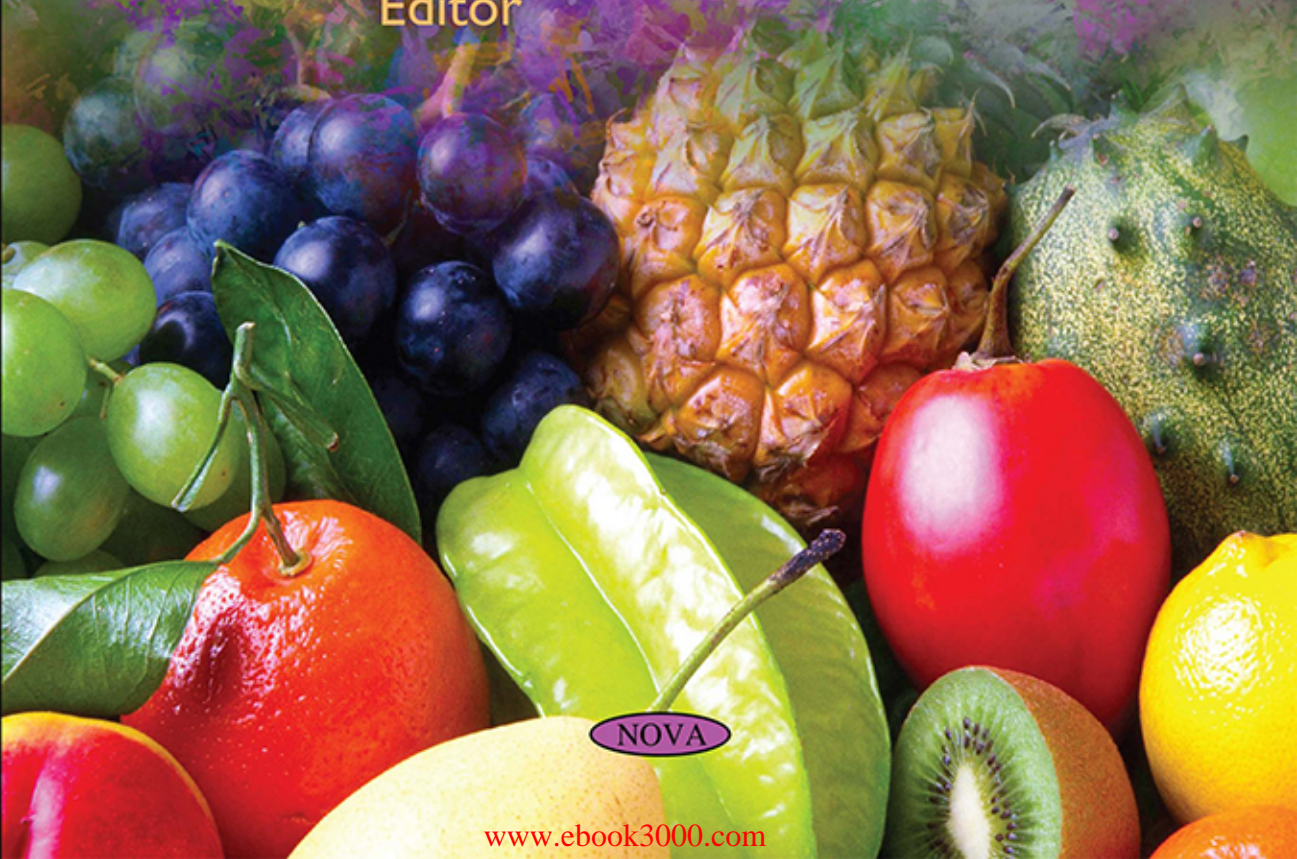


Microbiology Research Advances

Food Microbiology

Fundamentals, Challenges
and Health Implications

Elaine Perkins
Editor



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MICROBIOLOGY RESEARCH ADVANCES

FOOD MICROBIOLOGY
FUNDAMENTALS, CHALLENGES
AND HEALTH IMPLICATIONS

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ELAINE PERKINS
EDITOR



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Additional color graphics may be available in the e-book version of this book.

Library of Congress Cataloging-in-Publication Data

ISBN: ; 9: /3/856: 5/634/6 (eBook)

Library of Congress Control Number: 2015945237

Published by Nova Science Publishers, Inc. † New York

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PREFACE

The authors of this book discuss the most recent advancements in food microbiology research. Chapters include a review on the factors which help to choose the conditions that assure food microbial stability and contribute to food safety and quality; an examination of the prevalence of one of the most important food-borne pathogens, *L. monocytogenes*, particularly in fruits and vegetables; emerging bacteria detection methods in food and culture media using mass spectrometry (MS); detection techniques of *Salmonella*, of which infections from animal food play an important role in public health and particularly in food safety; and case studies of yeasts in fruit wine fermentations, which can have important implications for developing fruit wine and can contribute to an important advancements in any fermentation products.

Chapter 1 - The ability of microorganisms to grow on foods depends on storage conditions, food composition, presence of additives and food structure. The latter modify the way of microorganism growth, in the case of liquids, it occurs planktonically. But, in structured foods the mobility is restricted; microorganisms are immobilized and grow as colonies. It must be considered that most of food products present some degree of structure, such as the case of emulsions, gels and solid foods. However the effect of structure on microbial growth is scarce evaluated specially when dealing with spoilage flora. Trends observed about the effect structure on microbial growth are diverse. On one hand many studies postulate that structure acts as an additional stress factor and therefore lower growth is expected in these kinds of products. On the other hand other studies show that structure increases growth probability. The main objective of this chapter is to review the bibliography concerning the effect of structure on microbial growth and on the activity of stress factors with special emphasis on water activity (a_w) depressors and on antimicrobial agents. This information will help to choose the conditions that assure food microbial stability and contribute to improve the food safety and quality.

Chapter 2 - *L. monocytogenes* is recognized as one of the most import food-borne pathogens. A wide spectrum of *L. monocytogenes* habitats points to its high adaptation capabilities. *L. monocytogenes* is a bacterium often isolated from the following habitats: foods, water and soil. It is capable of producing VNC forms (viable not culturable), which may explain the phenomenon of its long-term survivability in unfavorable conditions, e.g., up to one year in humid soil and for over two years in arid soil. Soil is a habitat which plays a key role in this bacterium spreading to plants and animals, but also to foods, especially fresh fruits and vegetables.

The aim of the study described in this Chapter was to determine the prevalence of *L. monocytogenes* in soil samples with reference to the type of fertilizers (natural and chemical) and the distance from places intensively exploited by men, as well as to determine the relationship between *L. monocytogenes* presence in the soil and its occurrence in fruits and vegetables.

Our study demonstrates that the most frequent cause of soil contamination with *L. monocytogenes* is manure, which is linked with this bacterium transmission to crops. In most cases, it concerns fruits and vegetables that have a direct contact with contaminated soil (e.g., strawberries or root vegetables). The presence of *L. monocytogenes* was confirmed in 50% of beetroot samples, 25% of carrot samples, 15% of tomato and potato samples, as well as in 5% of parsley samples and 25% of strawberry samples. A correlation was determined between the prevalence of *L. monocytogenes* in fruits and vegetables and the type of fertilizer applied. *Listeria* sp. and particularly *L. monocytogenes* were found in the soil from arable lands fertilized with manure; pastures (areas fertilized with feces of domestic animals); and forests (again areas fertilized with feces of animals, not domestic but wild). The bacteria were not found in the soil samples collected at chemically-fertilized arable lands; waste lands (areas that were not fertilized with manure or animal feces).

Three degrees of risk were determined on the basis of bacterial contamination, also pointing out to the hazard posed by *L. monocytogenes* ingestion.

This Chapter presents also an overview of factors that facilitate *L. monocytogenes* occurrence in soil, fruits and vegetables as well as the circulation of *Listeria* sp. and *L. monocytogenes* in the environment, considering the possibility of the emergence of atypical strains.

Chapter 3 - Conventional microbiological assays have been a valuable tool for specific enumeration of indicative bacteria of relevance to food and public health, but these culture-based methods are time-consuming and require tedious biochemical and morphological identification. In recent years, various rapid detection methods have been developed. The ability of bacteriophages to specifically infect bacteria and amplify nearly a hundred-fold in 1-2 hours has been exploited by different groups. Bacteriophage amplification is integrated with mass spectrometry (MS) for quantitation of phage-specific peptides. This method has potential as a rapid tool for detection of bacterial contamination during food bioprocessing and distribution to safeguard public health. Section 1 of this chapter will briefly describe the basic biological properties of bacteriophages that are relevant to their application for detection of bacteria in environmental and food samples. Section 2 gives a brief summary on current triple-quadruple MS technologies and how lytic phage is utilized together with this technology. Advantages and shortcomings of this method are discussed in comparison to traditional microbiological and molecular methods. Section 3 describes several studies of this new rapid detection methods that use phage to detect bacteria in different food and culture media using MS.

Chapter 4 - *Salmonella* infections from animal food play an important role in public health and particularly in food safety, as food products of animal origin are considered to be the major source of human *Salmonella* infections. The primary reservoir is the intestinal tract of animals and the colonization is favored by intensive animal production. Poultry products are the most frequent vehicles in the transmission of *Salmonella* infections. Eggs and poultry meat are recognized as the major vehicles of human illness due to the high prevalence of infection in poultry. Information needed to make food safety risk management decisions must

be obtained from accurate risk assessments, which rely on the sensitivity of the isolation techniques used to identify *Salmonella*. Foods are complex matrices of fats, carbohydrates, proteins, preservatives, and other chemicals. Although hurdles associated with processing of a sample are overcome, foodborne pathogens are often present at extremely low levels, complicating the detection process. Another consideration is whether the examination is for routine monitoring or epidemiological purposes. The analyst may choose to augment the method for epidemiological purpose with additional enrichment procedures and culture media, two incubation temperatures, intensified picking of colonies from plates, and/or rapid screening methods. Therefore, better characterization of the *Salmonella* isolation and identification techniques will be warranted. Culture based methods are still the most widely used as detection techniques and remain the gold standard for the detection of *Salmonella* due to their selectivity and sensitivity. For instance, the US Food and Drug Administration (FDA), the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA), and the International Organization of Standardization (ISO) require an isolated *Salmonella* organism as unambiguous proof of contamination. Depending on the approach, standard culture methods typically require 5–7 days to obtain a result as they rely on the ability of *Salmonella* to grow and form visible colonies, which can then be characterized by performing additional biochemical and/or serological tests. Due to their widespread use, numerous and varied bacteriological media (selective enrichment broths and selective agar plates) are applied to best monitor *Salmonella* in food and food ingredients. The media and methods that are best for one particular sample type may not necessarily be optimal for other samples. Therefore particular procedures need to be evaluated for different types of samples. This chapter will provide an overview of various culture based methods currently available for the detection of *Salmonella* in different poultry product samples and agencies recommendations for these samples.

Chapter 5 - Eggs are laid by female animals of many different species, including birds, reptiles, amphibians, and fish, and they have been eaten by humans for thousands of years. Eggs consist of a protective eggshell, albumen (egg white), and vitellus (egg yolk), that are contained within various thin membranes. Chicken eggs are the most commonly eaten eggs. There are huge variations in egg consumption levels from country to country, depending on the countries levels of wealth. However, overall global consumption has tripled in the last forty years. Eggs from laying hen contain high-quality proteins, and lipids, as well as valuable minerals, carbohydrates, and vitamins. They were also widely used in the food industry due to their multifunctional properties. Although, avian eggs contain the basic elements for life and almost all the albumen proteins are antimicrobial, they can be contaminated by pathogenic bacteria, such as *Salmonella*. Infections of poultry with this bacteria can be grouped into three categories, but paratyphoid salmonellae (PT) are the most important for human being. More often, PT *Salmonella* infections in chickens are characterized by asymptomatic and sometimes persistent colonization of the intestinal tract and internal organs, potentially leading to contamination of the finished carcass. Vertical transmission of this bacteria may result in internal or external contamination of eggs. Egg shells are often contaminated with PT salmonellae by fecal contamination during oviposition. The penetration of salmonellae into or through the shell and shell membranes can result in direct transmission of infection to the developing embryo or can lead to exposure of the chick to infectious *Salmonella* organisms, when the shell structure is disrupted during hatching. Some PT serotypes, particularly *Salmonella* Enteritidis, can be deposited in the contents of eggs before

oviposition. The principal *Salmonella* serovar associated with infections linked to eggs and egg products in the UK, most European countries and North America is *S. Enteritidis*. However, other serovars have also been implicated in a number of egg-associated outbreaks, most notable *Salmonella* Typhimurium exhibiting a range of phage types. *Salmonella* prevalence on the eggshell and in the egg content vary, depending on the fact whether investigations were based on randomly sampled table eggs or eggs from naturally infected hens. Furthermore, other factors like sample size, timing of sampling, site(s) within the egg that were tested, techniques used, investigations of eggs laid by artificial or naturally infected hens could explain this variability. Studies on naturally infected layer flocks show mostly a prevalence below 3%. Control measures to limit the incidence of *S. Enteritidis* and *S. Typhimurium* in poultry flocks are vital.

Chapter 6 - Wine is a plant-derived alcoholic beverage, produced from raw materials that contain sugars or compounds that can be hydrolyzed to sugars. Fruits are increasingly used for wine making because of the diversity of raw materials, their uniqueness and health benefits. Many fruits which possess attractive color, aroma and taste, including temperate (i.e., apple, strawberry, pear, peach, cherry, raspberry) and tropical fruits (i.e., pineapple, orange, mango, banana) have been used for wine making. Quality and specific characteristics of the fruit wines depend mainly upon types of raw materials, wine maker's techniques, and particularly microbial strains and fermentation processes. Fleshy fruits such as berry, pepo, hesperidium, drupe and pome can all be used for wine making. However, process of making fruit wine is different from grape wine based on their specific physiological property, texture of fruit pulp and chemical composition of the juice. Unlike grape, fruits especially tropical fruits are usually low in sugars and other nutrients, yet high in acidity, which are not suitable for wine fermentation process. Therefore, adjusting sugar content, adding water to dilute the acidity and enhancing selected minerals to fruit juice prior to fermentation have generally been practiced. Consequently, these modifications affect the yeasts associated with fermentations. For fruit wines, the specific art or technology used to improve the wine quality is still at the initial development stage. The use of commercial strains of *S. cerevisiae* as an allochthonous starter culture at ambient temperature is a common practice in fruit wine production. Nevertheless, many winemakers still prefer wines produced by spontaneous fermentation because spontaneous/autochthonous yeasts, wild yeasts, provide unique flavors and exceptional quality of traditionally produced fruit wines. Local yeasts selected from spontaneous fermentation system are likely to be of superior quality compared to selected foreign yeasts and can be developed to be an autochthonous starter. The selected local yeasts should be able to adapt to the conditions of fruit juice, effectively dominate the fermentation, and become the most significant group of microbes in wine-making process. Selection of the suitable local yeasts can ensure the retaining of typical sensory properties of fruit wines produced in any given region. This chapter proposes case studies of yeasts in fruit wine fermentations to reflect impacts of allochthonous and autochthonous starters on wine quality. The work appears to have important implications for developing fruit wine and can contribute to an important advancement in any fermentation products.

Chapter 7 - The beginning of cheese-making processes dates back more than 8,000 years ago. Currently, there are more than 1,000 varieties of cheese throughout the world, each one possessing unique characteristics regarding its form and flavour. The history of cheese making in Brazil started with the Portuguese colonisation, which was responsible for introducing the first cattle herds and for initiating the manufacturing of cheeses similar to the

cheese “Serra da Estrela” that had only been produced in Portugal. The traditional Minas cheese is one of the oldest cheeses produced in Brazil and is responsible for generating income for a great number of small farmers. This traditional cheese is produced in the regions of Minas Gerais, Southeastern Brazil. Among other typical Brazilian cheeses, the Coalho cheese and the Butter cheese are strictly produced in the Northeast, while the Serrano cheese and the Colonial cheese are restricted to the South. The traditional production of cheese in Brazil involves the fermentation of raw cow's milk by lactic acid bacteria, which occurs naturally via the indigenous microbiota from milk and by the addition of commercial rennet. The microbial diversity contributes to the great differences in the organoleptic characteristics found in these traditional cheeses, and the main genera of lactic acid bacteria involved in the production processes are *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Enterococcus*. The raw-milk cheeses are of considerable importance in public health, given their particular conditions of production, and may become an important route of transmission of numerous pathogens, such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., *Shigella* spp. and *Listeria monocytogenes*, among others. Given the economic, social and cultural importance of artisanal cheeses produced in Brazil, together with the poor information available on the microbiological characteristics of these products, the understanding of the microbiota involved in the manufacturing processes, including the traditional technologies, as well as of the role played by the contaminant microorganisms are of great value. In this chapter, an overview about the traditional cheeses produced in Brazil is highlighted, focusing on the production technologies, the microbiota involved in the processes and the implications for public health.

Chapter 1

EFFECT OF FOOD STRUCTURE ON MICROBIAL GROWTH AND ON THE ACTIVITY OF STRESS FACTORS

*Carmen A Campos^{1,2}, María F Gliemmo^{1,2}, Aldana L. Zalazar^{1,2},
Marcela P Castro^{2,3} and Laura I Schelegueda^{1,2}*

¹Departamento de Industrias, Facultad de Ciencias Exactas y Naturales,
Universidad de Buenos Aires, Buenos Aires, Argentina

²Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)
de la República Argentina

³Departamento de Ciencias Básicas y Aplicadas, Universidad Nacional del Chaco
Austral, Sáenz Peña, Chaco, Argentina

ABSTRACT

The ability of microorganisms to grow on foods depends on storage conditions, food composition, presence of additives and food structure. The latter modify the way of microorganism growth, in the case of liquids, it occurs planktonically. But, in structured foods the mobility is restricted; microorganisms are immobilized and grow as colonies. It must be considered that most of food products present some degree of structure, such as the case of emulsions, gels and solid foods. However the effect of structure on microbial growth is scarce evaluated specially when dealing with spoilage flora. Trends observed about the effect structure on microbial growth are diverse. On one hand many studies postulate that structure acts as an additional stress factor and therefore lower growth is expected in these kinds of products. On the other hand other studies show that structure increases growth probability. The main objective of this chapter is to review the bibliography concerning the effect of structure on microbial growth and on the activity of stress factors with special emphasis on water activity (a_w) depressors and on antimicrobial agents. This information will help to choose the conditions that assure food microbial stability and contribute to improve the food safety and quality.

INTRODUCTION

Foods are in general dispersed systems and most of them exhibit a structure. The latter is provided by the presence of vegetal or meat tissues or by the inclusion of hydrocolloids and lipids in order to get viscous, gelled or emulsified food products. Some examples of the main structuring agents used in food products are detailed in Table 1.

The ability of microorganisms to grow on foods depends on storage conditions, food composition, presence of additives and food structure (Wilson et al., 2002). Food structure modifies water mobility and distribution of solutes such as acidulants, a_w depressors and preservatives (Brocklehurst et al., 1993; Castro et al., 2003; Wimpenny et al., 1995). Furthermore, it affects the mobility of microorganisms. It is well known that the site of microbial growth is the aqueous phase and that in liquids, it occurs planktonically. The medium surrounding microorganisms is uniform; transport of nutrients to the cell occurs freely and also the metabolites produced during growth are able to diffuse into the medium (Brocklehurst 2004). On the contrary, in structured foods the mobility is restricted; microorganisms are immobilized and grow as colonies. As a result of the close spatial distribution, colonies can compete for nutrients and oxygen; besides, their metabolic end products can be accumulated near colonies affecting growth (Wilson et al., 2002). Moreover, susceptibility to stress factors is modified (Castro et al., 2009; Skandanamis et al., 2000).

As previously mentioned, most food products present some degree of structure, such as the case of emulsions, gels and solid foods. However, the effects of structure on microbial growth and on the effectiveness of stress factors have been partially evaluated specially when dealing with spoilage flora. Briefly, trends reported about the effect structure on microbial growth are diverse. Many studies postulate that structure acts as an additional stress factor and therefore lower growth is expected. For example, Meldrum et al. (2003) found that *Listeria monocytogenes* in a gel made with gelatin grew more slowly than in broth. Brocklehurst (2004) reported that a_w depression with sodium chloride in a gelatin gel was more effective than in broth for decreasing the growth rate of *Bacillus cereus*. Conversely, other studies show that structure increases growth probability. For example, Wilson et al. (2002) reported that the addition of sucrose to broth promoted a decrease in *Staphylococcus aureus* growth, but when sucrose was added to a gelatin gel, *S. aureus* growth rate remained unaffected. The main objective of this chapter is to review the bibliography concerning the effect of structure on microbial growth and on the activity of stress factors with special emphasis on a_w depressors, pH adjustment and on antimicrobial agents. This information will help to choose the conditions that assure food microbial stability and contribute to improve the safety and quality of foods.

Table 1. Examples of structuring agents present in food

Structure agent	Food
Hydrocolloids with gelling ability (polysaccharides and proteins)	Jams, marmalade, dairy and fruit based desserts, jellies, cream cheeses, pates
Oil in water	Salad dressings, sauces, milk products containing cream
Water in oil	Butter, margarine, fat spread
Cellulose, Hemicelluloses and pectins	Vegetable tissues
Miofibrillar proteins	Meat tissues

EFFECT OF GELS ON MICROBIAL GROWTH AND ON THE ACTIVITY OF STRESS FACTORS

Gels represent one of the simplest structured systems. Gelling agents are incorporated into food formulations in order to modify their microstructure and texture (Antwi et al., 2006). However, the effects may be wider. It must be noted that in gelled products, microorganisms are immobilized and grow as micro-colonies (Robins and Wilson 1994; Wilson et al., 2002). Mentioned immobilization affects bacterial growth rate, as well as microbial response to environmental conditions, as a consequence of the reduced metabolic activity found in some regions of the colony (Koutsoumanis et al., 2004; Robins and Wilson 1994). Compared to cells in suspensions, bacteria immobilized in solid media have to overcome an additional stress to be able to initiate their growth. The lower microbial growth observed in solid than in liquid media may be related to nutrient diffusion, oxygen availability, the rate and profile of end-products production and cell to cell communication (Koutsoumanis et al., 2004; Skandamis et al., 2000).

Gelled foods are obtained by the addition of hydrocolloids which are polysaccharides or proteins (Antwi et al., 2006; Wilson et al., 2002). In addition to their uses as texturizing agents in the food industry, they are used to model solid food products for research purposes. The gelling agents most frequently selected are agar and gelatin. Agar is a polysaccharide obtained from seaweed and gelatin is a fibrous protein derived from collagen. Gelatin has a low melting point (25-37°C), as a consequence, it is possible to inoculate a microorganism at low temperature, without its inactivation. In addition, it is easy to remelt the gel for subsequent analysis. On the other hand, agar solidifies at 45°C and melts at 85°C. The high temperature at the inoculation step may be stressful for microorganisms, but the obtained gel is less influenced by temperature changes. As an advantage, agar is nontoxic and physiologically inert toward microorganisms (Mertens et al., 2009; Theys et al., 2010). Both gelling agents are generally recognized as safe (GRAS) food ingredients (FDA, 2014). To evaluate microbial growth in model gelled systems, the inoculated gelling agent (mainly agar or gelatin) is placed in Petri dishes, microplates or in a Gel Cassette. The latter consists of a frame holding a layer of gel. Mentioned frame is sealed with a plastic film, which is gas-permeable (Wilson et al., 2002). This system can be used to study microorganism's growth on the gel surface as well as within the gel matrix by measuring microbial growth by plate count (Brocklehurst et al., 1997; Mertens et al., 2009). Plate count is a time consuming technique. To solve up this problem, Mertens et al. (2011) develop the well scan method, which involves the determination of the optical density of the inoculated gel contained in microplate wells at nine different positions in each well. The average of the nine values is calculated and it is used to construct growth curves.

The role of structure on the inhibition of microorganisms' growth was widely demonstrated. A compilation of the studies on this subject is shown in Table 2. As an example, Antwi et al. (2006) observed that *L. innocua* growth rate decreased as gelatin concentration increased and that micro-colonies morphology changed as gelatin concentration was varied. However, gelling could not be used as the principal stress factor in food. The combination with other preservation factors is necessary to guaranty food safety. In reference to a_w , it was reported that its depression decreased the growth rate or the maximum population reached by bacteria and that the magnitude of the effect was dependent on the type

of solute used. Although the effect of a_w was greater in liquid media, the presence of solutes produced significant changes on bacterial development in gelled systems (Brocklehurst et al., 1997; Meldrum et al., 2003; Theys et al., 2008). As an example, Meldrum et al. (2003) reported that sucrose produced a decrease in *L. monocytogenes* Scott A growth rate in gel cassettes. In addition, Brocklehurst et al. (1997) observed that the maximum population reached by *Salmonella* Typhimurium during growth decreased as the concentration of NaCl or sucrose increased. Furthermore, it was shown that lowering a_w from 0.990 to 0.970 produced the elongation of lag phase and the decrease of growth rate and the maximum population reached by *S. Typhimurium* at different pH values or gelatin concentrations (Theys et al., 2008). Concerning the effect of pH, Meldrum et al. (2003) observed that the minimum pH at which *L. monocytogenes* Scott A was able to initiate its growth was higher for an immobilized culture than for a planktonic one. Similar results were obtained by Brocklehurst et al. (1997). Moreover, this trend is enhanced at low a_w values (Theys et al., 2008). This effect is related to the fact that the presence of gelling agents increases the buffering capacity of the medium, which offers protection to microorganisms (Antwi et al., 2006).

As regards the combined effect of mentioned stress factors, Koutsoumanis et al. (2004) studied the effect of structure, pH and a_w on bacterial growth and observed that the minimum values that allowed the growth in agar were higher than in broth, being even higher when temperature was decreased. Moreover, it has been observed that refrigerated incubation, at low pH, low a_w and immobilization may prevent *L. monocytogenes* Scott A growth and even cause the loss of cell viability (Meldrum et al., 2003).

Table 2. Compilation of studies about the effect of structure on microbial growth in gelled systems

Gel	Microorganism inoculated	Additional stress factor	Reference
Gelatin	<i>S. Typhimurium</i>	a_w	Brocklehurst et al., 1997
Agar	<i>Zygosaccharomyces bailii</i>	pH, a_w , temperature	Dang et al., 2010
Agar	<i>L. monocytogenes</i>	pH, a_w , temperature	Koutsoumanis et al., 2004
Gelatin	<i>L. monocytogenes</i>	pH, a_w	Meldrum et al., 2003
Gelatin	<i>S. Typhimurium</i>	pH, essential oil	Skandamis et al., 2000
Agar, Carbopol, carboxymethyl cellulose, gellan gum, locust bean gum, xanthan gum	<i>Z. bailii</i>	pH, a_w	Mertens et al., 2009
Carbopol, xanthan gum	<i>Z. bailii</i>	pH, a_w , temperature	Mertens et al., 2011
Gelatin	<i>S. Typhimurium</i>	pH, a_w	Theys et al., 2010
Gelatin	<i>Yersinia enterocolitica</i>	pH	Robins and Wilson 1994
Gelatin	<i>L. innocua</i> , <i>Lactococcus lactis</i>	pH	Antwi et al. 2007
Gelatin and dextran	<i>Escherichia coli</i>	pH, a_w	Boons et al., 2014
Gelatin, xanthan gum and carrageenan	<i>E. coli</i> , <i>S. Typhimurium</i>	pH, a_w	Boons et al., 2013

Gel	Microorganism inoculated	Additional stress factor	Reference
Gelatin and dextran	<i>Saccharomyces cerevisiae</i>	a _w	Boons et al., 2015
Xanthan gum	<i>Z. bailii</i> , <i>Z. rouxii</i>	Essential oils	Gliemmo et al., 2015
Gelatin	<i>L. monocytogenes</i>	Essential oil	Gill et al., 2002
Agar	<i>Acinetobacter baumannii</i> , <i>Aeromonas veronii</i> , <i>Candida albicans</i> , <i>Enterococcus faecalis</i> , <i>E. coli</i> , <i>Klebsiella pneumonia</i> , <i>Pseudomonas aeruginosa</i> , <i>Salmonella</i> , <i>Serratia marcescens</i> , <i>S. aureus</i>	Essential oils	Hammer et al., 1999

Antimicrobial effectiveness in solid media depends on the spatial relationship between colonies since microorganisms' growth is developed as discrete colonies. In general, antimicrobial action is lower in structured than in liquid media. This may be related with the proximity of the antimicrobial agent to bacteria or antimicrobial agent diffusion both limited by the solid medium (Gliemmo et al., 2015; Hammer et al., 1999; Skandamis et al., 2000). In the case of essential oils, different trends were reported. In the presence of oregano essential oil, the growth rate and the maximum population level of *S. Typhimurium* in gelatin gel, supplemented with yeast extract, were higher than in Tryptone soya broth (Skandamis et al., 2000). A level of 6% cilantro oil immersed in gelatin gel covering a ham, allowed the reduction of 1.3 log CFU/cm² on *L. monocytogenes* growth. This level is higher than 0.074% that is the minimal inhibitory concentrations (MIC) value of cilantro oil in broth (Gill et al., 2002). Hammer et al. (1999) obtained lower values of MIC of diverse essential oils against various Gram-positive and Gram-negative bacteria in broth than in agar. However, they observed the opposite trend for patchouli and sandalwood against *C. albicans* growth. These discrepancies could be due to the different sensitivity of microorganisms to the action of the essential oil, differences in solubility of essentials oils and the nature of the emulsifier used for oils dispersion. Other hypothesis would be that the oil droplet size in gel may limit oil diffusion toward microorganism colonies resulting in the reduction of antimicrobial action or that the growth as a colony protects cells located within the colonies from antimicrobial action (Burt 2004; Skandamis et al., 2000).

EFFECT OF EMULSIONS ON MICROBIAL GROWTH AND ON THE ACTIVITY OF STRESS FACTORS

Oil in Water Emulsions

Many foods are oil in water emulsions from the physical point of view. The oil concentration varies between 3-5% in the case of milk, from 10 to 40% for salad dressings and can be as high as 85% for mayonnaise. The oil phase consists of polydispersed droplets with a diameter of 0.15-10 µm (Brocklehurst and Wilson 2000). When droplets concentration is high, the space between droplets can be of the same order as the diameter of the droplets. This trend limits the space available for microorganism to grow (Brocklehurst et al., 1995).

