g of muscle tissue (Dubey, 2010). The method is based on the digestion process by which the parasite is released from tissue cysts to invade its host. In this method, the homogenization of muscle tissue occurs by digestion with porcine pepsin

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solution, HCl, NaCl and distilled water at 37°C for 1 hour. Subsequently, a series of centrifugations allow the parasites to concentrate and be more likely to find their genetic material when purification is performed. At the end of centrifugation the product has to be buffered with bicarbonate to prevent degradation of the nucleic acids contained in the homogenate [22].

Pepsin requires acidic pH for activation; it breaks the bonds between tyrosine and phenylalanine partially degraded proteins. Pepsin polypeptides of different sizes and some amino acids are obtained without degrading completely, so this procedure is commonly followed by the enzymatic action of another protease [52].

5.1.4. Proteinase K

Proteinase K is most often used because it is the widest-spectrum (degrades all proteins) protease and it is often used with buffers containing SDS and EDTA [50]. Proteinase K is a protease obtained from the fungus saprophyte *Tritirachium album* and is particularly suitable for digestion in a short time. Proteinase k acts on the carboxyl group of amino acids and is highly stable in a wide range of temperatures and pH values, having its greatest activity at elevated temperatures [53].

5.2. DNA purification methods

The DNA purification methods can be classified into two major branches: traditional protocols and by commercial kits [51].

5.2.1. Conventional protocols

They were developed in the 1950s; organic solvents used to separate proteins and DNA, once suspended in the aqueous phase by ethanol precipitation isolate [51]. In the case of *T. gondii*, more purification methodology used in the traditional methods is performed by phenol/chloroform.

5.2.2. Phenol/chloroform

The phenol/chloroform purification is a method of liquid-liquid organic extraction consisting of separate mixtures of molecules, which is based on the difference in solubility of individual molecules in two different liquids [52,53]. The nucleic acid extraction with phenol/chloroform involves adding equal volumes of phenol/chloroform aqueous cell lysate or tissue homogenate, mixing the two phases and allowing to separate by centrifugation (Alejos et al., 2014). The phenol/chloroform method ensures the separation of liquids in two phases (organic and aqueous lower than), because chloroform is miscible with phenol due to its higher density (1.47 g/cm3) phenol [54,55].

Nucleic acids are soluble in the upper aqueous phase because of their negatively charged phosphate backbone, while proteins and lipids are separated in the organic phase [55]. Phenol causes precipitation of proteins and polymers (including carbohydrates) that are contained in the interface between the two phases (often as a white supernatant); in the case of lipids, these

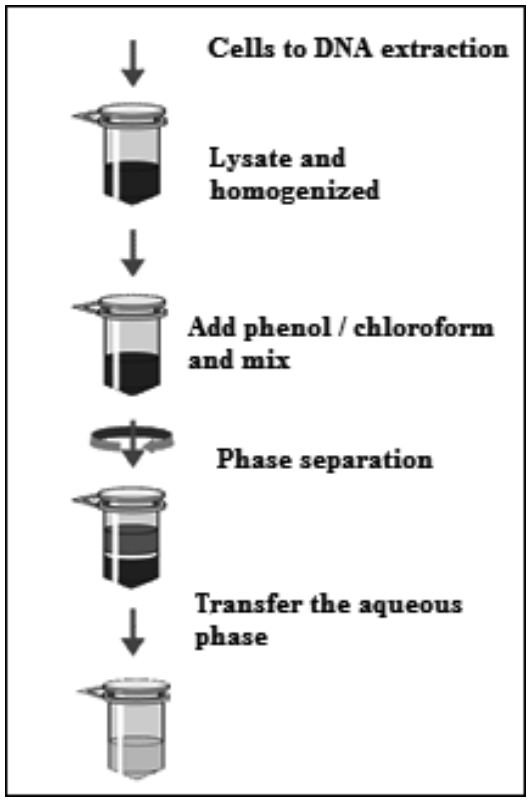

Figure 1. DNA purification with phenol/chloroform [55].

are dissolved in the lower organic phase. The separation between the aqueous and organic phase by centrifugation allows isolation of the DNA in the aqueous phase (Karp, 2009; Soma, 2010). Subsequently the DNA is recovered from the aqueous phase with ethanol and is insoluble, causing centrifugation to precipitate it [51].

5.3. DNA extraction using commercial kits

From the 1990s were introduced to market commercial purification kits; these kits commonly used membranes or inorganic matrices to which the DNA will bind to specific conditions (Karp, 2009). Often these arrays are stacked into small columns in centrifuge tubes so that the binding steps, washing and elution can be performed efficiently by applying a centrifugal force. Some of the advantages of using commercial kits are to increase efficiency of DNA recovery, to obtain inhibitor-free extract and to decrease the time spent for purification {51,54]

The purification procedure with commercial kits can be summarized in three steps: 1) homogenization of tissues to facilitate the selective attachment of DNA to the matrix; 2) washing to remove DNA contaminants and 3) recovering the DNA from the matrix using an eluting buffer [51].

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5.3.1. Silica matrix

This method is based in selectively adsorption/desorption of nucleic acids and it has proven more efficient for the recovery of pure DNA beside biological samples (i.e., blood, tissue). Silica extraction methods produce increased DNA yield while efficiently removing PCR inhibitors; those protocols usually include a small-scale silica-based spin column. The selective adsorption/desorption occurs when, by the action of ethanol, the DNA loses its humectant layer, exposing its phosphate groups and thereby facilitating the adsorption of the molecule to the positively charged membrane. Lipids and proteins are not related to the membrane and are removed using the wash solution and a cycle of centrifugation, while the genetic material remained is bound to the matrix [51].

5.3.2. Magnetic beads

Extraction methodology using magnetic beads or a magnet that attracts magnet to separate the beads from solutions in which they are suspended is applicable. In this case, the lysis buffer solution at acidic pH, which allows to positively charge the beads, favoring DNA binding [51,53], is added.

The methodology for the extraction and purification of DNA from *T. gondii* in tissue with magnetic beads was described by Opsteegh et al. [56]. Tissue homogenization is carried out in a bag filter Stomacher400 containing a lysis buffer (100 mM Tris HCl pH 8.0, 5 mM EDTA pH 8.0, 0.2% SDS, 200mMNaCl and 40mg/L Proteinase k), by reaction using 2.5 mL of lysis buffer per gram of sample for 2 minutes at high speed. The homogenate is placed in incubation at 55°C overnight and, after the incubation, the extract obtained is homogenized again for 1 minute; 50 mL of the homogenate is transferred to a 50-mL tube and is centrifuged at 3500 g × 45 min; 12 mL of the supernatant (crude extract) is collected for use in the purification of the genetic material of *T. gondii*.

The first step in the purification process based on magnetic beads is the removal of free biotin in the sample because this method is based on the target binding sequence labeled with biotin to the magnetic beads that are coated with streptavidin (protein high affinity for biotin). To remove free biotin from the sample streptavidin sepharose should be added to allow the biotin precipitation and to form a pellet by centrifuging and 10 mL of the supernatant are finally used for purification process [56,57].

To mark the sequences of interest with biotin is needed the addition of specific primers that are marked with this molecule [57]. The bonding occurs during hybridization, so it is first necessary to denature the sample nucleic acids by heating at 95°C for 15 min and then lower the temperature to allow hybridization; if the proposed protocol is by Opsteegh et al. [56]. the hybridization temperature is 55°C for 45 min. Once labeled primers are hybridized to the target sequence, we proceed to introduce the magnetic beads in the sample and proceed to incubate at room temperature for 60 minutes to allow binding sequences labeled with magnetic beads. They are separated from the beads using a magnet and then the DNA of the beads was recovered by washing with buffer B & W (Binding & Washing) provided in the kit extraction

and finally the DNA recovered is resuspended in distilled water. Magnetic capture process is presented in Figure 2.

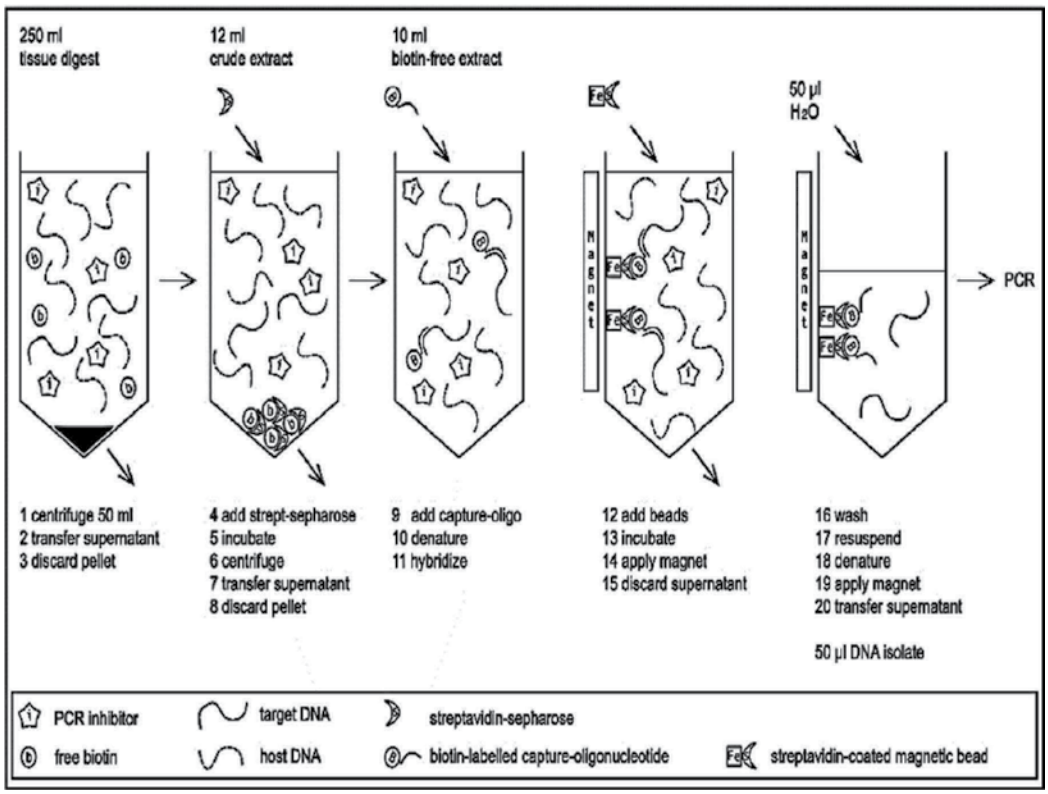

Figure 2. DNA purification from tissue employing magnetic beads [56].

Today there are also cases that implement business purification methods with organic solvents. Usually these methods are based on the phenol/chloroform by adding guanidine or any other part that improves the extraction and purification of nucleic acids. Examples of these traditional methods are converted to commercial kits extraction and extraction with TRIzol® DNAzol® [58,59]. These commercial kits based home methods are usually cheaper than commercial kits based on inorganic matrices, although purification performance is very similar in some cases, there is a risk of contamination of samples with phenol and inhibition of amplification in the PCR process [60]. Another important factor to consider is security, since this type of extraction involves the use of corrosive and irritant substances and therefore requires some experience to handle; accidental contact with the reagents that can burn skin and vapors inhalation could cause damage to the respiratory system, in both cases, medical assistance is required. Table 1 presents the main commercial kits used for the extraction and purification of DNA from *T. gondii*.

Commercial kit	Fundamental	Column material	Amount of recovered DNA	Reference
Universal Genomic DNA Extraction Kit (TaKaRa, USA)	Absorption/ desorption	Silica	10μg (2–30 mg of tissue)	[61].
Easy-DNA®Kit (Invitrogen)	Organic extraction	N/A	150μg (50 mg of tissue) 5–10 ng/μL blood	[62].
High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim Germany)	Absorption/ desorption	Glass fiber filter	3–9 μg (200–300 μL blood) 25–50 mg of tissue (variable amount recovered depending the tissue tested)	[6]. [63].
QIAamp DNA Mini Kit (QIAGEN, Valencia, CA)	Absorption/ desorption	Silica	4–12μg (200μL blood) 25–45μg (25mg of cardiac tissue)	[21,47,64–68].
TRIzol® LS Reagent (Invitrogen, Paisley, the United Kingdom) (Fenol-Cloroformo)	Organic liquid-liquid extraction	N/A	2–3μg (for each mg of sample)	[69].
Phenol-Chloroform	Organic liquid-liquid extraction	N/A	---	[70–76]
NucleoSpin Tissue Kit (Macherey Nagel, Czech Republic)	absorption/ desorption	Silica	20–35μg (25mg of tissue)	[77].
Biotin labeled capture oligonucleotides and Streptavidin labeled magnetic beads (Invitrogen)	magnetic capture	Magnetic beads	Recovered DNA from 5–100pb	[5,47,56]
NucliSens easy MAG system (BIOMERIEUX)	magnetic capture	Magnetic Silica	---	[49,78,79]
Genomic Prep Blood DNA isolation kit (Amersham Pharmacia Biotech, the United Kingdom)	absorption/ desorption	Silica	Recovered DNA from 100–200 kb	
DNAzol® (Life technologies)	organic extraction	N/A	3–5μg/mg from tissue	[80].
Qiaquick y PCR purification kit (QIAGEN, Valencia)	absorption/ desorption	Silica	10 μg DNA recovered from 100pb–10kb	[81,82]
DNeasy Blood & Tissue Kit (QIAGEN)	absorption/ desorption	Silica	10–30ug (25mg of tissue)	[83,84]

Table 1. Leading commercial kits used for DNA extraction and purification from different tissues for *T. gondii* detection

6. Methods for molecular detection of *T. gondii* in meat for consumption

The presence of *T. gondii* in biological samples can be diagnosed by molecular techniques aimed at detecting its genetic material [74,85] (Switaj et al., 2005; Garcia et al., 2006). A specific fragment of the parasite genome can be amplified by PCR to visualize on a polyacrylamide or agarose gel, to stain, to sequence directly in real time. The sensitivity and specificity of PCR-based techniques rely on an appropriate method of purification of genetic material from the samples, the characteristics of the DNA sequences chosen for amplification and the parameters of the amplification reaction [50,85]. Various amplification methods for *T. gondii* will be explained below.

6.1. Conventional PCR (endpoint)

The PCR is a molecular biology technique developed by Dr. Kary B. Mullis in 1985 [54,86]. The impact of his discovery was such that Dr. Mullis received the Nobel Prize for Chemistry in 1993 (Welch, 2012). PCR is a technique "*in vitro*" used to amplify enzymatically a specific region of DNA located between two regions of DNA whose sequence is known [86]. PCR simulates what happens during cell replication taking advantage of features of the chemical structure and semiconservative DNA replication [50].

DNA is a polymer formed from two complementary strands anti-parallel, each chain consists of nucleotide units which in turn are comprised of a nitrogenous base (adenine = A, guanine = G, cytokine = C and thymine = T) attached to sugar deoxyribose and a triphosphate group [50,87]. To replicate the DNA separates its two complementary strands, serving each mold or template for the "de novo synthesis" of its complementary strand, the specificity of pairing of the nitrogenated bases (TA, CG) to obtain two identical DNA molecules, each consisting of an original and a new chain. The enzyme that performs this process is called DNA polymerase [54,87]. PCR synthesis of new DNA strands is performed by mixing: containing DNA or fragments to be amplified; polymerase; primers (DNA fragment of 15 to 30 nucleotides flanking the region to be amplified and to provide the free 3 'end to initiate transcription); deoxynucleotides (dNTPs); magnesium chloride (MgCl2) or other cofactor necessary to work polymerase [86]. Generally, the PCR begins with denaturation or separation of the double helix of DNA by heating the sample at a temperature between 94 and 96°C to break the hydrogen bonds that bind them, so as each string is a template for synthesis A new complementary strand of DNA [88].