The study of microbial growth in emulsions is more difficult than in gels due to the fact that emulsions are opaque and microscopy and absorbance based methods cannot be applied.

To solve up this problem, Parker et al. (1995) developed a method that removes the oil phase of the emulsion with a mixture of methanol and chloroform prior to scanning electron microscopy, light microscopy or transmission electronic microscopy evaluation. Using these techniques, they found that *L. monocytogenes* and *Y. enterocolitica* grew in the form of colonies in emulsions containing hexadecane (30-83%) or double cream (33% fat) with or without agarose. In the case of dairy cream, it was found that bacteria were associated with particulate material, probably with casein and that little fat content was trapped among bacteria. Many regions of the sample were sterile and bacteria were concentrated in small areas. This fact has to be taken into account when evaluating microbial stability of these products.

Brocklehurst et al. (1995) evaluated the effect of hexadecane and sunflower oil concentrations and droplet diameter on the form of *L. monocytogenes* and *Y. enterocolitica* growth using the previously described method. They found that when mean droplet was around 2 μm and concentration of oil was high -in order to have close packed droplets-, bacteria grew as colonies and growth rates were lower than in liquid media. The increase in oil droplets to 15-25 μm removed the inhibitory effect on growth rate but the population at the stationary phase remained lower than the one found in liquid media.

Castro et al. (2003, 2009) studied the effect of oil concentration (11-23 and 46%) on rheological properties and on *Z. bailii* and *Lactobacillus fructivorans* survival in model salad dressings and they reported that the emulsions containing 11 or 23% oil behaved as viscoelastic systems and the ones with 46% oil behaved as a gel. *Z. bailii* was able to grow in emulsions containing 11 or 23% oil but it was inactivated in the presence of 46% oil, suggesting that the solid character of the media could affect growth and decrease nutrient availability. However, in the emulsions inoculated with *L. fructivorans*, the population remained constant for all oil levels suggesting that the change in structure produced by the use of 46% oil did modify *L. fructivorans* growth or survival.

Prachaiyo and McLandsborough (2003) studied the growth of *E. coli* in emulsions with 5 to 40% of hexadecane. They found that generation time of bacteria increased with the level of hexadecane, also the population reached at the stationary phase decreased as the level of hexadecane increased. They ascribed these trends to the space limitations between the droplets since their size (1-2 μm) was similar to the size of bacterial cells.

Naitali et al. (2009) evaluated the growth of two strains of *L. monocytogenes* in emulsions containing 80% oil simulating a mayonnaise and in a liquid media at acidic and neutral pH. They found no effect of the emulsion on the growth rate and maximum population at the stationary phase of both *Listeria* strains independently of pH. The authors attributed this lack of effect of oil to the fact that the studied emulsions presented a fluid structure despite their high content of oil.

It must be stressed that independently of its role as a structuring agent, the presence of oil affects the physiology of microorganisms. For example, Prachaiyo and McLandsborough (2003) reported that *E. coli* cells grown in emulsions with 40% hexadecane produced extracellular filaments as it could be seen by scanning electron microscopy images. This type of formation is usually induced when cells were exposed to stress factors such as low temperature or nutrient starvation (Prigent-Combarent et al., 2001). Moreover, thermal resistance studies showed that bacteria grown in 40% hexadecane emulsions exhibited high tolerance to heat since D value at 55 $^{\circ}\text{C}$ increased from 9.9 ± 1.3 to 18.6 ± 0.9 when cells submitted to the thermal treatment had been grown in an emulsion. Naitali et al. (2009)

reported that *L. monocytogenes* grown in a neutral emulsion exhibited an increase in hydrophobicity. As a consequence a weak affinity for dichlororoisocyanuric acid -hydrophilic compound- and a higher affinity for didecyldimethylammonium bromide -cationic amphiphile compound- were verified. These tendencies have to be considered when selecting a disinfectant.

Stress factors applied for the preservation of oil in water emulsions such as salad dressings, sauces and mayonnaises are mainly a_w depression by the addition of NaCl or glucose, depression of pH by the addition of acidulants (organic acids such as acetic, citric and lactic) and preservatives such as sorbic and benzoic acids. It is known that these compounds interact with the lipid phase and, consequently, their distribution is modified. Undissociated organic acids tend to partition between the aqueous and the lipid phase, hence the amount of the organic acid in the aqueous phase decreases and the magnitude of this depends on partition coefficient value. In the case of food preservatives such as sorbic and benzoic acids, partition coefficients are in the range of 3-6 and therefore most of the preservative is present in the lipid phase, fact that can compromised microbial stability (Brocklehurst and Wilson 2000). Moreover, emulsifiers are added to stabilize the emulsion; they are located at the interfacial phase and can also interact with the preservative (McClements and Decker 2000). In summary, the preservative added to an emulsion will not be entirely available for antimicrobial activity, some of the preservative will partition into the oil phase whereas some will interact with the interfacial phase. Thus, the knowledge of the distribution of the preservative is necessary to determine its required amount in order to protect food emulsions from microbial contamination since only the preservative which exists in its free form in the aqueous phase will be active (Wedzicha et al., 1991).

The most common emulsifiers used in the food industry are amphiphilic proteins, phospholipids, and small surfactant molecules. The nature of the interfacial membrane formed by these emulsifiers can have a large impact on the antimicrobial activity of preservatives. The characteristics of the interface depend on the type and concentration of the surface-active molecules present. Generally, this interface consists of a narrow region -few nanometers thick- surrounding each oil droplet, though the spatial fraction it occupies within an emulsion depends on the droplet size. A significant portion of preservative molecules may therefore partition into this interfacial region, particularly if they are present at relatively low concentrations. Moreover, when surfactants are used to stabilize emulsions, their amount in the aqueous phase far exceeds its critical micellar concentration, thus only a fraction of them actually surround the droplets, with the rest remaining in the aqueous phase, usually as surfactant micelles (Kurup et al., 1991a; Wedzicha and Ahmed 1994). These micelles entrap some of the preservative, resulting in less free preservative available to act against microorganisms. The presence of surfactants also reduces the interfacial tension between the oil and aqueous phases. Controversially, Kurup et al. (1991b) suggested that both the preservative and the microorganisms would be attracted to the interface where the accumulated preservative molecules could destroy microorganisms at a faster rate.

The effect of the interaction between potassium sorbate, oil and the surfactant Tween[®] 20 on the growth and inhibition of *Z. bailii* in salad dressings was studied by Castro et al. (2003). The presence of potassium sorbate (500 ppm) inhibited *Z. bailii* growth in emulsions containing 11 and 23% (v/v) of oil. When the oil content was raised to 46% (v/v), a sharp death rate curve was observed and potassium sorbate antimicrobial activity was overlapped by the inhibitory action of the high oil level. Tween[®] 20 effect depended on the amount of oil;

in emulsions containing 11 or 23% (v/v) a depression in the activity of potassium sorbate was observed. This fact was attributed to the decrease in the free form of the preservative due to its partition between surfactant micelles and water. However, when the oil content was 46% (v/v), potassium sorbate activity was enhanced in spite of the decrease in the amount of its free form. These results highlight the significant impact of the distribution of molecules within an emulsion on ingredient interactions, and hence, on its preservation.

Moreover, a decrease in the antimicrobial activity of the bacteriocin nisin was verified in milk with varying fat content (Bhatti et al., 2004; Jung et al., 1992; Zapico et al., 1999). Jung et al. (1992) and Bhatti et al. (2004) also found that the non-ionic emulsifier, Tween[®] 80, partially counteracted the decreases of nisin activity in milk caused by the high fat content. Nevertheless, Henning et al. (1986) confirmed an antagonistic effect of emulsifiers on the antimicrobial efficacy of nisin. The interaction of nisin with surfactants in a food matrix is relatively ambiguous and it still needs to be elucidated.

A research conducted by Castro et al. (2009) made this ambiguity even more obvious. They formulated emulsions with different oil contents with potassium sorbate and nisin as preservatives. When both antimicrobial agents were added together to an emulsion containing 110 g/kg of oil, the presence of potassium sorbate exerted an antagonistic action on nisin effectiveness, while in emulsions with higher levels of oil, a synergistic action on the bacteriocin activity against *Lactobacillus fructivorans* was verified. However, when the bacteriocin was exclusively present, it produced different effects which depended on system composition. Addition of Tween[®] 20 did not affect bacterial survival for emulsions free of additives or containing only potassium sorbate as preservative. Conversely, when nisin was present, the emulsifier effect was entirely dependent on oil content. It is noteworthy what these authors postulated regarding emulsion formulation. The addition of the surfactant turned the emulsions more fluid or solid, depending on the oil content of the systems. Consequently, they found that the structure of the food matrix appeared as an additional factor which could influence either the growth of the microorganisms or the functionality of the preservatives.

As previously commented, food emulsions comprise an abundant sort of products; ranging from liquids to solids, many are the examples to be mentioned: milk, fruit beverages, soup, cream, mayonnaise, salad dressings, ice-cream, butter, margarine, Frankfurters (Vienna-type sausages), structured ham, etc. Antimicrobial agents used to preserve them showed to be strongly dependent on system composition. Outcomes shown herein highlight the importance of considering the effect of the structure, ingredient interactions, and composition, when evaluating microbial stability of these food systems.

Water in Oil Emulsions

Several foods such as butter, margarine and fat spreads are water in oil emulsions in which droplets of aqueous phase (0.30-30 μm) are dispersed in the oil phase (Brocklehurst and Wilson 2000). Microbial growth takes place in water droplets therefore space and nutrient availability restrict growth (Verrips and Zaalberg 1980). A smaller droplet size and its separate distribution enhanced microbial stability (Brocklehurst and Wilson 2000). Based on commented trends, mechanistic models were developed and applied successfully to predict the potential for bacterial growth based on droplet size and bacterial energy demands (ter Steeg et al., 1995; Verrips et al., 1980). Mentioned models show that

bacteria growth is under control if emulsion structure is stable but if droplet coalescence takes place bacteria can grow. Regarding moulds, whose growth is not only restricted to the droplets, ter Steeg et al. (2001) proposed another model to evaluate the effect of green antifungal, droplet size distribution and temperature on mould outgrowth in fat spread taking into account movement of moulds to the lipid phase.

It must be stressed that microbial growth is not the main deteriorative reaction in water in oil emulsions but, if microbial growth takes place, high number of microorganisms can be found within the droplets and probably they died when concentration of metabolic end products become toxic or nutrients were not available (Brocklehurst and Wilson 2000).

EFFECT OF STRUCTURED FOODS ON MICROBIAL GROWTH AND ON THE ACTIVITY OF STRESS FACTORS

In meat and vegetable tissues growth occurs at the surface. Different model systems manufactured with gelling agents were used to study this issue. As an example, a solid surface made with agar was used to evaluate the effect of gas atmosphere composition on the growth of food-borne pathogens (Bennik et al., 1995). In surfaces, growth occurs in colonies and constraints on growth were the same as in gels. But, diffusion limitations and accumulation of protons under the colony are greater than in gels; as a consequence, microorganism growth rate becomes lower in surface colonies than in immersed colonies (Wilson et al., 2002). Furthermore, many foods contain micro-architectures and growth of microorganisms can take place planktonically, in colonies -immersed or at the surface- depending on the localization of the microorganisms. Water is located within the microstructure and its distribution is not uniform, in this way different a_w values can be found providing a heterogeneous environment for microorganism (Møller et al., 2013). As a_w gives information of the global available water, its determination must be complemented with the information of the different populations of water within the structure. The latter can be evaluated using proton nuclear magnetic resonance (NMR). These measurements were done and used to evaluate their relationship with food structure in cheese (Møller et al., 2013). In the following sections, the effect of structure on microbial growth in vegetables, dairy products, meat and meat products are discussed.

Vegetables

Increased consumption of minimally processed fruits and vegetables has lead to an increase in the number of outbreaks related to these products. The outbreaks were specifically associated with leafy green commodities and with *Salmonella* (Patel and Sharma 2010), *B. cereus* (Elhariry 2011), *E. coli* O157:H7 (Kroupitski et al., 2011) and *L. monocytogenes* (Ells and Hansen 2006). Since these products are often consumed raw or minimally processed, it is essential to understand the initial stages of pathogen attachment to vegetables in order to apply strategies to avoid it. Attachment ability depends on the pathogen, the surface morphology of the vegetables, the temperature and the integrity of the tissue (Ells and Hansen 2006). It is a complex mechanism that is linked to physicochemical properties of both

bacterium and plant surfaces (Hirano and Upper 2000; Ukuku and Fett 2002). A correlation between the negative surface charge and hydrophobicity of several bacterial pathogens and the strength of their attachment to cantaloupe rind surfaces was previously stated by Ukuku and Fett (2002). For example, when materials possess pits and cavities on surfaces such as asparagus, spores can penetrate in these areas (Park and Beuchat 1999). Moreover, bacteria with greater hydrophobic membrane may attach to the cuticle or plant surfaces (Patel and Sharma 2010). Main results of some studies about the topic will be commented in the next paragraphs.

Patel and Sharma (2010) evaluated the ability of five *Salmonella* serovars to attach to and colonize intact and cut lettuce (iceberg, romaine) and cabbage surfaces. They found that all *Salmonella* serovars attached rapidly on intact and cut produce surfaces. But, *Salmonella* spp. attached to romaine lettuce at significantly higher numbers than those attached to iceberg lettuce or cabbage. Attachment strength was significantly lower on cabbage followed by Iceberg and Romaine lettuce. Cabbage, intact or cut, did not support attachment of *Salmonella* as well as romaine lettuce.

Elhariry (2011) investigated the ability of six *B. cereus* strains to attach and form biofilm on cabbage and lettuce surfaces. The highest biofilm formation on cabbage and lettuce surfaces was obtained by spores and vegetative cells of all tested strains on the 4th hour of the incubation period. This trend highlights the importance of hygienic preparation and handling to avoid attachment and assure safety of green-leafy vegetables. The strength attachment of both spores and vegetative cells of the strains to the lettuce surface was higher than that of the cabbage surface confirming the dependence of surface morphology on attachment.

Kroupitski et al. (2011) analyzed the distribution of green-fluorescent protein-labeled *S. Typhimurium* on artificially contaminated romaine lettuce leaves in order to understand initial pathogen–leaf interactions. They reported that bacteria attachment to different leaf regions was highly variable and a higher attachment level was observed on leaf regions localized close to the petiole compared to surfaces at the far-end region of the leaf blade. Finally, attachment to surfaces located at a central leaf region demonstrated intermediate attachment level. Moreover, *Salmonella* were also visualized underneath stomata within the parenchymal tissue, suggesting that the bacteria can also internalize romaine lettuce leaves. Comparison of attachment to leaves of different ages showed that *Salmonella* displayed higher affinity to older compared to younger leaves. Scanning electron microscopy revealed a more complex topography on the surface of older leaves, as well as on the abaxial side of the examined leaf tissue supporting the notion that a higher attachment level might be correlated with a more composite leaf landscape.

Ells and Hansen (2006) evaluated the ability of different *Listeria* strains to attach and colonize intact or cut cabbage tissue which were exposed to different temperatures. Results showed that all strains exhibited more attachment to cut tissues compared to intact leaf surfaces. Furthermore, scanning electron microscopy revealed the presence of increased cell numbers on the cut edges with numerous cells located within folds and crevices. Cells found on the intact surfaces were randomly distributed with no apparent affinity for specialized surface structures. The culture growth temperature significantly affected the strength of attachment during the first 4 h of exposure to intact surfaces, being cells cultivated at 37°C more easily removed from leaf surfaces than those cultivated at 10 or 22°C. However, after 24 h, binding was not significantly different between temperatures and increasing exposure

time to the cabbage resulted in increased attached cell numbers as well as increased binding strength.

Dairy Products

Microorganisms are present in dairy products as natural microflora or as starter cultures. In fermented products flocculation of casein induced the formation of a gel and, according to the product, different microstructures can be found. Location of microorganisms during the processing of cheese and yoghurt is of interest due to the role of the microorganisms and their enzymes on ripening (Belitz and Grosch 2009). Non destructive microscopic methods such as confocal laser scanning and scanning electron microscopy are very useful to visualize the location of microorganisms (Hickey et al., 2015).

Different studies evaluated the distribution of microorganism in cheese. Hickey et al. (2015) reported that microorganisms are entrapped in the protein matrix being necessary the diffusion of nutrients to bacterial colony and also the diffusion of metabolic product to assure bacterial growth. Microscopy shows that bacteria are preferentially located at the fat-protein interface and sometimes within whey pockets. Parker et al. (1998), using light and electronic microscopy, found four populations of microorganisms in mature Serra Cheese depending on the position within the cheese and these trends were related to changes in flavor and texture.

Modification in dairy products formulations -such as the substitution of lipids by proteins- could modify the potential of growth of bacteria. It is known that microbial development during ripening or storage can be limited to aqueous micro-zone within a lipid-protein matrix. However, in light dairy products the aqueous phase is the continuous phase and microbial growth can take place. Guerzoni et al. (1994) modeled the growth of *L. monocytogenes* and *Y. enterocolitica* in food model systems with different levels of NaCl and lipids and in dairy products and compared the results obtained. They observed that individual or interactive effects of pH, salt and lipid content were not enough to predict bacteria growth and that microstructure played an important role.

Meat and Meat Products

Meat tissue consists of long, thin, parallel cells arranged into fiber bundles which are surrounded by elastin fibers. Its surface was defined by Wilson et al. (2002) as being the simplest form of micro-architecture affecting the growth of microorganisms. The aqueous phase is structured among the network of meat fibers and the crevices of the surrounded tissue. Hence, microorganisms are immobilized and constrained to grow as colonies. This fact limits nutrients diffusion and produces depletion of oxygen and accumulation of protons beneath the colony; this results in local changes in the concentration of substrates and acidic metabolic end-products, which leads to decreasing growth rates. Despite this overall unfavorable scenario, pathogens and spoilage microorganisms manage to colonize, survive and proliferate in meat surfaces. Bacteria that are trapped and attached firmly to these structures are hard to remove by decontamination or rinsing (Noriega et al., 2010). In general, surface composition, roughness, charge and hydrophobicity of both surface and cells influence the adhesion of bacteria to a surface (Treese et al., 2007). Bacterial cells attach to

and colonize surfaces which are more elastic, porous and rougher surfaces as compared to dense and smooth surfaces (Katsikogianni and Missirlis 2004; Wan Norhana et al., 2009). It should also be considered that microorganisms will tend to form microcolonies in a solid or particulate food, e.g., minced meat, or grow in the form of slime-covered biofilms on meat surfaces. Kumar and Anand (1998) made an exhaustive revision of the protective effect of biofilms against antimicrobial substances in the food industry.

Weak organic acids -mostly acetic and lactic acids- are frequently used as a cheap and effective means to reduce number and prevalence of bacterial pathogens on meat. Application of organic acids might be done as spray wash or dipping solution. The application of 2% lactic acid spray solution on beef carcasses and chicken breasts reduced *E. coli* O157:H7 population for more than 1.5 log CFU/cm² (Anang et al., 2007; Bosilevac et al., 2006; Kalchayanand et al., 2008). When used as carcasses or meat cuts decontamination rinses, possible color discoloration, flavour and/or odour due to the treatment should be evaluated since these could affect sensory quality of meat (Pipek et al., 2005; Theron and Lues 2007). Blagojevic et al. (2015) applied hot lactic acid (HLA) for the decontamination of incoming beef in the manufacturing of dry sausages. The process significantly reduced *E. coli* O157:H7 and *Salmonella* counts (but not *L. monocytogenes*); however, sensorial quality of finished sausages produced with HLA-decontaminated beef was somewhat reduced. Apparently, the effectiveness of the treatments depends on the extent of cell attachment; cells can be both actively attached to -and also potentially trapped in- the irregular structure and crevices on skin or the network of collagen and reticular fibers in meat surfaces (Gill et al., 1984; Thomas and McMeekin 1980). Thomas et al. (1987) suggested that the cells can be drawn in with water into the fiber tissues and migrate to a depth of about 25 mm. Furthermore, the buffering capacity of meat, i.e., its ability to modulate acidic or alkaline pH towards neutral and to withstand rapid pH fluctuations, has a great influence on these treatments (Goli et al., 2007). Tan et al. (2014) suggested that the difference in fat composition and other components, such as proteins, may increase the vulnerability of attached cells to acetic acid.

The use of bacteriocinogenic lactic acid bacteria (LAB) for the biopreservation of meat surfaces has been one of the novel techniques developed in the last decades. The efficacy of bacteriocins in meat and meat products critically depends on interactions with meat constituents (mainly fat, proteins and enzymes), and the distribution of bacteriocin molecules in the food matrix. For instance, application of nisin in meat products faces several limitations resulting from its interaction with phospholipid emulsifiers and other food components (Aasen et al., 2003; Henning et al., 1986; Jung et al., 1992), poor solubility at pH above 6.0, and inactivation by formation of a nisin-glutathione adduct (Ross et al., 2003). Nevertheless, inactivation is lower in cooked meats due to the loss of free sulphhydryl groups during cooking as a result of the reaction of glutathione with proteins (Stergiou et al., 2006). Antilisterial activity of sakacin Q produced by *Lb. curvatus* ACU-1 on the surface of cooked pork meat was assessed by Rivas et al. (2014). The use of freeze-dried reconstituted cell free supernatant (CFS) was effective to control *Listeria* growth reducing its population to undetectable levels after two weeks of refrigerated storage. Despite this effectiveness, the CFS suspension showed a lower antimicrobial potency. The authors attributed this phenomenon to the adsorption of the bacteriocin to fat and meat tissues since hydrophobicity of bacteriocins from LAB could lead to an unspecified union of bacteriocin molecules to the hydrophobic surface of fat particles (Holzapfel et al., 1995). Furthermore, Kouakou et al. (2009) found that high-fat content meat antagonizes the antilisterial effect of bacteriocinogenic *Lb. curvatus* CWBI-

B28wt in pork meat systems. The latter trends could be explained by the study of Aasen et al. (2003) which showed that the activity of the bacteriocins in a medium depends not only on the fat content and type of bacteriocin, but also on the physical state of the medium; i.e., liquid or solid.

Meat in its natural condition might differ from disintegrated states. Considering just meat composition would give us only limited information regarding the physical state, structure or engineering properties of meats. Ground meats have a chemical composition similar to their sources but quite different physical properties, rheological behavior and sensorial attributes (Aguilera 2005). Besides, fermentation, emulsification, or cooking -among other processing operations- will condition meat structure and its general behavior.

Comminuted Meat Batters - the So-Called “Meat Emulsions”

Finely comminuted meat products -sometimes referred to as meat emulsions- are mainly composed of water, protein, fat and salt; being the most representative products, Frankfurter sausages and bologna. The aqueous phase of the meat emulsion is the one that holds up microbial growth, as such is the case of other food emulsions. The availability of water determines how and where microbial proliferation will proceed in the meat batter. As a good example, the work of Terjung et al. (2015) can be cited. These authors found that the antimicrobial effectiveness of a spice ferment against *L. innocua* LTH 3096 and *Lb. curvatus* LTH 683 was higher on restructured ham compared to emulsion type sausages. They attributed this effect to the lower fat content - hence, higher a_w value – of restructured ham.

Microbial contamination of these products occurs during post-processing steps, i.e., handling, packaging, storage, etc.; hence, the colonization of the food matrix mainly takes place on the surface. The type of preservation method to be used is focused mainly on the protection of the outer layer of the products. Consequently, antimicrobial agents are added to rinsing or spray solutions, protective coatings, or packaging. The effectiveness of these preservatives is influenced by the solid food structure (Wilson 2000), which provokes a chemical redistribution of food preservatives (Brocklehurst et al., 1993; Brocklehurst and Wilson 2000). In addition, concentration gradients of nutrients and metabolites, and also O_2 and pH, within and around the colonies affect microbial growth, as a consequence of diffusional limitations in solid systems. Baka et al. (2015) found differences on the lag estimates of *L. monocytogenes* at 4°C on Frankfurters with different fat concentrations (‘type 1’: 19%; ‘type 2’: 14.5%). They assumed that the undissociated organic acids (preservatives) -being lipophilic compounds- migrated to the lipid phase of the product; which was driven by the presence of solutes, such as NaCl, in the water phase (Gooding et al., 1955; Sofos and Busta 1981). Therefore, in the ‘type 1’ sausages, with higher fat concentration, the action of undissociated organic acids might have been diminished. This would explain the more extended lag phase found on the ‘type 2’ sausages.

High-pressure processing (HPP) is an attractive preservation technology which has a good potential for the meat industry in particular. The resistance of the microorganisms is variable depending on the strain and the meat matrix to be treated (Garriga et al., 2004). As a consequence, antimicrobial agents are used together with HPP in ready-to-eat (RTE) meat products so as to contribute with their preservation. In a research conducted by Pal et al. (2008) on refrigerated RTE frankfurters, potassium lactate and sodium diacetate were added

to the meat batter and then final products were vacuum-packaged and subjected to HPP (400 MPa, 15 minutes). Although both antimicrobial agents had a bactericidal effect on background microbiota, a bacteriostatic effect had merely been displayed against *L. monocytogenes*. It is presumed that the application of HPP could have structurally degraded some muscle cell components and released additional nutrients for *L. monocytogenes* growth, contributing to its survival along shelf-life of the product.

The results showed herein comprise a condensed group of examples chosen to illustrate the wide-ranging influence of meat structure on several preservation methods. This evidences that: i) microbial growth data from liquid media could only characterize the isolated effect of the preservatives' levels, but not the stress from the 'solid environment'; ii) chemical nature of preservative agents -amphiphilic molecules (microbial peptides such as bacteriocins); lipophilic organic acids (e.g., sorbic and benzoic acids)- condition the distribution of these substances on the different phases of the meat matrix (aqueous-proteinaceous phase or lipoid phase); iii) processing mechanisms associated with changes (disrupters) of the cells within meat tissues could result in nutritional changes favorable for microbial growth. On the whole, meat and meat products have to be considered as complex architectures regarding food matrices, and therefore should be addressed as such when choosing the best way to preserve them.

PREDICTIVE MICROBIOLOGY IN STRUCTURED MEDIA

Predictive microbiology pretends to reduce time-consuming and costs involved in challenge tests through the model of microbial growth as a function of time (primary models) and as a function of a few environmental factors (secondary models). The last factors include the traditional ones such as temperature, pH, a_w , and others like antimicrobials, organic acids and oxygen. However, sometimes microbial growth cannot be predicted by these models since some factors such as background flora, microbial competition, stress factors, medium structure and environmental changes produced by microbial growth are not taken into account. This omission is the main source of error in predictive microbiology and it is called as the *completeness error* (McMeeckin and Ross 2002).

Concerning about this, Boons et al. (2014, 2015) studied the effect of increase complexity of the structured medium on *E. coli* and *S. cerevisiae* growth. They included heterogeneous systems and NaCl as a stress factor mimicking the inhomogeneous composition and structure of foods. Microbial dynamics was affected by medium structure complexity since the microorganisms showed higher growth in complex than in liquid medium. However, the behavior of both microorganisms was different in the same structured medium. A secondary model including the effect of medium structure on *S. Typhimurium* growth rate, previously developed by Theys et al. (2008), was successfully validated in pasteurized milk and cheese (Theys et al., 2009a). Also, this model described *L. innocua* and *Lb. lactis* growth as a function of gelatin level.

Different models such as Fickian diffusion model, to predict the diffusion of nutrients and metabolites, and Buffering Theory, to predict local pH changes, have being developed to be incorporated into an integrated modeling methodology to predict growth in structured systems

(Van Impe et al., 2005; Wilson et al., 2002). Regarding to this, Van Impe et al. (2013) explain that traditional predictive models consider the behavior of average population and fails in the description of colony dynamics since the local competition for nutrients causes a different behavior of the individual colony cells and does not have a normal distribution. They propose considering the effects of environmental conditions on cell metabolism and growth dynamics by using Metabolic Flux Analysis. They suggest that this information will allow improving precision of predictive models for more complex systems like structured media. More recently, Tack et al. (2015) applied an individual based model on *E. coli* growth in gel from cellular parameters reported in bibliography. The model included the local nutrient competition, individual cell differences and intercolony interaction. It successfully reproduced single colony dynamics, simulated interaction between colonies and demonstrated that nutrient diffusion and local cellular glucose competition produced emergence of a starvation zone in the center of the colony. From the knowledge of cellular parameters of microorganisms in structured media, this model contributes to microbiological quality and safety of structured foods.

On the other hand, there are several methods that combine microscopy and image analysis that pretend to be used in predictive food microbiology. In this regard, several authors developed microscopy techniques for monitoring individual colony dynamics. Skandamis et al. (2007) found the correlation between growth rate values of *E. coli* O157:H7 in gel cassette obtained by plate count and those obtained by light microscopy and image analysis. Also, they could fit a secondary model with growth rates obtained via image analysis. Theys et al. (2009b) compared colony volume dynamics and cell number of *S. Typhimurium* colonies in gel cassette and they found that there were dead and growing cells in stationary phase. However, these methodologies have some disadvantages since they require the design of the matrix to control experimental conditions (gel cassette in these cases) and a complex data processing procedure. This led to the search of faster and more versatile methodologies to study the structure effect on the colony growth. In this regard, Mertens et al. (2011) studied the effect of pH, a_w , acetic acid concentration, temperature and medium structure (Carbopol or xanthan gum as thickening or gelling agents) on the growth/no growth boundaries of *Z. bailii* in model systems of acidic sauces through optical density measurements. They could observe differences in growth probability of yeast between liquid and structured media. In connection with the use of optical density measurements, a novel method was developed by Mertens et al. (2012) for monitoring the individual dynamics of colonies growth in solid medium as a function of time by scanning the area of growth of an individual colony through optical density measurements in microtiter plates. Particularly, they studied *E. coli* growth in Brain Heart Infusion broth plus 5% agar but the methodology could be extended to other microorganisms. This methodology is promising because it allows to minimize the difficulties related to inoculation and sampling and to obtain rapid data collection. However, the transparency of the medium is required, which is a disadvantage when applying to other food matrices. Therefore, more research is needed to predict the effect of the structure on microbial growth in solid media by using methodologies as fast and versatile as this.