Once separated the chains of DNA primers (initiators or primers) are aligned in complementary-specific sites of the single strands in the region to be amplified; for this to happen it is necessary to lower the temperature between 40 and 60°C, allowing binding (hybridization or alignment) of the primers. Finally, a new strand is synthesized in the 5′ to 3′ for which the temperature is increased, generally at 72°C, which is the optimum temperature to work Taq polymerase [89]. These three stages — 1) denaturation, 2) hybridization and 3) elongation of DNA — are repeated successively in each new cycle and amplified the region of interest of the two complementary strands [86] simultaneously. The essential equipment for the process to take place is the thermal cycler, which has a heating pad where each reaction is placed and

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where temperature changes are accurate and can be pre-programmed in three stages by several cycles [50, 86].

Detecting the PCR product is usually accomplished by electrophoresis. Separation matrices (agarose, polyacrylamide) at various concentrations are used depending on the size of the amplification product and the resolution desired. The posterior viewing can be done with ethidium bromide with a UV lamp, silver staining, fluorescence or radioactivity light. The sizes of the PCR products are determined by comparing them with markers containing DNA fragments of known size, which are run in a gel with PCR products.

6.2. Nested PCR

Nested PCR (nested) consists of two successive processes of amplification, using the product of the first amplification as template for the second [2]. In the second amplification primers used should be different from the first amplification and are targeted to amplify a smaller fraction contained within the product of the first PCR [90]. This methodology increases both the sensitivity and specificity of the test. Furthermore, the risk of contamination increases significantly due to the increased amount of amplification products and work steps involved [91]. In this type of test validation it is always recommended by both negative and positive controls, ensuring that the positive controls are highly diluted to avoid contamination of the samples [90].

6.3. PCR-LAMP

This is a variant of PCR developed for parasites of the phylum Apicomplexa, among which is *T. gondii*. The amplification method LAMP (Loop-mediated isothermal amplification) is based on a displacement auto cyclic reaction chain using a set of four oligonucleotides (primers) which recognize six sequences within the genomic DNA target region and form a loop structured amplicon, the polymerase that performs this function is Bst polymerase having activity displacement [92,93].

6.4. Real-time PCR (qPCR)

Real-time PCR is a technique used to quantify specific nucleic acid sequences in a sample of interest. The assay is based on generating a fluorescent signal that is directly proportional to the amount of target DNA. Real-time PCR is able to monitor the fluorescence emission that occurs during the reaction progress, so is said to be in real time [94]. Among the major fluorophores used for determination of *T. gondii* load are as follows:

6.4.1. SYBR Green™

It is an intercalator that binds to dsDNA resulting fluorescence increased with increasing the amount of PCR product. An important aspect to consider is that the SYBR Green™ can also join primer dimers and nonspecific amplification products, resulting in an overestimation of the concentration of target DNA. The detection of DNA of *T. gondii* using SYBR Green it could be useful mainly during experimental infections. It has been used for monitoring parasitemia

loads in animal models, however for clinical diagnosis or monitoring any other natural infection (i.e., in animals for consumption) the performance it is not enough sensitive to detect the presence of low amount of the target in the sample.

6.4.2. TaqMan™

TaqMan probes are hydrolysis probes which allow increase the specificity of quantitative PCR. They have attached a reporter (a fluorophore) and a quencher. When both (fluorophore and the quencher) are in proximity, the reporter emits no signal, however, when the probe hybridizes to the sequence of interest during PCR, the endonuclease activity of Taq polymerase to short photochromic other reporter probe, allowing emission of a fluorescent signal. The reporter fluorescent signal is cumulative in each of the subsequent cycles [79].

7. Determination of parasite load in meat by qPCR

7.1. Absolute quantification

Absolute quantitation is a quantization strategy based on comparison of the test samples against a standard curve created from a template of known concentration (Sivaganesan et al., 2010). This template of known concentration is used to make serial dilutions and generate a curve from the CT values (threshold cycle) obtained for each concentration. The curve can interpolate directly the CT values of the test samples and get your concentration by the equation $Q = 10\left(\frac{CT - b}{m}\right)$ where: Q = amount shown, CT = amplification cycle, b = insertion axis "and" m = gradient of the line. In traditional protocols creating absolute quantification standard curve in each qPCR experiment conducted it suggested, however, can also be employed master calibration curve generated from multiple experiments qPCR [95]. The absolute quantification method is conceptually easy, however, requires a reliable source as a template of known concentration.

7.2. Relative quantification

Such quantification measures changes in the basal state of a gene of interest versus constant gene expression that acts as a control. The difference lies in the absolute quantification that are not part of a known amount of DNA, but an endogenous housekeeping gene control or reference "housekeeping". Because the absolute amount of internal standard is unknown, can be determined only relative changes of the gene of interest with reference to the endogenous gene. Some of the most commonly used reference genes include glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, hypoxanthine guanine phosphoribosyl transferase (HPRT) and 18S ribosomal RNA.

The advantage of using mass units and normalizing the experimental design is that it is conceptually simple, but requires accurate quantitation of starting material to be used as a normalizer. There are some methods and models to determine the amount of DNA that are based on comparison of the CT values using the efficiency of PCR reaction as a correction

factor. However, there is also a model that does not require the reaction efficiency of accessing a correction factor, assuming 100% efficiency in the PCR reaction in real time, both study gene as reference gene, this Method 2 is delta-delta CT (2-ΔΔCT) (Vinuesa, 2009; Aguilera et al, 2014.). Method 2-ΔΔCT expresses the ratio obtained from the relationship between the CT values of the sample and control values CT. Assay validation is made by serial dilution problem for both the endogenous gene to gene. ΔCt values (CT gen – CT endogenous) are obtained. These are plotted on the "y" axis versus the logarithm of the concentration in each of the dilutions in the "x" axis. The slope of the line should be less than or equal to 0.1 so that the method is valid [69]. Table 2 shows the most commonly used primers for the detection by different variants of PCR and also are listed the ones used to estimate the parasite load of *T. gondii* in tissues.

Amplification target	**primers**	**Sequence 5′–3′**	**Amplicon size**	**Reference**
PCR endpoint				
529pb Repeat Element (*RE*)	Toxo4	CGCTGCAGGGAGGAAGACGAAAGTTG	529pb	[62,74,75]
	Toxo5	CGCTGCAGACACAGTGCATCTCGGATT		
Gen B1	Tg1	AAAAATGTGGGAATGAAAGAG	469pb	[3,21,64]
	Tg2	ACGAATCAACGGAACTGTAAT		
Gen B1	F	AGCCTCTCTCTTCAAGCAGCGTA	300pb	[71,83]
	R	TCGGAGAGAGAAGTTCGTCGCAT		
Nested PCR				
Gen B1	S1	TGTTCTGTCCTATCGCAACG	580 pb	[49]
	AS1	ACGGATGCAGTTCCTTTCTG	530pb	
	S2	TCTTCCCAGACGTGGATTTC		
	AS2	CTCGACAATACGCTGCTTGA		
Gen GRA6	GRA6-F1x	ATTTGTGTTTCCGAGCAGGT	546pb	[76]
	GRA6-R1	GCACCTTCGCTTGTGGTT	351pb	
	GRA6-F1	TTTCCGAGCAGGTGACCT		
	GRA6-R1x	TCGCCGAAGAGTTGACATAG		
Gen B1	B1F1	TCAAGCAGCGTATTGTCGAG	194pb	[96]
	B1R1	CCGCAGCGACTTCTATCTCT		
	B1F2	GGAACTGCATCCGTTCATGAG		
	B1R2	TCTTTAAAGCGTTCGTGGTC		
529 pb *RE*	REF1	TGACTCGGGCCCAGCTGCGT-3′	164pb	[93,96]
	RER1	CTCCTCCCTTCGTCCAAGCCTCC-3′		
	REF2	AGGGACAGAAGTCGAAGGGG-3′		
	RER2	GCAGCCAAGCCGGAAACATC-3′		
PCR LAMP				
529bp *RE*	F3	ACGAGAGTCGGAGAGGGA	202pb	[93, 96]
	B3	TGGATTCCTCTCCTACCCCT		
	FIP (F1 c-F2)	GGATCGCATTCCGGTGTCTCTTAAGATGTTTCCGGCT		
	BIP (B1c-B2)	TGGC		

Amplification target	primers	Sequence 5′–3′	Amplicon size	Reference
		GACGACGCTTTCCTCGTGGTCAAGCCTCCGACTCTGT CT		
529bp *RE*	F3	CCACAGAAGGGACAGAAGTC	202pb	[96,97]
	B3	TCCGGTGTCTCTTTTTCCAC		
	FIP	TCCTCACCCTCGCCTTCATCTAGGACTACAGACGCGA		
	BIP	TGC		
	LF	TGGTTGGGAAGCGACGAGAGTTCCAGGAAAAGCAG		
	LB	CCAAG		
		TCCAAGACGGCTGGAGGAG		
		CGGAGAGGGAGAAGATGTTTCC		
Gen B1	BIP	TCGCAACGGAGTTCTTCCCAGTTTTGGCCTGATATTA	212 pb	[96]
	FIP	CGACGGAC		
	F3	TGACGCCTTTAGCACATCTGGT		
	B3	TTTTGATGCTCAAAGTCGACCGC		
		GGGAGCAAGAGTTGGGACTA		
		CAGACAGCGAACAGAACAGA		
qPCR				
Gen B1	SYBR	BE-F: CTCCTTCGTCCGTCGTAATATC	451pb	[47]
	Green™	BE-R: TGGTGTACTGCGAAAATGAATC		
SAG 1	SYBR	F: CTGATGTCGTTCTTGCGATGTGGC	128pb	[61]
	Green™	R: GTGAAGTG-GTTCTCCGTCGGTGT		
Gen B1	SYBR	F: CGTCCGTCGTAATATCAG	98pb	[47]
	Green™	R: GACTTCATGGGACGATATG		
529pb *RE*	TaqMan™	F: CACAGAAGGGACAGAAGT	94pb	[47,63,69]
		R: TCGCCTTCATCTACAGTC		
		RE probe: (JOE[e])-CTCTCCTCCAAGACGGCTGG-(TAMRA[f])		
Gen B1	TaqMan™	GENE_B1_TG-TX2F: CTAGTATCGTGCGGCAATGTG	62 pb	[79]
		GENE_B1_TG-TX2R: GGCAGCGTCTCTTCCTCTTTT		
		GENE_B1_TG-TX2M1: 6-FAM)-CCACCTCGCCTCTTGG-(NFQ-MGB)		
Gen B1	TaqMan™	BE-F: CCCCACAAGACGGCTGABE-R:	248pb	[47]
		TGGTGTACTGCGAAAATGAATC		
		TaqMan probe: (6-FAM)-CATTTGCAAAACAGCGGCAGCGTCT-(DQ)		
529pb *RE*	TaqMan™	TOX-9F: AGGAGAGATA TCAGGACTGT AG	524pb	[5,47,56,77]
		TOX-11R: GCGTCGTCTC GTCTAGATCG		
		TOX-TP1: (6-FAM)-CCGGCTTGGC TGCTTTTCCT-(BHQ1)		

Table 2. Amplification targets, primers and probes used in the diagnosis and quantification of *T. gondii* in meat.

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Although there are several methods for the diagnosis of toxoplasmosis, the present work focused on the most used by researchers working with *T. gondii* in animal tissue samples. In this aspect the molecular, especially the PCR technique and its variants, has been a major technique for diagnosing the presence and in some cases abundance of the parasite in the tissues of the various productive species that are commonly consumed tool.

The sensitivity of PCR techniques is influenced by several factors, among which are tissue type, sample handling, the process employed in the extraction and purification of nucleic acids and the type of card selected amplification. All these factors cause variation sensitivity in these tests of about 20% to over 80% in some cases. Variations of the PCR most commonly used for the diagnosis of toxoplasmosis are conventional PCR, LAMP-PCR, nested PCR and real time PCR, considered as the most sensitive nested PCR and PCR techniques in real time, can detect the presence of nucleic acids of *T. gondii* from a single parasite in the sample.

The techniques of DNA extraction and purification significantly affect the development of PCR techniques; the presence of contaminants may inhibit the amplification process. Nowadays, there are different methodologies used for DNA extraction and also, there is a wide variety of commercial options available to perform the procedures, however, even almost all of them have been designed to obtain as much amount of DNA as possible from any specimen while minimizing manual handling and co-extraction of PCR inhibitors. Methodologies based in small-scale silica-based spin column, has proven more efficient for the recovery of pure DNA in comparison with phenol/chloroform extraction, because they have showed several disadvantages besides being unable to remove potential PCR inhibitors efficiently.

The sensitivity of PCR assays depends heavily on the number of copies of the target sequence; in the case of *T. gondii* although various cards amplification as is the case of SAG1, SAG2, SAG3 and GRA6 genes, most widely used are those which are directed to highly repetitive genes as gene B1 is repeated 35 times in the genome of *T. gondii* or repeated 529pb fraction, which is repeated 200 to 300 times in the parasite genome. Although the B1 gene is considered to be the gene of choice for the diagnosis, repeated fraction 529pb has begun to have a greater impact due to it being repeated more times increases the sensitivity of PCR testing. This trend toward the use of these cards amplification is observed in most variants of the PCR.

An important aspect for the selection of the PCR technique used in the diagnosis of toxoplasmosis has been the cost involved, the techniques of real-time PCR are usually much more expensive due to the use of probes and specialized equipment for reading the results, so this type of quantitative PCR are mainly used for research.

It is important to consider that molecular methods allow us to estimate whether the presence (when we use some variants as endpoint PCR or nested PCR) or quantity (by qPCR) parasite DNA in the evaluated sample. However, these findings do not allow for determination whether the parasites are viable and capable of producing infection.

Tissues that tend to have higher parasite loads are often brain, heart and spleen, but it is feasible to detect the presence of the parasite in other tissues intended for human consumption. Loading and distribution of parasites depends on biotype and density involved in the environment, as well as the animal's age and the type of production system. Due to this high variability, it is important to determine which tissues have higher loads and biotypes which are involved in order to avoid risks of transmission to consumers of meat from different regions and emphasize the importance of giving proper management to meat products (such as cooking or freeze) to reduce the risk of acquiring infection by eating them.

8. Conclusion and recommendation

Toxoplasmosis is a zoonotic disease transmitted by common foods; the occurrence of cysts of *T. gondii* in meat for human consumption represents a potential health risk, especially if the meat is consumed raw or cooked at lower temperatures of 67°C. In this sense the existence of accurate and reliable diagnostic tests is essential for the detection, monitoring and control of infections in intermediate hosts and to minimize the risk of infection in humans.

For the diagnosis of infection in tissue samples from naturally infected animals intended for consumption, it is desirable to use the technique Taqman qPCR probes since it is a more sensitive alternative to the same qPCR detection when used with SYBR Green dye. The format for performance this technique (qPCR with a Taqman probe) is versatile, allowing to evaluate both few samples (in a research environment for example) and in environments where it is required to test a greater number of specimens in a short time period (i.e., specialized laboratories for monitoring food safety), and increasingly, inputs for this procedure are more affordable.

Author details

G.F. Dzib Paredes[1], A. Ortega-Pacheco[2], J.A. Rosado-Aguilar[2], K.Y. Acosta-Viana[3], E. Guzmán -Marín[3] and M. Jiménez-Coello[1*]

*Address all correspondence to: mjcoello@correo.uady.mx

1 Institutional Postgraduate Agricultural Science and Resource Management, CCBA/UADY, Mérida, Yucatán, México

2 Department of Animal Health and Preventive Medicine, CCBA/UADY, Merida, Yucatan, Mexico

3 Laboratory of Cell Biology, CIR, Dr. Hideyo Noguchi, CA Biomedicine of Infectious and Parasitic Diseases, Autonomous University of Yucatan, Mérida, Yucatán, México

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Chapter 9

Spread and Control of Prion Diseases in the Food and Feed Chains

Qinchun Rao and Yun-Hwa Peggy Hsieh

Additional information is available at the end of the chapter

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Abstract

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are a group of chronic, progressive, and fatal neurodegenerative disorders that affect a variety of mammalian species. This chapter discusses the issues raised by two foodborne prion diseases, namely bovine spongiform encephalopathy (BSE) and variant Creutzfeldt–Jakob disease (vCJD), particularly those related to their spread in cattle and humans, the contamination of specified risk material (SRM) in meat, the relevant regulations, and appropriate detection methods for surveillance.