CONCLUSION

Microorganism's development in food is not only determined by its environmental and storage conditions but also by its structure. Concerning this influence, the following facts need to be remarked:

- Microbial growth in structured systems is inhibited compared to liquid systems. Therefore, the response of microorganisms to different stress factors is modified with respect to planktonic growth and different trends can be observed depending on the stress factor applied, the composition of the system and the microorganism.
- Independently of its role as a structuring agent, the presence of oil affects the physiology of microorganism and the distribution of lipophilic additives. Hence, the effectiveness of lipophilic antimicrobial agents can be decreased.
- There is some information available about the effect of the structure itself and the stress factors such as pH and a_w in systems modeled by gels. However, this information is scarce in emulsified systems. This trend can be linked with the difficult for studying growth in emulsions due to their opacity which ruled out the use of microscopy and absorbance based methods.
- The effect of medium structure has been introduced as a factor in predictive microbiology models in the last years. However, further research is needed in this area.
- Many foods contain micro-architectures and growth of microorganisms can take place planktonically, in colonies -immersed or at the surface- depending on the localization of the microorganisms.
- In vegetables, it is essential to understand the factors affecting pathogen attachment in order to apply strategies to avoid the growth. Commented studies demonstrated that attachment ability depends on the pathogen, the surface morphology of the vegetables, the temperature and the integrity of the tissue.
- In dairy fermented products, structure determines the location of microorganisms during the processing. The role of microorganisms and their enzymes on ripening processes is a key factor on the quality of these products, underlying the importance of this feature.
- In meat and meat products, many are the structures -from fiber structure to meat emulsions- which can exert several effects on microbial growth. Mainly, they can modify the action of preservatives and condition the distribution of compounds in different phases.

Outcomes shown herein highlight the importance of considering the effect of the structure on microbial growth when evaluating microbial stability of these food systems.

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Chapter 2

THE EFFECT OF SOIL TYPE ON THE OCCURRENCE OF *L. MONOCYTOGENES* IN FRUITS AND VEGETABLES

Barbara Szymczak^{*}

Department of Applied Microbiology and Biotechnology,
Faculty of Food Sciences and Fisheries,
West Pomeranian University of Technology in Szczecin, Poland

ABSTRACT

L. monocytogenes is recognized as one of the most important food-borne pathogens. A wide spectrum of *L. monocytogenes* habitats points to its high adaptation capabilities. *L. monocytogenes* is a bacterium often isolated from the following habitats: foods, water and soil. It is capable of producing VNC forms (viable not culturable), which may explain the phenomenon of its long-term survivability in unfavorable conditions, e.g., up to one year in humid soil and for over two years in arid soil. Soil is a habitat which plays a key role in this bacterium spreading to plants and animals, but also to foods, especially fresh fruits and vegetables.

The aim of the study described in this Chapter was to determine the prevalence of *L. monocytogenes* in soil samples with reference to the type of fertilizers (natural and chemical) and the distance from places intensively exploited by men, as well as to determine the relationship between *L. monocytogenes* presence in the soil and its occurrence in fruits and vegetables.

Our study demonstrates that the most frequent cause of soil contamination with *L. monocytogenes* is manure, which is linked with this bacterium transmission to crops. In most cases, it concerns fruits and vegetables that have a direct contact with contaminated soil (e.g., strawberries or root vegetables). The presence of *L. monocytogenes* was confirmed in 50% of beetroot samples, 25% of carrot samples, 15% of tomato and potato samples, as well as in 5% of parsley samples and 25% of strawberry samples. A correlation was determined between the prevalence of *L. monocytogenes* in fruits and vegetables and the type of fertilizer applied. *Listeria* sp. and particularly *L. monocytogenes* were found in the soil from arable lands fertilized with

^{*} Department of Microbiology and Applied Biotechnology, West Pomeranian University of Technology, Papieża Pawła VI 3, 71-459 Szczecin, Poland, e-mail: barbara.szymczak@zut.edu.pl.

manure; pastures (areas fertilized with feces of domestic animals); and forests (again areas fertilized with feces of animals, not domestic but wild). The bacteria were not found in the soil samples collected at chemically-fertilized arable lands; waste lands (areas that were not fertilized with manure or animal feces).

Three degrees of risk were determined on the basis of bacterial contamination, also pointing out to the hazard posed by *L. monocytogenes* ingestion.

This Chapter presents also an overview of factors that facilitate *L. monocytogenes* occurrence in soil, fruits and vegetables as well as the circulation of *Listeria* sp. and *L. monocytogenes* in the environment, considering the possibility of the emergence of atypical strains.

Keywords: fruits, *L. monocytogenes*, prevalence, soil, vegetables

INTRODUCTION

The genus *Listeria* comprises ten species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, *L. marthii* (Graves et al. 2010), *L. rocourtiae* (Leclercq et al. 2010), *L. weihenstephanensis* (Halter et al. 2012), and *L. fleischmanni* (Bertsch et al. 2013).

New seven species have been recently described: *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. grandensis*, *L. riparia* (den Bakker et al. 2014), *L. booriae*, and *L. newyorkensis* (Weller et al. 2015).

Among these species, only *L. monocytogenes* is pathogenic to man and animals, and *L. ivanovii* to animals, by inducing listeriosis. For a long time, listeriosis has been classified as zoonosis (anthropozoonosis). Already works describing numerous epidemics of listeriosis in the 1970ies that indicated food contaminated with *L. monocytogenes* bacilli as the source of these outbreaks made that listeriosis was included amongst “food-borne diseases.” Although *L. monocytogenes* is not the most ubiquitous bacterium found in food, it is much more hazardous than other pathogens as 20-30% of all cases of listeriosis are fatal (Reissbrodt 2004).

Propagation of a healthy lifestyle including encouragement for the consumption of fruits and vegetables from organic crops (no fertilizers and herbicides, use of exclusively natural fertilizers) contributes to a growing demand for products of this type. This, in turn, increases the risk of food poisonings induced by pathogenic microflora present in fruits and vegetables having contact with soil.

L. monocytogenes is widely distributed in nature including vegetation (Weis et al. 1975, Welshimer 1968), water (Lyautey et al. 2007), sediment (Colburn et al. 1990, El Marrakchi et al. 2005) and soil (Weis et al. 1975, Welshimer 1975).

Although *L. monocytogenes* is ubiquitous in the environment, humans and animals are likely to be its important reservoirs (Wesley 1999, Grif et al. 2003, Borucki et al. 2005).

L. monocytogenes has been isolated from livestock, domestic and wild animals in both infections and latent states (Wesley 1999, Gray et al. 1966), in animal feces and in the close environment of animals (Nightingale et al. 2004). The occurrence of *L. monocytogenes* is generally higher in fecal samples of healthy cattle (33.0%) than in sheep (8.0%) or pig (5.9%) (Wesley 1999).

Microflora of plant materials originates mainly from soil and depends on plant genus, soil type, climatic conditions of growth and harvest, as well as conditions of transportation and storage.

The risk of contamination with soil-derived microflora depends on plant position in respect of the substratum. The closer a plant is to the soil, the more contaminated it becomes. The first noted cases of food poisoning with vegetables contaminated with *L. monocytogenes* were described in 1983 in Canada after consumption of a salad made of white cabbage grown on a field fertilized with manure from listeriotic sheep (Schlech et al. 1983), in 1986 in the USA after consumption of a fresh vegetable salad (Ho 1986) and in Sweden after consumption of common mushroom (Nguyen-the and Karlin 1994). The last epidemic of listeriosis was noted in the USA after consumption of melon.

A wide spectrum of *L. monocytogenes* habitats points to its high adaptation capabilities. *L. monocytogenes* is capable of producing VNC forms (viable not culturable), which may explain the phenomenon of its long-term survivability in unfavorable conditions (Doyle et al. 1997).

Listeria grows in a wide range of temperatures (1-45°C) and pH values (4.5-9.0), therefore it develops in many food products. These bacteria were frequently isolated from cold-stored and from frozen food products. A high concentration of sodium chloride (10-28%) has no negative effect upon the survivability of *L. monocytogenes* (Lovett 1990). *Listeria* strains preserved complete viability after storage for 12 months in the medium containing 16% NaCl, at pH 6.0 and a temp. of 37°C. They were also shown to grow at reduced oxygen content and in the presence of CO₂ in the medium (Lammerding, Doyle 1990). According to Welshimer, *Listeria* may survive even for 295 days in soil, for over half a year in straw, for up to one year in humid soil, and for over two years in arid soil. Welshimer (1968) isolated *L. monocytogenes* from various parts of plants like stem or leaves of maize. There may be various sources of vegetables contamination, e.g., as a result of irrigation with contaminated water or fertilization with manure. This study was aimed at determining factors that affect the prevalence of *L. monocytogenes* in fruits and vegetables grown on various types of soil with consideration given to: class of soil, type of fertilization, degree of utilization by man, season of the year and soil pH.

MATERIALS

Soil

One thousand soil samples collected in the years 2008-2011 in Poland were analyzed in the study. The samples were collected in two seasons: in the spring (March-May) and in the autumn (September-November), on the 250 km² area in Poland. Sampling sites were selected based on the extent of their use by man (arable lands – waste lands), type of fertilization (natural with manure – chemical), and soil class. Information on soil classes was derived from a land record map published by the Center for Geodetic and Cartographic information. Soil samples preparation and storage followed ISO and AFNOR standards: NF ISO 10381-1 and ISO 10381-6.

Measurement of Soil pH

Soil pH was measured at the sampling site using a CP-104 pH-meter (Elmetron, Poland) according to producer's instructions.

Fruits and Vegetables

Analyses were conducted for 210 samples of vegetables (beetroot, cabbage, carrot, parsley, tomato, lettuce and potato, 30 samples of each) and for 140 samples of fruits (blueberries – 20, blackberries – 20, raspberries – 40, and strawberries – 60). Fruits and vegetables were purchased directly from farmers who provided information on the fertilization method of soil the products were grown on. Hence, the possibility of microbiological contamination of fruits and vegetables during transport and storage was excluded. Before microbiological analyses, the fruits and vegetables were washed under running water and peeled with a sterile knife. Strawberries were deprived of stems, the peeled fruits and vegetables were again washed under running water. 25-g samples of each fruit and vegetable were collected for analyses.

Reference Strains

Use was made of reference strains from the ATCC collection: *L. monocytogenes* (19114) and *Listeria* sp. (*L. grayi* 25401, *L. seeligeri* 35967, *L. innocua* 33090, *L. ivanovii* 19119, *L. welshimeri* 35887). The strains were enliven in a liquid BHI medium (CM 1135, Oxoid), incubated for 24 h at 37°C, and then sieved into BHI agar medium (CM 1136, Oxoid) and again incubated for 48 h at 37°C.

Storage of *L. Monocytogenes* Strains

The analyzed strains were archived, by storing them frozen in Tryptone Soy Broth (Soybean–Casein Digest Medium U.S.P.) (TSB, CM129, Oxoid, England) with the addition of 10% glycerol (Sigma Aldrich, Germany). Before further analyses, the strains were enliven in a liquid BHI medium (CM 1135, Oxoid).

METHODS

Isolation of *L. monocytogenes* from Soil, Fruits and Vegetables

L. monocytogenes was isolated from samples of soil, fruits and vegetables according to the Polish Standard PN–EN ISO 11290–2:2000 “Horizontal method for detection of the presence and determination of the count of *Listeria monocytogenes* – Method for bacteria count determination considering the amendment of PN – EN ISO 11290–2: 2000/A1.

Isolation from Soil

A soil sample (25 g) was placed in a glass flask with 225 mL of semi-Fraser broth (Fraser without selective agents, Fraser Broth 835, Oxoid) and shaken with sterile glass beads for 10 min in a WU-4 universal shaker (Premed, Poland). Afterwards, the samples were incubated at 30°C for 24h. After incubation, 1 mL of the culture was transferred to a test tube with 10 mL of Fraser with Fraser-Selective Supplement (SR 156, Oxoid), and incubated at 37 °C for another 48 h. Simultaneously, a culture into single colonies was performed on the Listeria Selective Agar medium (LSA, CM 856, Oxoid) with Listeria Selective Supplement (SR 140, Oxoid) and incubated at 30°C for 48h. The last stage of isolation included streaking from Fraser medium onto LSA medium.

Isolation from Fruits and Vegetables

A sample of fruit or vegetable (25 g) was placed in a sterile foil bag, homogenized with 225 mL of semi-Fraser broth for 2 min in a stomacher 400 (Colworth, England), and incubated at 30 °C for 24 h. Further procedures were identical as during *L. monocytogenes* isolation from soil.

Characteristics of Listeria Colonies Growth on Listeria Selective Agar (LSA) Medium

Once colonies with typical growth on the LSA medium were isolated, further identification analyses were carried out for up to five typical strains. If one of the analyzed colonies turned out to be *Listeria* sp., the sample was recognized as positive.

On the LSA medium, *Listeria* sp. grows in the form of black, round colonies 0.3-2.3 mm in diameter with a concave centre, whereas the change of medium color from yellow-green to black is caused by esculin degradation.

Characteristics of Listeria Sp. and L. monocytogenes Colonies Growth on Oxoid Chromogenic Listeria Agar (OCLA) Medium

Oxoid Chromogenic Listeria Agar (OCLA, CM 1084, Oxoid) allows for differentiation of pathogenic *Listeria* (*L. monocytogenes* and *L. ivanovii*) from non-pathogenic ones. On this medium, identification is carried out based on the activity of two enzymes (lecithinases): phospholipase C (PCPLC) and specific phospholipase C (PIPLC) (Reissbrodt 2004). These enzymes are responsible for pathogenicity.

On the OCLA medium, *Listeria* sp. grows in the form of turquoise colonies developed as a result of degradation of glucosidase contained in this medium by β -glucosidase enzyme. In turn, *L. monocytogenes* and *L. ivanovii* are further differentiated based on the capability for the synthesis of a lecithinase enzyme - phospholipase. This enzyme hydrolyzes lecithin from the culture medium, and develops an opaque zone (halo) around colonies (Reissbrodt 2004).

Preparations Stained with Gram's Method

Five colonies from each sample were seeded from the LSA medium onto BHI (CM 1136, Oxoid) and preparations were made by staining with the Gram's method. In the microscopic

picture, *Listeria* is characterized by Gram-positive short bacilli arranged individually or in the form of palisade clusters in the shape of X, Y and Z letters or short chains.

Catalase Detection Test

The test was carried out on defatted microscope slide, by applying colonies onto droplets of hydrogen peroxide with an inoculation loop. Characteristic foaming is indicative of the positive result (catalase-positive). *L. monocytogenes* is catalase-positive.

Motility

The motility of *Listeria* strains was evaluated by inoculating the colony onto semi-liquid nutrient agar with the addition of 1% solution of tetrazolium red (TTC, 2,3,5 – triphenyl tetrazolium chloride, Sigma Aldrich). The growth of colonies in the shape of a characteristic red umbrella was identified as a positive result.

Evaluation of Hemolytic Activity of Listeria Strains

For species identification, the strains of *Listeria* sp. were seeded from LSA medium onto nutrient agar enriched with 4% of human blood and incubated at 37 °C for 24 h. A narrow halo around colonies (β - hemolysis) was identified as a positive result.

Capability for Carbohydrates Degradation

The first stage of the evaluation of the capability for carbohydrates degradation included seeding the colonies from LSA onto TSYEB (CM 0862, Oxoid) and incubation at 25° C for 24 h. Once noticeable opacity has been achieved, 0.1 mL of the culture was transferred into broth with bromocresole purple enriched with 0.5% solutions of individual carbohydrates (L-rhamnose or D-xylose), and incubated at 37 °C for 5 days.

The change of medium color from violet into yellow (medium acidification) was identified as a positive result.

Isolation of DNA

DNA was isolated according to Szymczak et al. (2013) using a DNA Genomic Mini AX *Bacteria* kit (A and A Biotechnology, DNA, Poland). The concentration and purification degree of DNA were determined spectrophotometrically (NanoDrop DN-1000, Biotech, Poland).

Multiplex PCR Reaction

Identification to the genus *Listeria* was conducted with the use of starters specific for the 16S rRNA gene sequence fragment: U1 (5' - CAG CMG CCG CGG TAA TWC -3') and LI1 (5' - CTC CAT AAA GGT GAC CCT -3'). Starters *iap1* (5' - CGA ATC TAA CGG CTG GCA CA -3') and *iap2* (5' - GCC CAA ATA GTG TCA CCG CT -3'), specific for the *iap* gene sequence, were used for species identification of *L. monocytogenes*. The sizes of amplified DNA sequences were 938 bp which indicated affiliation of *Listeria* genus and 287 bp which indicated affiliation to *L. monocytogenes* species (Szymczak et al. 2014). Owing to a higher number of strains to be identified, the multiplex PCR was applied. It was conducted in a volume of 50 μ L of the reaction mixture containing: 500 mM KCl, 100 mM Tris-HCl (pH 8.3 at 25°C), 2.5 mM MgCl₂, 0.3 mM of each nucleotide, 30 pM/mL of each starter

(Table 1), 2.5 U *Taq* DNA polymerase (Eppendorf), and 5 μ L of DNA matrix in a *Mastercycler Gradient* thermocycler (Eppendorf). The thermal profile consisted from the following stages: preliminary denaturation – 60 seconds/95°C and 35 cycles including: denaturation – 30 seconds/94 °C, primers annealing – 20 seconds/51°C and extension – 30 seconds/72°C. The amplification ended with extension – 8 minutes/72°C.

The product of PCR reaction with the addition of 0.037g Bromophenol Blue (ICN Biomedicals Inc., USA) in a 1.5% saccharose solution was separated electrophoretically under standard conditions (5V/cm) in 2% agarose gel (Prona Agarose Plus). The gel was stained with ethidium bromide (0.5 μ g/ML) in 1xTBE buffer, observed under UV light and archived. The size of the product was evaluated by comparison with the mass marker XVI (Roche, Germany).

RESULTS AND DISCUSSION

Analyses conducted according to PN–EN ISO 11290–2:2000 demonstrated that 19.5% of the 1000 soil samples examined were contaminated with *Listeria* sp., whereas multiplex PCR confirmed that only 5.5% of them were infected with *L. monocytogenes*.

« *Listeria* is present in the environment worldwide »- is a statement frequently appearing in works describing *Listeria* bacilli. It is commonly believed that this bacterium is a permanent resident of soil habitat and, therefore, isolated from fruits and vegetables that had contact with contaminated soil.

A question arises, then, whether *L. monocytogenes* is indeed a microorganism of the soil habitat and whether it is so ubiquitous in soil? The pioneering work by Welshimer presented the first evidence that soil is an environmental niche for *L. monocytogenes* and occurrence of the bacterium was observed in three out of 12 sampled farms (Welshimer 1968, Welshimer and Donker Voet 1971).

Weis and Seeeliger surveyed the occurrence of *L. monocytogenes* in 746 soil samples collected in Southern Germany. Weis and Seeliger reported *Listeria* sp. prevalences in vegetation samples ranging from 9.7 to 44% for samples from agricultural areas and from 21.3 to 23.1% for samples from nonagricultural areas.

In turn, Dowe et al. 1997 demonstrated that *L. monocytogenes* was more frequently isolated from waste lands (30.8%) than from arable lands (8.3%). Sauders et al. (2012) isolated *L. monocytogenes* from 19% of the samples originating from the natural environment and from 30% of the samples from urban environments. Locatelli et al. 2013 demonstrated that farm environments were potential sources of *L. monocytogenes* and might contribute to the contamination of vegetables at the preharvest stage. *L. monocytogenes* is frequently isolated from a large variety of vegetables collected in farms (Aytac et al. 2010, Jeyaletchumi al. 2011). In this study, the pH value of soil was measured during sample collection and ranged from 5.0 to 6.7.

Because acidity was at a similar level (acidic soils) and due to difficulties in measurements (drought in the further stages of the study), pH measurements were neglected as the pH value had no effect upon the prevalence of *L. monocytogenes*.

In this study, analyses were also conducted to determine dependency between *L. monocytogenes* occurrence and season of the year.

It was assumed that the level of soil contamination with *Listeria* bacilli would be higher in the autumn than in the spring, because better conditions for bacteria survival and proliferation in soil occur in the summer than in late autumn or early spring.

Although Welshimer (1968) suggests that *L. monocytogenes* is capable of surviving under unfavorable conditions even for over 295 days, considering the conditions (arid soil) this process shall be determined as successive decay of the culture rather than its survival. In mild winter and in the early spring, bacteria are subjected to alternate processes of freezing and defrosting, which is very destructive for cells.

MacGowan et al. (1994) reported that *Listeria* sp. is more frequently isolated from samples originating from the period since July till August than from other months of the year. Sauders et al. 2012 isolated *L. monocytogenes* more often in the summer than in the other seasons of the year. In another report of a 3-year survey on fruit and vegetable farms, prevalence was higher in winter except during 1 year (Strawn et al., 2013, Vivant et al. 2013) and correlation between season and occurrence is not a clear-cut. Our research demonstrated no correlation between the occurrence of *L. monocytogenes* and season of the year. In the springtime and in the autumn, *L. monocytogenes* was isolated from 4.8% and 5.8% of the analyzed soil samples, respectively (Table 1).

The sampling sites of soil were selected considering the extent of their utilization by man (arable lands – waste lands) and type of fertilization (natural with manure – chemical).

The study demonstrated that the occurrence of *L. monocytogenes* in soil was affected to the greatest extent by the anthropogenic factor. The highest level of contamination with *L. monocytogenes* was noted in the samples from meadows used for intensive grazing of dairy cattle (27.8%), from the area approximating a meat processing plant (25%) and from forests being part of a hunting district (24%). *Listeria* sp. does not occur on the areas with intensive artificial fertilization and on waste lands (Table 2). Similar results were obtained by Zaytseva et al. (2007) who did not isolate *Listeria* sp. and *L. monocytogenes* from 76 samples of soil originating from eastern Russia. This author emphasizes that these areas are far from large urban agglomerations, processing plants and animal production plants.

Isolation of *L. monocytogenes* bacilli in the proximity of processing plants is not surprising, because other authors have already emphasized a serious problem posed by listerias in meat processing plants and other plants linked with food processing. For instance, Tompkin (2002) isolated *L. monocytogenes* from floors of a processing plant. It suggests that the bacilli were “brought in” on shoes of the staff, because *L. monocytogenes* had been earlier isolated from swabs from floors and walls of a production hall and from working table tops.

Analyses were also carried out on a specific territory of enclosed wild animal breeding (fallow deer, wild boar, roe deer, deer) with the area of ca. 20 ha and population density of ca. 100 animals per 1 ha. *L. monocytogenes* was detected in 15.6% of the analyzed samples. Similar results were reported by Kalorey (2006), who isolated *L. monocytogenes* from 16% of feces samples collected from animals living in a zoo in India. In turn, Bauwens et al. (2003) demonstrated contamination with *L. monocytogenes* in 14 (7%) samples of feces of various game species (including 6.7% of feces samples from mammals and 8.6% from birds) living in the zoo in Antwerp. Wild animals living in low-populated areas like, e.g., northern Norway, are free from *Listeria* as indicated in the study by Aschfalk et al. 2003 conducted on samples of reindeer feces. This author emphasizes that those territories are very extensive and that reindeers are wandering animals, hence the possibility of infection spreading from one animal to the other is low.

Table 1. Occurrence of *L. monocytogenes* depending on the season of year and soil class

Sampling site			Number of samples analyzed in the spring	Count of <i>L. monocytogenes</i> ² / % ³	Number of samples analyzed in the autumn	Count of <i>L. monocytogenes</i> / %
Arable lands	soil class	type of fertilization				
		III	natural	13	0	53
chemical			13	0	53	0
IV		natural	13	1 / 7.7%	53	0
		chemical	13	0	53	0
		waste lands	10	0	50	0
V		natural	13	0	28	0
		chemical	13	0	28	0
		waste lands	10	0	50	0
Garden plots			16	1 / 6.3%	31	4 / 12.9%
Orchards			14	1 / 7.1%	4	0
Meadows		intensive grazing of dairy cattle	18	4 / 22.2%	18	5 / 27.8%
		intensive grazing of meat cattle	22	0	22	4 / 18.2%
		usable	20	3 / 15%	n.d.	n.d.
		non-usable	24	0	54	0
Forests		deciduous	16	0	56	9 / 16.1%
		coniferous	16	1 / 6.3%	56	1 / 1.8%
Forest area with lake		50	0	n.d.	n.d.	
Area of hunting district		n.d. ¹	n.d.	29	7 / 24.1%	
Reserve of wild animals		n.d.	n.d.	32	5 / 15.6%	
Area around meat processing plant		18	4 / 22.2%	18	4 / 22.2%	
TOTAL		312	15 / 4.8%	688	40 / 5.8%	

¹ n.d.- not determined.

² *L. monocytogenes* confirmed with multiplex PCR.

³percentage of analyzed samples.

Table 2. Occurrence of *L. monocytogenes* depending on the extent of utilization by man

Sampling site	Number of analyzed samples	Count of <i>Listeria</i> sp. ¹ /% ²	Count of <i>L. monocytogenes</i> ³ /%
Naturally-fertilized lands	173	51 / 29.5%	2 / 1.2%
Chemically-fertilized lands	173	0	0
Waste lands	120	0	0
Garden plots	47	25 / 53.2%	5 / 10.6%
Orchards	18	3 / 16.7%	0
Meadows	intensive grazing of dairy cattle	36	10 / 27.8%
	intensive grazing of meat cattle	44	13 / 29.5%
	usable	20	5 / 25%
	waste lands	78	0
Forests	hunting district	29	21 / 72.4%
	deciduous	72	25 / 34.7%
	coniferous	72	7 / 9.7%
Forest area with lake	50	5 / 25%	0
Reserve of wild animals	32	21 / 65.6%	5 / 15.6%
Area around meat processing plant	36	9 / 25%	9 / 25%
TOTAL	1000	195 / 19.5%	55 / 5.5%

¹ Count of *Listeria* sp. confirmed with PN EN ISO 11290: 2000.

² percentage of analyzed samples.

³ Count of *L. monocytogenes* confirmed with multiplex PCR.

Manure constitutes the main source of contamination with *L. monocytogenes* bacilli. In this study, also atypical strains were isolated from soil samples. In terms of morphological and biochemical traits, these strains resembled *Listeria*, whereas products obtained in genetic studies were far from the expected ones. In addition, as demonstrated in this study, *Listeria* sp. and *L. monocytogenes* were isolated only from these habitats that are subjected to intensive anthropopressure. It may, therefore, be speculated that soil as the medium being the poorest in nutrients indispensable for bacteria growth, including *Listeria* sp. and *L. monocytogenes*, among the aforementioned environments, causes damage to bacteria cells. Feces of man and animals as well as food processing environment provide fine conditions for the growth of *L. monocytogenes*. In soil, however, these bacteria are exposed to various stress factors, which results in permanent metabolic damages (bacteria still maintain their traits after seeding onto rich BHI medium). Owing to this, they are recognized as atypical *Listeria*. After re-passaging through the gastrointestinal tract of animals, they are probably eliminated therein. The potential sources of *L. monocytogenes* transmission to soil and plant materials were elaborated by Beuchat (1996), who based on findings of other researchers (including Welshimer and Donker-Voet 1971) and own studies demonstrated the possibility of soil-vegetables and vegetables-soil contamination with this pathogen. In the reported study, *L. monocytogenes* was isolated from fruits and vegetables originating only from garden plots and from a farm, i.e., from areas fertilized only with manure. Also Alghazali and Alazawi (1990) demonstrated that pathogenic bacilli were isolated in Bagdad from crops fertilized with natural fertilizers. For this reason, the possibility of soil contamination from vegetables is highly unlikely, although Beuchat suggests that *L. monocytogenes* may survive on plants for 10-12 years. Likewise in our study, this author found that animal feces are the main source of terrestrial environment contamination. In turn, MacGowan et al. (1994) indicate wastewater as the second source of contamination with *L. monocytogenes*. These authors investigated wastewater in the Great Britain in the years 1991-1992 and isolated *L. monocytogenes* and/or *L. innocua* from 84-100% of the analyzed samples that constituted the source of water, soil and vegetables contamination with pathogenic bacilli. In British fresh livestock manure, prevalence of *Listeria* sp. (including *L. monocytogenes* and *L. ivanovii*) is globally higher in cattle (29.8%) and sheep (29.2%) than in pig (19.8%) and poultry (19.4%) wastes with levels ranging from 2×10^2 to 1×10^3 *Listeria* spp. per gram manure (Hutchison et al. 2005). In this study, the degrees of risk were determined and correlation analyses were performed to assess the relationship between the occurrence of *L. monocytogenes* in soil and in fruits and vegetables.

Soil samples were classified according to the three degrees of risk on the basis of proportional occurrence of bacteria (Table 3). Vegetables and fruits collected from examined soils were classified into one of the three groups, depending on the degree of soil contamination. They were also sorted by proportional occurrence of *L. monocytogenes*. The division into anthropopression classes was made mainly on the basis of results obtained with biochemical analyses, used as standard in identification of *L. monocytogenes* in food.

The first group includes soils not cultivated by man or soils fertilized artificially. These soils do not pose a health threat of listeria food poisoning, since these bacteria were not present on fruits and vegetables. The second degree (class) of anthropopression relates to soils exploited by man for grazing cattle and those fertilized naturally. *L. monocytogenes* was present in 25 - 50% of the samples.

Table 3. Degrees of soil anthropopression determined on the basis of percentage of occurrence of *L. monocytogenes* in soil, fruit and vegetables (Szymczak et al. 2014)

Degree	Type of soil	[%]	Fruit	[%]	Vegetables	[%]
I	Chemically-fertilized land	0/0	raspberry, strawberry	0/0	potato, parsley, carrot, beetroot, tomato, lettuce, cabbage	0/0
	Waste lands	0/0				
	Meadows - wasteland	0/0				
	Coniferous forests	9.7/2.8	blueberry	0/0		
	Orchards	16.7/0				
II	Usable meadow	25/0				
	Forests around the lake	25/0				
	Areas near food-processing plants	25/25				
	Meadows with dairy cattle	27.8/27.8				
	Naturally fertilized land	29.5/1.2	strawberry	15/10	lettuce, cabbage carrot	0/0
					tomato	5/0
					potato	15/15
					parsley	15/5
beetroot					25/0	
Meadows with meat cattle	29.5/13.6					
Deciduous forests	34.7/12.5	blackberry	0/0			
III	Garden plots	53.2/10.6	strawberry	25/10	lettuce, cabbage	0/0
					parsley	5/5
					tomato	15/0
					potato	15/15
					carrot	25/0
					beetroot	50/0
	Reserve of wild animals	65.6/15.6				
Forests of hunting district	72.4/24					

Moreover, depending on the type of vegetables and fruits collected from these soils, *L. monocytogenes* was present in 0 – 25% of the samples of fruits and vegetables (Szymczak et al. 2014).