Keywords: Bovine spongiform encephalopathy, variant Creutzfeldt–Jakob disease, specified risk material, central nervous system tissues, regulations, detection methods

1. Introduction

Prion diseases or transmissible spongiform encephalopathies (TSEs) are a group of chronic, progressive, and fatal neurodegenerative disorders that affect a variety of mammalian species. They include scrapie in sheep and goats, chronic wasting disease in deer and elk, bovine spongiform encephalopathy (BSE or so-called "mad cow disease") in cattle, and variant Creutzfeldt–Jakob disease (vCJD) in humans.[1] Since the term "prion" was coined by Dr. Stanley Prusiner in 1982,[2] the extensive research on prion diseases has investigated their molecular biology,[3] causes,[4] pathogenesis,[5] genetics,[6] types,[7] biochemical mechanisms,[8] and therapies,[9] among other aspects. A prion is a proteinaceous infectious particle that lacks nucleic acid.[7] Abnormal prions (PrP^{Sc}) are the pathologic isoforms of prions and are expressed in specified risk material (SRM) such as bovine central nervous system (CNS) tissue.[10] PrP^{Sc} has a high β-sheet content, is extremely resistant to heat and proteases, and is insoluble in non-denaturing detergents.[11, 12] Infection of prion diseases occurs naturally via

the oral route as well as by blood transfusion and maternal routes.[13] This chapter reviews the research into two foodborne prion diseases, BSE and vCJD, related to their spread in cattle and humans, the contamination of SRM in meat, the relevant regulations, and the detection methods available for surveillance.

2. Spread of BSE and vCJD

Research into clinical BSE cases has reported that 84.3% of the BSE infectivity is associated with bovine CNS tissue.[14] Another study found that the CNS tissue carries 89.7% of the infectivity.[15] With regard to the pathogenesis of BSE, animal by-products such as meat and bone meal (MBM), which have been used as nutritional supplements in livestock feedstuffs for more than 100 years, are major carriers of the infectious agent PrP^{Sc}.[16, 17] Significant changes in the manufacturing processes of feedstuffs that took place in the 1970s, including the introduction of mechanical systems that permitted continuous flow production and solvent extraction of fats, enabled PrP^{Sc} to enter the livestock feed chain.[18, 19] The recycling of animal by-products from BSE-infected animals caused a wide spread infection of BSE in cattle,[19] subsequently affecting the food chain through the consumption of the PrP^{Sc}-contaminated meat products.[20] To date, more than 190,000 BSE cases have been reported in 28 countries since it was first identified in the UK in around 1986,[21] with approximately 97% of the cases being reported in the UK (Figure 1).[22] It has been estimated that the total number of BSE-infected cattle is around one million, of which around 75% entered the human food chain during the 1980s and early to mid-1990s.[23, 24] BSE has had a huge impact on the beef industry worldwide.[25-30] In the USA, estimates of the total loss of beef and offal exports ranged from $3.2 billion to $4.7 billion in 2004 after the nation's first BSE case was confirmed at the end of 2003.[31] Although the link between BSE and vCJD through eating prion-contaminated meat has not been conclusively proven, the consumption of food of bovine origin contaminated with PrP^{Sc} has been strongly linked to the occurrence of vCJD in humans.[20] Both vCJD and BSE share the same infectious agent, PrP^{Sc}.[32, 33] A total of 229 vCJD patients have been identified in 12 countries since it was first discovered in the UK in 1996 (Figure 1),[34, 35] of whom about 77% were in the UK (Figure 1).[36] It should be noted that following the successful containment of the BSE epidemic through the imposition of strict feed controls for ruminant animals, the numbers of cases of both BSE and vCJD in the UK have declined significantly since 1992 and 2000, respectively (Figure 1).

3. Contamination of SRM in meat products

During the animal slaughter process, two major SRM, brain and spinal cord material, can easily contaminate meat products.[37-40] In general, there are four main pathways for the contamination of CNS-based SRM in meat products, which can occur at any stage during these processes: animal stunning, animal pithing, carcass dressing, and advanced meat recovery. The risks associated with each process have been studied and are discussed in turn below in

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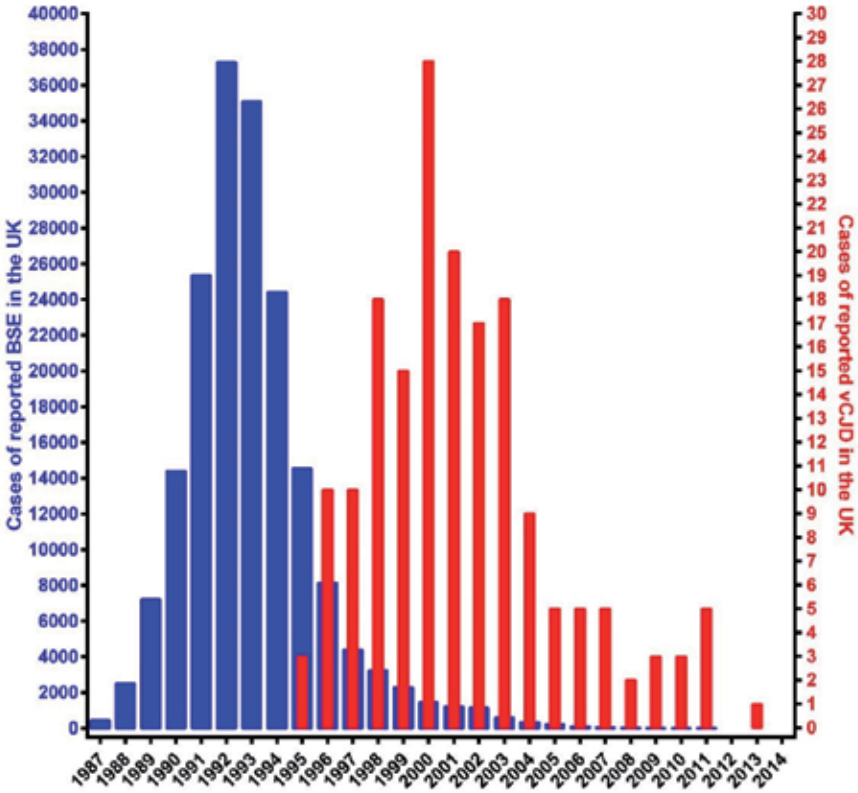

Figure 1. Number of reported BSE-cases (blue) and number of reported vCJD-cases (red) in the UK (1987-present)[22, 36]

the following sections. The major results of the relevant studies for each of these four pathways are summarized in Table 1.

3.1. Animal stunning

The stunning method has been widely used in many countries and areas of the world for some time as it slaughters the animals humanely. Stunning renders the livestock unconscious before slaughter, but because the heart of the stunned animal continues pumping for several minutes after stunning, during this time any CNS tissue that enters the jugular venous blood could still be spread throughout the body, contaminating both muscle and bone marrow via the blood circulation.[41] Several CNS markers that have a molecular weight similar to the PrP^{Sc} (MW: 30 kDa),[3] including syntaxin 1B and glial fibrillary acidic protein (GFAP),[42, 43] have been detected in non-CNS tissue after stunning. It thus seems likely that PrP^{Sc} could also be present in the edible carcass after stunning. Two types of captive bolt stunner, penetrative and non-penetrative, are widely used to stun domestic animals prior to bleeding (Figure 2). Most abattoirs prefer to use penetrative captive bolt stunners, either with or without air injection, to render cattle quickly and painlessly unconscious before slaughter.

Routes by which meat becomes contaminated by SRM			Location of SRM	SRM-contaminated samples (%)	Species		Detection methods						References
					Cattle	Sheep	Microscopy	Macroscopy	Immunoassay				
									ELISA[a]	IB[b]	IHC[c]	ICH[d]	
Animal stunning	Penetrative stunning with air injection		Left and right branches of the main pulmonary artery	2.5–5	√			√					[46]
			Right ventricle	33 (n^h=1050)	√			√					[51]
			Jugular vein	26.7 (n=15)	√		√		Syntaxin 1-B Annexin V			S100β	[48]
			Jugular vein	13.3 (n=15)		√	√		Syntaxin 1-B			S100β	[49]
			Aorta	18.2 (n=11)		√						NF[e] S100	[47]
			Heart, lung, liver and kidney					√					[52]
	Penetrative stunning without air injection	Pneumatic-powered stunning	Heart	12 (n=450)	√			√					[51]
		Cartridge-operated stunning	Heart	1 (n=480)	√			√					[51]
			Jugular vein	13.3 (n=15)		√						S100β	[49]
			Pulmonary artery and right ventricle	8.5 (n=412)				√					[56]
			Jugular vein	4 (n=100)	√		√		GFAP[f]			S100	[55]
			Jugular vein	1 (n=360)	√				GFAP				[178]
			Carcass surface	100 (n=30)	√				GFAP				[43]
	Non-penetrative stunning		Jugular vein	2 (n=100)	√		√		GFAP			S100	[55]
			Carcass surface	83.3 (n=30)	√				GFAP				[43]
Animal pithing			Jugular vein	6.3 (n=16)	√		√		Syntaxin 1-B Annexin V			S100β	[48]
			Pulmonary artery and right ventricle	4.1 (n=314)				√					[56]
Carcass dressing			Hand-held screen, tray, apron, and internal surfaces of the carcass along the cut vertebral surface		√				S100β				[69]
			Sawing water, areas of thoracal and cervical regions of the carcass		√					NSE[g]			[56]

Routes by which meat becomes contaminated by SRM	Location of SRM	SRM-contaminated samples (%)	Species		Detection methods					References
			Cattle	Sheep	Microscopy	Macroscopy	Immunoassay			
							ELISA[a]	IB[b]	IHC[c]	ICH[d]	
	Captive bolt, floor of the receiving platform, aprons, hands, knives, captive bolt aperture, head wash water, spinal cord channel, saw, table striploin, conveyor belt, and beef hide		√				Syntaxin 1B GFAP				[42]
	Captive bolt gun/hole, apron, hands, saw, spinal cord channel, and meat		√				Syntaxin 1B GFAP				[67]
	Carcasses, loin, forerib, minced meat, worktable surface, saw, and air samples		√				S100β GFAP				[179]
	Split vertebral face and saw		√				GFAP				[69]
AMRS[i]	Meat	35	√								[77]
	Meat	41.2 (n=17)	√						NF Synapto physin GFAP		[75]
	Meat	17.2 (n=279)	√						GFAP		[76]

[a]ELISA, enzyme-linked immunosorbent assay; [b]IB, immunoblotting; [c]IHC, immunohistochemistry; [d]ICH, immunocytochemistry; [e]NF, neurofilament; [f]GFAP, glial fibrillary acidic protein; [g]NSE, neuron-specific enolase; [h]n, sample size; [i]AMRS, advanced meat recovery system.

Note: NF is heteropolymer intermediate filaments that are composed of three neurofilament subunits (NF-L, NF-M and NF-H), which are expressed mainly in neurons.[180]

S100 protein (MW: 10.5 kDa) is a calcium-binding protein found predominantly in the vertebrate nervous system.[181] Syntaxin 1B (MW: 33 kDa) which is an integral membrane protein exclusively and abundantly expressed in nervous tissues.[182]

Table 1. Summary of the results of SRM contamination through the four major routes during animal slaughtering and meat processing

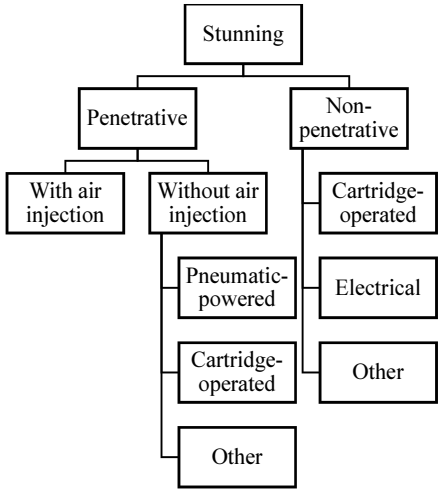

Figure 2. Classification of animal stunning techniques

3.1.1. Penetrative stunning with air injection

This stunning technique involves the injection of compressed air into the cranium of cattle to effectively disrupt the brain structure. In 2001, it was estimated that 15% of cattle were stunned using an air-injection stunner in the USA.[44] However, penetrative stunning with air injection poses a major risk of spreading the PrP^{Sc} from the BSE-infected CNS tissue to the edible carcass. Similarly, it is well known that severe penetrating injuries to the human brain, such as a gunshot wound, can cause acute brain damage and produce cerebral emboli that end up in the lung.[45]

For each BSE-infected animal stunned with this type of stunner, there is a 31.2, 16.3, 3.3, and 0.7% probability that a portion of the PrP^{Sc} would be transferred to the blood, heart, lung, and liver, respectively,[44] which is significant because it has been estimated that 60% of the liver, 50% of the heart, 25% of the kidney, and 5% of the blood are potentially available for human consumption.[44] Studies have also found that an air-injection stunner forced visible pieces of CNS tissue into the circulatory system of stunned cattle and sheep.[46, 47] This device has been shown to cause the formation of grossly visible brain tissue in the left and right branches of the main pulmonary artery in 2.5–5% of cattle,[46] in the jugular venous blood of cattle and sheep,[48-50] and in the aortic blood from sheep.[47] Particles of brain tissue as large as 20 μm in diameter can pass through the ovine pulmonary capillary network and enter the systemic arterial circulation during stunning, suggesting that this technique could easily disperse PrP^{Sc} throughout the edible carcass.[47] It has also been reported that CNS tissue has been observed in 33% of cattle hearts,[51] and in the kidneys of cattle stunned with air-injection stunners.[52]

Another factor to consider is that on occasion when operation of air-injection stunners fails, leading to an increase of CNS emboli formation and deposition, the probability of PrP^{Sc} transfer

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could be nearly 50% higher in blood, twice as high for the heart, and 10 times higher for the lung and liver.[44] Furthermore, a slaughterer who uses this type of stunner for too long or uses it successively to immobilize the same cattle could significantly increase the CNS tissue dispersion in blood vessels of the carcass.[51] It is also common for this type of stunner to press brain material out of the brain cavity through the bolt hole in the animal skull and splash onto the operator's face.[41, 53] Based on these results, the use of this type of stunner has been prohibited in many BSE-reporting countries and areas.

3.1.2. Penetrative stunning without air injection

This type of captive bolt stunner has a sharp-rimmed steel bolt and is powered by either compressed air (pneumatic-powered) or a blank cartridge that produces sufficient penetration force to initiate trauma to the cortex. It is the preferred tool for stunning cattle in the EU; 79 and 96% of the slaughterhouses in Europe use this method for cattle and calves, respectively.[54] However, the use of pneumatic-powered stunners and cartridge-fired stunners produced visible CNS clots in 12 and 1% of the cattle heart samples, respectively.[51] In addition, after using this type of stunner, CNS tissue was detected in the jugular venous blood from sheep,[49, 50] in venous blood from cattle,[55] in bovine pulmonary arterial system,[56] and on the surface of beef carcasses.[43]

In a study designed to trace the spread of CNS tissue in the cattle body after stunning with a cartridge-fired stunner, a marker microorganism, *Pseudomonas fluorescens*, was inoculated into the bovine brain via the stunning procedure.[57] The researchers reported finding this bacterium in the animal's blood, spleen, liver, kidney, lymph node, lung, and spinal cord. Using a similar approach, two microorganisms, *Escherichia coli* and *P. fluorescens*, have been detected in the blood, liver, lung, spleen, lymph node, longissimus muscle, and on the carcass surface of stunned sheep.[58] These studies demonstrate that the use of penetrative stunning without air injection could still spread CNS tissue throughout other portions of the carcass.