The last third degree of risk refers to soils extensively used by man. Occurrence of *L. monocytogenes* in these soils was from 50 to more than 70%, and in samples of fruits and vegetables it reached 25% and 50%, respectively. Consumption of fruits and vegetables from soils from the third class poses the highest risk of infection with *L. monocytogenes*. In turn, the reported study indicates manure to be the most frequent cause of soil contamination with *L. monocytogenes* bacteria, which is linked with bacteria transmission to crops. In majority of cases, it concerns these fruits and vegetables that have a direct contact with contaminated soil for example: strawberries, root vegetables etc. (Szymczak and Dąbrowski 2015).

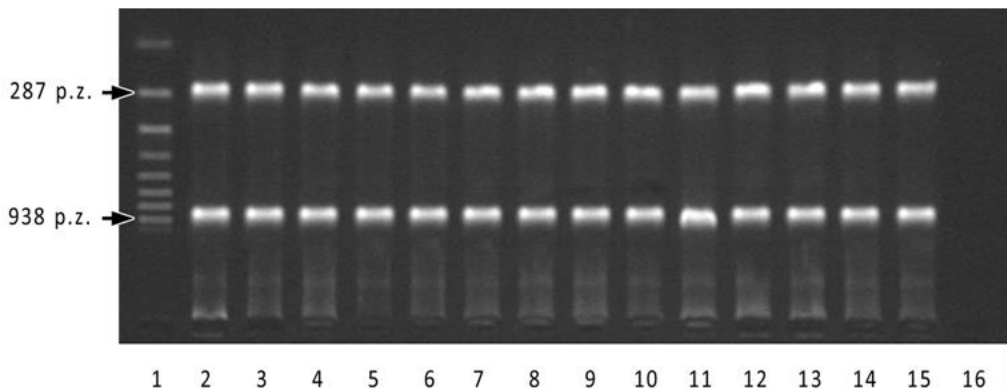


Figure 1. Identification of *L. monocytogenes* with the multiplex PCR method.

Bands: 1 – marker XVI (Roche, Germany), 2 – reference strain 19114 (ATCC), from 3 to 6 – strains isolated from soil, from 7 to 11 – strains isolated from fruits, from 12 to 15 – strains isolated from vegetables, and 16 – control sample (without DNA).

Bands: from 2 to 15 – *L. monocytogenes*.

CONCLUSION

The genus *Listeria* comprises 17 species. Out of these, only *L. monocytogenes* is hazardous to man. This bacterium occurs in various environments and possesses excellent adaptation capabilities. *L. monocytogenes* rarely occurs in soil, its presence is linked with anthropogenic activity or with the presence of domestic and wild animals. No correlation was determined in the study between soil class, season of the year, soil pH and the occurrence of *L. monocytogenes* in soil. The greatest risk of these bacteria occurrence is in fruits and vegetables originating from crops fertilized with manure.

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Chapter 3

EMERGING TRENDS OF BACTERIA DETECTION FROM FOOD USING PHAGE-BASED MASS SPECTROMETRY

Mazlina Banu and Dave Siak-Wei Ow**

Microbial Cells Group, Bioprocessing Technology Institute, A*STAR, Agency for
Science, Technology and Research, Singapore, Centros, Singapore

ABSTRACT

Conventional microbiological assays have been a valuable tool for specific enumeration of indicative bacteria of relevance to food and public health, but these culture-based methods are time-consuming and require tedious biochemical and morphological identification. In recent years, various rapid detection methods have been developed. The ability of bacteriophages to specifically infect bacteria and amplify nearly a hundred-fold in 1-2 hours has been exploited by different groups. Bacteriophage amplification is integrated with mass spectrometry (MS) for quantitation of phage-specific peptides. This method has potential as a rapid tool for detection of bacterial contamination during food bioprocessing and distribution to safeguard public health. Section 1 of this chapter will briefly describe the basic biological properties of bacteriophages that are relevant to their application for detection of bacteria in environmental and food samples. Section 2 gives a brief summary on current triple-quadruple MS technologies and how lytic phage is utilized together with this technology. Advantages and shortcomings of this method are discussed in comparison to traditional microbiological and molecular methods. Section 3 describes several studies of this new rapid detection methods that use phage to detect bacteria in different food and culture media using MS.

* Corresponding authors: Email mazlina_jaikubali@bti.a-star.edu.sg, dave_ow@bti.a-star.edu.sg.

1. INTRODUCTION

The World Health Organization estimates that each year up to one-third of the populations of developed countries is affected by foodborne illness caused by bacterial contamination. Food related diarrhoeal diseases are one of the top five leading causes of death worldwide with an estimated 4.7% of death in 2011 alone (World Health Organization 2014). This problem is likely to be more extensive in developing countries. Therefore, microbial analysis of food is an integral part in food bioprocessing and food safety. Bacterial detection methods are essential for the prevention and identification of food problems impacting health and safety. In recent years, various rapid bacterial detection methods have been developed. The ability of bacteriophages to specifically infect bacteria and amplify nearly a hundred-fold in 1-2 hours has been exploited by different groups (Pierce et al., 2012; Martelet et al., 2014, Banu et al., 2014). Bacteriophage amplification is integrated with mass spectrometry (MS) for quantitation of phage-specific peptides. This method has potential as a rapid tool for detection of bacterial contamination during food bioprocessing and distribution to safeguard public health.

Bacteriophages make a great tool for bacteria detection because of several distinctive features. Bacteriophages are obligate parasites of bacteria that rapidly multiply within their specific host cells upon infection (Hagens and Loessner 2007; Smartt and Ripp 2011). Bacteriophages are specific to their target host. This is referred to as the host range of the bacteriophage. They generally infect only specific host cells, thereby confirming their presence to the bacteria of interest. Successful phage infection and amplification requires metabolically active bacteria allowing differentiation between live and dead host cells (Campbell 1996; Heineman and Bull 2007). Bacteriophages have been used in assays for the detection of *Mycobacterium tuberculosis* in pulmonary tuberculosis diagnosis (McNerney et al., 2004), *Pseudomonas aeruginosa*, *Salmonella typhimurium* (Stewart et al., 1998) and *Escherichia coli* (Tanji et al., 2004). Another feature of bacteriophages is that they self-replicate upon infecting the host bacteria. Within minutes, phage proteins are synthesized to a high copy number and hundreds of newly-formed phages are released from each lysed cell. The number of infectious phages released per bacterial cell after infection and growth by one phage is known as burst size. The burst size serves as signal amplification in which each phage-infected bacterium can release hundreds of progeny phages that are assembled from multiple copies of different structural proteins (up to 1000 copies per particle) (Campbell 1996; Shabani et al., 2008, Brovko et al., 2012). This lowers detection limits with mass spectrometry as the bacteriophage amplification will be selective and proportional to the target bacteria. Phage amplification in host bacteria is a complex process that also involves non-structural proteins, which are encoded by the phage genome but not assembled in viral particles (as opposed to structural proteins). Bacteriophages have comparatively simple protein make-up and are less affected by factors that influence bacterial growth like sample preparation, temperature and nutrients. They are relatively inexpensive to produce in large quantities and have a strong resistance to heat, pH, solvents, acids, alkali and chemicals (Arya et al., 2011). Hence, as diagnostic agents, bacteriophages have the advantage of extensive shelf-lives and low production cost.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has emerged as a mainstream quantitation analytical tool in many environmental and food safety

laboratories largely due to its superior sensitivity, molecular specificity and multiplexing capability. Two strategies, “bottom up” and “top down,” have both been applied for protein profiling. The “bottom up” approach is more widely used for protein identification and characterization. In this method purified proteins, or complex protein mixtures, are subjected to enzymatic digestion prior to analysis by mass spectrometry. When “bottom up” strategy is applied for the analysis of a complex protein mixture, it is referred to as “shotgun proteomics.” It includes four common steps: (1) proteolytic digestion, (2) peptide separation, (3) peptide fragmentation in the mass spectrometer and (4) data analysis. “Shotgun proteomics” can be used for biomarker discovery including phage markers. On the other hand, in the “top down” method the whole protein remains intact for analysis.

Targeted protein analyses are typically performed using triple quadrupole mass spectrometers, which dates back to the development work first reported by Yost et al. (1978). Today, such analytical instruments are capable of a wide range of data acquisition techniques but the primary function most frequently used for high sensitivity targeted quantitation is the multiple reaction monitoring (MRM) approach. MRM is a mass spectrometric technique that relies on triple quadrupole mass spectrometers to act as mass filters to selectively isolate targeted analytes and one of its fragments (known as transitions). Each MRM transition specifies the targeted molecular precursor mass (m/z value) and its most abundant fragment ion generated after a fragmentation process known as collision-induced dissociation. Liquid chromatography-multiple reaction monitoring tandem mass spectrometry (LC-MRM-MS/MS) can monitor multiple transitions, which allows multiplexed analysis of numerous peptides in a single run. The peak areas for MRM transitions serve as the basis for quantitative comparisons and are integrated as measures of peptide abundance (Perry et al., 2008; Olsen et al., 2009). The advantages of bacteriophage amplification with MRM technology include sensitivity, speed and the ability to quantify hundreds of peptides in one LC-MS/MS run. Although the instrumentation is expensive in capital terms, the cost of sample preparation is considerably lower than cost of sample preparation involving molecular methods.

Traditional microbiological plate-culture methods for the detection of bacterial contamination like *E. coli* require two days or more. Despite being specific and sensitive in the detection of targeted bacteria, it is greatly hampered by the long culture time. Confirmation of species identity requires additional morphological testing or further subculturing in differential media (El-Hadedy and El-Nour 2012). The time constraint and inconveniency of traditional methods severely limits the ability to provide a rapid response to the presence of virulent bacteria (Manafi 1996). This is an obvious problem particularly in the food sector where fast-response approaches are often required. Other than standard cultural and biochemical methods, rapid culture-independent methods such as those based on polymerase chain reaction (PCR) are also used for identifying bacteria in food samples. Examples of such molecular detection methods include PCR, reverse transcription-polymerase chain reaction (RT-PCR), and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). These DNA-targeted PCR-based methods detect the presence of genes specific to bacterial species or strains and they offer good sensitivity and speed using minimal amount of sample (Cui et al., 2003; Odonkor and Ampofo 2013). Real-time PCR is being increasingly used as a rapid detection tool as it is able to continuously monitor the progress of PCR in each sample after each cycle. However, PCR is unable to discriminate dead from viable bacteria as it amplifies DNA from biological materials regardless of viability (McLain

et al., 2011). They are also susceptible to ambiguous and false positive results that can only be verified through microbiological or other methods. Therefore, alternative rapid methods allowing sensitive detection and quantification of bacteria from food are needed (Table 1).

Quantification using LC-MS/MS can be achieved by adding a known amount of stable isotope-labelled protein or peptide to a sample as an internal standard and comparing instrument response to an unlabelled counterpart. The species tagged with heavy isotopes differ from the unlabelled light ones in terms of their masses but show almost identical physiochemical properties such as MS/MS fragmentation patterns, ion yields and retention times. Labelling strategies include metabolic isotope labelling using heavy amino acids, using chemical reactions to introduce an isotopic or isobaric tag at specific functional groups on polypeptides, and, introducing stable isotope tags via enzymatic reactions. Each of these methods has its own strengths and drawbacks. Metabolic labelling of stable isotopes in whole organisms using cell culturing in heavy media is attractive as it allows labels to be introduced at the earliest time point possible, during protein synthesis, and offers the most complete way to cover proteomes entirely. Heavy isotopes, such as ^{15}N and ^{13}C , in media fed to organisms during growth result in incorporation of heavy labels into all proteins over the course of replication. Bacteriophage can be easily labelled with heavy isotopes such as ^{15}N by simple cultivation in ^{15}N -rich synthetic media (Figure 1).

Table 1. Advantages and shortcomings of bacteriophage-based MS method in comparison to traditional microbiological and molecular methods (Adapted from Mandal et al., 2011)

	Microbiological assays	Polymerase chain reaction	Phage-based MS detection
Speed	Long culture time ~48 hours	Rapid ~6 hours	Rapid ~4 hours
Specificity	Good	Moderate	Excellent
Sensitivity (CFU/ml)	1	$\sim 10^3$	$\sim 3.0 \times 10^3$
Detect viable cells	Yes	No (High chances of false-positives)	Yes
Follow-up tests	Often requires biochemical analysis/ subculturing	Culturing	Not required

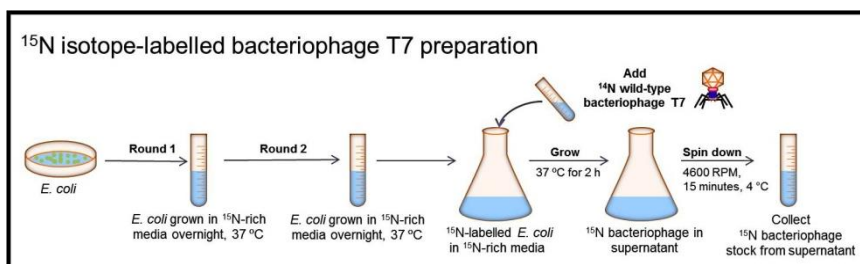


Figure 1. A simplified workflow of heavy isotope labelled bacteriophage T7 preparation. ^{15}N bacteriophage T7 is generated by infecting ^{15}N -labelled *E. coli* with T7 in ^{15}N -cell growth media. The process is repeated twice to ensure effective heavy isotopic labelling of the phages.

2. APPLICATIONS OF BACTERIOPHAGE AMPLIFICATION COUPLED WITH LC-MS/MS FOR DETECTION AND QUANTIFICATION OF BACTERIA

Three different studies have exploited bacteriophage amplification coupled with LC-MS/MS for the detection and quantification of bacteria in various food matrices and culture media. The first study used a bottom-up approach for the detection of *Staphylococcus aureus* using bacteriophage 53 where as the second study used both the bottom-up and top-down approaches to detect *E. coli* and *Bacillus subtilis* using bacteriophage T4 and SPP1 respectively. The last study used a bottom-up approach to detect and also quantify *E. coli* using bacteriophage T7 over a larger dynamic range.

Two of these studies used heavy isotope ^{15}N -labelled bacteriophage as input phage for quantification of bacteria. The objective of using ^{15}N -labelled bacteriophage is twofold. First, it allows parent bacteriophage (^{15}N -labelled input) to be distinguished from progeny bacteriophage (^{14}N wild-type output) by their mass differences. This gives more confidence in the mass spectrometric analysis as the parent and progeny can be differentiated by mass. Second, by using the ^{15}N -labelled phage as an internal standard, the number of bacteria can be quantified. A high concentration of ^{15}N bacteriophage can be used to ensure at least one infective ^{15}N bacteriophage is attached to each bacterium, preventing any further bacterial growth. This reduces the time of analysis and improves detection limits with LC-MRM-MS/MS for the accurate quantification of bacteria (Edgar et al., 2006).

2.1. Study 1 – Detection of *Staphylococcus aureus* by Bacteriophage 53 (Pierce et al., 2012)

A multiple reaction monitoring liquid chromatography method with tandem mass spectrometric detection for quantification of *S. aureus* via phage amplification detection (PAD) was described by Pierce et al. A "bottom-up" approach that combines PAD, stable isotope metabolic labelling, and LC-MRM-MS/MS was used to quantify peptides of the capsid head protein of *S. aureus*-specific lytic bacteriophage 53 (Figure 2). The presence of the target peptides was indicative of viable *S. aureus* concentration.

A known amount of isotope-labelled ^{15}N -reference bacteriophage, used as the input phage and as the internal standard for quantification, was spiked into *S. aureus* samples. This is the first study employing ^{15}N phage amplification combined with LC-MS/MS to quantify target bacteria. Following a 2 h incubation of the input phage and *S. aureus* sample, the sample was subjected to a 3 min rapid trypsin digest and analyzed by high-throughput LC-MS/MS detection targeting peptides unique to both the ^{15}N (input phage) and ^{14}N (progeny phage) capsid protein. Quantification was performed by comparing peak areas of target peptides from the metabolically labelled ^{15}N bacteriophage peptide internal standard with that of the wild-type ^{14}N peptides that were produced by phage amplification. This approach is based on the principle that labelled species differs from the unlabelled one in terms of mass but exhibits similar chemical properties. A calibration curve for *S. aureus* concentration was constructed with standards ranging from 5.0×10^4 to 2.0×10^6 colony forming units (CFU) per ml in LB media, with the ^{15}N reference phage spiked at a concentration of 1.0×10^9 phage

forming units (PFU) per ml. The ability to inoculate with high titers of ^{15}N -labelled phage (higher than the LC-MS/MS detection limit) allows *S. aureus* cells to be infected concurrently, thus offering time-saving advantages over standard methodologies in quantification of *S. aureus*.

In conclusion, the rapid identification and accurate quantification of *S. aureus* required:

- 1) Developing a rapid ^{15}N phage amplification step that could concurrently infect all *S. aureus* cells
- 2) Developing a rapid and efficient proteolytic digestion method
- 3) Employing high-throughput LC-MS/MS for rapid, sensitive, and specific quantification

2.2. Study 2 – Detection of *Escherichia coli* and *Bacillus subtilis* by Bacteriophage T4 and SPP1 Respectively (Martelet et al., 2014)

Martelet et al. reported indirect specific detection of bacteria in food samples using unlabelled phage amplification coupled to mass spectrometry analysis using the model phage systems T4 and SPP1. The well-known lytic phages T4 and SPP1 were chosen for detection of Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria respectively. In this study simultaneous identification of phage structural and non-structural proteins was performed to be used as markers for indirect detection of bacteria.

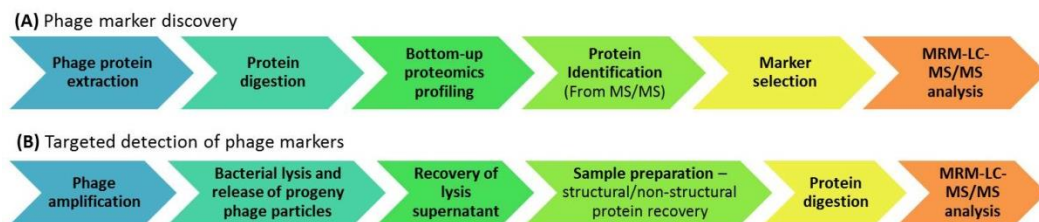


Figure 2. A general overview of “bottom-up” strategy used in detection and quantification of bacteria using isotope-labelled bacteriophage amplification coupled to liquid chromatography-multiple reaction monitoring tandem mass spectrometry. (A) Strategy for MS-based phage protein marker discovery. Phage proteins were extracted and digested into peptides for untargeted bottom-up analysis and identification using the Mascot algorithm. Results obtained provided suitable peptides for targeted marker detection with an MRM-LC-MS/MS method. (B) Strategy for the targeted detection of phage markers. Detection of phage markers was carried out after 2 h bacteriophage amplification in bacterial cells. Phage protein markers were recovered, digested, and analyzed using the MRM-LC-MS/MS method.

Mass spectrometry (including “bottom-up” and “top-down” approach) was used for the discovery of specific phage markers from phage-infected but non-lysed host cells. The advantage of this strategy is that all proteins encoded by the phage genome are available for in-depth analysis by high resolving power MS. Two protein families were identified as phage markers: structural proteins that are assembled in viral particles and non-structural proteins that are encoded by the phage genome but absent of the infective particles. Phage markers

that give the best signal amplification during phage infection of the targeted bacterium were selected mainly based on “bottom-up” approach. Intact proteins were also analyzed, by a targeted “top-down” analysis to confirm the robustness of selected markers for sensitive detection.

Two protein markers were chosen for each of the phage model. Internal protein I and small outer capsid protein were selected for T4 phage, and coat protein gp13 and scaffold protein gp11 were selected for SPP1 phage. Two peptides were selected for each marker, with the exception of SPP1 gp13, for which only one peptide was employed. Isotopically labelled form of each peptide was synthesized to serve as internal standard. Then, targeted detection of these markers was performed using LC-MRM-MS/MS. A new approach using unlabelled phage amplification for the detection of bacteria in media and complex food matrices was performed. A drawback with using unlabelled phages is the difficulty to distinguish proteins of the input phage added to infect bacteria from the progeny phages produced through infection. This method thus requires the amount of input unlabelled phage to be below the instrumentation detection limit but at the same time sufficient to infect bacteria for reproducible detection of progeny phage. Another weakness of using a low quantity of input phages is that it increases the infection time before successful analysis can be achieved. This problem can be solved by detecting an abundant non-structural protein. A high amount of input phage can be added to increase infection efficiency because the non-structural protein is absent in the infectious phage particle. Scaffold protein gp11 of SPP1 was selected as a non-structural protein for this study. *B. subtilis* and SPP1 lysis supernatants were analyzed using targeted MRM method. Protein gp11 was well-detected in the lysis supernatant obtained after infections. Hence, detection of non-structural proteins yielding high signal intensity functions as a new alternative for phage infection monitoring.

E. coli at 1.0×10^5 , 5.0×10^5 , and 1.0×10^6 CFU/ml concentrations was successfully detected in LB media, orange juice (liquid matrix), and French bean stew (solid matrix) respectively by monitoring phage T4 structural protein markers after 2 h infection. The limit of detection (LOD) of *E. coli* in complex food matrices was found to be 5.0×10^5 CFU/ml using this strategy with MRM monitoring.

2.3. Study 3 – Detection of *Escherichia coli* by Bacteriophage T7 (Banu et al., 2014)

Banu et al. modified the methods of Pierce et al. (2012) and Martelet et al. (2014) to specifically detect and quantify *E. coli* over a wider dynamic range of cell count using bacteriophage T7 in LB media, coconut water and apple juice. This was achieved by exploiting the biological amplification process based on the intrinsic lytic infection cycle of T7 phage for *E. coli* and using ^{15}N -labelled T7 as an internal standard. Bacteriophage T7 is a lytic phage that infects common strains of *E. coli* including the laboratory K-12 strains and pathogenic strains of O157: H7 (Molineux 2005).

Bacteriophage amplification is integrated with LC-MRM-MS/MS for quantitation of phage-specific peptides. In order to identify target phage proteins for quantification, trypsin-digested T7 samples were first subjected to a global proteomic profiling.

The target protein was selected based on the following criterion:

- 1) A robust protein hit with high signal intensity peptides that can be monitored using MRM;
- 2) Protein is unique to bacteriophage T7, and typically, this will be a structural protein which is more abundant than non-structural proteins.

From the shot-gun proteomic analysis the major capsid protein 10A was selected. “Best” peptide targets with high confidence scores were determined from the shotgun proteomic analysis of the digested major capsid protein using a separate hybrid high resolution mass spectrometer based on the orbitrap mass analyzer. Two peptides (SAQFPVLGR and AALTDQVALGK) were chosen for further quantification using LC-MRM-MS/MS.

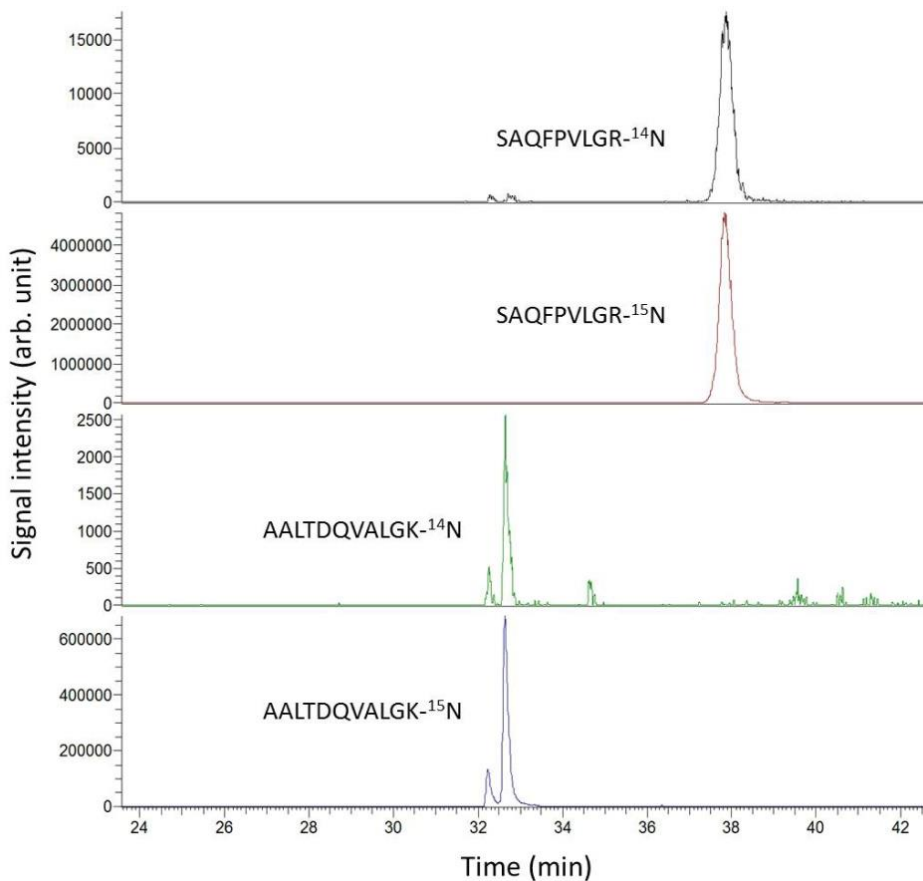


Figure 3. Extracted ion chromatograms from LC-MRM-MS/MS of SAQFPVLGR and AALTDQVALGK quantitative MRM transitions and their corresponding heavy isotopically-labelled forms showing similar retention time in ^{15}N bacteriophage T7.

The peptides were selected based on the following criterion:

- 1) High signal intensities;

- 2) Between 5 to 25 amino acids in length;
- 3) Unique for the target protein. In particular, the peptides should not be present in other organisms associated with water and food contamination.
- 4) Peptides are selected for optimal stability. Peptides that contain multiple adjacent cleavage sites such as arginine/lysine, lysine/lysine or arginine/proline, which are likely to give missed cleavage, and peptides containing cysteine and methionine are avoided as these residues are susceptible to modifications such as oxidation, which would affect the accurate peptide quantification and lead to inconsistent mass spectrometry analysis.

Heavy isotope ^{15}N -labelled bacteriophage T7 was introduced as the inoculum phage and internal standard. The ^{15}N -labelled SAQFPVLGR and AALTDQVALGK peptides are expected to shift by 13 Da compared to the unlabelled peptides as the ^{15}N -labelled peptides are heavier than naturally-occurring ^{14}N peptides. Transitions from the heavy isotope-labelled peptides were confirmed by having the same retention times of the ^{14}N -unlabelled T7 peptides in the LC run (Figure 3).

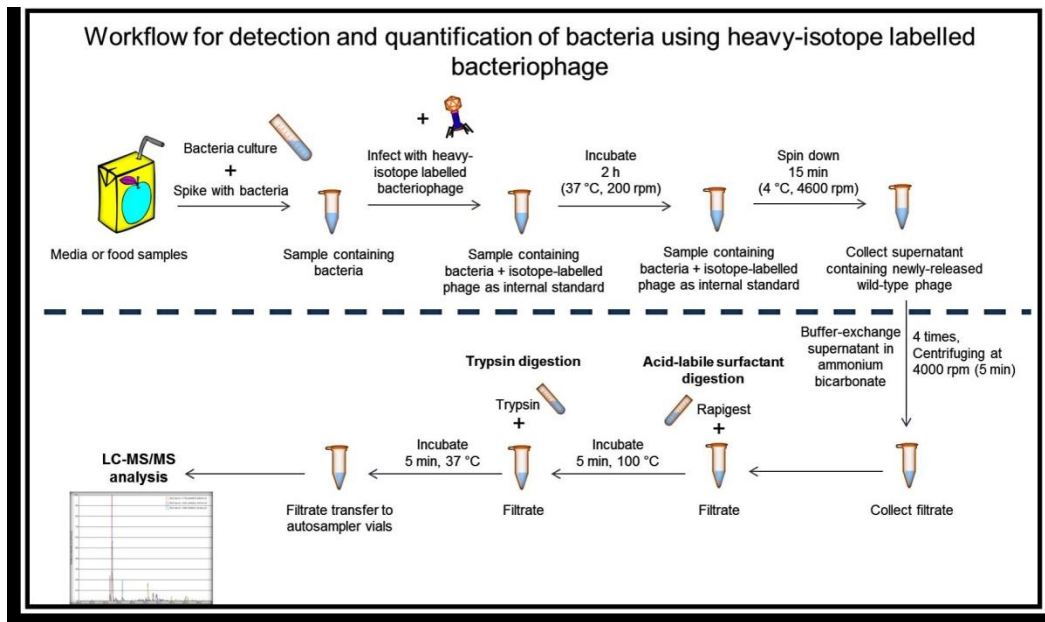


Figure 4. A simplified workflow of the method used for detection and quantification of bacteria using heavy isotope-labelled bacteriophage in media and food samples. Media or food samples spiked with *E. coli* were infected with 100 μl of ^{15}N bacteriophage T7 (1.0×10^9 PFU/ml) and incubated for 2 h. Samples were centrifuged and the supernatant containing phage particles was recovered and buffer-exchanged with four volumes of ammonium bicarbonate. The concentrated filtrate was collected for subsequent enzymatic digestion, followed by LC-MS/MS analysis.

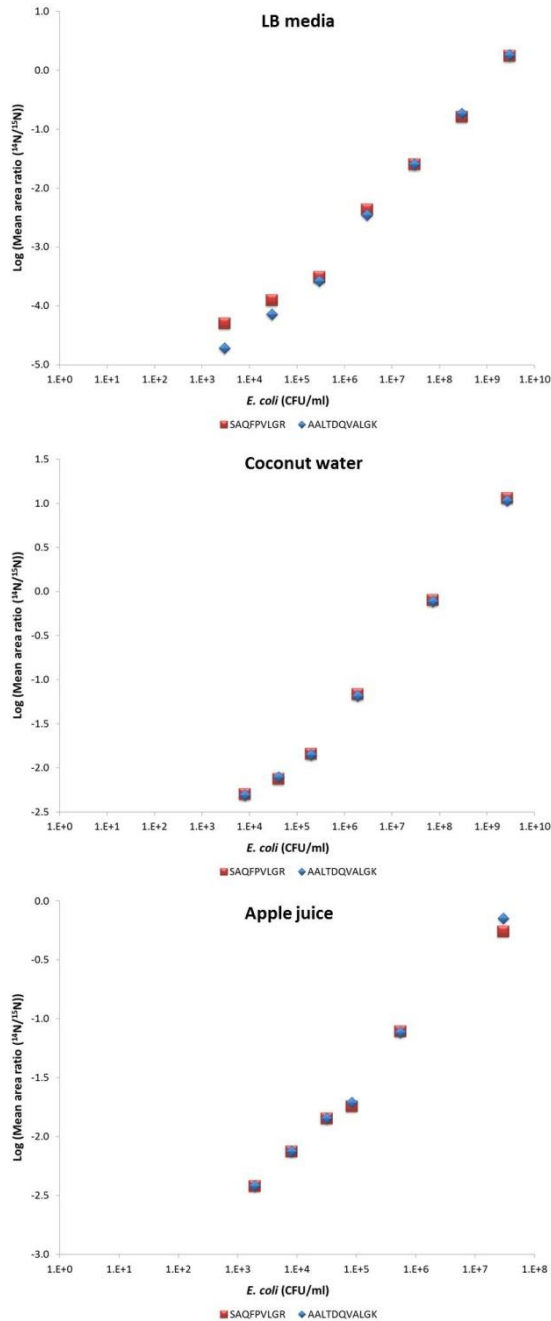


Figure 5. Quantification of *E. coli* in LB media, coconut water and apple juice using LC-MRM-MS/MS. Samples were spiked with serial dilutions of *E. coli* in LB media, coconut water and apple juice. Bacteriophage amplification was performed using an inoculum ^{15}N phage of $\text{MOI} \geq 5$ ($\leq 10^9$ PFU/ml). Mass spectrometry analysis were performed for each sample 2 h after infection and the log mean area ratios (^{14}N -unlabelled bacteriophage/ ^{15}N heavy isotope-labelled bacteriophage) for each quantitative peptide were plotted against *E. coli* numbers. The curves for SAQFPVLGR and AALTDQVALGK were linear (R^2 values 0.98-0.99) between 3.0×10^3 to 3.0×10^9 CFU/ml range in LB, 4.1×10^4 to 2.7×10^9 CFU/ml in coconut water and 1.9×10^3 to 3.0×10^7 CFU/ml in apple juice.

E. coli was spiked into samples at a concentration between 10^3 to 10^9 CFU/ml and a standard curve was constructed with decreasing *E. coli* numbers in LB media, coconut water and apple juice. The samples were spiked with ^{15}N inoculum bacteriophage at a standard concentration of 1.0×10^9 PFU/ml with an estimated multiplicity of infection (MOI) ≥ 5 . Multiplicity of infection is the ratio of infectious bacteriophage to bacteria. A high MOI is needed to ensure that at least one infective ^{15}N -bacteriophage to each *E. coli* bacterium even in the presence of a high numbers of dead *E. coli* cells. Under high MOI, it can be assumed that all metabolically active and viable bacteria are infected, leading to accurate quantification. Following 2 h incubation with inoculum ^{15}N bacteriophage, the samples were subjected to a rapid 5-min trypsin digestion, clean-up and analysis by liquid chromatography tandem mass spectrometric detection targeting peptides of both the ^{15}N and ^{14}N bacteriophage major capsid proteins (Figure 4). Quantification was based on peak areas of target peptides from the ^{14}N peptides that were produced by phage amplification in *E. coli* normalized to the ^{15}N -labelled T7 standard. The number of ^{14}N progeny bacteriophage will be proportionate to *E. coli* numbers. The more cells present in the sample, higher number of cells will be infected by ^{15}N inoculum bacteriophage. And the infected cells will release ^{14}N bacteriophage that is proportional to the number of infected cells. Therefore, bacteriophage amplification is expected to be proportional to bacteria numbers. The log of mean ^{14}N -unlabelled and ^{15}N -labelled mass spectrometry area ratios for each quantitative peptide was plotted against *E. coli* concentrations upon MRM analysis (Figure 5).

Table 2. Summary of the three studies using bacteriophage amplification coupled with LC-MS/MS for the detection and quantification of bacteria

	Study 1 (Pierce et al., 2012)	Study 2 (Martelet et al., 2014)	Study 3 (Banu et al., 2014)
Bacteria – bacteriophage used	Detection of <i>S. aureus</i> using bacteriophage 53	Detection of <i>E. coli</i> and <i>B. subtilis</i> using bacteriophage T4 and SPP1 respectively	Detection of <i>E. coli</i> using bacteriophage T7
Heavy isotope-labelling	^{15}N isotope labelled input phage	Unlabelled phage but used isotope labelled peptides	^{15}N isotope labelled input phage
Strategy used for protein identification	Bottom-up approach	Top-down & Bottom-up approach	Bottom-up approach
Number of proteins & peptides used for quantification	1 protein – 3 peptides	2 proteins – 2 peptides (structural & non-structural proteins)	1 protein – 2 peptides
Duration of enzymatic digestion	3 min trypsin digestion	O/N trypsin digestion	5 min trypsin digestion
Matrix used	LB media	LB media, bean stew, orange juice	LB media, coconut water, apple juice
Limit of Detection (CFU/ml)	$<5.0 \times 10^4$	Food: $<5.0 \times 10^5$	LB: 3.0×10^3 , Coconut water: 4.1×10^4 , Apple juice: 1.9×10^3

This approach could perform quantification of *E. coli* over a linear dynamic range of 6 log orders. A broad dynamic range of 6-log orders ranging from 3.0×10^3 to 3.0×10^9 CFU/ml is attained in LB media, while a range between 4.1×10^4 to 2.7×10^9 CFU/ml and 1.9×10^3 to 3.0×10^7 CFU/ml was enumerated in coconut water and apple juice respectively within 4 h (Figure 5). Regression analysis for quantification in LB media showed good linearity ($R^2 = 0.98-0.99$) over the 3.0×10^3 CFU/ml to 3.0×10^9 CFU/ml range. The LOD was as low as 3.0×10^3 CFU/ml, which is an improvement over the results of Pierce et al. (2012) who reported a LOD of 5.0×10^4 CFU/ml in LB media for *S. aureus*. Martelet et al. (2014) reported a LOD of 5.0×10^5 CFU/ml *E. coli* in orange juice using T4 phage. This study shows, that in apple juice using T7, the LOD for *E. coli* can be significantly lower at 1.9×10^3 CFU/ml. Linear regression analysis on the standard curve for coconut water and apple juice showed good linearity ($R^2 = 0.99$) over a broad dynamic quantification range (from 4.1×10^4 to 2.7×10^9 CFU/ml in coconut water and from 1.9×10^3 to 3.0×10^7 CFU/ml in apple juice) for both peptides.

This method offers rapid analysis time (less than 4 h total analysis time), sensitivity (LOD: 3.0×10^3 CFU/ml), specificity and dynamic quantification of bacterial cells across 6-log order of magnitude. This method can be a rapid tool for the diagnosis and control of bacterial contamination for food safety and public health.

CONCLUSION AND PROSPECTS

This chapter describes the development of an approach allowing the identification of bacteriophage proteins by exploiting the use of isotopically labelled phage amplification, then used as markers for indirect detection and quantification of living bacteria by LC- MRM-MS/MS. The coupling of heavy-isotope labelling bacteriophage amplification to MS-based method provides quantitative results with short detection times, enhanced specificity, and improved sensitivity (Table 2). MS-based method's current level of complexity makes it more compatible for specialized laboratories. However, isotopically labelled phage amplification coupled to MS offers unique capabilities that are well-matched to be incorporated into the workflow of microbiological laboratories easily. Mass spectrometers are also becoming more common in laboratories and this trend will likely to continue. These instruments are becoming simple to operate, have improved selectivity and sensitivity than traditional assays, and offer universal detection and multiplexing capabilities that support rapid analyses and a cost-effective investment. The simplicity of bacteriophage cultivation preparation and the low cost of reagents required make this approach a very practical outlook.

This approach is potentially transferable to virtually any bacterium and in particular, to foodborne pathogens such as *Salmonella*, *Listeria*, or *E. coli* O157:H7. This method can be a highly effective diagnostic tool for prevention and control of foodborne diseases. Moreover, it is possible to detect multiple bacterial pathogens in a single assay using a cocktail of specific phages. The MRM method described in this chapter combines the advantages of quantifying proteins from phage amplification in different bacterial environments. Detection of several phage amplification events using multiplexed MS analysis can be performed in a single run. It thus provides a versatile framework to develop detection assays of different bacteria in complex microbial communities like those found in food, environmental and clinical samples.

The low limit of detection (LOD: 3.0×10^3 CFU/ml) also suggests direct analysis from such samples. However, the presence of extraneous proteinaceous constituents, surfactants, salts and other impurities in the samples can affect the LC-MS/MS performance by suppressing chromatographic separation or molecular ionization. Phage-based sensing methods for detecting target bacteria also need analysis of phage specificity, especially when used in co-infected samples and complex, non-sterilized matrices, which contain competitive flora.

ACKNOWLEDGMENTS

The authors would like to thank the Biomedical Research Council of A*STAR (Agency for Science, Technology and Research) and A*STAR Joint Council Office (Grant No. 1131A018) for funding the project, and members of the Microbial Cells and Proteomics groups at Bioprocessing Technology Institute for their competent support and contribution. We are also grateful to Lim Pei Yu and Ho Pooi Leng for their insightful comments on the manuscript.

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Chapter 4

CULTURE BASED METHODS TO DETECT *SALMONELLA* FROM DIFFERENT POULTRY PRODUCTS

Mario A. Soria and Dante J. Bueno*

Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental
Agropecuaria, Concepción del Uruguay, Entre Ríos, Argentina

ABSTRACT

Salmonella infections from animal food play an important role in public health and particularly in food safety, as food products of animal origin are considered to be the major source of human *Salmonella* infections. The primary reservoir is the intestinal tract of animals and the colonization is favored by intensive animal production. Poultry products are the most frequent vehicles in the transmission of *Salmonella* infections. Eggs and poultry meat are recognized as the major vehicles of human illness due to the high prevalence of infection in poultry. Information needed to make food safety risk management decisions must be obtained from accurate risk assessments, which rely on the sensitivity of the isolation techniques used to identify *Salmonella*. Foods are complex matrices of fats, carbohydrates, proteins, preservatives, and other chemicals. Although hurdles associated with processing of a sample are overcome, foodborne pathogens are often present at extremely low levels, complicating the detection process. Another consideration is whether the examination is for routine monitoring or epidemiological purposes. The analyst may choose to augment the method for epidemiological purpose with additional enrichment procedures and culture media, two incubation temperatures, intensified picking of colonies from plates, and/or rapid screening methods. Therefore, better characterization of the *Salmonella* isolation and identification techniques will be warranted. Culture based methods are still the most widely used as detection techniques and remain the gold standard for the detection of *Salmonella* due to their selectivity and sensitivity. For instance, the US Food and Drug Administration (FDA), the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA), and the International Organization of Standardization (ISO) require an isolated *Salmonella* organism as unambiguous proof of contamination. Depending on the approach, standard

*Corresponding author: dantejb@yahoo.com.ar.

culture methods typically require 5–7 days to obtain a result as they rely on the ability of *Salmonella* to grow and for visible colonies, which can then be characterized by performing additional biochemical and or serological tests. Due to their widespread use, numerous and varied bacteriological media (selective enrichment broths and selective agar plates) are applied to best monitor *Salmonella* in food and food ingredients. The media and methods that are best for one particular sample type may not necessarily be optimal for other samples. Therefore particular procedures need to be evaluated for different type of samples. This chapter will provide an overview of various culture based methods currently available for the detection of *Salmonella* in different poultry product samples and agencies recommendations for these samples.

1. INTRODUCTION

Salmonellosis is an important zoonosis associated with food consumption of animal origin (EFSA 2012). The motile non-typhoid *Salmonella* serotypes, which include some 2,500 different serotypes, are widely distributed in nature, including in the gastrointestinal tracts of mammals. Most clinical infections of human are transmitted from healthy carrier animals to human through food. Several other factors influence *Salmonella* to cause a clinical infection in human. Strain to strain variation, the number of microorganisms, the physical and chemical properties of the vehicle, and host factor are all significant (Mølbak et al., 2006).

The reduction of the level of human illness from food-borne pathogens is a public health goal in many countries worldwide. Epidemiological studies show that poultry meat and eggs are important sources for consumer exposure to zoonotic pathogens such as *Salmonella*, which are involved in human salmonellosis outbreaks, constituting an important threat to public health (Dunkley et al., 2009; Luber 2009; Marin and Lainez 2009). The prevalence of *Salmonella* in eggs is usually much lower than in broiler chickens and meat. Nonetheless, eggs are commonly recognized source of *Salmonella* outbreaks. This is related to the fact that eggs are often served raw or undercooked and may be pooled during the preparation of dishes, thereby exposing to a large number of people (De Medici et al., 1998; Mølbak et al., 2006).

Many serious factors are implicated in the occurrence of *Salmonella* in broiler flocks, such as inadequate cleaning and disinfection, and presence of rodents and insects. Moreover, cross-contamination during processing and inadequate sanitation in slaughterhouse together with the consumption of undercooked poultry meat could serve as the most frequent causes of infection by *Salmonella* reported in humans (Zdragas et al., 2012).

Often, microorganisms in food are referred as “the good, the bad, and the ugly.” The bad ones cause spoilage, and the ugly ones cause human illness. Determining the type of microorganisms in a food is an important aspect for food microbiology (Montville and Mathews 2008). Foods come in many physical forms (powder, liquid, gel, solid, semi-solid, etc) and contain different combination of ingredients like carbohydrates, proteins, fats, oils, and chemicals, some of which can interfere with mixing, resulting in heterogeneous samples (Feng 2006). Microbiological methods can differ widely in their comparative advantages and disadvantages. These relative benefits and limitations may influence the choice of method for a particular task (Blackburn 2007).

A large variety of different media for isolation and detection of *Salmonella* are available, and many different combinations of media and culture conditions have been developed to

isolate *Salmonella* from different types of products and samples. It is, however, important to note that there is no single method that is optimal to all type of samples. Finding the best method for a given type of sample may require extensive literature studies, and often comparative testing of different methods (Van der Zee 2003; Mølbak et al., 2006). Due to their widespread use, numerous and varied bacteriological media (selective enrichment broths and selective agar plates) are applied to best monitor *Salmonella* in food and food ingredients. The media may contain inhibitors in order to stop or delay the growth of non-target organisms, or particular substrates that only the target bacteria can degrade, or that confer a particular color to the growing colonies (Manafi 2000).

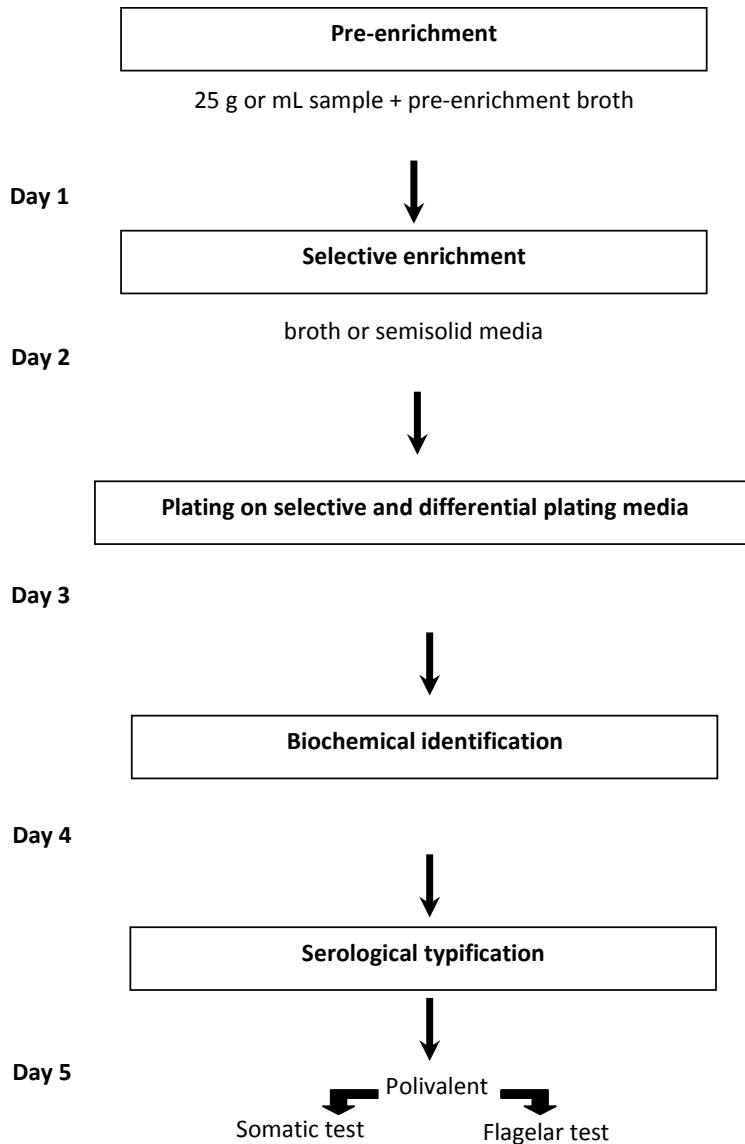


Figure 1. Flow chart of analysis for detection of *Salmonella* in foods using presence/absences method.

The conventional technique for *Salmonella* isolation in samples, when there are low initial cell numbers, or when the cells are stressed due to physical or chemical injury, requires the following steps: pre-enrichment in non-selective broth, enrichment in selective broth or semisolid media, isolation on selective and indicative (differential) agar media, biochemical characterization, serological characterization and final identification (Figure 1). This technique is applicable to most environmental and food samples, and requires at least four days for a negative result and six to seven days for the identification and confirmation of positive samples. The presence of *Salmonella* has to be determined in at least 25g or mL of sample (Soumet et al., 1997; Van der Zee 2003; Mølbak et al., 2006; Schönenbrücher et al., 2008; Castiglioni Tessari et al., 2012).

Culture based methods are still the most widely used detection techniques and remain the gold standard for the detection of *Salmonella* due to their selectivity and sensitivity. Different organizations, such as the International Organization of Standardization (ISO), the American Association for Analytical Chemist (AOAC), the US Food and Drug Administration (FDA), the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA), the International Dairy Federation (IDF), American Public Health Association, British Standards (BSI), French Standards Association (AFNOR), and the Nordic Committee on Food Analysis (NMKL) have developed reference methods for the isolation of *Salmonella* (Table 1). These methods require an isolated organism as unambiguous proof of *Salmonella* contamination in food (ISO 2002; Andrews et al., 2014; FSIS/USDA 2014).

As the conventional method for *Salmonella* detection are so laborious and time consuming, more rapid method have been developed. Often these methods are a combination of an enrichment culture step and a polymerase chain reaction (PCR) and/or an enzyme-linked immunosorbent assay (ELISA) method (Mooijman 2012).

Begum et al. (2010) reported only 0.3% were positive to *Salmonella* (one was *Salmonella* Typhimurium-ST- and 2 were *Salmonella* Enteritidis –SE-) from 1,000 poultry eggs, using the FDA/BAM methodology. Donado-Godoy et al. (2012) showed 27% prevalence of *Salmonella* in retail market chicken carcass-rinses in Colombia tested, using methodology recommended by the USDA/FDA. In contrast, Jiménez et al. (2002) reported 20% prevalence in carcasses in Argentina and Fruzhara et al. (2000) showed a 42% in Brazil. The differences in *Salmonella* detection or prevalence among these studies could be attributed to difference in sampling scheme, and the *Salmonella* detection protocol. Therefore, this chapter will provide an overview of various culture based methods currently available for the detection of *Salmonella* in different poultry product samples. Organizations recommendations for these kinds of samples are also included.

2. ENRICHMENT

Typically, samples that have been dried, heated, irradiated or otherwise processed require the use of non-selective pre-enrichment for optimal recovery of *Salmonella*. In these samples, *Salmonella* may be present, but are ‘damaged’ or ‘sublethally injured’ (Corry et al., 1969).

Enrichment is a critical step of presence/absence tests for three reasons:

- a. The population of pathogens in the samples is normally lower than the detection limit of plate counts. Therefore, it is necessary to increase the number of cells to detectable quantities.
- b. In most industrially processed foods, the cells of the target microorganism are injured by processing, thus require a period of time under optimal growth conditions to reactivate the metabolic pathways responsible for multiplication.
- c. Normally, the competing microflora in the sample is present in much higher numbers than the target microorganism, making it necessary to inhibit the growth of this population in order to give the target an opportunity to multiply.

For *Salmonella* monitoring tests, enrichment is done in two steps. The first is common to call pre-enrichment or primary enrichment, while the second is called selective enrichment (Da Silva et al., 2013).

2.1. Pre-Enrichment

The objective of pre-enrichment is to repair injured cells, offering conditions for their recovery, rehydration, and dilution of toxic or inhibitory substances; but at the same time, without favoring too much the growth of competing microflora. In general, injured cells do not grow under highly selective conditions, and for that reason, pre-enrichment broths are normally either not selective or only moderately selective (Waltman 2000; Gaitan Herrera 2001; Da Silva et al., 2013). Injured cells may be defined as a loss of the reproductive and/or metabolic capability of microbial cells following exposure to some nonsterilizing treatment. This loss is evidenced by the failure of injured cells to grow or reproduce under cultural conditions that are satisfactory for unexposed cells (Chipley 1987).

The optimal growth temperature of *Salmonella* is about 37°C. However, with naturally contaminated samples the selective benefits of incubation at higher temperatures may offset the reduced growth potential. Incubating the pre-enrichment for 18-24 h is recommended to allow resuscitation of *Salmonella* before transferring into selective-enrichment media. The recommended incubation temperature for pre-enrichment is 35-37 °C. Since the purpose of pre-enrichment is the resuscitation of damaged *Salmonella*, a higher temperature should be avoided (Waltman 2000).

Several pre-enrichment media have been advocated: nutrient broth (NB), lactose broth (LB), buffered peptone water (BPW), trypticase soy broth (TSB), Salmosyst Broth Base (SBB), and Universal Pre-Enrichment Broth (UPEB). NB is used for the cultivation of many species of nonfastidious microorganisms (Anonymous 2009). In spite of LB was, perhaps, the first to receive widespread use, the fermentation of lactose and resulting acidity would allow the pH to fall to a level that is inhibitory or lethal to *Salmonella* (Waltman 2000). Other pre-enrichment media have been formulated without a fermentable sugar and with greater buffering capacities, BPW (Bayle and Cox 1992). Juven et al. (1984) compared LB and BPW and found that BPW were better than LB. The pH of the LB cultures after incubation ranged from 4.8 to 5.5, whereas the ranges for BPW were 5.8 to 6.4.

Sometimes for specific cases, modified pre-enrichment procedures are required, e.g., when attempting to shorten the pre-enrichment phase (Fung et al., 1993) or investigating eggs and eggs products for SE (Van der Zee 2003). The addition of ammonium-iron (III)-citrate to BPW facilitated the isolation of salmonellae from eggs and egg products, and in particular SE from the yolk of fresh hen-eggs. In the presence of the iron-chelating albumen protein ovotransferrin, pre-enrichment of pooled egg contents in TSB, supplemented with 35 mg/l ferrous sulphate (TSBF) has been recommended to ensure adequate iron availability to support bacterial growth (Gast 1993, Gast and Holt 1995). Soria et al. (2012) compared BPW and TSBF based on ISO 6579 and FDA/BAM methodology in yolk and albumen contaminated artificially by motile non-typhoid *Salmonella* strains, and they reported that detection limit were the lowest dilution strains tested for both culture methods (5 to 54 cfu/25 mL) of each technique.

Table 1. Example of culture media for *Salmonella* isolation from food, proposed by different regulatory organizations (Herrera 2001; Van der Zee 2003; Mølbak et al., 2006; Da Silva et al., 2013)

Organization	Culture media		
	Pre-enrichment (broth)	Enrichment (broth)	Plating Media (agar)
International Organization for Standardization (ISO 6579:2002)	Buffered Peptone Water	Rappaport-Vassiliadis soya; Muller Kauffmann Tetrathionate Novobiocin	Xylose Lysine Desoxycholate, 2 nd optional
Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA)	Buffered Peptone Water, Trypticase soy, Universal Preenrichment	Rappaport-Vassiliadis or Rappaport-Vassiliadis soya; Tetrathionate Hajna	Brilliant Green Sulfa; Xylose Lysine Tergitol 4 or Double Modified Lysine Iron
US Food and Drug Administration	Lactose, Trypticase soy	Rappaport-Vassiliadis; Tetrathionate	Hektoen Enteric; Xylose Lysine Desoxycholate, Bismute Sulfite
International Dairy Federation (ISO 6785 IDF 093:2001)	Buffered Peptone Water	Tetrathionate; Selenite Cystine	Brilliant green; Bismute Sulfite
American Public Health Association	Lactose	Selenite Cystine; Tetrathionate	Salmonella-Shigella; Brilliant green; Hektoen Enteric
British Standards (BSI)	Buffered Peptone Water	Rappaport-Vassiliadis; Selenite Cystine	Brilliant green or other media
French Standards Association (AFNOR)	Buffered Peptone Water	Rappaport-Vassiliadis; Selenite Cystine	Brilliant green; Xylose Lysine Desoxycholate; Hektoen Enteric; Deoxycholate citrate
Nordic Committee on Food Analysis (NMKL)	Buffered Peptone Water	Rappaport-Vassiliadis	Brilliant green; Xylose Lysine Desoxycholate

Stephenson et al. (1991) reported that LB was less productive than TSB, or BPW; and that TSB was the best for *Salmonella* recovery from eggs yolks. Hara-Kudo et al. (2001) reported that a procedure including BPW was superior to other broths for liquid egg samples both naturally and artificially contaminated with *Salmonella*. Zhang et al. (2013) compared the effectiveness of pre-enrichment broths TSB, TSBF, and BPW for the detection and isolation of *Salmonella* from shell eggs. TSB was proven to be superior to the other broths studied.

On the other hand, Salmosyst Broth Base (SBB) is a non-selective pre-enrichment where all microorganisms present in the sample material grow. Following the addition of the selective reagents in the form of a Salmosyst Selective Supplement tablet (SSST), the growth of the accompanying organisms is inhibited, but the salmonellae continue to grow (Anonymous 2012). Pignato et al. (1995) evaluated the use of SBB in artificially contaminated food (chicken parts, and eggs) with a pure culture of 4 motile serotypes of *Salmonella* that were exposed to heat injury. These authors found that limit detection between 10^0 and 10^2 cfu/mL, when the pre-enrichment was followed by an enrichment carried out with a SSST.

Another traditional broth to recover *Salmonella* spp. from food is the Universal Pre-Enrichment Broth (UPEB) formulated by Bailey and Cox (1992) to permit resuscitation of sublethally injured *Salmonella*. The broth medium provides sufficient buffering capacity to prevent rapid decreases in pH, and allows for repair of injured cells that might be sensitive to low pH values or inhibitory substances. The UPEB is used as the pre-enrichment for *Salmonella* in food different from poultry products (orange and apple juice, cider, cantaloupes, tomatoes, and mamey pulp) according to FDA/BAM methods (Andrews et al., 2014).

2.2. Selective Enrichment

The objective of selective enrichment is to inhibit the competing microflora present in the samples and to enhance the growth of the selected microorganism. This is achieved by using selective agents and/or restrictive conditions for the growth of the competing flora, which may include: the pH of the culture medium, the temperature and/or atmosphere of incubation, the addition of antibiotics (polymixin B, novobiocin, vancomycin) and the addition of chemical compounds (brilliant green, sodium selenite, bile salts).

The ideal selective enrichment should repress competing organisms and allow the *Salmonella* to multiply without restriction (Gaitan Herrera 2001; Van der Zee 2003; Da Silva et al., 2013). Several enrichment media have been advocated. Three types of selective enrichment media are in common use: tetrathionate, selenite and Rappaport (Waltman 2000; Van der Zee 2003).

The tetrathionate broth (TT) base is used as a selective enrichment for the cultivation of *Salmonella* species that may be present in small numbers and compete with intestinal flora. *Salmonella* organisms may also be injured in food-processing procedures although injured cells may not form colonies on selective media, but they can, if ingested, cause disease. TT was originally described by Mueller who found that the medium selectively inhibited coliforms, thereby permitting enteric pathogens to grow virtually without restriction. The

enzymatic digest of casein and enzymatic digest of animal tissue provides nitrogen, carbon, vitamins, and amino acids in TT. The selectivity is accomplished by the combination of sodium thiosulfate and tetrathionate, which suppresses commensal intestinal organisms. Tetrathionate is formed upon addition of the iodine and potassium iodide solution in the medium. Organisms which possess the enzyme tetrathionate reductase will proliferate in the medium. Bile Salts, a selective agent, suppress coliform bacteria and inhibits Gram-positive organisms. Calcium carbonate neutralizes and adsorbs toxic metabolites (Knox et al., 1942; Harvey and Price 1979; Anonymous 2009).

There are several formulations of TT broth. These include Mueller-Kauffmann tetrathionate (MKTT) as formulated by Mueller (1923), who described that combined iodine and sodium thiosulphate form TT. Kauffmann modified this medium in 1935, adding brilliant green dye and bile salts as selective agents. Jeffris (1959) modified the TT enrichment by adding 40 µg ml⁻¹ novobiocin (MKTTn). Hajna and Damon (1956) modified the conventional TT by adding yeast extract as a growth stimulant, glucose and mannitol, decreasing the bile salts concentrations and increasing the sodium thiosulphate concentration. The TT-Hajna is recommended in the FDA/BAM method for the environmental sampling and detection of *Salmonella* Enteritidis in poultry houses. The selectivity of TT media depends on the presence of the enzyme tetrathionate reductase in the selected microorganism. *Salmonella* spp. as well as some other organisms such as *Proteus* spp. possess the enzyme, therefore *Proteus* spp. growth has to be suppressed by the addition of novobiocin and/or brilliant green to the medium (Van der Zee 2003). On the other hand, the National Poultry Health Plan in Argentina (National Agrifood Health and Quality Service 2002) recommends the incubation of TT broth with novobiocin at 37°C, or at 42°C if this antibiotic is not used.