As with air-injection stunning, multiple use of this type of stunner also poses a high risk of spreading PrP^{Sc} from the infected brain to the edible carcass. In 2004, as few as 71% of slaughterhouses in the USA using penetrating captive bolt stunning successfully stunned 99–100% of the cattle with one shot and only 50% of abattoirs in Canada achieved the same target.[59] In 2005, this percentage decreased to 55% in US beef plants.[60] Since then, the percentage has increased gradually in both countries, reaching 91% in the USA by 2011.[61] In the EU, about 4–6.6% of captive bolt stunning in cattle requires a second stun.[54] Such practices could increase the risk of CNS tissue contamination in meat products. Although this type of stunner exerts less impulsive force on the stunned animal compared with air-injection stunners, it remains a threat to meat safety because this type of stunner is still in use worldwide.

3.1.3. Non-penetrative stunning

Non-penetrative stunning devices include the non-penetrating cartridge-operated stunner and the electrical stunner. Several studies have reported that no CNS tissue was detected in either aortic or venous blood samples collected from cattle or sheep after using these two devices.[48-50] However, other studies have detected brain tissue in venous blood samples

from stunned cattle,[55] and on the surface of beef carcasses[43] after non-penetrating stunning. There also is an increased risk to operators as some animals may be inadequately stunned with non-penetrating stunning and recover their consciousness during slaughtering. Electrical stunning is the main alternative method for stunning cattle, but it is expensive to install and meat quality defects such as petechial hemorrhages have been linked to electrical stunning in sheep.[62]

3.2. Animal pithing

Pithing is the insertion of an elongated rod-shaped instrument into the cranial cavity of a stunned animal to further lacerate the CNS tissue.[63, 64] This operation prevents the animal stunned by a penetrating captive bolt from recovering permanently.[52] Pithing is considered by the industry to be a more effective technique for maintaining the safety of the operator and was used in 70% of UK abattoirs as recently as 1997.[50] However, in 2001, this technique, which has never been used in the USA,[52] was banned in the EU.[65] Brain tissue has been detected in venous blood[48, 50] and arterial blood samples[56] from cattle after pithing.

Based on scientific evidence of the dispersion of CNS-based SRM in response to various stunning and pithing techniques, the EU has ranked the stunning methods used in ruminants, from the most hazardous to the least dangerous, as: (1) penetrative stunning with air injection; (2) penetrative stunning without air injection; (3) captive bolt stunner with pithing; (4) captive bolt stunner without pithing and free bullet; (5) non-penetrative stunner and electro-narcosis. [66] Two techniques, air-injection stunning and pithing are now prohibited worldwide. However, there is still a 50% probability that emboli will be deposited in the blood of an animal stunned with a non-air-injection stunner.[44]

3.3. Carcass dressing

During animal slaughtering, the general sequence followed during the dressing procedure is stunning followed by exsanguination, dehiding, evisceration, splitting, washing, and chilling. [42] Of these steps, stunning is not the only point at which the carcass can potentially be contaminated with CNS tissue; other steps in the slaughtering procedure also have a high probability of dispersing CNS-based SRM across the carcass (Table 1). CNS tissue has been found in samples collected at a number of points along the slaughter line, including on the captive bolt gun, on the aprons worn by the operators, on their hands and the saws used, and in the longissimus muscle.[67]

In a study tracking two marker microorganisms, *E. coli* and *P. fluorescens*, both bacteria were transferred from the first stunned sheep to the stun wounds of the next 10 sheep stunned using the same contaminated penetrative cartridge-operated pistol.[58] These bacteria were also detected in the sheep's blood and on their carcass surfaces.[58] Perhaps most worryingly, the marker organisms were found in the air, and on the hands and aprons of the operator.[58] In another similar study,[57] the marker organism *P. fluorescens* was detected in the slaughter environment immediately after stunning and at each subsequent stage of the slaughter-dressing process, including on the hands of the operators, the slaughter equipment (captive bolt gun and knife), the cattle hide, and the carcass splitting saw.

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Spinal cord material is easily spread to bovine carcasses during carcass splitting. The majority of the CNS contamination was found on the internal surfaces of the carcass and along the cut vertebral surface, with lesser levels of contamination inside the body cavity.[68] CNS tissue was also detected in the surroundings during splitting, including on the hand held screen, the tray and apron, the captive bolt, the captive bolt aperture, and the floor of the receiving platform.[42, 69] Spinal cord tissue can also be transferred to subsequent bovine carcasses during carcass splitting. The main risk of subsequent carcass contamination comes from the splitting saw.[68] Overall, these studies demonstrate that the practice of carcass dressing, especially splitting, can lead to the extensive spread of SRM within the abattoir environment, contaminating equipment, surfaces, operators, and edible carcasses. There are also grounds for concern regarding operator safety when dealing with CNS tissue, particularly due to the aerosol of cerebrospinal fluid and spinal cord produced during the splitting process.

3.4. Advanced meat recovery

Mechanical systems have been developed to separate meat from bone by scraping, shaving, or pressing the meat from the bone, for example, via advanced meat recovery systems (AMRS). AMRS enables processors to remove any remaining muscle tissue from beef carcasses without breaking the bones.[70] About 70% of fed cattle and hogs, and 60% of cows slaughtered in the USA were processed using AMRS in 1998[71] and around 5,000 metric tons of mechanically recovered meat (MRM) were produced yearly during the period 1980–95 in the UK.[72] Although AMRS makes deboning more efficient and considerably faster than using a knife, if spinal cord material is attached to the spinal column as it enters these machines, it can be incorporated into the meat product produced.

A number of studies have detected CNS tissue in MRM products (Table 1).[73-76] In 2002, about 35% of US bovine MRM contained CNS tissue[77] and other studies detected spinal cord tissue in 29%[77] and 33.3%[75] of bovine MRM samples. Dorsal root ganglia tissue was found in 10% of the MRM samples.[77] Schmidt *et al.*[74] reported that 50% of the bovine MRM samples from 14 slaughter plants that they tested were positive for CNS protein. In a later study by the same group,[76] 17.2% of the MRM samples tested were contaminated by CNS tissue.

By the 1990s, about 90% of the beef MRM produced was being used in burgers in the UK.[78] Around 75–80% of individuals surveyed in France consumed MRM products from burgers, and the consumption of burgers increased by 40% over the period 1980–1995.[79] Frequent consumption of beef and beef products, including burgers and meat pies, containing the MRM or head meat has been linked to an increased risk for vCJD;[80] infection with vCJD is thought to be predominantly due to exposure to BSE in beef MRM and head meat products.[81]

Looking at the results of the present review (Table 1), every stunning method except for electrical stunning could potentially contaminate meat products by dispersing CNS-based SRM, including any PrP^{Sc} present in sub-clinically infected cattle. Although the use of air-injection stunning, pithing, and AMRS has now been prohibited during animal slaughtering and meat processing (Table 2), penetrative stunning without air-injection and non-penetrative stunning are still in widespread legal use. Slaughter conditions and procedures such as carcass

dressing, especially the splitting process, can also result in widespread contamination within abattoirs, contaminating equipment, surfaces, operators, and carcasses destined for human consumption. CNS contamination of bovine carcasses simply cannot be eliminated by the slaughtering technology currently used.

4. The regulation of SRM

The contamination of animal carcass by SRM is not only a food and feed safety issue, but also of global economic importance, leading countries or areas such as the UK, the EU, Japan, Canada, and the USA to institute strict regulations to prevent SRM entering the human food/animal feed chain (Table 2). Although SRM has different definitions in different countries and areas, bovine CNS tissue (brain and spinal cord) tops every list of prohibitive materials due to its high prion infectivity.

Prohibited activity	UK	EU	Japan	USA	Canada
Air-injection stunning	April 1, 2001	April 1, 2001	N/A*	January 12, 2004	July 24, 2003
Pithing	April 1, 2001	January 1, 2001	N/A	Never be used	July 24, 2003
AMRS	December 15, 1995	October 1, 2000	N/A	January 12, 2004	July 24, 2003
SRM in food	December 17, 1997	January 1, 1998	July 4, 2002	January 12, 2004	August 23, 2003
SRM in feed	January 1, 1998	January 1, 2001	October 2001	April 27, 2009	July 12, 2007

* N/A, not available

Table 2. Legislation related to prion disease control

Given that it was the first and most seriously area affected, it is not surprising that the early legislation to control the spread of prion diseases came from the UK. In 1995, the UK banned the use of MRM from bovine vertebral column for human consumption[82] and in 1996, the "over thirty months" rule came into force, which automatically banned older cattle from entering the human food chain.[83] In 1997, the UK enacted a comprehensive set of SRM regulations,[84] which classified SRM into specified sheep or goat material and specified bovine material. It also emphasized that no person should use or sell any SRM, or any food containing SRM, for human consumption. To further prevent SRM from entering the human food chain, the UK prohibited the practice of pithing in 2001.[85] The EU was also quick to take action; since the consumption of BSE-infected feedstuffs by ruminants was rapidly identified to be the main BSE transmission channel, in 1994 the EU banned the use of proteins originating from mammalian tissues for feeding ruminants.[86] Largely as a result of this feed ban, the UK's BSE risk status dropped from high to low between 1996 and 2012. Since 2013, the regulations have been relaxed somewhat and cattle aged over 72 months (O72M) are now permitted to enter the food chain if they have tested negative for BSE.[87]

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In 2001, the World Health Organization recommended that all countries should introduce risk management procedures such as the identification and removal of entire bovine heads and/or prohibiting the harvesting of all MRM. All tissues that have been shown to be capable of carrying BSE infectivity should be removed and destroyed. If the risk is high, additional precautions should be taken such as prohibiting cattle over a certain age from entering the food chain.[88] The World Organization for Animal Health suggests that when countries import beef products from a BSE risk country or area, meat processing should not use air-injection stunning or pithing.[89]

In the EU, the use of air-injection stunning has been banned for slaughtering cattle since 2001; [90] it has also prohibited pithing since 2001.[65] In 1997, the EU enacted strict regulations prohibiting the use of SRM,[91] including the skull, brain, eyes, tonsils and spinal cord of cattle, sheep, and goats aged over 12 months and the spleens of sheep and goats. This was later extended to include bovine intestines in 2000[92] and vertebral columns in 2001.[93] Since 2000, all the member states of the EU, including the UK, are prohibited from producing MRM from bones of the head and vertebral columns of bovine, ovine, and caprine animals,[65] and this was extended to include all types of bones from these three species in 2001.[93] From 2001 onwards, SRM was excluded from the feed chain as a result of an EU-wide ban on the feeding of processed animal protein to all farmed animals.[94] In 2013, BSE testing in the EU has been changed to the O72M rule.[87]

Among the Asian countries, Japan has been the most severely affected by TSE, with about 36 cases of BSE and one case of vCJD being confirmed since 2001.[21, 95] In 2002, Japan announced that bovine MBM could not be used as an ingredient in animal feedstuffs and prohibited the use of specified materials from cattle.[96] However, the bovine vertebral column can still be consumed as food if it is derived from cattle originating from a BSE-free country or zone.[97] Because the BSE risk status has moderated somewhat, the age of cattle subject to inspection for BSE in Japan was revised upward from zero months to 21 months in 2005.[98] In 2013, it was further revised and is now only required for cattle over 48 months of age.[99]

In Canada, the first cases of BSE and vCJD were reported in 2003 and 2002, respectively, triggering a food directorate policy on SRM in the food supply that was implemented in 2003. [100] This required the removal of SRM from all cattle during the slaughtering process and prohibited its sale or import for human consumption.[101] The vertebral column from all cattle aged 30 months or older must now be removed as an inedible product and cannot be used in the preparation of MRM.[102] Canada has also prohibited the use of air-injection stunning and the pithing technique for cattle.[102]

The USA banned the use of proteins originating from ruminant tissues for feeding ruminants in 1997.[103] Later, the USA issued a prohibition of SRM consumption as food in January 2004, adopting a definition for SRM that is similar to that used in Canada.[104, 105] To ensure that AMRS do not become a means of spreading CNS tissue into meat products, the USA has also prohibited the use of brain, spinal cord, dorsal root ganglia, trigeminal ganglia, and significant amounts of bone solids or marrow of all cattle, as well as the skull and vertebral column of cattle 30 months of age and older, in AMRS.[106] The National Animal Identification System, an efficient and effective animal identification program, has been implemented in the USA

since 2004 to support animal disease monitoring, surveillance, and eradication programs.[107] The USA has also prohibited the use of a number of cattle materials, including CNS-based SRM, in animal feedstuffs since 2009.[108] The USA currently prohibits the slaughter of cattle that are unable to stand or walk ("downer" cattle) when presented for pre-slaughter inspection[109] and in January 2004 also banned air-injection stunning of cattle; pithing has never been used.[52] From January 2004 to May 2005, the USDA performed inspection and verification procedures in about 6,000 meat and poultry establishments and found 1,036 procedures (< 1%) that were not in compliance with the regulations related to SRM.[110] In 2008, about 1.5% of the US companies handling materials prohibited from use in ruminant feed (n=7,876) committed technical violations, mostly consisting of minor recordkeeping lapses or conditions involving non-ruminant feeds.[111] However, in January 2006, Japan suspended all US beef imports after discovering SRM in beef products exported from the USA.[112] In South Korea, US beef imports did not resume until June 26, 2008, after having been suspended in 2003 after the first BSE case was reported in the USA.[113]

Based on statistical information related to MBM,[114] it is clear that the problems with SRM-contaminated feed should not be forgotten. In 2000, the total amount of mammalian MBM produced in the USA was about three million metric tons.[115, 116] Ruminant tissues have been prohibited in ruminant feed in the USA to prevent the spread of BSE since 1997[103] and about 41,520 metric tons of animal feed in the USA were recalled between 2006 and 2007 due to the omission of the cautionary BSE statement on the label or as a result of ruminant MBM contamination during the feed processing procedure.[117] As the data shown in Table 1, it is very easy for meat products to become contaminated by bovine CNS-based SRM when the animal is slaughtered and during meat processing. In 2012, about 53% of the red meat produced in the USA. was beef,[118] but fewer than 1.2% of the cattle slaughtered are tested for BSE each year.[119, 120] As part of the regulation enforcement, about 65,693 metric tons of ruminant SRM-contaminated cattle products in the USA have been recalled since 2003.[121]

5. Detection methods for surveillance of prion diseases

As international trade continues to increase, in order to successfully enforce food and feed safety regulations and avoid economic loss, many nonclinical methods for the detection of the contaminated products have been developed. Overall, these detection methods can be classified into two types: non-immunochemical detection (i.e., chromatography, spectroscopy, and polymerase chain reaction (PCR)) and immunochemical detection methods.

Among the non-immunochemical detection methods, chromatography is typically used to separate molecules based on differential absorption and elution, which involves the flow of a fluid carrier over an immobile absorbing phase. Using chromatography, brain-specific fatty acids such as lignoceric acid and the *cis*/*trans* isomers of nervonic acid[122, 123] had been used as the analyte for the detection of the presence of CNS tissue in meat products through their characteristic component patterns. Spectroscopic techniques are based on the unique absorbance profiles of the sample components at specific wavelengths of the electromagnetic spectrum.