Leifson (1936) formulated the first selenite enrichment, commonly known as selenite F (SF). North and Bartram (1953) modified SF by adding cystine (selenite cystine -SC-). Stoke and Osborne (1955) modified SF by changing the carbohydrate source from lactose to mannitol and adding sodium taurocholate and brilliant green (selenite brilliant green -SBG-). The formulation was changed to suppress the overgrowth of *Proteus* spp. and coliforms. Later, it was found that SBG did not work well with eggs samples. Some disadvantages that have resulted in less frequently used of selenite enrichment media include their relative shorter-life in comparison to TT or Rappaport-Vassiliadis (RV) enrichments (Waltman 2000). Also the potential workplace hazard due to their toxic effect, sodium acid selenite is reduced to the toxic heavy metal selenium, which has been shown to be toxic to embryos, and produce growth abnormalities and damages to kidneys, liver and spleen (Robertson 1970; Andrews 1996; Goyer 1996).

The SC broth is used as a selective enrichment medium for the isolation of *Salmonella* from feces, foods, water and other materials of sanitary importance. North and Bartram (1953) modified SF by adding cystine (selenite cystine -SC-). It had been reported by Leifson (1936) that selenite selectivity was enhanced under reduced conditions. SC broth performs better than SF in the presence of organic material (Waltman 2000). Ramya et al. (2012) founded that the incident of *Salmonella* was 56% in 25 chicken meats tested by using BPW as pre-enrichment; TT, SF, SC, and RV as enrichment, where RV and TT broths were superior over SF and SC broths.

The Gram negative broth Hajna (GNHB) was developed by Hajna as an enrichment medium for the recovery of *Salmonella* and *Shigella* from clinical and nonclinical specimens.

GNHB currently is recommended for use in the microbiological examination of foods. The peptones provide amino acids and other nitrogenous substances to support bacterial growth. Mannitol and dextrose are sources of energy. Mannitol is provided in a higher concentration than dextrose to enhance the growth of mannitol-fermenting species, such as *Salmonella* and *Shigella*, and limit the growth of *Proteus* and other dextrose-fermenting bacteria. Phosphate buffers are incorporated to maintain the pH of the medium. Sodium citrate and sodium desoxycholate are added to inhibit gram-positive and some gram-negative bacteria. *Proteus*, *Pseudomonas* and coliforms do not overgrow *Salmonella* and *Shigella* in GNHB during the first 6 hours of incubation (Anonymous 2009). Cox et al. (1972) found that GNHB was less effective than RV and SC broths for isolating *Salmonella* from egg, and meat.

The Rappaport medium contained malachite green and magnesium chloride as selective agents (Rappaport et al., 1956), based on the ability of *Salmonella* to survive relatively high osmotic pressures (concentration of hexahydrate magnesium chloride of 28.6 g/L in the final medium); multiply at relatively low pH (pH 5.2); survive in malachite green and grow with minimal nutritional requirements. Later, Vassiliadis et al. (1981) modified the medium by reducing the concentrations of malachite green. A modification, using soya peptone instead of tryptone, was reported to improve recovery rates of *Salmonella* (Van Schothorst and Renaud 1983; Van Schothorst et al., 1987) and is in use as Rappaport-Vassiliadis Soya peptone (RVS) broth.

Modification of selective enrichment procedures can include the use of motility enrichment media like the semi-solid *Salmonella* media Modified Semisolid Rappaport Vassiliadis (MSRV) medium and Diagnostic *Salmonella* Medium (DIASALM) (De Smedt et al., 1986; Holbrook et al., 1989). The use of MSRV and DIASALM are widely recognized as effective methods for detecting motile salmonellae in contaminated products. MSRV medium showed that a semi-solid medium in Petri dishes could be used as a rapid and sensitive test for isolating motile *Salmonella* from food products following pre-enrichment or selective enrichment. The semisolid medium allows motility to be detected as halos of growth around the original point of inoculation when is incubated at 41.5-42°C for only 18-24 h (Davis and Wray 1994; Wiberg and Norberg 1996, Anonymous 2009).

Delayed secondary enrichment (DSE) is the process whereby the original selective-enrichment broth (usually TT) is held at room temperature after the initial 24 h incubation and subsequent culture. If the initial subculture is negative for *Salmonella*, the enrichment broth is left at room temperature for 5–7 days (Waltman 2000). Moreover, Waltman et al. (1993) compared incubation for 24 h and 48 h and with a 3-day and 5-day DSE. The number from which *Salmonella* were isolated was 32, 48, 58 and 65, respectively. Incubation times of 24 and 48 h would have failed to detect 55% and 32% of the total *Salmonella*, respectively. This study also showed that the longer, 5-day, DSE was better than the shorter, 3-day, DSE. On the other hand, it was reported that an incubation time longer than 24 h was more important (more positive results after 48 h) for selective enrichment medium in chicken meat and in poultry and poultry environmental samples (Waltman et al., 1991; Kuijpers et al., 2008). Soria et al. (2012) incubated the TT and MKTTn broth at $35 \pm 2^\circ\text{C}$ for 5 days, but they could not find any difference for *Salmonella* isolation between these two media.

3. ISOLATION IN SELECTING DIFFERENTIAL PLATING (TABLE 2)

The objective of isolation in differential plating is to differentiate and separate the selected or target microorganism from the competitive microflora. This is done by inoculating the culture on a solid medium, which also allows obtaining pure cultures to be later used in tests to confirm microbial identity. In general, isolation media are selective and differential to suppress part of the competing microflora and distinguish the target from the remaining microorganism (Gaitan Herrera 2001; Hanes 2003; Da Silva et al., 2013).

The main components of selective isolation media include (Waltman 2000; Van der Zee 2003; Da Silva et al., 2013):

- a. Nutrient substrates: soya or met peptone, yeast extract and/or carbohydrates (lactose, mannitol, xylose, etc).
- b. Selective agents: bile salts, deoxycholate, brilliant green, novobiocin, etc.
- c. Dyes and indicators: bromothymol blue, and phenol red.
- d. Detectors: ferric citrate, ammonium ferric citrate or ammonium ferric sulphate, which stain colonies black by reacting with hydrogen sulphide. These compounds produce ferrous sulphide, a black and soluble precipitate that diffuses into the growth medium causing blackening of the medium.

Table 2. Color of *Salmonella* colony on selective–differential agar

Media	Color of <i>Salmonella</i> colony
Bismuth sulphite agar	Black colonies surrounded by a brown to black zone that casts a metallic sheen
Brilliant green agar	Pink colonies surrounded by red zone
Eosin-methylene agar	Translucent amber to colorless colonies
Salmonella-Shigella agar	Colorless colonies, black centered due to H ₂ S production
Mac Conkey agar	Colorless, transparent colonies
Xylose lysine desoxycholate agar	Black centered red colonies with or without H ₂ S production
Xylose lysine tergitol 4 agar	Yellow to red with black centre
Deoxycholate citrate agar	Colorless with black centered due to H ₂ S production
Hektoen enteric agar	Blue to blue green colonies, most with black centers (H ₂ S)
EF-18 agar	Green, blue-green, or blue
Double modified lysine iron agar	Clear colonies with black centers (H ₂ S)
Salmonella agar acc. to ÖNÖZ	Yellow with black center
Rambach agar	Red

Some of the selected microorganism strains may be sensitive to the selective conditions of plate culture media. In these cases, it is a common practice to use more than one medium (Waltman 2000; Van der Zee 2003; Da Silva et al., 2013). Each should contain different selective agents and indicator systems or differential characteristics in order to detect a wide range of *Salmonella* serotypes from different situations (Fricker 1987; Andrews 1996, Waltman 2000). Temelli et al. (2010) evaluated the *Salmonella* serogroup detection in poultry meat samples by examining multiple colonies from selective plates following the FDA/BAM and ISO 6579 methods. They founded multiple serogroups (D and E4; B and C2; C1; G; and E1 and F) and concluded that a selective plate for *Salmonella* culture method can harbor more than one serogroup, and that the FDA and ISO methods could detect different serogroups from chicken and turkey meats. On the other hand, they suggested screening multiple suspect colonies from each plate, if possible, and considering the collective and comparative use of the FDA and ISO culture methods and/or including several selective and differential media to ensure the detection of *Salmonella* and the possible detection of multiple serogroups from samples.

Selective and differential media used for isolation of *Salmonella* include different plates agar media like: Bismuth sulphite (BS) agar, *Salmonella-Shigella* (SS) agar, Xylose lysine desoxycholate (XLD) agar, Deoxycholate citrate agar (DCA), Brilliant green agar (BGA). These media are highly selective media and may inhibit growth of some *salmonellae* isolates. Several media are less selective, but primarily differential, like MacConkey (MC) agar, Hektoen enteric (HE) agar, and eosin-methylene (EMB) agar. Plating media should be incubated at 35-37°C for 18-24 h (Waltman 2000; Herrera 2001; Hanes 2003; Van der Zee 2003).

The BS agar is a strongly selective and differential medium for *Salmonella*, including *S. Typhi* isolated from food. Typical *S. Typhi* surface colonies are black, surrounded by black or brown-black zone with or without a metallic sheen. This zone may be several times the size of the colony. Other strains of *Salmonella* produce black to green colonies with little or no darkening of surrounding medium. The metallic shining surface characteristic feature of *Salmonella* spp. colonies is the result of the production of hydrogen sulfide, forming a metallic-black sheen in reaction with iron ions. The growth of Gram-positive bacteria and other *Enterobacteriaceae*, including *Shigella* spp., are strongly inhibited by brilliant green and bismuth sulfite present in the medium (Van der Zee 2003; Salem Imen et al., 2012). A major disadvantage of BS agar involves its stability; some investigators recommend using within a few days of preparation, while others recommend “ageing” the medium (Waltman 2000). Moats (1981) found inhibitory properties of BS agar during storage. Therefore, *Salmonella* have been shown to grow better on BS agar which had been storage in the refrigerator for few days.

Since there are *Salmonella* strains that ferment lactose or do not produce H₂S, it is important that the selection of the second or third plating medium should not be based on any of these two characteristics. One of these options is BGA, which is based on the fermentation of lactose, but not on the production of H₂S, and BS agar, which is based on the production of H₂S (colonies appearing black with a characteristic black sheen), but not on the fermentation of lactose (Waltman 2000; Da Silva et al., 2013). On the other hand, the selectivity of the BGA derives from the presence of brilliant green dye, lactose and sucrose, which are the basis for the differential capabilities of the media. Almost all *Salmonella* fail to ferment either

lactose or sucrose and their colonies appear either pink to red, with reddening of the media (Waltman 2000).

Eosin methylene blue (EMB) agar was developed by Holt-Harris and Teague. The medium contains lactose and sucrose with two indicator dyes, eosin Y and methylene blue. The use of these two indicators produced sharp and distinct differentiation between colonies of lactose fermenting and nonfermenting organisms. Sucrose is included to detect coliforms that ferment sucrose more readily than lactose. EMB agar is selective due to the presence of an inhibitor and differential based on the ability of some organisms to ferment carbohydrates with the absorption of an eosin Y and methylene blue complex (Holt-Harris and Teague 1916, Anonymous 2009). Levine (Levine EMB agar) described a modification, which he claimed gave better differentiation between *Escherichia* and *Enterobacter* species. Both agars differ in that Levine EMB agar does not contain sucrose (Anonymous 2009). Colonies of lactose fermenters are visualized as blue-black with or without a green metallic sheen. *E. coli* colonies typically are dark centered and usually have a green metallic sheen. Colonies of *Salmonella* and *Shigella* are colorless, transparent or amber (Anonymous 2009).

SS agar was formulated to inhibit coliforms, while allowing the recovery of *Salmonella* and *Shigella*. The selective agents include bile salts brilliant green dye and, to a lesser degree, sodium citrate. The differential agents are lactose fermentations and the presence of an H₂S indicator system. Typical *Salmonella* colonies are colorless with black centers.

MC agar use bile salts and crystal violet as selective agents. However the concentration of bile salts is lower than in other media. The differential ability of MC agar lies in the fermentation of lactose, most *Salmonella* do not ferment lactose and, as a consequence, the colonies are colorless and translucent.

The selective agent of XLD agar is a sodium desoxycholate and the differential ability comes from the combination of the sugars xylose, lactose, and sucrose, the amino acid lysine and H₂S indicator system. Most *Salmonella* ferment xylose, but not lactose and sucrose, decarboxylate lysine and produce H₂S, and typical *Salmonella* colonies are red with black center (Waltman 2000). Bayu et al. (2013) studied the presence of *Salmonella* species in whole eggs purchased from local markets in Addis Ababa, Ethiopia by using a modified protocol of ISO 6579 (2002). They founded, from 384 samples, 5.21% positive samples for *Salmonella* Enteritidis, using SC and RV broth as liquid media and XLD, MC and SS agar for selecting differential plating media.

Xylose-Lysine-Tergitol 4 (XLT-4) agar is an XLD modification, where the sodium desoxycholate found in the XLD media is replaced by tergitol 4. The surfactant tergitol 4 (7-ethyl-2-methyl-4-undecanol hydrogensulphate sodium salt) is added to the xylose-lysine agar base as a selective inhibitor of *Proteus* species and other non-*Salmonella* organisms (Miller et al., 1991; 1995). *Salmonella* spp. appear as black colonies, and can be easily differentiated from colonies of *Citrobacter* spp. which appear as yellow colonies and are reduced in sizes on the XLT-4 medium.

On the other hands, DCA is a medium to isolate *Salmonella* and other enteric pathogens, such as *Shigella*. The selectivity of DCA involve sodium deoxycholate and to a lesser extent, sodium citrate. The presence of lactose and an H₂S indicator system provide the differential characteristics of the agar. Since *Salmonella* usually do not ferment lactose, but produce H₂S, their colonies are clear with black center (Waltman 2000).

The HE agar for the isolation of *Shigella* and *Salmonella* was originally developed by King and Metzger (1968) from the Hektoen Institute of Medicine (Chicago, USA). It relies on

the use of bile salts for selective inhibition and two indicator systems: (i) bromothymol blue and acid fuchsin as indicators of carbohydrate dissimilation (involve the sugars lactose, sucrose, and salicin) and (ii) ferric iron as an indicator of the formation of hydrogen sulfide from thiosulphate. HE agar allows good growth of *Shigella* spp. because the bile salt inhibition of these organisms is reduced by the addition of relatively large amounts of peptone and carbohydrates. The medium provides good colonial differentiation and inhibits some coliforms and other nonlactose-fermenting bacteria, thereby facilitating the identification of *Salmonella* and *Shigella* from food products. Most *Salmonella* do not ferment the three sugars, but produce H₂S, which results in bluish-green colonies with black center (Waltman 2000; Van der Zee 2003).

The EF-18 medium is highly selective and contains bile salts, crystal violet, sulphapyridine and novobiocin. The differential properties are conferred by the presence of sucrose and lysine, and *Salmonella* colonies appear blue-green (Waltman 2000). Warburton et al. (1994) encountered problems with the use of this medium for *Salmonella* isolation, because the colonies were reduced in size and overgrown by other bacteria.

The Double Modified Lysine Iron agar (DMLIA) is recommended for the selective and differential isolation of *Salmonella* spp. from food. Edwards and Fife designed LIA in 1961 to presumptively identify *Salmonella* species, including lactose fermenting *Salmonella* Arizonae, which has been implicated in food-borne outbreaks of gastroenteritis (Edwards and Fife 1961). However, some *Salmonella* spp. could be overlooked on LIA due to their atypical appearance and the growth of non-salmonella *Enterobacteriaceae*. LIA was modified to provide better detection of hydrogen sulfide-positive and -negative strains through the addition of novobiocin, bile salts, lactose, and sucrose. Differentiation of *Enterobacteriaceae* on DMLIA is based on a color change from purple to yellow if lysine is not decarboxylated and lactose or sucrose or both are fermented. Typical salmonellae decarboxylate lysine and do not ferment lactose or sucrose; therefore the purple color of the medium is maintained. H₂S-positive salmonellae grow with black-centered colonies (Rappold and Bolderdijk 1979). Rappold and Bolderdijk (1979) used DMLIA in routine analysis of food together with BGA, deoxycholate lactose agar, HE agar, and XLD agar. Nineteen percent of isolations were made from DMLIA exclusively; in 36%, DMLIA showed more *Salmonella* colonies than the other media; and in 45% of the isolations all media gave equal results. The USDA FSIS lists DMLIA as an alternative to Brilliant green sulfa agar and XLT-4 agar as a means of identifying *Salmonella* spp. (United States Department of Agriculture, 2014).

Other medium used for *Salmonella* isolation is *Salmonella* agar according to ÖNÖZ (SAO). This medium was originally published as enabling an immediate distinction of *Proteus* and *Citrobacter* and increasing positive *Salmonella* yield from feces specimens compared with Leifson agar and SS agar (Onöz and Hoffmann 1978). The growth of Gram-positive bacteria is almost completely inhibited while lactose- and sucrose-positive *Enterobacteriaceae* are partially suppressed. Furthermore, their colonies can be differentiated, by means of the different shades of color produced, in the presence of the indicators neutral red and aniline blue. *Proteus* colonies can be differentiated, because they deaminate phenylalanine to give phenylpyruvate, which forms a dark brown complex with iron ions. Phenylalanine also neutralizes chloramphenicol, so that detection of salmonellae is affected only slightly during treatment with this compound (Anonymous 2012).

New media have been developed incorporating chromogenic substances to create a better differentiation of target organisms from the accompanying flora on agar media (e.g., red

colonies for salmonellae). The Rambach agar medium for the differentiation of *Salmonella* spp. from other members of the family *Enterobacteriaceae* was described by Rambach (1990). It exploits a novel phenotypic characteristic of *Salmonella* spp.: the formation of acid from propylene glycol. This characteristic is used in combination with a chromogenic indicator of 13-galactosidase to differentiate *Salmonella* spp. from *Proteus* spp. and from other members of the *Enterobacteriaceae*. Deoxycholate is included in the medium as an inhibitor of Gram-positive organisms. *Salmonellae* other than *Salmonella* Typhi-yield distinct- bright red colonies on the medium, allowing easy identification and unambiguous differentiation from *Proteus* spp. (Van der Zee 2003).

On the other hand, HiCrome Miller-Mallison agar (HiCrome MM) was formulated by Miller and Mallison for specific isolation and detection of *Salmonellae*. This medium is superior to XLT-4 agar in supporting growth of *Salmonella* due to the presence of appropriate proportion of four sugars. Most differential and selective media are formulated with one or more sugars and pH indicators, respectively. The utilization of sugars by organisms results in pH-changes. This is used as a means of distinguishing *Salmonella* from competing bacteria based on the colony color. *Salmonella* usually are unable to ferment those sugars (Miller et al., 1991) that supports growth of competing bacteria. Thus other bacteria tend to overgrow *Salmonellae*, masking their presence. The inclusion of sugars like mannitol, cellobiose and trehalose stimulate the better initial growth of *Salmonella* cells. However, the low concentrations of these sugars do not interfere with the utilization of protein and H₂S production. Presence of lactose suppresses H₂S production by non-*salmonellae* like *Citrobacter freundii*. The chromogenic mixture, present in this medium helps to differentiate between lactose fermenters and nonfermenters. Lactose fermenters give bluish green colored colonies, which would have been impossible to differentiate with an indicator based on pH change. Inclusion of tergitol 4 in the medium suppresses the presence of *Proteus* and *Providencia* colonies. Peptic digest of animal tissue and beef extract provide essential nitrogen compounds (Anonymous 2011).

Others medium have been developed based on biochemical characteristic of *Salmonella* such as α -galactosidase activity in the absence of β -galactosidase activity, C8-esterase activity, catabolism of glucuronate, glycerol and propylene glycol, hydrolysis of X-5-Gal, and H₂S production, e.g., SMID agar (BioNerieux, France), Rainbow *Salmonella* agar (Biolog, USA), CHROMagar *Salmonella* (CHROM agar, France), chromogenic *Salmonella* esterase agar (PPR Diagnostics Ltd, UK), Compass *Salmonella* agar (Biokar diagnostics, France), and chromogenic ABC medium (Lab M. Ltd., UK) (Maciorowski et al., 2006; Manafi 2000; Perry et al., 2007; Schonenbrucher et al., 2008).

4. BIOCHEMICAL IDENTIFICATION TESTS

Biochemical tests aid in identifying bacterial species based on the differences in the biochemical activities of the target microorganism. Most commonly used are biochemical characteristics (Da Silva et al., 2013; Waltman 2000) and their results for *Salmonella* isolation are shown in Table 3.

Table 3. Biochemical reaction of *Salmonella* (Andrews et al., 2014)

Substrate	Positive reaction	Negative reaction	<i>Salmonella</i> species reaction ^a
Glucose (TSI)	Yellow butt	Red butt	+
H ₂ S (TSI, LIA, and SIM)	Blackening	No blackening	+
Lactose/Sucrose (TSI)	Yellow slant	Red slant	-
Lysine decarboxylase	Purple butt	Yellow butt	+
Urease	Purple red color	No color change	-
Purple broth base 0.5% dulcitol	Yellow color and/or gas	No gas, no color change	+ ^b
Voges-Proskauer	Pink to red color	No color change	-
Methyl red test	Diffuse red color	Diffuse yellow color	+
Simmons Citrate	Growth, blue color	No growth, no color change	v
ONPG	Yellow	Uncolored	-
Indol (SIM)	Red color	No color	-
Motility (SIM)	diffuse turbidity surrounding the puncture line	Growth along the puncture line	v
Malonate	Blue color	No color change	- ^c
Phenylalanine deaminase	Green color	No change color	-
Jordan's Tartrate agar	Yellow	No color change	v

^a +: 90% or more positive in 1 or 2 days; -: 90% or more negative in 1 or 2 days.

^b Majority of *Salmonella* *Arizonae* cultures are negative.

^c Majority of *Salmonella* *Arizonae* cultures are positive.

V: variable.

4.1. Triple Sugar Iron (TSI) Agar and Kligler Iron Agar (KIA)

The carbohydrate fermentation tests aid in identifying whether a bacterium is able to ferment certain carbohydrates, producing acid with or without visible gas (Da Silva et al., 2013). Hajna (1945) developed the formulation for TSI agar by adding sucrose to the double sugar (dextrose and lactose). Carbohydrate fermentation is detected by the presence of gas and a visible color change (from red to yellow) of the pH indicator, phenol red. The production of hydrogen sulfide is indicated by blackening of the butt medium in the tube (Anonymous 2009).

To facilitate the detection of organism that only ferment dextrose, the dextrose concentration is one-tenth the concentration of lactose and sucrose. The small amount acid produced in the slant of the tube during dextrose fermentation oxidizes rapidly, causing the medium to remain red or revert to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube because it is under lower oxygen tension. After depletion of the limited dextrose, organisms able to do so will begin to utilize the lactose or sucrose. So TSI agar is a differential medium that can distinguish between a number of Gram-negative enteric bacteria based on their physiological ability (or lack thereof) to: metabolize lactose

and/or sucrose; conduct fermentation to produce acid; produce gas during fermentation; generate H₂S (Anonymous 2009; Da Silva et al., 2013).

Similar to TSI agar, KIA is used for the differentiation of family members of the *Enterobacteriaceae* on the basis of their ability to ferment dextrose and lactose and produce gas and/or H₂S. The KIA, in addition to casein and meat peptones, contains lactose and dextrose which enable the differentiation of species of enteric bacilli due to the color change of the phenol red pH indicator in response to the acid production during the fermentation of those sugars. The dextrose concentration is only 10% of the lactose concentration. The combination of ferric ammonium citrate and sodium thiosulfate enables the detection of hydrogen sulfide production (Anonymous 2009).

There are different reactions for bacteria inoculated into TSI agar and KIA. *Salmonella* typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H₂S (blackening of agar) in both TSI agar and KIA (Andrews et al., 2014; Caffer et al., 2008).

4.2. Lysine Iron Agar (LIA)

The LIA is used for the differentiation of enteric organisms based on their enzymatic ability to decarboxylate (lysine decarboxylase) or deaminate (lysine deaminase) lysine and to form hydrogen sulfide. Decarboxylation depends on ability of the microorganism to produce decarboxylation enzymes, specific for each individual amino acid. Only the amino acids that have at least one chemically active group, in addition to the amine and carboxyl groups, can be subjected to or are capable of decarboxylation (Anonymous 2009; Da Silva et al., 2013).

The LIA aids in the differentiation of enteric pathogens on the basis on their ability to decarboxylate or deaminate lysine and produce hydrogen sulfide. It is designed for use with other media (e.g., TSI) in appropriate identification schemes. Dextrose serves as a source of fermentable carbohydrate. The pH indicator, bromocresol purple, is changed to a yellow color at or below pH 5.2 and is purple at or above pH 6.8. Ferric ammonium citrate and sodium thiosulfate are indicators of H₂S formation. Organisms that produce lysine decarboxilase produce an alkaline reaction (purple color) in the butt of the medium. Organisms that deaminate the lysine cause the development of a red slant over an acid butt (Anonymous 2009).

In LIA, *Salmonella* typically produces alkaline (purple) reaction in butt of tube. It should be consider only distinct yellow in butt of tube as acidic (negative) reaction. It is recommended to not eliminate cultures that produce discoloration in butt of tube solely on this basis. Most *Salmonella* cultures produce H₂S in LIA. Some non- *Salmonella* cultures produce a brick-red reaction in LIA slants (Andrews et al., 2014).

All cultures that give an alkaline butt in LIA, regardless of TSI reaction, should be retained as potential *Salmonella* isolates and submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an alkaline slant and acid butt in TSI should also be considered potential *Salmonella* isolates and should be submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an acid slant and acid butt in TSI may be discarded as not being *Salmonella* (Andrews et al., 2014).

4.3. Simmons Citrate Agar

The objective of the citrate test is to verify whether the bacterium is able to use citrate as the sole source of carbon for its growth. If the organism has the metabolic apparatus necessary to assimilate citrate, multiplication will occur and the medium will become alkaline, indicated by a change in color of the pH indicator (bromothymol blue) from green (neutral) to blue (alkaline) (Anonymous 2009, Da Silva et al., 2013). A positive reaction means the presence of growth, usually accompanied by color change from green to blue. Most cultures of *Salmonella* are citrate-positive. On the other hand, negative reaction is showed as no growth or very little growth and no color change (Andrews et al., 2014).

4.4. SIM Medium

The SIM is a semisolid media used to differentiate enteric pathogens on the basis of sulfide production, indole formation and motility. Sulfide production is shown by a blackening in those areas of the medium in which microbial growth has occurred. The casein peptone is rich in tryptophan. Bacteria that possess the enzyme tryptophanase can degrade the tryptophan to indole. Indole reacts with added Kovacs reagent (p-dimethylamino benzaldehyde), following the incubation period, to produce a red color (Indole positive reaction). Motility is detected by a diffuse turbidity of the culture medium surrounding the puncture line. In case of nonmotile organisms, growth takes place solely along the puncture line. (Anonymous 2009).

Most *Salmonella* cultures produce H₂S, are motile and do not produce indole. The typically non-motile salmonellas, *Salmonella* Gallinarum and *Salmonella* Pullorum, produce fowl typhoid (FT) and Pullorum disease (PD), respectively, which are septicemic diseases affecting primarily chickens and turkeys, but other birds such as quail, pheasants, ducks, peacocks, and guinea fowl are also susceptible. Rare cases of PD in humans have resulted from massive exposure following the ingestion of contaminated foods or experimental challenge. The clinical signs are characterized by a rapid onset of acute enteritis, followed by prompt recovery without treatment. *S. Gallinarum* is rarely isolated from humans and is of little public health significance (Shivaprasad 2008).

4.5. Methyl Red /Voges-Proskauer (MR/VP) Test

The objective of the MR test is to verify whether the fermentative metabolism of the bacterium is of the mixed-acid type. Fermentation of glucose by the bacteria may result in different final fermentation products, with the type of fermentative metabolism being a characteristic of the species. In mixed-acid fermentation, the final product is a mixture of acids (lactic, acetic, formic acids), which reduce the pH of the medium to a value lower than 4.5. This pronounced reduction in pH, and which exceeds the buffering capacity of the phosphate buffer present, may be detected by adding to the culture a few drops of a methyl

red solution, a pH indicator that changes color below 4.5 (Anonymous 2009; Da Silva et al., 2013). Most *Salmonella* cultures give positive test, indicated by diffuse red color in medium. A distinct yellow color is negative test (Andrews et al., 2014).

The objective of the VP test is to verify whether the bacterium produces butylene-glycol (butanediol) as final fermentation product of glucose. In butylene glycol fermentation, the final product is preceded by an intermediate precursor, acetoin (acetyl methyl carbinol), converted into butylene-glycol by action of diacetyl reductase. Acetoin may be detected in the VP test, by the addition of the reagents for this test (Anonymous 2009; Da Silva et al., 2013). Most cultures of *Salmonella* are VP-negative, indicated by absence of development of pink-to-red color throughout broth (Andrews et al., 2014).

4.6. Purple Broth Base with 0.5% Dulcitol

The purple broth base media consist of carbohydrate-free peptone with the pH indicator bromcresol purple. Specific carbohydrates are added in a concentration of 0.5-1%. This concentration is recommended to ensure against depletion of the carbohydrate and reversal of the fermentation reaction. When the media are inoculated with an organism that is able to ferment the carbohydrate present, acid or acid and gas are produced. A Durham tube is provided in tubed broth media to collect the gas produced during fermentation. The indicator in the media changes from purple to yellow when the amount of acid produced by carbohydrate fermentation is greater than the alkaline end products from peptone utilization. If the carbohydrate is not fermented, the color will remain unchanged or become more alkaline (darker purple) due to degradation of the amino acids in the medium. Most *Salmonella* species give positive test, indicated by gas formation in inner fermentation vial and acid pH (yellow) of medium (Anonymous 2009, Andrews et al., 2014).

4.7. Malonate Broth

Malonate Broth contains ammonium sulfate, which is the sole source of nitrogen in the medium; sodium malonate is the sole source of carbon. Dipotassium phosphate and monopotassium phosphate provide buffering capability. Sodium chloride maintains the osmotic balance of the medium. Increased alkalinity resulting from malonate utilization causes the indicator, bromthymol blue, to change color from green to blue. Most *Salmonella* species cultures give negative test (green or unchanged color) in this broth (Andrews et al., 2014).

4.8. ONPG Test

The test is important in differentiating among the *Enterobacteriaceae* which are commonly classified according to their ability to ferment lactose. The ONPG (o-nitrophenyl- β -D-galactopyranoside) test is used to determinate the presence or absence of the enzyme β -galactosidase in an organisms. The presence of two enzymes, permease and β -galactosidase, are required to demonstrate lactose fermentation. Permease allows the lactose to enter the

bacterial cell. In lactose-fermenting bacteria the breakdown of lactose to glucose and galactose involves the enzyme β -galactosidase. True lactose non-fermenters do not possess either of these enzymes. Late lactose fermenting organisms do not have permease, but do possess β -galactosidase. ONPG is similar in structure to lactose. If β -galactosidase is present, the colourless ONPG is split to galactose and o-nitrophenol, a yellow compound (Public Health England, 2014). Most *Salmonella* species cultures give negative test, without changing the color of the saline water.

4.9. Phenylalanine (PA) Deaminase Test

The objective of this test is to verify whether the organism is able to deaminate the amino acid phenylalanine to phenylpyruvic acid. Deamination occurs in the presence of oxygen, by action of an amino acid oxidase, a flavoprotein that catalyzes the conversion of a molecule of phenylalanine into a molecule of phenylpyruvic acid and molecule of ammonia. Phenylpyruvic acid may be detected in the culture medium by adding ferric chloride, which reacts with phenylpyruvic acid forming a colored compound, phenylhydrazone (Da Silva et al., 2013). *Salmonella* species cultures give negative test, without changing the color of the media after adding ferric chloride (Barrow and Feltham 1993).

4.10. Urease Test

The objective of the urease test is to verify whether the organism produces the urease enzyme, responsible for hydrolyzing urea into ammonia. Urease test is an enzyme of the amidase type, which catalyzes the hydrolysis of amides such as urea. The hydrolysis of each molecule results in two ammonia molecules, which increase the pH of the culture medium and may be detected by phenol red, a pH indicator that changes color at pH 8.4 from yellow to pink (Da Silva et al., 2013). Most *Salmonella* species cultures give negative test, without changing the color of the media (Andrews et al., 2014).

4.11. Jordan's Tartrate Agar

The utilization of the organic salt, sodium tartrate, may be used to differentiate enteric bacilli. Tartrate fermentation acidifies the medium, indicated by the development of a yellow color in the lower portion of the tube. Phenol red is incorporated as an indicator of acid production. The test is positive if the lower portion of the medium has turned yellow. If there is no change in the color of the medium, the test is negative (Anonymous 2009). Most *Salmonella* species cultures give positive test.

4.12. Other Tests

Furthermore, biochemical tests can be determinates using API 20E test kit. The plastic strips holding twenty mini-test tubes were inoculated with saline suspensions of the cultures according to manufacturer's directions. This process also rehydrated the desiccated medium in each tube. A few tubes were completely filled (CIT, VP and GEL), and some tubes were overlaid with mineral oil such that anaerobic reactions could be carried out (ADH, LDC, ODC, H₂S, URE). After incubation in a humidity chamber for 18-24 hours at 37°C, the color reactions were read (some with the aid of added reagents as supplied by the kit). The data were analyzed by the manufacturer's software and positive results with $\geq 89\%$ probabilities were confirmed as *Salmonella* (Salem Imen et al., 2012).

5. CONFIRMATORY SEROLOGICAL TESTS

The identification of a bacterium as *Salmonella* is not difficult but, with that done, two problems arise. The first is the complex antigenic analysis and often phage typing needed to identify strains in sufficient detail to be helpful in tracing the source of infection; the second is how to label (by name or antigenic formula) the strain in the report that must be made to the clinician and to public health officials. Complete antigenic analysis and phage typing are not routine procedures for clinical and food laboratories, but reference laboratories are available in most countries (Barrow and Feltham 1993).

The genus *Salmonella* is characterized serologically by specific antigenic components. The antigens are divided into somatic (O), flagellar (H), and capsular (Vi), using agglutination tests with polyvalent antisera. These antisera should contain antibodies for the factors most commonly encountered and which, in the case of the somatic serological test, belong to the serogroups "A" to "E" (Da Silva et al., 2013; Gaitan Herrera 2001). Up to date, over 2,500 serotypes of *Salmonella* has been identified and classified in the Kauffmann-White scheme. This scheme differentiates between O (somatic) antigens of the cell surface, H1 and H2 (flagellar) antigens of the phase 1 or phase 2, respectively (Selander et al., 1996), and the Vi (capsular) antigens which, however, may only be present in very few serotype, such as Typhi, Paratyphi C or Dublin (Montville and Mathewa 2008; Salem Imen et al., 2012).

Each *Salmonella* serogroup has a group specific O-antigen. Within each O-group, different serovars are distinguished by the combination of O- and H-antigens that are present (Table 3). Each serotype has a specific antigenic formula where the O-antigens are indicated by Arabic numbers, the H1-antigens by lower case letters and the H2- antigens again by Arabic numbers. In these formulas, underlined antigens may only be expressed once the culture is lysogenised by the corresponding converting phage whereas letters or numbers in brackets indicate antigens, which may be present or absent without relation to phage conversion (Le Minor 1984, Montville and Mathews 2008). The detection of the presence of *Salmonella* O- and H- antigens were tested by slide agglutination with the commercially available antisera. One loop of appropriate antisera was dropped onto a cleaned glass slide. One loop of overnight culture grown on agar was dispersed in the drop to obtain a homogeneous and turbid suspension (Salem Imen et al., 2012).

Several rapid latex agglutination assay tests are widely used for the rapid detection of *Salmonella*. These assays however, are primarily used as a confirmation screen for presumptive *Salmonella* colonies after culture isolation from selective agar plates, with further confirmation and identification work carried out on those organisms giving a positive latex reaction. An aliquot of a colony suspension or enrichment broth is simply mixed with the latex reagent and after a few minutes rotation, the results are clearly visible. If the test is negative, the latex remains in smooth suspension and retains its original color. A positive result is indicated by distinct color agglutination against an altered background. These tests save time and resources and allow negative results to be reported at least 24 hours earlier than by conventional culture methods. However, depending on the antibodies used they may lack specificity due to non-specific agglutination of some organisms (Cheesbrough and Donnelly 1996).

Some commercial kits include Remel Wellcolex Color tests for the presumptive identification of *Salmonella* serogroups A, B, C, D, E, and G, and the Vi antigen using just two reagents. Similar tests include Oxoid *Salmonella* latex test, Microgen *Salmonella* Latex test, and Denka-Seiken, among others (Odumeru and León-Velarde 2012).

6. REGULATORY ORGANIZATIONS THAT SPECIFY DETECTION PROCEDURE FOR *SALMONELLA* ISOLATION RELATED TO POULTRY PRODUCTS

During several decades standardized methods for detection of *Salmonella* in food and food ingredients have been independently developed in both the United State and Europe. The basic procedures for different international regulatory organizations are similar. Differences exist in the selection of the culture media and incubation conditions, and the way in which the samples are to be prepared. All basically follow four steps that can be applied to any type of food (Feldsine et al., 2003; Odumeru and León-Velarde 2012; Da Silva et al., 2013). It is, however, important to note that there is no single method that is optimal to all types of samples. Finding the best method for a given type of samples may require extensive literature studies, and often comparative testing of different methods. Procedures are available from a number of standard-setting organizations. The choice of method may be dictated by regulation or requirements from trading partners. Standard methods published for detection of *Salmonella* in poultry products include the International Organization for Standardization, the Bacteriological Analytical Manual (BAM-8) from FDA, and the Laboratory Guide book from the United States Department of Agriculture (Mølbak et al., 2006, Lee et al., 2015).

The International Organization for Standardization method (Table 5) is applicable to products intended for human consumption or for the feeding of animals, and environmental samples in the area of food production and food handling. It may not recover all *Salmonella* Typhi and Paratyphi (ISO 2002). From the BPW, two selective enrichments are inoculated. The ISO 6579 (2002) use RVS broth, which is highly effective for the recovery of *Salmonella* from foods with a high level of background contamination. In addition, this method replaces the formerly used selenite broth with Mueller-Kauffmann tetrathionate novobiocin broth (MKTTn) for the isolation of serotypes of *Salmonella* that are inhibited by constituents from

RVS broth. From each enrichment broths two selective solid isolation media are inoculated, XLD agar is specified. For the other selective agar, e.g., BGA can be used (ISO 2002).

Table 4. Examples of the antigenic formulas of *Salmonella enterica* subsp. *enterica* serotypes according to Kauffmann-White scheme (Poppoff and Le Minor 2001)

Serotype	Somatic (O) antigen	Flagellar (H) antigen	
		H1-antigen(s)	H2-antigen(s)
<i>S. Enteritidis</i>	1, 9, 12	[f], g, m, [p]	[1, 7]
<i>S. Dublin</i>	1, 9, 12 [Vi]	g, p	-
<i>S. Gallinarum</i>	1, 9, 12	-	-
<i>S. Typhimurium</i>	1, 4, 5, 12	i	1,2
<i>S. Virchow</i>	6, 7	r	1,2
<i>S. Infantis</i>	6, 7, 14	r	1,5

Table 5. Guide for pre-enrichment broths for *Salmonella* isolation related to poultry products, using the methods proposed by the International Organization for Standardization (2002), US Food and Drug Administration (Andrews et al., 2014), and the US Department of Agriculture (USDA, 2014)

Organization	Sample	Pre-enrichment broth	Variation in the procedure
International Organization for Standardization	Poultry products	Buffered peptone water	-
Food and Drug Administration	Shell eggs	Trypticase soy broth with 35 mg/l ferrous sulfate	Decontaminate the shells in a 3:1 alcoholic solution of iodine
	Hard-boiled eggs (chicken, duck, and others)	Trypticase soy broth	Decontaminate the shell (peel) in a 3:1 alcoholic solution of iodine, if it is intact
	Liquid whole eggs (homogenized)	Trypticase soy broth with 35 mg/l ferrous sulfate	-
	Dried egg yolk, dried eggs whites, dried eggs whole	Lactose broth	Add the enrichment broth gradually and under constant agitation, to prevent lumping
US Department of Agriculture	Raw Poultry Products	Buffered peptone water	-
	Pasteurized Liquid, Frozen or Dried Egg Products	Buffered peptone water	-
	Poultry Carcass	Buffered peptone water	-

On the other hand, a FDA method (Table 5) is applicable to all foods intended for human consumption. The pre-enrichment uses are LB or TSB. From frozen foods, it is not recommended to thaw the samples before pre-enrichment, not only to prevent injuries to *Salmonella* cells, but also to reduce multiplications of competitors. For the analyses of powdered eggs, it is recommended to add the pre-enrichment broth (LB) gradually and under constant agitation to avoid lumping. For the analyses of the internal content of fresh eggs, it is recommended to wash the eggs under running water and immerse and keep the eggs for 10s in a 3:1 alcoholic solution of iodine. Break the shells aseptically, and transfer the internal content to a sterile plastic bag. Boiled eggs with their shells intact should be disinfected and prepared in the same way. Sample of pooled eggs should inoculate with TSBF. From de pre-enrichment, according to the type of food, two selective enrichments are inoculated: RV medium, and TT broth. From the culture obtained from RV and TT, it should streak a loopful onto BS, XLD and HE agars (Andrews et al., 2014).

The Method of Microbiological Laboratory Guidebook, Food Safety and Inspection Service, United States Department of Agriculture (Table 5) describe in chapter 4.08 the methodology to isolate and identify *Salmonella* from meat, poultry, pasteurized egg, and catfish products and carcass and environmental sponges. It is not intended for the isolation and identification of *Salmonella* Typhi. This methodology uses BPW as pre-enrichment. For chicken carcasses, the carcass has to be transferred to a sterile plastic bag and weigh. Then, add BPW to the bag and to the body cavity of the carcass, agitate the liquid inside the bag with shaking and rotating movements. For *Salmonella* analysis, the rinse fluid is inoculated into BPW. For ready-to-eat-foods containing the poultry component separated from the non-meat ingredients, only the representative meat/poultry portion is analyzed by FSIS. When the meat/poultry is combined with other ingredients to form the product, the combined ingredients are analyzed together. After the pre-enrichment period, it should inoculate into Rappaport-Vassiliadis (R-10) broth or RVS broth, and TT-Hajna broth. From the culture obtained from these media, streak a loopful onto a plate of Brilliant Green Sulfa and XLT-4 or DMLIA (United States Department of Agriculture 2014).

CONCLUSION

A wide range of methods for the detection of *Salmonella* has been developed in the last decade and significant progress has been made in sample preparation techniques to improve isolation and detection of *Salmonella* in foods and food ingredients. For conventional methods to detect *Salmonella* spp., there are a lot of culture media than those mentioned in the reference methods that are still considered to be the “Gold Standard.” Sometimes it can be advisable to use modifications, because the pure standard method cannot be the optimal method for some types of poultry products.

ACKNOWLEDGMENTS

We thank MLS (ASCP)^{CM}SBB^{CM} Marina U. Bueno (National Institute of Health, USA) for reading the manuscript.

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Chapter 5

SALMONELLA'S CONTAMINATION IN EGG

Francisco Procura^{1,2} and Dante J. Bueno^{2,*}

¹Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

²Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria, Concepción del Uruguay, Entre Ríos, Argentina

ABSTRACT

Eggs are laid by female animals of many different species, including birds, reptiles, amphibians, and fish, and they have been eaten by humans for thousands of years. Eggs consist of a protective eggshell, albumen (egg white), and vitellus (egg yolk), that are contained within various thin membranes. Chicken eggs are the most commonly eaten eggs. There are huge variations in egg consumption levels from country to country, depending on the countries levels of wealth. However, overall global consumption has tripled in the last forty years. Eggs from laying hen contain high-quality proteins, and lipids, as well as valuable minerals, carbohydrates, and vitamins. They were also widely used in the food industry due to their multifunctional properties. Although, avian eggs contain the basic elements for life and almost all the albumen proteins are antimicrobial, they can be contaminated by pathogenic bacteria, such as *Salmonella*. Infections of poultry with this bacteria can be grouped into three categories, but paratyphoid salmonellae (PT) are the most important for human being. More often, PT *Salmonella* infections in chickens are characterized by asymptomatic and sometimes persistent colonization of the intestinal tract and internal organs, potentially leading to contamination of the finished carcass. Vertical transmission of this bacteria may result in internal or external contamination of eggs. Egg shells are often contaminated with PT salmonellae by fecal contamination during oviposition. The penetration of salmonellae into or through the shell and shell membranes can result in direct transmission of infection to the developing embryo or can lead to exposure of the chick to infectious *Salmonella* organisms, when the shell structure is disrupted during hatching. Some PT serotypes, particularly *Salmonella* Enteritidis, can be deposited in the contents of eggs before oviposition. The principal *Salmonella* serovar associated with infections linked to eggs and egg products in the UK, most European countries and North America is *S. Enteritidis*. However, other serovars have also been implicated in a number of egg-

*Corresponding author: dantejb@yahoo.com.ar.

associated outbreaks, most notable *Salmonella* Typhimurium exhibiting a range of phage types. *Salmonella* prevalence on the eggshell and in the egg content vary, depending on the fact whether investigations were based on randomly sampled table eggs or eggs from naturally infected hens. Furthermore, other factors like sample size, timing of sampling, site(s) within the egg that were tested, techniques used, investigations of eggs laid by artificial or naturally infected hens could explain this variability. Studies on naturally infected layer flocks show mostly a prevalence below 3%. Control measures to limit the incidence of *S. Enteritidis* and *S. Typhimurium* in poultry flocks are vital.

INTRODUCTION

The egg is a biological structure intended by nature for reproduction. It protects and provides a complete diet for the developing embryo, and serves as the principal source of food for the first few days of the chick's life. The egg is also one of the most nutritious and versatile of human foods. Popular choices for egg consumption are chicken, duck, quail, roe, and caviar, but the egg most often consumed by humans is the chicken egg (Abanikanda and Leigh 2007).

Egg weigh is highly variable, between 34 and 92.1 g. (Soria et al., 2013). Of this weight, the shell (including shell membrane) constitutes 9.5%; the albumen (egg white) 63%; and the yolk 27.5% (Kovacs-Nolan et al., 2005). The yolk is well-centered in the albumen, on opposite sides of the yolk are two, twisted, whitish cord-like objects known as chalazae. Their function is to support the yolk in the center of the albumen. The yolk of one large egg contains approximately: 2.7 g. protein, 210 mg. cholesterol, 0.61 g. carbohydrates, and 4.51 g. total fat. All of the fat-soluble vitamins (A, D, E, and K) are found in the egg yolk. This is one of the few foods naturally containing vitamin D (USDA, 2010).

The egg white forms around either fertilized or unfertilized egg yolks. The primary natural purpose of egg white is to protect the yolk and provide additional nutrition for the growth of the embryo (when fertilized). Egg white consists primarily of about 90% water into which is dissolved 10% proteins (including albumins, mucoproteins, and globulins). Unlike the yolk, which is high in lipids (fats), egg white contains almost no fat, and carbohydrate content is less than 1% (Hosen et al., 2013). Is an alkaline solution and contains approximately 40 different proteins. On the other hand, membrane is a clear film lining the eggshell. Eggshell membrane is primarily composed of fibrous proteins such as collagen type I. It provides efficient defense against bacterial invasion.

The eggshell is the outer covering of a hard-shelled eggs. An eggshell is covered with as many as 17,000 tiny pores. The chicken eggshell is 95-97% calcium carbonate crystals, which are stabilized by a protein matrix (Yves et al., 2004). Without the protein, the crystal structure would be too brittle to keep its form and the organic matrix is thought to have a role in deposition of calcium during the mineralization process. The eggshell, a thin mineral structure, protects the egg contents against mechanical impact, dehydration, and microorganism contamination. At the same time, the eggshell is permeable to gases and water necessary for the development of the chick embryo (Nys et al., 1999; Hincke et al., 2012).

Increased egg production and consumption in the developing world could significantly improve food and nutrition security, especially for growing minds. There are huge variations in egg consumption levels from country to country. Depending largely on the countries'

levels of wealth, annual consumption of eggs ranges from as low as 300 g. per person in African countries like Burundi, Rwanda, Chad and Niger to 19.1 kg. in Japan. Out of 43 countries in sub-Saharan Africa, only 9 countries have an average consumption that is higher than 2 kg, while in most of Asia and the Americas, people eat at least twice that amount (Food and Agriculture Organization 2013)

Egg can be contaminated by different bacteria, like *Salmonella* sp. Infections of poultry with salmonellae can be grouped into three categories, one is the infection with the two nonmotile biovars, *S. Gallinarum* biovar Pullorum and *S. Gallinarum* biovar Gallinarum, which are generally host-specific for avian species. Another is the infection with the numerous motile and non-host-adapted *Salmonella* serotypes/serovars referred to collectively as paratyphoid salmonellae. Found nearly ubiquitously in wild and domestic animals, this diverse group of serotypes is principally of concern as a cause of food-borne disease in humans. Although paratyphoid infections of poultry are very common, they seldom cause acute systemic disease except in highly susceptible young birds subjected to stressful conditions. More often, paratyphoid *Salmonella* infections of chickens and turkeys are characterized by asymptomatic and sometimes persistent colonization of the intestinal tract and internal organs, potentially leading to contamination of the finished carcass. Some serotypes, especially *S. Enteritidis* (SE), can be deposited in the contents of clean and intact eggs laid by systemically infected hens. The other infection is that produces by *S. enterica* subspecies *arizonae*, a motile serotype which was formerly designated *Arizona hinshawii*. This organism, although biochemically distinct, causes an acute septicemic disease that is not clinically distinguishable from other *Salmonella* infections (Gast 2008). Therefore, this chapter will review the presence of *Salmonella* spp. in eggs for human consumption and some strategies to reduce contamination of eggs with this bacteria.

EGGS AS SOURCE OF *SALMONELLA* INFECTION IN HUMAN

Foodborne diseases are a major public-health concern worldwide. The burden of pathogens commonly transmitted through foods has increased substantially in the last decades (Greger 2007). *Salmonella* spp. is one of the most common and widely distributed foodborne pathogens in the European Union (EFSA 2008a). SE and *Salmonella* Typhimurium (ST) are the most frequently reported serovars, but a wide range of other serovars frequently cause disease in humans and thus are of public health significance (Vieira et al., 2008; EFSA 2010a). The most important route of transmission is fecal-oral; humans can get infected through direct contact with infected individuals or indirectly through the consumption of contaminated food or water. The foodborne route appears to be the most common. The food categories posing the greatest risk to public health include raw meat, raw or undercooked products of poultry meat, eggs and products containing raw eggs. In addition, fruits and vegetables are becoming increasingly important sources of salmonellosis. A risk assessment conducted by FAO and WHO (FAO/WHO 2002) noted that the human incidence of salmonellosis transmitted through eggs and poultry meat appeared to have a linear relationship to the observed *Salmonella* prevalence in poultry. This means that, when reducing the prevalence of *Salmonella* in poultry with 50% it is estimated that the incidence of salmonellosis in humans will fall with 50% assuming all other conditions stay constant.

Nearly 84% of food-borne human illnesses in Scotland between 1980 and 1989 and about 81% of food-borne disease outbreaks in Italy between 1991 and 1994 were attributed to *Salmonella*. The incidence of human *Salmonella* infections (especially involving SE and ST) increased in many nations between 1985 and 1995. Culture-confirmed human cases of salmonellosis reported to the Centers for Disease Control and Prevention (CDC) in the United States have increased steadily, rising from 26,326 in 1972 to 39,033 in 1996. According to CDC, salmonellae are responsible for an estimated 1.34 million illnesses, 16,430 hospitalizations, and 582 deaths each year. In *Salmonella* outbreaks, widespread distribution of contaminated foods can sometimes involve huge numbers of consumers. For example, a 1994 SE outbreak attributed to ice cream in the United States affected 224,000 people. *Salmonella* outbreaks can have particularly severe consequences for highly vulnerable populations in locations such as daycare centers and nursing homes. More than one-third of food-borne salmonellosis outbreaks in humans in the United States between 1983 and 1987 were associated with poultry meat or eggs. Between 1985 and 1996, 79% of SE outbreaks in the United States that could be attributed to a specific food vehicle were associated with eggs.

Eggs were estimated to be the most important source of disease, and pork, chicken, the general category meat and poultry, and dairy followed in importance. The relative contribution of eggs for *Salmonella* outbreaks increased from 2007 (56.1%) to 2008 (61.6%), but a substantial decrease was observed in the following year, when the proportion of outbreaks attributed to this source was estimated to be 34.5%. Pork was estimated to be the second most important source of salmonellosis in 2007 (5.4%) and 2008 (6.1%), whereas poultry meat (10.9%) was the second contributor for disease in 2009 (Pires et al., 2011). The laying hen reservoir (i.e., eggs) was estimated to be the most important source of salmonellosis in 13 countries (Austria, Czech Republic, Estonia, Germany, Greece, Hungary, Latvia, Lithuania, Luxembourg, Slovenia, Slovakia, Spain and the United Kingdom).

The principal *Salmonella* serovar associated with infections linked to eggs and egg products in the UK, most European countries and North America is SE. However, other serovars have also been implicated in a number of egg-associated outbreaks, most notable ST exhibiting a range of phage types. In parts of the world where SE historically has not penetrated laying hen breeding flocks, egg-related salmonellosis is a problem associated specifically with non-SE serovars (Threlfall et al., 2014).

SE is the most commonly detected serovar in human salmonellosis in Europe. It has declined substantially in recent years, most likely as a result of the successful control measures implemented for laying hens and egg production. In 2012, SE accounted for 179 outbreaks and 2,177 human cases (37.6% of all cases in *Salmonella* outbreaks). Most of these SE outbreaks were attributed to eggs and egg products. In the same year, egg and egg products were implicated in 168 outbreaks (22%) out of 763 outbreaks reported at EU level, of which 93.5% were caused by *Salmonella* spp. The majority of these outbreaks were associated with SE (66.7%), as in previous years. Recently, sporadic or outbreak cases of SE reported by Austria, France, Germany and the United Kingdom, in addition to one case reported in Luxembourg in a patient residing in France, appear to be linked by time of symptom onset and microbiological characteristics of isolates. Cases in Austria, France and Germany share an epidemiological link to the same egg packaging centre in southern Germany. Isolates from contaminated eggs identified in France originating from the implicated German egg packaging centre share similar molecular characteristics to the human

cases (European Centre for Disease Prevention and Control, European Food Safety Authority 2014).

The history of eggs as a source of human illness has almost exclusively concerned bacteria of the genus *Salmonella*. Until the late 1960s, human salmonellosis was commonly attributed to table eggs with cracked or dirty shells or to egg products that had not been heated sufficiently during processing to completely destroy pathogens. In the USA, the 1970 Egg Products Inspection Act prohibited the sale of cracked and dirty table eggs and mandated reliably effective pasteurization standards for liquid egg products, thereby leading to a very dramatic reduction in the frequency with which human illness was linked to eggs in the years that followed. However, by the mid-1980s, a newly emerging public health issue again focused attention on eggs as a source of *Salmonella* transmission (St Louis et al., 1988). In this, more recent version of the story of eggs and *Salmonella*, human illness was associated primarily with clean and intact, Grade A table eggs. Moreover, the vast majority of these disease outbreaks involved a single serotype, SE. An international surge in human SE infections has been principally connected to contaminated eggs (Angulo and Swerdlow 1999; van de Giessen et al., 1999; Wall and Ward 1999). In the USA, approximately 80% of the human SE outbreaks for which a food source could be identified have been attributed to eggs or egg-containing foods (Patrick et al., 2004). Accordingly, developing and implementing effective programmes to diminish the likelihood that consumers will be exposed to contaminated eggs has become an important objective for both government and industry on several continents (Hogue et al., 1997b; Cogan and Humphrey 2003).

According to the Food and Public Health Branch of the Food and Environmental Hygiene Department of Hong Kong Government (2004), 252 confirmed *Salmonella* food poisoning outbreaks (affecting 1628 persons) occurred during 1998 to 2002. Egg and egg products (including desserts) were identified as the incriminated food in 90 (36%) out of 252 cases, involving 415 persons. Further analysis revealed that 55 (61%) out of 90 of these cases were caused by consumption of desserts. Among the 55 cases, tiramisu and pudding were identified as incriminated food items in 21 and 25 outbreaks respectively. Tiramisu and pudding are non-heat-treated type desserts which may contain raw egg ingredients and are prepared without involving any pathogen reduction steps, like heat treatment. The major contributing factors of these cases include the consumption of raw food (eggs) and poor personal hygiene of food handler.

Generally, there are two possible routes of egg contamination by *Salmonella*. Eggs can be contaminated by penetration through the eggshell from the colonized gut or from contaminated feces during or after oviposition (horizontal transmission). The second possible route is by direct contamination of the yolk, albumen, eggshell membranes or eggshells before oviposition, originating from the infection of reproductive organs with SE (vertical transmission). It is not yet clear as to which route is most important for SE to contaminate the egg contents (Gantois et al., 2009).

SALMONELLA PREVALENCE IN LAYING HENS AND EGG

The observed prevalence on the eggshell and in the egg content can be variable. There are a number of factors that could explain this variability, such as sample size, timing of

sampling, site (s) within the egg that were tested, technique used, investigations of egg laid artificially or naturally infected hens, etc (Humphrey 1994). The percentage of eggs, naturally infected with *Salmonella*, varies in different public health laboratory reports. Most studies show the percentage to be below 3% (Kinde et al., 1996). In artificially infected hens the percentage can range from 0% to 27.5% (Keller et al., 1995).

Molting of flocks and penetration of *Salmonella* through the outer shell of the egg are considered as factors contributing to SE prevalence. Molting is not a practice in Europe, as it is a stress for the bird. The incidence of egg-associated salmonellosis in Europe is much higher than in the USA and, thus, there is compelling reason to believe that cessation of molting would substantially reduce the incidence of infection in humans (Guard-Petter 2001).

The frequency of *Salmonella*-positive samples within egg-laying flocks is often far less than the overall incidence among flock. The distribution of *Salmonella* within contaminated laying houses is not necessarily uniform. The number of *Salmonella* found in individual environmental samples for laying houses is typically relatively low, although somewhat high levels can be present at the beginning of egg production and molting (Gast 2013).

In a 1995 report, 191 eggs were contaminated with SE out of 738,000 eggs tested, whereas ST was isolated from only one egg (Anon., 1995a). In an earlier study, oral nor intravenous challenge of laying hens with ST resulted in the contamination of eggs (Baker et al., 1980). However, experimental infection with ST DT104 can result in the contamination of intact eggs (Williams et al., 1998; Leach et al., 1999). Hassan and Curtiss (1997) found that egg contents were almost equally frequently infected when an oral challenge of laying hens with ST was compared with SE. However, Soria (2012) studying the prevalence of *Salmonella* in eggshells and egg content from Entre Rios, Argentina, found that 1.8% of samples were positive to this bacteria, and 8 different *Salmonella* serovars were isolated, 66% of the positive samples for *Salmonella* spp. corresponded to ST, while 14% to SE. And a study of the prevalence of *Salmonella* on eggshells in France resulted 1.05%, and the serotyping of *Salmonella* strains revealed five different serovars including Enteritidis, Typhimurium, Virchow, Infantis, and Montevideo (Chemaly et al., 2009).

Field studies of commercial poultry in the USA observed SE egg contamination frequencies of less than 0.03% from environmentally positive flocks. The U.S. Department of Agriculture estimated the overall national incidence of egg contamination with SE at approximately 0.005% (Gast 2008). On the other hand, the Food Standards Agency's from UK (2007) carried out a survey of *Salmonella* contamination of raw shell eggs used in catering premises over a period of 14 months between November 2005 and January 2007. Six pooled samples were found to be contaminated with *Salmonella* spp. on the shell of the egg giving a prevalence of 0.38%. Of these SE was detected in 5 samples with a prevalence of 0.31%, with SE PT4 at 0.19%.