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Near-infrared spectroscopy[124] and attenuated total reflectance Fourier transform infrared spectroscopy[125] have both been used to analyze bovine spinal cord in ground beef. Some CNS fatty acids have also been used as markers in gas chromatography–mass spectrometry (GC–MS).[126, 127] However, these methods require expensive instruments and reagents and highly trained staff; their sophisticated nature and the laborious sample preparation involved have limited the utility of these instrumental methods for routine analysis. Alternatively, its ability to detect GFAP mRNA makes PCR a sensitive technique for the detection of bovine CNS tissue in meat products.[128-130] However, although PCR can achieve a very low detection limit, it suffers from serious drawbacks, once again requiring expensive instrumentation and reagents and expert technicians. PCR methods are also prone to contamination.[130-132]

Immunochemical detection methods based on the specific immunoreactions between an antibody and its target antigen have been extensively employed for the detection of CNS contamination in meat (Table 1), generally in the form of an enzyme-linked immunosorbent assay (ELISA),[76, 128, 133-143] immunoblotting[137-140, 144-149] or immunohistochemistry.[145, 146, 150-152] There are a number of advantages associated with using an immunoassay. For example, no serious instrumentation is required; it is easy to operate and it employs minimal reagents. In the case of ELISA, it not only has a large-scale screening and field test capability, but is also a rapid, specific, and sensitive technique. Several markers have been explored to detect bovine CNS tissue in meat products with immunoassays, either using monoclonal (mAb) or polyclonal (pAb) antibodies.

5.1. CNS markers for non-immunochemical detection methods

5.1.1. Cholesterol

Cholesterol (MW: 386.7 g/mol) is a sterol component of cell membranes, hormones, and bile acids. It has been reported that 85 g of brain tissue may contain as much as 2,640 mg of cholesterol, while the same amount of a meat sample will only contain up to 85 mg cholesterol.[153] Although cholesterol is not specific to CNS, it can serve as a useful marker for a screening test due to the low-cost and rapid procedures available for measuring it. Lucker *et al*.[146] pioneered the use of cholesterol to analyze CNS tissue in meat products, using it to detect the cholesterol content of 402 heat-treated meat products from different food markets in Germany. Sixteen field samples (4%) were identified as being possibly contaminated with CNS tissue using this procedure, but the presence of CNS tissue was confirmed by immunoblotting for both neuron-specific enolase (NSE) and GFAP in just 7 of these 16 meat products. This suggests that cholesterol is not a reliable marker for CNS tissue due to its non-tissue specificity. It can, however, be used to roughly screen for CNS residue in meat products even though the accuracy is much lower than that of markers such as GFAP and NSE.

5.1.2. Nervonic acid

Nervonic acid (15-tetracosenoic acid, MW: 366.6 g/mol), a monounsaturated long chain fatty acid (C 24:1), is enriched in nervous tissue and is mainly present in the sphingolipids of the brain. As it is seldom found in non-CNS tissue, nervonic acid has been used as a marker

indicating the presence of CNS for the detection of SRM in meat products by chromatographic techniques. Based on the different ratios of *cis*/*trans* isomers of nervonic acid in different animal species, nervonic acid has also been used to differentiate CNS tissue from various animal species in meat products using an on-line liquid chromatography–gas chromatography (LC–GC) method developed by Barcarolo *et al*.[123] The authors reported that the ratio of the *cis*/*trans* isomers can be used to provide a rough estimate of the age of the animal but may not be an accurate method for the detection of CNS adulterant. Biedermann *et al*.[154] analyzed the concentrations of fatty acids typical in bovine CNS, including docosahexaenoic acid (C22:6), lignoceric acid (C24), nervonic acid, and cerebronic acid (C_{24}OH), using GC–MS to determine the CNS content The detection limit for CNS using this GC–MS method was 0.01%. In 2003, Agazzi *et al*.[122] reported an enhanced GC method for the detection of isomers of nervonic acid in meat products, reporting that the ratio of the *cis*/*trans* isomers of nervonic acid not only exhibit species specificity but also show a significant difference between cow and calf, and between pig and piglet.

Biedermann *et al*.[127] went on to study the structural characterization of nervonic acid as a marker for SRM using GC–MS and found that the ratio of *cis*/*trans* isomers used previously should be replaced by the ratio of positional isomers, i.e., the ratio of ω9/ω7-nervonic acid (15c-C24:1/17c-C24:1). Overall, although nervonic acid may be a specific and stable marker, the GC–MS-based CNS method is costly, requires a lengthy analysis time, and the data interpretation is highly complex. In addition, the lack of species and age specificity of nervonic acid for the detection of CNS disqualifies the GC–MS-based method as an effective method.

5.1.3. Glial Fibrillary Acidic Protein (GFAP) mRNA

In the CNS, GFAP (MW: about 50 kDa) is the major component of the filaments found in the astrocytes that support the functions of nerve cells.[155] In the peripheral nervous system (PNS), GFAP is mainly expressed by Schwann cells. The PCR method has been used in several studies to detect GFAP mRNA as a potential marker of CNS tissue contamination in meat products.

Seyboldt *et al*.[130] developed a reverse transcription-PCR (RT-PCR) assay coupled with restriction fragment length polymorphism for the detection of GFAP mRNA from bovine CNS tissue. Although this approach suffered from cross-reactions with unheated heart and skeletal muscle tissues, heat treatment (70 °C for 20 min) prior to RNA extraction was found to reduce the cross-reaction with a detection limit of 0.5% (g/g) heated bovine brain in bovine minced meat. In another study[143], a similar method was able to detect 0.25% (g/g) bovine CNS tissue in pork liver sausage (after a heat treatment of 80 °C for 80 min) that had been stored up to 28 days at 4 °C, although native peripheral nerves showed positive results.

Abdulmawjood and co-workers[129, 131, 156] developed a quantitative real-time RT-PCR to detect GFAP mRNA. Their system was capable of detecting down to 0.1% of CNS tissue in strongly heated (120 °C for 15 min) or medium-heated (80 °C for 90 min) meat samples (50% pork and 50% beef), and 0.2% of CNS tissue in pork liver sausages (80 °C for 1 h). However, low levels of GFAP could still be detected in peripheral nerves (sciatic and axillary nerves) and non-neuronal tissues (parotid gland, pancreas and adrenal gland).

5.2. CNS Markers for immunochemical detection

5.2.1. GFAP

The immunochemical differences between CNS-GFAP and PNS-GFAP are well known [157], and GFAP was first exploited to indicate the presence of brain or spinal cord tissues in meat as early as 1999 [134]. It has since been utilized as a marker for the detection of CNS tissue in meat products in a number of immunoassay systems [74, 76, 133, 141, 158]. Schmidt *et al*. were the first to develop a colorimetric sandwich ELISA in 1999 [134] and went on to develop an improved fluorescent sandwich ELISA in 2001 [135]. Although this fluorescent method was capable of detecting the presence of as little as 0.05% (g/g) bovine brain and spinal cord in beef, once again this assay suffers from a cross-reaction with PNS-GFAP, which is present in sciatic nerve and cervical ganglion tissue and is not considered an SRM [135]. These results suggest that the anti-GFAP pAb used in their assay was not actually specific to the CNS tissue.

GFAP has also been used as a CNS marker in a number of immunoblotting techniques [137, 145-147]. In a study by Lucker *et al.* [146], the immunoresponse of heat-treated samples was reduced significantly, however, and the sensitivity of the assay was inadequate when intensively heat-treated meat products were analyzed. Several other studies have also shown that the ability to detect GFAP is diminished when meat samples are subjected to severe heat treatment [133, 135].

5.2.2. Neuron-Specific Enolase (NSE)

NSE (MW: 48 kDa) is an enzyme that is found in both neurons and neuroendocrine cells. The immunological CNS specificity of γγ-enolase has been used in clinical research for a long time [159], and in 1999 Lucker *et al*. were the first to adapt this clinical method to permit the use of NSE as a marker for the detection of CNS tissue in meat products using immunoblotting [148]. The detection limit of this assay was 1% (g/g) spiked brain in cooked (100 °C for 1 h) sausages of the emulsion type. They suggested that the sensitivity of the assay could be dramatically improved by removing the sample's fat content (30 to 40%) using Soxhlet extraction, although this is exceptionally time-consuming, requiring 8 h to extract the fat.

Several commercial immunochemical test kits have been developed [160]. Hughson *et al*. [140] compared the performance of two commercial kits, the RIDASCREEN GFAP kit and the Brainostic NSE kit (Table 3), for the detection of CNS tissue in meat products; these kits were based on the methods proposed by Schmidt *et al*. [134] and Lucker *et al*. [148], respectively. Hughson *et al*. [140] reported that levels down to 0.1% (g/g) of ovine spinal cord tissues could be detected in raw lamb meat using the GFAP kit, while 1.0% (g/g) was detected by the NSE kit. The detection limits of the GFAP and NSE methods for cooked samples (80 °C for 40 min) were 0.2% (g/g) bovine brain in pork and 0.1% (g/g) bovine spinal tissue in pork, respectively. They noted that the GFAP assay was easier to use and to interpret the data than the NSE assay. In a similar study by Agazzi *et al*. [138], the sensitivity of the NSE test kit was reported as being 0.5% (g/g) CNS tissue in raw and medium-heated (80 °C for 20 min) meat samples, rising to 2.0% (g/g) in strongly heated (120 °C for 20 min) meat materials. Another study [146] also found that the NSE immunoreactivity in samples of pure brain and brain muscle dropped signifi-

cantly with time at 100 °C, almost disappearing after a 2 h heat treatment. The properties of these commercial kits are summarized in Table 3.

Company	ScheBo Biotech AG	R-Biopharm		ELISA Technologies	Neogen	
Commercial kit name	Brainostic Test (GFAP-ELISA)	RIDASCREEN Risk Material	RIDASCREEN Risk Material 10/5	MELISA-TEK Ruminant Kit	Reveal for Ruminant in Feed	Reveal for Ruminant in MBM
Detection method	sELISA*	sELISA	sELISA	sELISA	LFA*	LFA
LOD (%)	0.1	0.2	0.1	0.5	1	2
Target products	Raw, processed (heated) meat and on contaminated surfaces	Processed (heated) meat and sausages	Raw meat, meat products and on contaminated surfaces	MBM	Ruminant	Ruminant
Sample preparation	Homogenization, extraction, swabs, dilution	Homogenization, extraction, centrifugation, dilution	Place the sampling swab into the sample dilution buffer	Extraction	Extraction	Extraction
Species selectivity	Unknown	Cattle, veal, sheep, goat, horse, poultry and pig	Cattle, veal, sheep, goat, horse, poultry and pig	Beef, sheep and water buffalo	N/A*	N/A
Tissue specificity	Unknown	No	No	Muscle	Muscle	Muscle
Minimum sample amount	50 mg	2 g	50 mg	5 g	10 g	10 g
Antigen†	GFAP*	GFAP	GFAP	Troponin-I	Troponin-I	Troponin-I
Test implementation	45 min	1 h	15 min	1 h	10 min	15 min
Tests possible per kit	42 (each in duplicate)	96 (including standards)	96 (including standards)	16 (each in triplicate)	25	25
Kit store temperature	4–8 °C	2–8 °C	2–8 °C	4–8°C	18–30 °C	18 – 30°C
Standards provided	0.1, 0.2, 0.4 and 1.0%	0, 0.2, 1.0 and 2.0%	0, 0.1, 0.2 and 0.4%	No	No	No

*sELISA: sandwich ELISA; GFAP: glial fibrillary acidic protein; LFA: immunochromatographic lateral flow assay; N/A: not available

Table 3. Commercial immunoassay kits for the detection of prohibited CNS and ruminant tissue contaminants in meat and feed products

5.2.3. Myelin proteolipid protein

In the CNS, myelin proteolipid protein (MPP, MW: 30 kDa) makes up approximately 50% of the myelin proteins [161]. This protein can be readily extracted from brain white matter using organic solvents [162]. Although MPP is the most abundant myelin protein in the CNS, mRNA for PLP is also expressed in the PNS and small amounts of MPP are synthesized [162]. This protein has also been used as a marker for the detection of CNS tissue in meat products [163]. The detection limit for CNS in raw meat can be as low as 0.025% using Western blot [164], and 0.01% bovine brain in raw minced beef using dot blot [165]. However, the antibody used in these studies was not species-specific and was unable to differentiate between mammalian species. Another problem is that MPP is not thermostable; the reaction signal decreased significantly when the heating time was increased to 95 °C. MPP was not detectable after 3 h of heat treatment at this temperature using dot blot [164].

5.2.4. Myelin Basic Protein (MBP)

MBP is located in CNS on the cytoplasmic face of myelin membranes in the white matter corresponding to the major dense line. MBP makes up about 30% of the total protein in the mammalian CNS myelin. It is the only structural protein found so far that is essential for the formation of CNS myelin [166]. As a water-soluble protein, MBP can be extracted with either acid or salt solutions [162]. There are four isoforms of MBP, each with a different molecular weight: 21 kDa, 18.5 kDa, 17 kDa and 14 kDa [162]. Different animal species have different isoforms. In particular, 18.5-kDa MBP, the major isoform protein in the CNS, is highly unfolded with essentially no tertiary structure in solution and the amino acid sequence of this major basic protein is similar in a number of animal species [162]. The sequence identity of the 18.5-kDa MBP in bovines and pigs is 93% homologous [167]. It has been reported that bovine 18.5-kDa MBP is not only very stable in solution at neutral pH (pH 7.2), but also highly thermostable [168]. Its immunoreactivity has been shown to remain clearly detectable even after severe heat treatment at 133 °C for 30 min [168].

MBP has been used as a marker for the detection of CNS contamination using immunoassays in several studies.[136, 143, 145, 147, 151] Levels as low as 1% (g/g) of bovine brain have been detected in a heated luncheon meat-type product (115 °C for 1 h) using a pAb in immunohistochemistry[151] and a mAb in immunoblotting.[147] In another study,[136] a detection limit of 10% was recorded for CNS tissue using a mAb in indirect competitive ELISA. Using an improved MBP extraction method,[143] compared with the detection limit reported using the same antibody and the same ELISA method,[136] the detection limit for brain tissue could be enhanced from 10 to 0.05% (~200 times). This improved extraction method will provide a useful analytical basis for the subsequent development of a specific immunoassay for the detection of bovine CNT in processed meat and feed products.

5.3. Detection methods for the control of prion diseases in feed products

To safeguard livestock, a number of non-immunochemical and immunochemical detection methods have been developed to detect the presence of banned animal materials in feeds. A

high-performance liquid chromatography method has been developed that utilizes dipeptide carnosine and related dipeptides as the markers,[169] and several other studies have used near-infrared reflectance spectroscopy (NIRS) to predict the amino acid content in animal feeds. [170-172] PCR has also been used for the detection of animal tissue in feeds. For example, Gao *et al.*[173] amplified a highly conserved eukaryotic DNA region of the 18S ribosomal gene using multiplex PCR (MPCR) and were able to detect levels as low as 0.02% bovine MBM in feedstuffs. Bellagamba *et al.*[174] detected 0.25% ruminant or pig adulterants in fish meal by MPCR.

A number of immunoassays have been developed to detect prohibited ruminant animal materials in feed. In our laboratory, a panel of species-specific mAbs have been produced for the detection of animal tissues in both food and feed products. [175-177] A skeletal muscle protein, troponin I (sTnI, 24 kDa), was first identified as a thermal-stable species marker protein; sTnI maintains its solubility and antigenicity even after undergoing severe heat treatment (126 °C for 120 min).[177] Given its even distribution in skeletal muscles, sTnI appears to be an ideal marker, allowing antibodies to be developed for the detection of animal tissues in severely heat-treated samples such as MBM. sTnI-specific mAbs have demonstrated their usefulness in an indirect non-competitive ELISA for the detection of rendered muscle tissues in animal feedstuffs, with detection limits of the mammalian and ruminant assays being between 0.3 and 2%.[175] In 2004, a sandwich ELISA was reported for the detection of ruminant proteins in feedstuffs.[176] This assay used a capture mAb specific to bovine and ovine sTnI and a biotin-conjugated detection mAb that reacts to all animal sTnI. The optimized assay achieved detection limits for bovine and ovine sTnI as low as 5.0 and 4.0 ng/ml, respectively. [176] Based on the detection of sTnI, several commercial immunoassay kits are now available for the enforcement of ruminant feed ban regulations (Table 3).

6. Conclusions

In order to maximize the economic profit from animal food production, humans made livestock animal's cannibals in the 1970s. Only a few years later, this backfired spectacularly, resulting in the rapid spread of infectious PrP^{Sc} through both the feed and food chains. These practices created a huge man-made disaster, mad cow disease, which went on to enter the human food chain through poor animal slaughtering and meat processing practices. The devastating impact of BSE on cattle has been called "a punishment from God" from which the global beef industry is only now beginning to recover. Although the negative impacts of the human version of mad cow disease, vCJD, are less significant, they continue to receive a great deal of publicity and the risk of recurrence of the disaster cannot be ignored. Most countries and areas have now enacted legislation to prohibit the use of SRM in the food and feed chain, and the goal of everyone concerned is to eventually eliminate the threat of BSE completely. Currently, through the strict implementation of feed bans and the enforcement of the regulations, both types of foodborne prion diseases appear to be under control. However, the prevention of prion diseases is not simply a matter of food or feed safety, but is also a significant factor affecting a number of political and economic issues. Effective detection methods for

ruminant SRM in rendered feedstuffs are still lacking, as currently none of the available assays can effectively differentiate ruminant CNS tissue from that of non-ruminant animal species, or detect bovine CNS tissue in excessively processed (e.g., 133 °C for 20 min) meat and feed products. It is therefore vital to continue to conduct scientific research in this area if we are to gain a better understanding of these destructive prion diseases and develop more effective surveillance techniques for this disease in both humans and animals. We must take to heart this solemn lesson if we are to avoid another such punishment from God.

Author details

Qinchun Rao and Yun-Hwa Peggy Hsieh*

*Address all correspondence to: yhsieh@fsu.edu

Department of Nutrition, Food and Exercise Sciences, Florida State University, Tallahassee, FL, USA

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Chapter 10

Herbal Extracts – Possibility of Preventing Food-Borne Infection

Biljana Damjanović-Vratnica

Additional information is available at the end of the chapter

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Abstract

Despite the high degree of awareness of food preservation methods, there is increasing occurrence of disease outbreaks caused by pathogenic and spoilage microorganisms in foods. Due to consumer awareness and negative perception of artificial preservatives in food, in recent years attention is shifting toward alternatives that the consumers recognize as natural. Thus, herbal extracts are now getting more space in food industry to prevent the propagation of bacteria that affect the spoilage of food or for the spread of so-called food-borne diseases. Herbal extracts, particularly essential oils (EOs), have complex composition that quality and composition depend on the method of extraction. There are now numerous reports of the in vitro antimicrobial activity of EOs in the scientific and medical literature: EOs are found to have broad-spectrum inhibitory activities against various food-borne Gram-positive and Gram-negative pathogenic bacteria. In this chapter, definition, history, and economic importance of aromatic herbs and herbal extracts, particularly EOs, are described. Also, attention has been paid to techniques for extraction, as well as chemical composition and antimicrobial activity of herbal extracts. This chapter demonstrates the possibility of usage of herbal extracts in preventing food-borne infection through literature survey and original results.

Keywords: Aromatic herbs, herbal extracts, food-borne disease, food preservation

1. Introduction

In recent years, there has been a dramatic increase of reported cases of food-borne illness because of consumption of foods contaminated with pathogenic bacteria. The presence of various microorganisms in food followed by inappropriate storage results not only in a reduction of food quality but also in spoilage of food.

Food contaminants, such as harmful parasites, bacteria, viruses, prions, and chemical or radioactive substances, cause more than 200 diseases – ranging from infectious diseases to cancers [1]. Consequently, there is considerable interest in ways to stop this upward trend and reduce the incidence of food poisoning. The development of new and improved methods of food infectious intestinal disease preservation is of utmost importance [2]. The microbiological safety in ready-to-eat products is a cause of big concern not only for the consumers and food industries but also for the regulatory agencies. It can be said that the food industry faces a constant problem in providing the food where there are no food-borne pathogens present [3, 4]. The number of documented outbreaks of food-borne diseases has increased in the last decade, with *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* being responsible for the largest number of outbreaks and deaths [5].

Due to consumer awareness and negative perception of artificial preservatives in food, in recent years attention is shifting toward alternatives that the consumers recognize as natural, for example, herbal extracts. It can be said that herbal extracts have been relatively neglected as soon as modern antibiotics were discovered; they became the primary means of treating bacterial infections. Antibiotics have selective toxicity and that many important bacteria were exquisitely susceptible to them. However, bacterial infections have gone up even after the discovery of many antibiotics, mainly due to the reduced susceptibility to conventional antimicrobial agents shown by many important pathogens. Because of that, studies of the antimicrobial activity of plant extracts are intensified [6] and became prominent in science, this area has suddenly become more important in the scientific literature, and this issue is currently most attractive. Therefore, at the beginning of the 21st century, herbal extracts are getting more space in food industry to prevent the propagation of bacteria that are responsible for the spoilage of food or the spread of so-called food-borne diseases [7].

Herbal extracts, particularly essential oils (EOs) have complex composition, containing from a few dozen to several hundred constituents, especially hydrocarbons (terpenes and sesquiterpenes) and oxygenated compounds (alcohols, aldehydes, ketones, acids, phenols, oxides, lactones, ethers, and esters). The greatest use of EOs in the European Union (EU) is in food (as flavorings), perfumes (as fragrances and aftershaves), and pharmaceuticals (for their functional properties). The aroma oil is the result of the combination of the main components in the oil, but trace components are also important. Thus, it is significant that the quality and composition of an EO are maintained during isolation from herb matrix, where the method of extraction plays a crucial role [8]. There are now numerous reports of the in vitro antimicrobial activity of EOs in the scientific and medical literature: EOs are found to have broad spectrum inhibitory activities against various food-borne Gram-positive and Gram-negative pathogenic bacteria.

Hydrodistillation has traditionally been applied for EO recovery from plant materials, but, during isolation, heat-sensitive compounds can easily be destroyed and quality is extremely impaired. Thus, the extraction of EO components using solvents at high pressure, or supercritical fluids, has received much attention in the recent years, especially in food, pharmaceutical, and cosmetic industries, because it presents an alternative to conventional processes such as organic solvent extraction and steam distillation [9].

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In this study, the antimicrobial properties of herbal extracts obtained by hydrodistillation and supercritical carbon dioxide (CO_2) extraction from plants originated from Montenegro, sage (*Salvia officinalis*), rosemary (*Rosmarinus officinalis*), oregano (*Origanum vulgare*), and savory (*Satureja montana*), were investigated against five pathogenic bacteria important in food industry.

2. Aromatic plants and herbal extracts

Aromatic plants and herbal extracts have been used for thousands of years as incense, perfumes, and cosmetics. After the discovery of plants' medicinal properties, natural flora became a valuable source of health improvement in the ancient civilizations.

Literature dating from around 2000 BC lists over 700 substances, including ginger, myrrh, and coriander, used for therapeutic purposes in India. Chinese ancient herbal tradition was recorded in Yellow Emperor's *Book of Internal Medicine*, dating more than 2000 BC. However, the most famous and richest associations concerning the first aromatic materials are those surrounding the ancient Egyptian civilization. Through thousands of recipes, Egyptian papyruses showed that, for example, coriander and castor oils were used as cosmetics, for medicinal applications, and as preservatives [10].

Therapeutic uses of herbal plants were described by several scholars, during Greek and Roman period, namely Hippocrates, Galen, Dioscorides, and many others [11]. Romanians are known for their use of medicinal herbs since very long; thus, Herodotus was the first to mention the method of distillation of turpentine, in about 425 BC. These great Graeco-Roman works were translated into Persian, Arabic, and other languages, and at the end of Byzantine Empire, their knowledge was passed to the Arab world. However, in 1975, archeological expedition in Pakistan found perfectly preserved perfume containers and terracotta apparatus similar to distillation apparatus, from about 3000 BC. This discovery suggests that the Arabs simply revived or improved upon a process that had been known for over 4000 years. In 19th century, herbal products were introduced by Romanian pharmacopoeia, whereas in 1904 the first institute of medicinal herbs was established in Cluj city [10].

2.1. History and definition of bioactive compounds

The use of aromatic plants and herbal extracts in the past demonstrates the history of bioactive compounds usage. Typically, bioactive compounds of plants are produced as secondary metabolites [12]. Traditionally, secondary plant metabolites have been defined as all compounds synthesized by the plant that do not appear to be essential for plant growth and development and/or those compounds without an obvious function [13]. In different words, secondary metabolites are those metabolites that are often produced in a phase of subsequent to growth, have no function in growth (although they may have survival function), have unusual chemical structures, and are often formed as mixtures of closely related members of a chemical family [14]. They are not universally synthesized in all plants. In contrast, primary

metabolites are produced by all plants, usually are part of essential metabolic processes of growth and development, such as proteins, carbohydrates, lipids, and amino acids.

In recent years, as interest as well as investigation of secondary metabolites grow, this artificial and naive definition is changing. Natural functions of many secondary metabolites are unknown because they have never been investigated; this lack of evidence or knowledge is then interpreted as lack of function [15]. Recently, it was discovered that they can help plant to increase their overall ability to survive and overcome local challenges by allowing them to interact with their surroundings [16, 17]. Thus, floral species synthesize aroma to attract insect for their pollination and fertilization while synthesis of toxic chemical has evolved to pathogens as well as to herbivores for suppressing the growth of neighboring plants [18].

Among secondary metabolites, some of these substances have effect on biological systems, which are considered as bioactive. Thus, bioactive compounds in plants can be defined as follows: secondary plant metabolites eliciting pharmacological or toxicological effects in humans and animals [19]. Bioactive compounds of plants could be divided into three main categories: (1) terpenes and terpenoids (approximately 25,000 types), (2) alkaloids (approximately 12,000 types), and (3) phenolic compounds (approximately 8000 types) [13].

2.1.1. EOs as bioactive compounds

EOs are natural, volatile, complex plant compounds, oily or lipid-like in nature, and frequently characterized by strong fragrance [20, 21]. They have been known as herbal extracts to mankind for hundreds of years, even millenniums, but there are different opinions about historical origin of EO production [22]. According to some authors, China has been the cradle of hydrodistillation [23], whereas others claim, as already mentioned, that Arabs invented this process about 3000 BC. Also, there are other opinions that distillation as a method of producing EOs was first used in the East (Egypt, India, and Persia) more than 2000 years ago and improved in the 9th century by the Arabs [23, 24]. However, Villanova (ca. 1235–1311), a Catalan physician, was the first scientist who performed distillation of EOs and left authentic written account [23].

By the 13th century, EOs were being made by pharmacies and their pharmacological effects were described in pharmacopoeias [24], but their use does not appear to have been widespread in Europe until the 16th century when they were traded in the City of London [20]. In "Grete Herbal," published in 1526, some of the illustrations of the retorts and stills used for the extraction of volatile oils are presented [25].

While knowledge of the science of EOs did not increase during 17th century when pharmacies generally used 15–20 different oils, 18th century brought about only small progress in the design of equipment and in refinements of the techniques used [23]. At the end of the 18th century, the use of tea tree oil for medical purposes has been documented in Australia [26]. In 19th century, the first movable apparatus appeared, cooling methods were improved, and double-wall distillation plants appeared. The medical properties and application of increasing number of new EOs were analyzed and recorded by the pharmacists, and the first experimental

measurement of the bactericidal properties of EOs were performed [23]. With the scientific revolution in this century, chemists were able to identify for the first time the various constituents of EOs, and the most important investigation was performed by O. Wallach, an assistant of Kekule [27]. In 1876, Haarman and Reimer started the first production of synthetic aroma chemicals: vanillin, coumarin, anisaldehyde, heliotropin, and terpineol. These researches, partly, laid the ground for the development of the EOs' synthetic counterparts and the growth of the modern drug industry. By the middle of 20th century, the use of EOs in medicine gradually became secondary to their use for flavor and aroma in perfumes, cosmetics, and foodstuffs [28].

2.1.2. Taxonomy of EO-producing plants

EOs are complex mixture of volatile compounds produced by living organisms, isolated by steam distillation, hydrodistillation, or solvent extraction from a whole plant or plant part of known taxonomic origin [29]. Around 3000 EOs have been produced from around 2000 plant species that belong to various genera distributed to around 60 families. It is well known that all plants possess the ability to produce volatile compounds, however, quite often only in traces. Only 300 EOs are important from the commercial point of view, and "EO-bearing plants" in particular are those plant species delivering an EO of commercial interest [20]. Some of those plants have specialized anatomic structures (secretory cells, glandular trichomes, and cavities/ducts), which leads to accumulation of volatiles and higher concentration in the plant [30]. Others, such as rose (*Rosa* spp.), jasmine (*Jasminum sambac*), or tuberose (*Polyanthes tuberosa*), produce and emit the volatiles by the epidermal layers of their petals [31, 32]. Their EO yield is exceptionally small so special techniques have to be applied to recover volatile fragrance compound.

The term "EO" groups together a wide range of chemical compounds on the basis of their historic use and method of isolation and belies the variety and complexity of compounds found within them [33]. Some plant families are particularly well known for their oil-bearing species and ability to produce EOs of medicinal and industrial value. These include *Alliaceae, Apiaceae, Asteraceae, Cupressaceae, Lamiaceae, Lauraceae, Myrtaceae, Piperaceae, Poaceae, Rosaceae, Rutaceae, Santalaceae, Zingiberaceae*, and *Zygophyllaceae* [30, 34]. Those plant families are not restricted to one specialized taxonomic group but are distributed among all plant classes. Gymnosperms, for example, the families Cupressaceae (cedarwood, cedar leaf, juniper oil, etc.) and Pinaceae (pine and fir oils, etc.), produce EOs with significant biological activities [34] as well as angiosperms (*Magnoliopsida*, *Rosospida*, and *Liliospida* [35–37]).

2.1.3. Composition of EOs

EOs as herbal extracts are not simple compounds or even simple mixtures of several individual compounds. They may comprise up to approximately 100 components, but usually between 20 and 60 components [38–40]. Major components can constitute up to 85% of the EO, whereas other components are present only as a trace [24, 41]. Numerous publications have presented data on the composition of the various EOs [20, 21, 42–48].

The major volatile constituents are hydrocarbons (e.g. pinene, limonene, and bisabolene), alcohols (e.g. linalol and santalol), acids (e.g. benzoic acid and geranic acid), aldehydes (e.g. citral), cyclic aldehydes (e.g. cuminal), ketones (e.g. camphor), lactones (e.g. bergaptene), phenols (e.g. eugenol), phenolic ethers (e.g. anethole), oxides (e.g. 1,8 cineole), and esters (e.g. geranyl acetate) [45]. They are defined as substances composed of isoprene (2-methylbutadiene) hydrocarbons joined together in a repetitive head-to-tail manner (known as the isoprene rule) [46, 49]. Leopold Ruzicka, recipient of the 1939 Nobel Prize in Chemistry, proposed biogenetic isoprene rule [50], which emphasizes the single biochemical origin of terpenes.

The composition of EOs are influenced by many factors such as plant ecotype or variety, genetic variation, plant nutrition, application of fertilizers, geographic location of plants, surrounding climate, seasonal variation, stress during growth or maturity, and postharvest handling, and also by isolation technique applied [51–53].

The major components of EOs isolated from aromatic herbs examined in this chapter are presented in Table 1.

Common name of EO	Latin name of plant source	Major components	Approximate % composition	References
Sage	*Salvia officinalis* L.	Camphor	6-23%	[55-57]
		B-pinene	2-10%	
		1,8-cineole	6-14%	
		α-thujone	20-42%	
Rosemary	*Rosmarinus officinalis*	α-pinene	2-51%	[57-59]
		camphor	2-32%	
		Bornyl-acetate	0-17%	
		1,8 cineole	3-89%	
		Limonene	4-15%	
Oregano	*Origanum vulgare*	Carvacrol	2-80%	[55, 60-62]
		Thymol	1-64%	
		γ-terpinene	2-52%	
		p-cymene	Trace-52%	
		sabinene	1-48%	
Savory	*Satureja montana*	Thymol	4-38%	[54, 63,64]
		Carvacrol	5-96%	
		γ-terpinene	1-31%	
		p-cymene	3-27%	

Table 1. Major components of selected EOs

2.1.4. Economic importance of EOs

Use of herbal extracts, particularly EOs, for perfumery, additives in food/confectionary as well as for pharmaceuticals and cosmetics is a growing market trend. The huge production of EOs (>70,000 tons per annum) with estimated market value of more than 700 million $US indicates

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that production and consumption of EOs is increasing all over the world [45]. This production is achieved mainly by major cultivators and producers like the United States, Brazil, India, and China; although some other countries are important contributors of EOs; for example, vetiver/khus, clove, lemongrass, basil, and celery oils are mainly produced in India, whereas Spain and France are major producers of rosemary oil [52, 60].

Today, aromatherapy and use of herbal extracts as "natural" products are fast developing segment of the industry, and this is a return to what was common practice in ancient and medieval times [64].

The largest world consumer of EOs is the flavor industry, especially for soft drinks. However, this is limited to a few EOs, mainly citrus (orange, lemon, grapefruit, and lime), ginger, cinnamon, clove, and peppermint. Similar oils are used in confectionery, bakery, desserts, and dairy products, besides some fruity products and spices. Alcoholic beverage industry is the one of the largest consumers of various EOs as well as dairy, desserts, sweet bakery, confectionery, and cream manufacturers. The fast food and processed food industries use EOs with spicy and herbal flavors: oregano, basil, fennel, pepper, dill, etc [65].

3. Techniques for extraction of bioactive compounds from aromatic plants

When considering quality and composition of an EO, the method of extraction plays a crucial role. EO are extracted from different part of the plants – flowers, buds, seeds, leaves, bark, herbs, wood, fruits, and roots – and different extraction techniques should be used in different conditions for understanding the extraction selectivity.

Furthermore, separation, identification, and characterization of bioactive compounds are only possible by an appropriate extraction process of plant matrix.

Different techniques could be used to extract bioactive compounds; many of them remain almost same through hundreds of years. All these techniques have some common objectives: (a) to extract targeted bioactive compounds from complex plant sample, (b) to increase selectivity of analytical methods, (c) to increase sensitivity of bioassay by increasing the concentration of targeted compounds, (d) to convert the bioactive compounds into a more suitable form for detection and separation, and (e) to provide a strong and reproducible method that is independent of variations in the sample matrix [66].

3.1. Conventional extraction techniques

Bioactive compounds from plant materials can be extracted by various classical extraction techniques. Most of these techniques are based on the extracting power of different solvents in use and the application of heat and/or mixing. To obtain bioactive compounds from plants, the existing classical techniques are maceration [19, 67], expression [25], solvent extraction [19], and hydrodistillation.

Hydrodistillation is a traditional method for extraction of bioactive compounds and EOs from plants. Organic solvents are not involved, and it can be performed before dehydration of plant

materials. There are three types of hydrodistillation: water distillation, water and steam distillation, and direct steam distillation [68]. In hydrodistillation, first, the plant materials are packed in a still compartment; second, water is added in sufficient amount and then brought to boil. Alternatively, direct steam is injected into the plant sample. Constituents that are insoluble in the water but volatile enough to be driven off by the steam come over and are cooled, condensed, and collected in the receiving vessel. The condensed mixture flows from the condenser to a separator, where oil and bioactive compounds separate automatically from the water [69]. At a high extraction temperature, some volatile components may be lost. This drawback limits its use for thermolabile compound extraction. EOs extracted by hydrodistillation need further purification, especially drying, to remove water.

3.2. Nonconventional extraction techniques

It was found that the major drawbacks of conventional extraction are necessity of costly and high purity solvent, solvent or water removal, low extraction selectivity, thermal decomposition of thermolabile compounds and long extraction time [70]. In recent years, to overcome these limitations, so-called nonconventional extraction techniques are presented: ultrasound-assisted extraction [71], pulsed electric field–assisted extraction [19, 72], enzyme-assisted extraction [19, 73], microwave-assisted extraction [19, 74], and supercritical fluid extraction (SFE).

SFE was systematically investigated over the past decades [48]. SFE is performed by using fluids in supercritical state at temperature higher than their critical temperature and under the pressure higher than their critical pressure. Supercritical fluid possesses gas-like properties of diffusion, viscosity, and surface tension and liquid-like density and solvation power [7]. These characteristics enable easy penetration of the fluid in supercritical state into herbal material and the extraction of secondary herbal metabolites.

For several practical reasons, more than 90% of all analytical SFE is performed with carbon dioxide (CO_2). Apart from having relatively low critical pressure (74 bar) and temperature (32°C), CO_2 is relatively nontoxic, nonflammable, available in high purity at relatively low cost, and easily removed from the extract. The main drawback of CO_2 is its lack of polarity for the extraction of polar compounds [75]. The limitation of low polarity of carbon dioxide has been successfully overcome by the use of chemical modifier [76].

A basic SFE system consists of the following parts: a tank of mobile phase, usually CO_2; a pump to pressurize the gas; co-solvent vessel and pump; an oven that contains the extraction vessel; a controller to maintain the high pressure inside the system; and a trapping vessel. Usually, different type of meters such as flow meter and dry/wet gas meters could be attached to the system. The major variables influencing the extraction efficiency are temperature, pressure, particle size, packing density and moisture content of feed material, extraction time, flow rate of CO_2, and solvent-to-feed-ratio [9, 77]. By varying the extracting conditions, it is possible to obtain extract with the maximum content of desired active substances. The only disadvantage of industrial application of SFE, as opposed to conventional methods, is the larger investment in equipment due to working in conditions of elevated pressure. The costs of production,

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however, are significantly reduced; the process is simpler and more cost-efficient; and quality of the final product good [7].

Supercritical carbon dioxide (SC_{CO2}) is known as a good solvent for a wide range of natural bioactive principles. In the last 10 years, studies on the extraction of classical compounds like essential and seed oils from various sources: seeds, fruits, leaves, flowers, rhizomes, etc., with or without the addition of a co-solvent, have been published [78]. Extraction of bioactive compounds from plant material using SC_{CO2} has been indicated as a favorable technique for producing solvent-free extracts suitable for wide use in pharmaceutical, biomedical, cosmetic, and food industries [79].

4. Herbal extracts as antimicrobial agent

Even before the role of microorganisms in disease pathogenesis was understood, plant-based medicines were used for treating such illnesses. It is recognized that plant molecules have antimicrobial properties; especially, EOs exhibit broad spectrum inhibitory activities against various Gram-positive and Gram-negative bacteria pathogens [20, 38, 57].

However, herbal extract medicine diminished as soon as modern antibiotics were discovered. Renewed recent interest in their use has been attributed to several factors, including the desire for antimicrobial compounds with even better safety and toxicity profiles [80]. Also, severity of bacterial infections has gone up even after the discovery of many antibiotics, mainly due to the reduced susceptibility to conventional antimicrobial agents shown by many important pathogens. Therefore, infectious diseases caused by bacteria are still one of the leading causes of deaths [57, 81]. In addition, toxicity due to side effects limits the prolonged use of high concentrations of available antibacterial drugs [57].

Most EOs possess at least some degree of antibacterial activity. Generally, EOs with phenolics and aldehydes exhibit better antibacterial efficacies [38]. In few cases, a main component of the oil has been observed to possess activity better than the EO. For example, carvacrol and eugenol from clove oil, terpinen-4-ol in *Melaleuca alternifolia* (tea tree oil), or thymol from oregano oil displays greater efficacy than specific oil. Many of the plant molecules are effective against drug-sensitive as well as drug-resistant strains [58, 82].

The methods used for establishing antibacterial activities are, usually, disc diffusion methods or agar or broth dilution methods. Although disc diffusion methods are popular, the data they offer are less useful than others. Agar and broth dilution methods, in which serial dilutions of the test oil in agar or broth media are inoculated with a known concentration of test organism, allow minimum inhibitory concentrations (MICs) to be determined [83]. The MIC is generally defined as the lowest concentration of EO that inhibits growth of the test organism. Although solubilization or dispersion in these systems may be problematic, MICs can help establish safe and effective final concentrations in formulated products.

Two principal reasons for performing the in vitro tests are as follows:

- Identification of antimicrobially active compounds

- Control of microbial susceptibilities toward approved antibiotics and antimicotics

The procedure from identification of antimicrobially active compounds for their use in humans to treat infectious disease is a multistep pathway, which includes pharmacological (concentration of the active compound at the site of action, half-life time, serum levels, dose–response relationship, etc.) and toxicological (e.g. toxicity, allergic responses, and interactions) aspects [84].

4.1. Mode of action of bioactive compounds

The health benefits of medicinal plants are ascribed to their bioactive compounds, known as phytochemicals. It has been estimated that 74% of pharmacologically active plant-derived compounds were discovered after following up on ethnomedicinal use of the plants [85]. Various phytochemicals are recognized to possess antimicrobial, anti-inflammatory, analgesic, anesthetic, antioxidant, neuroprotective, and antitumor activity, thus providing medicinal plants with great therapeutic as well disease-preventive potential [86].

However, detailed knowledge about the mode of action of EOs and other bioactive compounds is still lacking. In general, the mechanism of action of EOs is to alter the structure of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents [87, 88]. The mode of action of phytochemicals depends on the type of microorganism and is generally related to their outer membrane arrangement as well as cell wall structure. For example, antimicrobial action of EOs depends on their hydrophilic or lipophilic character. Certain components of EOs can act as uncouplers, which interfere with proton translocation over a membrane vesicle and subsequently interrupt ADP phosphorylation [89]. Phytochemicals may also modulate transcription factors, redox-sensitive transcription factors, redox signaling, and inflammation [90].

4.1.1. Terpenoids

Specific terpenoids with functional groups, such as phenolic alcohols or aldehydes, interfere with membrane-integrated or associated enzyme proteins stopping their production or activity. It was found that some phenolic alcohols (e.g. carvacrol and thymol) cause a disruption of the lipopolysaccharide outer layer followed by partial disintegration of the outer membrane [89]. The interaction of thymol with the membrane affects membrane permeability and results in the release of K+ ions and ATP [91]. In some cases, thymol can induce the release of lipopolysaccharides, but it does not affect chelating cations [92]. Thymol integrates within the polar head groups of the lipid bilayer, inducing alterations of the cell membrane. Zengin and Baysal [93] found that α-terpineol 1,8-cineole and linalool alter the function of cell membranes and the permeability of outer membranes of *Staphylococcus aureus* and *E. coli*. Bard *et al.* [94] reported that geraniol enhances the permeability of whole cells of *Candida albicans* and also increases the fluidity of both *C. albicans* membranes and dipalmitoyl phosphatidylcholine DPPC liposomal membranes. Mendanha *et al.* [95] revealed that all the tested terpenoids (nerolidol, menthol, pulegone, carvone, (+)-limonene, α-terpineol, and 1,8-cineole) increase the fluidity of cell membrane and exert cytotoxic effects on fibroblast cells. Yin *et al.*

[96] found that borneol increases the fluidity of DPPC bilayer membranes. Terpenoid, isolated from purple prairie clover, petalostemumol, has significant activity against *Bacillus subtilis* and *S. aureus* [97]. Carvone is capable to disrupt pH gradient and membrane potential of cells. With increasing amount of carvone, Oosterhaven *et al.* [98] reported a decrease in the growth rate of *E. coli*, *Streptococcus thermophilus*, and *Lactococcus lactis* was caused by disturbing the metabolic energy status of cells.

4.1.2. Phenolics

Phenolic toxicity to microorganism is often explained by enzyme inhibition with the oxidized compounds, probably through reaction with sulfhydryl groups or in more nonspecific interactions with the proteins. The induced defense response includes formation of a lesion that limits the growth of the pathogen, where polyphenols and other antibiotic compounds accumulate [85].

Hydroxyl groups number as well sites in phenolics are thought to be related to their relative toxicity to microorganisms (increased hydroxylation results in increased toxicity) [85]. Thus, eugenol is considered bacteriostatic against both fungi and bacteria [88]. It was found that eugenol alters the membrane, affects the transport of ions and ATP, and changes the fatty acid profile of different bacteria [99]. It also acts against different bacterial enzymes, including ATPase, histidine carboxylase, amylase, and protease [100, 101]. Cinnamaldehyde is usually less powerful than eugenol [102], but extremely effective against *E. coli* and Salmonella *typhimurium* [92]. Catechin acts on different bacterial strains belonging to different species (*E. coli, Salmonella choleraesis, Klebsiella pneumonie, Serratia marcescens, B. subtilis, Pseudomonas aeruginosa*, and *S. aureus*) by generating hydrogen peroxide and by altering the permeability of the microbial membrane [85, 103]. It was found that polyphenols obstruct bacterial quorum sensing, that is, the production of small signal molecules by *E. coli, Pseudomonas putida,* and *Burkholderia cepacia* cells that trigger the exponential growth of a bacterial population [104, 105].

4.1.3. Other compounds

Flavonoids have been shown in vitro to be effective antimicrobial substances against a wide array of microorganisms [106]. Their activity is probably due to their ability to disrupt microbial membranes or to complex with extracellular and soluble proteins in bacterial cell walls [107, 108]. Moreover, Arora *et al.* [109] demonstrated that some flavonoids (naringenin and rutin) and isoflavonoids (genistein) decrease the membrane fluidity. Selvaray *et al*. [110] correlated bioactivities of flavonoids to their membrane localization and their induced changes in membrane fluidity. Thus, catechins inhibited in vitro *Vibrio cholerae*, *Streptococcus mutans*, *Shigella*, and other bacteria and microorganisms [97]. Several studies have documented the effectiveness of flavonoids such as swertifrancheside, glycyrrhizin (from licorice), and chrysin against multiple viruses [97, 106, 111]. It was found that membrane-interacting properties of flavonoids to modify permeability of cellular membranes could inhibit *E. coli* growth [112].

The mode of antimicrobial action of tannins could be related to their capability to inactivate enzymes, cell envelope, transport proteins, microbial adhesins, etc. Previous studies have

shown that tannins can be toxic to filamentous fungi, yeasts, and bacteria. It was found that condensed tannins prevent growth and protease activity by binding the cell walls of ruminal bacteria [97].

The potential range of quinones' antimicrobial effects is great, due to its ability to complex irreversibly with nucleophilic amino acids in proteins, often leading to inactivation of the protein and loss of function. Possible targets in the microbial cell are cell wall polypeptides, surface-exposed adhesins, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism [85]. Hypericin, an anthraquinone from *Hypericum perforatum*, has received much attention lately as an antimicrobial agent [97].

Several alkaloids have the properties to interact with artificial and biological membranes to change the fluidity in association with their pharmacological effects [113]. Berberine is one of isoquinoline alkaloids, which have been considered to possess anti-inflammatory and antimicrobial effects [114]. Also, it has been found that alkaloid sanguinarine possesses antibacterial activity against bacteria and pathogenic fungi [115].

The bacterial resistance is conferred by multidrug resistance pumps (MDRs), membrane translocases that extrude structurally unrelated toxins from the cell. These protect microbial cells from both synthetic and natural antimicrobials [116]. The mechanism of action of EOs depends on their chemical composition, amount of the single components, and their antimicrobial activity is not attributable to a unique mechanism but is instead a cascade of reactions involving the entire bacterial cell [20]. Also, the synergistic effects of antibiotics and herbal extracts can provide successful therapy against drug-resistant bacteria. The use of herbal extracts and phytochemicals can be of great significance in therapeutic applications and could help control the problem of multidrug-resistant organisms [85].

4.2. Effect of some herbal extracts on selected food-borne microorganisms

Here, some original results of antibacterial activity of EO and supercritical extracts from aromatic herbs (from Montenegro) – sage (*Salvia officinalis*), rosemary (*Rosmarinus officinalis*), oregano (*Origanum vulgare*), and savory (*Satureja montana*) – against some pathogenic bacteria important in food industry are presented.

Leaves from selected herbs were collected in the central southern part of Montenegro, air-dried and stored in double-layer paper bags at the room temperature, until further analysis.

The selected test organisms, used to evaluate the antimicrobial activity of the herbal extracts, were as follows: Gram-positive (*Bacillus cereus* ATCC 11778, *S. aureus* ATCC 25923, and *Listeria innocua* ATCC 33090) and Gram-negative (*Salmonella enteritidis* ATCC 13076 and *E. coli* ATCC 25922).

To obtain EOs, herb material was subjected to hydrodistillation in a Clevenger-type apparatus for 2 hours according to Yugoslav Pharmacopoeia IV. Supercritical CO_2 extraction (SCE) procedure is previously described in detail [117], while the extraction conditions were: temperature 40 °C, pressure 100 bar, extraction time 360 min; and CO_2 flow rate 0.3 kg CO_2/h.

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MIC values were determined for extracts displaying antimicrobial properties in screening studies, using a modified microdilution broth method [118]. Briefly, the extracts were first dissolved in DMSO, then diluted in sterile water and tested over a range of concentrations from 0.09 to 25 mg/ml against overnight broth cultures of selected bacteria grown to a population of 10^6 CFU/ml in tryptic soy broth (TSB). Microplates were incubated at optimum growth temperature for each bacterial strain, and growth was monitored by measuring absorbance at 600 nm every 45 min over 18 hours, using a microplate reader.

The antibacterial activity, for investigated herbal extracts against some pathogenic bacteria important in food industry, are summarized in Table 2.

Herb material	*Salvia officinalis*	*Rosmarinus officinalis*	*Origanum vulgare*	*Satureja montana*
Method of extraction	HD/SCE	HD/SCE	HD/SCE	HD/SCE
Bacterial strain	**MIC (mg/ml)**			
Bacillus cereus ATCC 11778	0.09/0.09	0.36/0.72	0.09/0.18	0.18/0.36
Staphylococcus aureus ATCC 25923	0.09/0.09	0.36/0.72	0.36/0.72	0.18/0.36
Listeria innocua ATCC 33090	0.18/0.18	3.13/6.25	0.18/0.36	1.57/3.13
Salmonella enteridis ATCC 13076	3.13/3.13	3.13/6.25	3.13/3.13	3.13/6.25
Eschericia coli ATCC 25922	0.18/0.18	0.36/0.72	0.36/0.36	1.57/3.13

Table 2. Antibacterial activity of isolates obtained by hydrodistillation and SCE from selected herbs against some pathogenic bacteria important in food industry

According to the results presented in Table 2, sage extracts had the highest antibacterial efficiency against tested bacteria strains (MIC=0.09–3.13 mg/ml) followed by oregano (MIC=0.09–3.13 mg/ml) extracts. In this study, the carriers of antimicrobial activity of the sage oil were probably α-thujone and camphor. High efficiency of oregano extracts could be attributed to the high content of compounds with known antimicrobial activity in examinated samples, such as phenolic components, thymol and its isomer carvacrol, as well as its precursors, γ-terpinene and p-cymene. Rosemary extracts showed somewhat smaller activity than expected, probably because rosemary is a cultivated herb, whereas all other examined herbs were wild growing.

Among tested bacteria, *B. cereus* and *S. aureus* were the most sensitive to presence of all tested extracts, especially to presence of sage extracts. Thus, Gram-positive bacteria seemed to be more susceptible to all tested herb extracts.

The results of the bioassays show that tested extracts obtained by SC-CO_2 extraction from different pretreated herb matrices exhibited the same or weaker antimicrobial activity when compared to the EO obtained by hydrodistillation.

The presented results in this study confirm the facts that plant molecules have significant antibacterial activity and therefore can be used as a strong antimicrobial agent. The use of EOs

in foods as preservatives is limited due to toxicological aspects, but also possible reasons for this limitation may be the strong smell and taste of these substances when used at effective doses. The SC-CO_2 extracts bear the closest natural smell and taste of original material, thus further investigation should point to various combinations of investigated extracts, which should improve the level of inhibition due to synergistic effects.

5. Potential application of herbal extracts in foods

The microbiological safety in ready to eat products is a cause of big concern not only for the consumers and food industries but also for the regulatory agencies. It can be said that the food industry faces a constant problem in providing the food where there are no food-borne pathogens present [3, 4]. The number of documented outbreaks of food-borne diseases has increased in the last decade with *Salmonella* spp., *L. monocytogenes*, and *E. coli* being responsible for the largest number of outbreaks and deaths [5]. The different diseases, caused by food-related pathogenic bacteria, such as listeriosis, hemorrhagic colitis, campylobacteriosis, and salmonellosis, are still reported. For example, in meat and meat products, spoilage bacteria could shorten the shelf life by causing off-odors, discoloration, gas production, etc. Recently, we have trend in reducing the level of synthetic antimicrobial agents, as well as salt levels, in ready meals because of the proven development of hypertension and increased risk of cardiovascular disease. In recent years, consumers prefer fewer chemicals and more natural foods. Therefore, there is growing interest in using natural antimicrobial compounds, including extracts of herbs and spices, as salt replacers or alternatives to synthetic compounds for food preservation [119].

As already emphasized, in addition to providing flavor and fragrance, spices and herbs also have antimicrobial potential and thus can be used for preventing food deterioration and shelf life extension. It was found that, however, well herbal extracts perform in antibacterial assays in vitro, generally, a higher concentration is required to obtain the same efficacy in foods. [120]. The basic properties of the food (fat/protein/water content, antioxidants, preservatives, pH, salt, and other additives) are the most relevant in this respect, although, the extrinsic elements (temperature, packaging in vacuum/gas/air, and characteristics of microorganisms) can also influence bacterial sensitivity [20, 121].

Utilization of packaging materials containing these herbal extracts as antimicrobial compounds is also becoming an attractive option in the food industry. If herbal extracts were to be more widely applied as antibacterials in foods, the organoleptic impact would be important.

5.1. Meat, meat products, and fish dishes

Activity of oregano, thyme, basil, marjoram, lemongrass, ginger, and clove EOs against bacterial strains inoculated experimentally in irradiated minced meat and against natural microbiota found in minced meat samples was tested [122]. $MIC_{90\%}$ values ranged from 0.05%v/v (lemongrass oil) to 0.46%v/v (marjoram oil) to Gram-positive bacteria and from 0.10%v/v (clove oil) to 0.56%v/v (ginger oil) to Gram-negative strains.

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Eugenol and coriander, clove, oregano, and thyme oils were found to be in inhibiting *L. monocytogenes, Aeromonas hydrophila*, and autochthonous spoilage flora in meat products, sometimes causing a marked initial reduction in the number of recoverable cells [123] whereas mustard, cilantro, mint, and sage oils were less effective or ineffective [124].

Carvacrol vapor antimicrobial activity was established against *S. enteritidis* on pieces of raw chicken [125]. The effectiveness of oils and vapors of lemon, sweet orange, and bergamot against *L. monocytogenes*, *S. aureus*, *B. cereus*, *E. coli* O157, and *Campylobacter jejuni* was investigated on chicken [126]. The results indicate that bergamot was the most inhibitory EO due to high content of citral and linalool.

The antimicrobial effect of extracts from *S. officinalis* L. and berries of *Schinus molle* L. against *Salmonella anatum* or *S. enteritidis* inoculated on minced beef meat was studied [127]. It was found that use of 0.1% or 1.5% *S. officinalis* with 6% or 4% NaCl or 0.1% or 1.5% *S. molle* with 4% or 8% NaCl could effectively eliminate *S. anatum* from refrigerated raw beef.

Effectiveness of eight EOs as antimicrobial agents for fish preservation on 18 genera of bacteria, which included some important food pathogen and spoilage bacteria, was investigated. Clove EO showed the highest inhibitory effect, followed by rosemary and lavender [128]. It was found that citrus EO incorporated into different edible biopolymer film has the potential to preserve fish fillets [129]. Also, using EO in a coating for shrimps appears effective in inhibiting the respective natural spoilage flora [130].

The antimicrobial effect of nine EOs on *Photobacterium phosphoreum* on the shelf life of modified atmosphere-packed cod fillets was determined. The antimicrobial effect of EO was studied in a liquid medium and in product storage trials. Oils of oregano and cinnamon had strongest antimicrobial activity, followed by lemongrass, thyme, clove, bay, marjoram, sage, and basil oils, whereas oregano oil (0.05%, v/w) reduced the growth of *P. phosphoreum* in naturally contaminated cod fillets and extended shelf life from 11–12 days to 21–26 days at 2°C [131].

5.2. Fruit and vegetable

The shelf life of unpasteurized fruit juices is limited by microbial enzymatic spoilage; moreover, these products could be contaminated by some pathogens. Some EOs could be used to prevent this kind of problem. Lemongrass and geraniol have been found effective against *E. coli*, *Salmonella* sp., and *Listeria* spp. in apple, pear, and melon juices [47, 132].

Carvacrol and cinnamaldehyde were very effective at reducing the viable count of the natural flora on kiwifruit but less effective on honeydew melon. It is possible that this difference is due to difference in pH between the fruits: the lower the pH, the more effective EOs and their components generally are [20, 133].

The antimicrobial activity of basil, caraway, fennel, lemon balm, marjoram, nutmeg, oregano, parsley, rosemary, sage, and thyme EO against food-borne pathogens and key spoilage bacteria pertinent to ready-to-eat vegetables was evaluated [134]. On a carrot model product, basil, lemon balm, marjoram, oregano, and thyme EOs were deemed organoleptically acceptable, but only oregano and marjoram EOs were deemed acceptable for lettuce. It was found

that selected EOs may be useful as natural and safe additives for promoting the safety and quality of ready-to-eat vegetables [135]

Listeria strains were more sensitive than spoilage bacteria, and oregano and thyme were the most active EOs using food model media based on lettuce, meat and milk [136]. This work shows that EOs might be more effective against food-borne pathogens and spoilage bacteria when applied to foods containing a high protein level at acidic pH, as well as moderate levels of simple sugars.

The antimicrobial potential of oregano EO on *S. typhimurium* ATCC 13311 on tomatoes was tested. Tomatoes treated with 100 ppm oregano oil resulted in 2.78 log reduction, after 20 min [137]. Oregano oil was effective at inhibiting *E. coli* O157:H7 and reducing final populations in eggplant salad compared to the untreated control. Also, it was found that, in vegetable dishes, the antimicrobial activity of oil decrease in storage temperature and/or a decrease in the pH increases [138].

5.3. Cereals and dairy products

Nielsen *et al*. [139] found that cinnamon, mustard, garlic and clove EO have strong effect in active packaging to prevent bread from fungal contamination. Although oregano oleoresin weakly prevent the growth of most important spoilage fungi of bread, vanilla EO had no preventative effect against these fungi [139].

It was found that sage oil was ineffective against *B. cereus* in rice was while carvacrol was very effective at extending the *B. cereus* lag phase, reducing the final population compared to a control [20, 140, 141].

Orange, lemon, grapefruit, madrine, terpeneless lime, orange, D-limonene, terpineol, and geraniol were tested against *Salmonella, E. coli, S. aureus*, and *Pseudomonas* spp. in different types of milk. Terpineol was the most effective oil in vitro, thus it was used in combination with orange oil for a validation in milk [142]. For mint oil, it was found that it is effective against *S. enteritidis* in cucumber salad and low-fat yoghurt [143].

6. Legal aspects of the herbal extracts use in foods and safety data

The new Regulation (EC) N°1334 of the European Parliament and of the Council on flavorings and certain food ingredients with flavoring properties for use in and on foodstuffs entered into force in 2009 [138].

The new Regulation stipulates new labeling requirements for both flavoring manufacturers and (final) food manufacturers. These include labeling as “natural flavoring substance(s)” may only be used for flavorings where the flavoring part contains exclusively natural flavoring substances.

The risk management of certain substances naturally present in certain food ingredients with flavoring properties (e.g. herbs, spices) and/or flavorings is based on the “major contributor

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approach": maximum levels are established for the presence of these undesirable substances in food, which contribute most to the human intake of these substances.

European Commission has been registered numerous herbal extracts' components for use as flavorings in foodstuffs. The flavorings registered are considered to present no risk to the health of the consumer and include, among other, carvacrol, carvone, cinnamaldehyde, p-cymene, eugenol, limonene, menthol, and thymol.

New flavorings may only be evaluated for registration after carrying out serious toxicological and metabolic studies, which could require a considerable financial expense. Also, the legislative is different in different countries, for example, estragole and methyl eugenol were deleted from the European Commission list in 2001 due to their being genotoxic. However, since today, estragole is on the EAFUS (Everything Added to Food in the United States) list. The EAFUS list is a list of substances that the United States Food and Drug Administration (FDA) has classified the as generally recognized as safe (GRAS) or as approved food additives.

Till today, many researchers found that a significant number of herbal extracts' components are GRAS and/or approved food flavorings. However, some research data indicate irritation and toxicity: cinnamaldehyde, carvacrol, carvone, and thymol appear to have no significant effects in vivo, whereas in vitro they exhibit mild-to-moderate toxic effects at the cellular level [20]. Also, it was found that eugenol, menthol, and thymol, when applied in root canal treatments, could cause irritation of mouth tissues [144]. Some oils used in the fields of medicine, paramedicine, and aromatherapy have been shown to exhibit spasmolytic or spasmogenic properties [145].

7. Conclusion

Due to consumer awareness and negative perception of artificial preservatives in food, in recent years attention is shifting toward alternatives that the consumers recognize as natural. Thus, herbal extracts, particularly EOs, are getting more space in food industry to prevent the propagation of bacteria that are responsible for the spoilage of food or for the spread of so-called food-borne diseases.

Herbal extracts have antimicrobial potential and thus can be used for preventing food deterioration and shelf life extension. However, if herbal extracts were to be more widely applied as antibacterials in foods, the cytotoxic property and organoleptic impact are extremely important issues to consider. They may vary according to extract composition where the method of extraction plays a crucial role.

Therefore, herbal extracts should be used very carefully and with considerable precautions about the concentrations and product application, target consumer, major constituents of the oil, and toxicology profile.

Author details

Biljana Damjanović-Vratnica*

Address all correspondence to: biljanad@ac.me

Faculty of Metallurgy and Technology University of Montenegro Podgorica, Montenegro

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Food-borne diseases are major causes of morbidity and mortality in the world. It is estimated that about 2.2 million people die yearly due to food and water contamination. Food safety and consequently food security are therefore of immense importance to public health, international trade and world economy. This book, which has 10 chapters, provides information on the incidence, health implications and effective prevention and control strategies of food-related diseases. The book will be useful to undergraduate and postgraduate students, educators and researchers in the fields of life sciences, medicine, agriculture, food science and technology, trade and economics. Policy makers and food regulatory officers will also find it useful in the course of their duties.

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