It seems to be a link between egg contamination and the infection of the laying hen, since SE is far more frequently isolated from naturally infected hens than any other serovar (Anon., 2003.) The ability of SE to colonize the reproductive organs may be a selective advantage over other serotypes. (Keller et al., 1997) and may be one of the reasons that egg contamination with SE has increased (Okamura et al., 2001b). Understanding the SE specific factors involved in the egg contamination process should be the basis for the development of control measures (De Buck et al., 2004).

The occurrence of SE within an egg depends on whether the hen that laid it was infected with SE. Although SE-contaminated eggs only come from infected hens, not all eggs

produced by infected hens are SE contaminated. Furthermore, infected hens are only found on farms in which SE is present, and on such farms, not all hens are infected. Thus, for an egg to be contaminated with SE three conditions must exist: SE must be present on the farm, SE must infect one or more hens, and SE-infected hens must be susceptible to producing SE-contaminated eggs. If an egg is laid with SE inside, these bacteria may die, remain dormant, or multiply. Multiplication depends primarily on time and temperature of storage. Higher temperatures (up to 37°C) favor SE growth, and longer storage times at temperatures permitting growth favor greater amounts of SE growth. Thus, the interaction of time and temperature determines how much SE growth occurs inside an egg (FSIS, 2005).

After processing, further growth of SE within an egg is possible, even in pasteurized eggs. Either some SE may survive pasteurization and grow or the egg may not be pasteurized and the SE inside continue to grow. An egg is shipped to retailers or wholesalers to be purchased for food. The egg may be stored for varying times and temperatures before shipment, during shipment, and after shipment. Furthermore, the egg will likely be stored for some time in a consumer's refrigerator at home before it is consumed. All of these steps could present additional opportunities for SE growth (FSIS, 2005).

A study, conducted on commercial large-scale egg-laying hen holdings with at least 1,000 laying hens (*Gallus gallus*) in the 25 European Union countries and Norway by the European Food Safety Authority, found a range of *Salmonella* levels in hens in these countries of between 0% and 79% (Anon., 2006). The results also showed that samples taken on 20% of all large-scale laying hen holdings in the European Union tested positive for SE and/or ST. A study across European countries has shown a high linear correlation between *Salmonella* in egg-laying hens and human illness (de Jong and Ekdahl 2006).

A baseline study on the prevalence of *Salmonella* in holdings of laying hen flocks conducted by the European Food Safety Authority (EFSA) revealed a *Salmonella* spp. prevalence of 30.8% ranging from a minimum of 0% (Luxembourg and Sweden) to a maximum of 79.5% (Portugal) (WHO, 2007). On the other hand, a study carried out in laying hen farms from Argentina (Entre Ríos) revealed *Salmonella* prevalence of the 60%. However, *Salmonella* was only isolated in 9.2% from 1,963 samples examined. The highest percentage of *Salmonella* isolations (32.5%) were found in the boot swab samples. In contrast, there were no *Salmonella* isolations from eggs (Soria 2013). This author found significant differences in relation to regional distribution of the houses respect to the presence of *Salmonella* sp., which could be explained by the farm density. Namata et al. (2008) also found that the likelihood of *Salmonella* infection increased by both flock size and age.

Housing systems for laying hens have been intensively investigated as potential SE risk factors. Huneau-Salaun et al. (2009) carried out a cross-sectional study to identify risk factors for *Salmonella* spp. contamination in French laying hen flocks at the end of the laying period. They found that a prevalence of this bacteria was significantly higher in caged flocks than in on-floor flocks. In caged flocks, the risk of *Salmonella* contamination increased with flock size and when delivery trucks passed near poultry-house entrances. In on-floor flocks, a higher risk of contamination was associated with multistage management (presence of hens of different ages on the farm) and contamination by SE of a previous flock kept on the farm. However, the use of a container for dead bird disposal was a protective factor. On the other hand, Gast et al. (2015) reported that the susceptibility of hens to intestinal colonization by SE can differ between conventional and enriched cage based production systems, although

this effect does not necessarily translate into a corresponding difference in the longer-term persistence of fecal shedding.

CONTAMINATION OF EGGS DURING EGG FORMATION

SE is the dominant serotype isolated from egg contents. There is no relation between SE contamination of the egg shell and that of the egg content. A study reported that SE was the only one of six serotypes tested that was deposited in egg yolks by experimentally-infected hens (Okamura et al., 2001a). Similarly, SE only found inside naturally-contaminated eggs, even though a wide assortment of serotypes was present on the shells of these eggs (Humphrey et al., 1991a). This may suggest that contamination of egg contents is likely to take place in the reproductive organs rather than by eggshell penetration. Examination of eggs from birds infected artificially found no relationship between fecal carriage of SE and the presence of the bacterium in egg contents. It is also possible to isolate SE from the reproductive tissue of infected hens, in the absence of intestinal colonization. This serovar has been found in both the yolk and albumen of eggs laid by infected hens. Different reports have also implicated *Salmonella* Heidelberg as an egg-transmitted pathogen (Hennessy et al., 2004), and an experimental-infection study documented the ability of some strains of this serotype to colonize reproductive tissues and be deposited inside eggs (Gast et al., 2004).

Albumen is the compartment most frequently contaminated by SE. Yolk contamination points to the ovary as site of origin of the egg contamination. Contamination of the albumen by this serovar is believed to occur during passage of the egg through the oviduct. Several studies even suggest that SE most frequently migrates into chicken eggs through the upper oviduct in association with albumen. SE has been found in association with secretory cells of the upper and lower magnum by immunohistochemical staining. This is also compatible with the hypothesis that the pathogen may contaminate forming eggs through the albumen. Keller et al. (1995) observed a higher contamination rate of forming eggs as compared to laid eggs.

The number of bacteria in the eggs is controlled before the egg is laid by factors within the eggs, such as antibodies, antibacterial enzymes, iron sequestering and bacterial protease-inhibiting proteins. Egg shell membrane and egg shell are produced in the lower reproductive tract. These compartments of the egg also may be contaminated during egg development. Contamination of egg shells and egg shell membranes by SE has been reported to occur frequently. In some studies it is even reported as the most infected component of contaminated eggs. However, since *Salmonella* bacteria can penetrate egg shells, it is difficult to distinguish between contamination during formation of the egg or after oviposition (De Buck 2004).

In experimental infection studies, laying hens have typically produced internally-contaminated eggs for only a few weeks following oral inoculation (Gast and Beard 1990a; Gast and Holt 2000a). However, in commercial laying flocks, the patterns of egg contamination over time are far more irregular, as infection spreads gradually through each house. Like most other paratyphoid (non-host-adapted) *Salmonella* serotypes, SE is usually introduced to chickens via the gastrointestinal tract. After oral ingestion from the environment, this serovar colonizes several regions of the tract, particularly the crop and caeca (Turnbull and Snoeyenbos 1974). Invasion through mucosal epithelial cells can then

lead to systemic dissemination to a wide array of internal organs, including reproductive tissues (Gast and Beard 1990b; Humphrey et al., 1993). By colonizing the ovary (the site of yolk maturation and release) and the oviduct (the site of albumen secretion around the descending yolk), SE appears to gain access to the contents of eggs (Miyamoto et al., 1997; Okamura et al., 2001a; De Buck et al., 2004). Some investigators have found SE inside pre-ovulatory follicles and in developing eggs removed from the oviducts of infected hens before oviposition (Thiagarajan et al., 1994; Keller et al., 1995).

The extensive permeability of the vascular endothelia observed in the ovary may contribute to the high colonization rate of the ovary (Griffin et al., 1984). In the majority of experimental studies in laying hens, a higher frequency of ovary colonization is reported, compared with the frequency of recovery from the oviduct (De Buck et al., 2004; Gantois et al., 2006; Gast et al., 2007). Therefore, it is strongly believed that SE must interact with the cellular components of the preovulatory follicles. It was indeed shown that SE can attach to developing and mature follicular granulosa cells exhibiting different attachment patterns (Thiagarajan et al., 1994).

The penetration of immature follicles has practical implications because it can lead to contamination of eggs after maturation and can cause continuous transovarian infection of eggs throughout the reproductive cycle. This statement is, however, questionable because not all small white follicles will mature and because the extensive growth of *Salmonella* in the nutrient-rich follicles will most likely lead to their degeneration (Kinde et al., 2000). The fact that *Salmonella* can interact with the cellular components of preovulatory follicles raises the question as to whether serotype Enteritidis harbours some intrinsic characteristics allowing it to specifically interact with these cells and, as a consequence, be transmitted to eggs. In a study by Okamura et al. (2001a, b), it was shown that among six different *Salmonella* serotypes, SE colonized ovaries and preovulatory follicles at significantly higher levels than five other serotypes after intravenous inoculation. Because samples in this study were only taken at 4 and 7 days postinfection, and bacteria were still persistent in the peripheral blood, it cannot be concluded, however, that SE displays a stronger interaction with follicles than other serotypes.

Based on the fact that systemic spread is a characteristic of most *Salmonella* serotypes, it is believed that ovarian colonization is not a specific trait allowing the serotype Enteritidis to contaminate eggs. However, the possibility that this serovar has a specific ability to interact and invade the preovulatory follicles cannot be ruled out. A large-scale study using multiple strains from different *Salmonella* serotypes should be carried out in order to provide more information regarding the serotype specificity of ovarian colonization and persistence. High levels of nutrients are available to bacteria invading ovarian follicles. Therefore, it is to be expected that this should lead to extensive replication of the bacteria, almost inevitably resulting in follicular degeneration. Because this is not a common phenomenon in naturally infected laying hens, as the laying percentage is usually not reduced, follicle colonization is not believed to be an important source of egg contamination (Gantois et al., 2009).

Reports point to albumen as the principal site of contamination in eggs (Shivaprasad et al., 1990; Humphrey et al., 1991b; Keller et al., 1995), indicating that SE is colonizing oviduct tissues. Miyamoto et al. (1997) observed that developing eggs in a highly contaminated oviduct are likely to be *Salmonella* positive. Colonization of the reproductive tract can be the result of an ascending infection from the cloaca (Reiber et al., 1995; Miyamoto et al., 1997), a descending infection from the ovary (Keller et al., 1995) and/or a

systemic spread of *Salmonella*. Depending on the site of contamination, i.e., the vagina, isthmus and magnum, *Salmonella* could be incorporated into the eggshell, the eggshell membranes or the albumen.

Really, the oviduct is a stressful and damaging environment for *Salmonella*, but the bacteria can counteract this by stress-induced protective and reparative responses, enabling the bacteria to survive in the hostile environment and/or escape the host defence reactions (Gantois et al., 2008). It is clear that the process of oviduct colonization is complex and depends on many factors including fimbriae, flagellae, lipopolysaccharide, cell wall structure and stress tolerance. Although most, if not all, bacterial factors, shown to play a role in reproductive tract colonization, are not specific to the serotype Enteritidis, a unique regulation of these known virulence factors in the reproductive tract environment could be one plausible explanation for the epidemiological association with hen's eggs (Gantois et al., 2009). Raspoet et al. (2011) hypothesize that the *Salmonella* *uspA* and *uspB* genes are involved in long term persistence of SE in harmful environments, such as in the oviduct and eggs, by conferring resistance against compounds that damage the bacterial cell membrane and DNA.

It was demonstrated that repeated *in vivo* passages through the reproductive tissues of chickens increase the ability of an SE strain to induce internal egg contamination, whereas serial passage through the liver and the spleen did not affect the ability of the strain to cause egg contamination (Gast et al., 2003). This is an indication that interaction of SE with the reproductive tissues may either induce or select for the expression of microbial properties important for egg contamination.

INTERNAL EGG CONTAMINATION AFTER PENETRATION OF THE EGGSHELL

Shell contamination is the first requisite for bacterial penetration. The possibility of *Salmonella* contamination of the shell after lay depends on intrinsic and extrinsic factors. For the extrinsic factors, the influence of bacterial strain and the number of organisms, temperature, moisture and immersion and storage conditions are important. On the other hand, the presence of cuticle, shell characteristics (shell quality, porosity, and shell defects) and membrane properties correspond to the intrinsic factors (Messens et al., 2005a).

A wide range of serovars has been recovered from egg shells, including SE (Poppe et al., 1992; de Louvois 1993b; Humphrey 1994; Schutze et al., 1996). The presence of many different *Salmonella* serotypes on the surface of the shells, as well as contamination of the contents of the egg, represents a potential threat to public health. Surface contamination may be the result of either infection of the lower reproductive tract or fecal contamination. Fecal contamination however is unlikely to occur during oviposition in a healthy laying hen. Indeed, when a healthy hen lays an egg, the hen's bearing everts the vagina beyond the alimentary tract. This protects the emerging egg from fecal contamination. Also, the stretching of the cloacal lining effectively makes the intestinal tract somewhat slit-like, further reducing the opportunity for contamination of egg shell. This is why most egg shells in healthy birds are not covered by feces at oviposition. Fecal contamination may however very well take place in the environment after oviposition. If contamination through contact with feces or the environment is important, then the hygiene in the chicken house and during

egg handling and processing is critical. The presence of chicken manure and other moist organic materials facilitates the survival and growth of *Salmonella* by providing the required nutrients and a degree of physical protection.

Penetration of egg shell and egg shell membranes by SE (Haigh and Betts 1991; Dolman and Board 1992; Schoeni et al., 1995; Miyamoto et al., 1998; Wang and Slavik 1998) as well as ST (Padron 1990; Schoeni et al., 1995; Berrang et al., 1998; Miyamoto et al., 1998; Berrang et al., 1999) and other serovars (Javed et al., 1994) has repeatedly been described under experimental conditions. These exclusively experimental penetration assays have led to the hypothesis that the contents of eggs can become contaminated immediately after laying through pores or cracks in the shell. However, this penetration of *Salmonella* bacteria does not seem to occur at the same rate in practice, since the spectrum of *Salmonella* serovars isolated from the egg surface does not correspond with that found in the egg contents, the latter being almost uniquely SE (De Buck et al., 2004).

Only few reports suggest that egg contents are more likely to become contaminated during passage through the cloaca than as a result of ovarian infection (Rodriguez et al., 1990; Barrow and Lovell 1991). When total egg shells are cultured, it is impossible to discriminate between surface contamination from the environment and contamination during formation of the eggs. Genuine egg surface contamination could be differentiated from shell and shell membrane contamination that took place inside the reproductive tract, by dipping eggs in culture broth before their surface is sterilized and the egg shells are cultured (De Buck et al., 2004).

Bacteria can easily penetrate through a cracked egg shell (Fajardo et al., 1995). The intact egg, however, possesses three physical barriers to bacterial penetration. These are the cuticle, which is a hydrophobic proteinaceous layer covering the eggshell and the pore openings, the crystalline eggshell and the shell membranes (Ruiz and Lunam 2002). Shell membranes consist of three different layers, i.e., the inner and the outer membrane, consisting of a network of randomly oriented fibres, and a homogenous third layer of electro-dense material called the limiting membrane, demarcating the membrane at the interface with the albumen (Wong-Liong et al., 1997). In addition to their function as a physical barrier, the eggshell and shell membranes also act as a chemical barrier. Although antibacterial proteins have been identified mainly in the albumen, proteins with well-known antibacterial properties have also been associated with the eggshell and shell membranes (Gantois et al., 2009).

In spite of the protective physical and chemical barriers, numerous researchers have demonstrated rapid penetration into the egg by various bacteria, including *Salmonella* (Williams et al., 1968; Humphrey et al., 1989, 1991b). Miyamoto et al. (1998a) observed that after exposing freshly laid eggs to a *Salmonella* suspension for 2 h at 25°C, the inner eggshell and egg contents were contaminated. The eggshell appears to be more easily penetrated immediately after the egg is laid (Sparks and Board 1985; Padron 1990; Miyamoto et al., 1998a). It is suggested that for the first minutes after oviposition, the cuticle is immature and some pores may be open. Moreover, when the egg is exposed to an environment cooler than the chicken body temperature (42°C), a negative pressure may develop and the bacteria migrate more easily through the eggshell and membranes (Board 1966; Bruce and Drysdale 1994). In addition, the cuticle in older eggs becomes dehydrated, resulting in its shrinkage, and the pores become more exposed to bacterial penetration (Mayes and Takeballi 1983). In recent studies (De Reu et al., 2006; Messens et al., 2007), it was reported that cuticle deposition is important for the prevention of penetration, and in the absence of cuticle

deposition, penetration is a frequent event. However, some reports (Nascimento et al., 1992; Messens et al., 2005b) observed no correlation between cuticle deposition and penetration of *Salmonella* through the eggshell. Additionally, bacterial penetration was found to be independent of the pore number (Nascimento et al., 1992; Messens et al., 2005b; De Reu et al., 2006).

As mentioned earlier, temperature is also an important factor affecting the penetration. Fast penetration is observed when a positive temperature differential is created between the egg (warm) and the bacterial suspension (cool) (Mayes and Takeballi 1983; Bruce and Drysdale 1994). It is believed that a positive temperature differential, combined with the presence of moisture, provides an ideal opportunity for the bacteria to penetrate the eggshell (Berrang 1999). On the other hand, the higher number of SE in the culture used for dipping the eggs, the higher the rate of contamination of the egg (Messens et al., 2005a).

THE INTERACTION BETWEEN *SALMONELLA* AND THE FORMING EGG

Inside the egg, several components of the albumen are directly or indirectly antimicrobial (Ricke et al., 2001). The most significant of these antibacterial albumen proteins is ovotransferrin, which binds iron to limit its availability to support microbial growth (Baron et al., 1997). Furthermore, the pH of albumen increases as it ages and thereby becomes more inhibitory to bacterial multiplication. Despite this considerable array of protective constituents, SE is able to survive and sometimes even grow slowly in albumen. Several investigators have reported that this serovar inoculated into separated albumen was able to persist during incubation at warm temperatures for days or even weeks (Lock and Board 1992; Gast and Holt 2000b, 2001b), although a decline in the numbers of SE cells in albumen has been noted during refrigerated storage (Stephenson et al., 1991). After inoculation into the albumen of whole eggs, at sites remote from the yolk, a modest degree of growth has sometimes been observed after several days of incubation at 20°C or higher (Gast and Holt 2000b; Cogan et al., 2001). Multiplication of SE proceeds faster in fresh than in stored albumen, possibly due to an increase in pH during storage (Messens et al., 2004). Perhaps by inactivating ovotransferrin and other antibacterial proteins, pasteurization has been found to render albumen less resistant to bacterial growth (Baron et al., 1999).

In egg yolk, nutrients are present in abundance and the antimicrobial albumen proteins are absent, so the growth of SE can be rapid and prolific (Clay and Board 1991). Even very small initial numbers of cells from this serovar can multiply to reach dangerously high concentrations within a single day, after inoculation into egg yolk (Gast and Holt 2000b). Temperature is the principal factor that affects SE growth in egg yolks. Extensive multiplication has been reported at 15°C and higher, whereas slower multiplication is evident at 10°C and growth ceases at around 4°C (Kim et al., 1989; Saeed and Koons 1993; Schoeni et al., 1995; Gast and Holt 2000b).

Even if SE is not located initially inside the yolk contents of contaminated eggs, but, instead, is deposited on the exterior surface of the vitelline membrane or in nearby areas of the albumen, bacterial penetration through the membrane could still result in extensive multiplication within yolks. Using various *in vitro* models for egg contamination, the penetration of SE through the yolk membrane has been reported to occur at a wide range of

frequencies (Hammack et al., 1993; Humphrey and Whitehead 1993; Braun and Fehlhaber 1995; Gast and Holt 2000b). However, in a similar study, no movement of *Salmonella* from the exterior to the interior of the yolk membrane was observed (Fleischman et al., 2003). The migration of this serovar across the vitelline membrane into the yolk has been shown to increase with the level of contamination, storage temperature and egg age (Braun and Fehlhaber 1995; Gast and Holt 2000b).

Another mechanism by which SE could eventually begin to multiply rapidly after deposition in the albumen involves the gradual degradation of the vitelline membrane, leading to the release of yolk nutrients into the albumen, as the egg ages (Humphrey 1994). This deterioration of the yolk membrane is accelerated by abusively high temperature conditions (Hara-Kudo et al., 2001; Latimer et al., 2002). In experimentally contaminated eggs, the growth of SE in areas of the albumen around the yolk increased with the age of the eggs at inoculation (Humphrey and Whitehead 1993). However, rapid growth of this serovar in albumen, due to yolk-membrane degradation, has been observed after only three weeks of storage at 20°C (Humphrey and Whitehead 1993).

Antimicrobial Components in the White Egg and Egg Yolk

Proteins in the egg white as phosvitin, lysozyme, ovotransferrin, and avidin have proven to exert numerous biological activities. Moreover, a specific protein in eggshell matrix shows unique activity; enhancement of calcium transportation in the human intestinal epithelial cells (Abdou et al., 2013). Another group of antimicrobial proteins are those showing proteinase-inhibiting activity. They include ovomucoid, ovoinhibitors (serine protease inhibitors), cystatin (a cysteine protease inhibitor) and ovostatin (Stevens 1991). Their function lies in inhibiting tryptic digestion of egg proteins by bacteria and thus protection of the antimicrobial activity of albumen proteins (Gantois et al., 2009).

The antibacterial activity of phosvitin could be attributed to the synergistic effects of the high metal-chelating ability and the high surface activity under the influence of thermal stress. Phosvitin and the phosvitin-galactomannan conjugate may represent safe anti-bacterial agents for foods (Hernández-Ledesma and Chia-Chien 2013).

Lysozyme was originally used to describe an enzyme which had lytic action against bacterial cells and it is one of the oldest egg components to be utilized commercially, after it was discovered by Alexander Fleming in 1922. It is a bacteriolytic enzyme commonly found in nature and is present in almost all secreted body fluids and tissues of humans and animals. It has also been isolated from some plants, bacteria and bacteriophages. Avian egg white is a rich and easily available source of lysozyme. The lysozyme content of a laying hen's blood is 10-fold higher than in mammals because it is being transferred to the egg white. Lysozyme constitutes approximately 3.5% of hen egg white. Egg white lysozyme consists of 129 amino acid residues with a molecular weight of 14.4 kDa. Because of its basic character, lysozyme binds to ovomucin, transferrin or ovalbumin in egg white. It has long been believed that lysozyme's antimicrobial action could only be attributed to its catalytic effect on certain Gram-positive bacteria, by splitting the bond between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan in the bacterial cell wall. Beside this well-known inactivation mechanism, a non-enzymatic antibacterial mode of action of lysozyme was achieved by denatured form of lysozyme without enzymatic action (Abdou et al., 2013).

Lysozyme demonstrates antimicrobial activity against a limited spectrum of bacteria and fungi. However, the antimicrobial activity of lysozyme is greater for certain Gram-positive bacteria. On the other hand, Gram-negative bacteria are less susceptible to the bacteriolytic action of the enzyme. The cell walls of different bacteria show varying degrees of susceptibility to digestion with hen egg white lysozyme. The walls of *Micrococcus lysodeikticus* were the most sensitive and the walls of *Staphylococci* were the less sensitive to the bacteriolytic action of lysozyme. Among Gram-negative bacteria, the walls of *Salmonella* and *Shigella* were the most sensitive (Hernández-Ledesma and Chia-Chien 2013). The susceptibility differences are believed to be due to the complex envelope structure of Gram-negative bacteria such as *E. coli*. The outer membrane serves to reduce the access of lysozyme to its site of action (peptidoglycan layer).

There is also extensive evidence of an antibacterial effect of ovotransferrin based on iron deprivation, iron being an essential growth factor for most microorganisms. The high affinity of transferrins for iron means that, in the presence of unsaturated transferrin (apotransferrin), iron will be sequestered and rendered unavailable for the growth of microorganisms. *In vivo*, ovotransferrin has been shown to have therapeutic properties against acute enteritis in infants. On the other hand, there is a direct interaction with the membrane and induction of damage to biological functions bacterial cytoplasmic membrane (Hernández-Ledesma and Chia-Chien 2013).

Functional avidin is found only in raw egg, as the biotin avidity of the protein is destroyed by cooking. The natural function of avidin in eggs is not known, although it has been postulated to be made in the oviduct as a bacterial growth-inhibitor, by binding biotin the bacteria need. As evidence for this, streptavidin, a loosely related protein with equal biotin affinity and a very similar binding site, is made by certain strains of *Streptomyces* bacteria, and is thought to serve to inhibit the growth of competing bacteria, in the manner of an antibiotic (Hendrickson et al., 1989).

Although the egg white is the main line of defense against invading microorganisms, a number of egg yolk components have also demonstrated antimicrobial activity. One of the most extensively studied is immunoglobulin (Ig) Y. IgY is the functional equivalent of IgG, the major serum antibody in mammals. It is transferred from the hen to the developing embryo, to give acquired immunity to the chick. Specific IgY can be produced by immunization of chickens with the target antigen and then purified from the egg yolk. It has been suggested that the antibodies may exert a sort of antimicrobial activity against pathogenic organisms, like *Salmonella*, by binding, immobilizing, and consequently reducing or inhibiting their growth, replication, or colony-forming abilities (Kovacs-Nolan et al., 2005).

GROWTH OF *SALMONELLA* IN EGGS POST-LAY

SE can grow in the contents of naturally contaminated eggs at room temperature; Cogan et al. (2001) observed growth after 8 days at 20°C in 7% of whole eggs inoculated in the albumen near the shell with as few as 2 CFU. It is clear that this implies a serious threat to human health because extensive growth in eggs does not lead to changes in the color, smell and consistency of the egg contents (Humphrey and Whitehead 1993). After experimental and

natural infections, some authors point to the albumen as being most frequently contaminated (Gast and Beard 1990; Humphrey et al., 1991b), while others point to the vitelline membrane as the most common contamination site (Bichler et al., 1996; Gast and Holt 2000a, 2001a; Gast et al., 2007).

The albumen is growth restricting for *Salmonella* because it contains multiple antimicrobial components, inducing bacterial cell wall and DNA damage. At temperatures 10°C, *Salmonella* bacteria are unable to grow in the albumen (Braun and Fehlhaber 1995; Schoeni et al., 1995). At room temperature, data are conflicting and it is difficult to compare the various studies because the inoculation size, strains, incubation temperatures and period, age of eggs and many other factors vary (Humphrey and Whitehead 1993; Braun and Fehlhaber 1995; Schoeni et al., 1995; Gast and Holt 2000b). Data showed that, at 20°C, upon inoculation with 39 CFU/ml albumen, both SE and non- SE strains are able to grow in separated fresh albumen samples up to 4,106 CFU/ml (Clavijo et al., 2006) and, on extending the incubation time, the number of samples with pronounced growth increased further. Numerous other studies also observed the growth of SE in egg albumen at room temperature (Braun and Fehlhaber 1995; Schoeni et al., 1995; Dubocage et al., 2001), indicating that the *Salmonella* bacteria harbor intrinsic characteristics to counteract the attacks of the antimicrobial components present in the egg albumen. Inoculation of bacteria in the egg albumen of whole eggs resulted in faster growth than separated egg albumen and also high numbers of *Salmonella* bacteria were detected in the yolk, indicating migration towards the yolk (Cogan et al., 2001; Messens et al., 2004).

Rehault-Godbert et al. (2010) analyzed the potential of egg white to inhibit growth of SE following storage at 4, 20, or 37°C for 30 days prior to inoculation. Egg white displayed higher anti-*Salmonella* activity after a few days of storage at 20 and 37°C. The rate of increase in activity was more rapid and pronounced at the higher temperature. However, egg white stored at 20°C retained higher antimicrobial activity than that of egg white stored at 4 or 37°C, when the entire storage period is taken in consideration. In contrast, storage of egg at 37°C for more than 14 days reduced the bacteriostatic potential of egg white. There was a correlation between pH and the antimicrobial activity of egg white. Moreover, diminished antimicrobial activity was associated with degradation of ovalbumin and ovotransferrin. However, the fluctuation in anti-*Salmonella* activity of egg white could not be related to any variation of trypsin-like, chymotrypsin-like, or gelatinolytic activities that potentially account for degradation of antimicrobial egg white proteins.

It is believed that *Salmonella* cells that are deposited in the albumen are able to migrate to and penetrate through the vitelline membrane in the egg post-lay, in order to reach the yolk and thus gain access to a pool of nutrients that are necessary for its survival and growth. Rapid and extensive multiplication of SE in the nutrient-rich egg yolks at 25°C has been reported (Gast and Holt 2000b). Data from contaminated eggs from either naturally (Humphrey and Whitehead 1992) or artificially (Gast and Beard 1992) infected hens suggest that there is a delay before yolk penetration and fast growth occurs in yolk, in eggs stored at room temperature. This is believed to be because the vitelline membrane in fresh eggs inhibits yolk invasion by *Salmonella*. Gradually, the integrity of the vitelline membrane will become lost during storage, resulting in leakage of nutrients into the albumen. This is considered to allow the bacteria to migrate to the vitelline membrane and multiply and invade the yolk (Humphrey and Whitehead 1993). Experimentally infected laying hens also often deposit SE

on the vitelline membrane (Bichler et al., 1996; Gast and Holt 2000a, 2001b; Gast et al., 2007).

Salmonella growth profiles seen in naturally contaminated eggs are different from those seen in eggs contaminated artificially. The latter suggests that growth is rapid in most eggs and that yolk invasion is common (Braun and Fehlhaber 1995; Chen et al., 1996). The experimental studies, however, used either high contamination levels (104 cells per egg), most likely not representative for naturally contaminated eggs, or buffered peptone water for the SE solution to be injected, which enhances bacterial growth in the albumen (Chen et al., 1996). Data obtained from artificial egg contamination models should thus be interpreted with caution. A model for artificial egg contamination mimicking the natural situation was developed by Cogan et al. (2001). Using low numbers of bacterial cells in a low-nutrient, low-iron suspension as inoculum, a low level of growth was detected in eggs, comparable to that seen in naturally contaminated eggs. When few *Salmonella* bacteria are deposited in the albumen, very little bacterial multiplication occurs and SE can persist there at supportive temperatures (Lock and Board 1992; Hammack et al., 1993; Gast and Holt 2000b). Humphrey and Whitehead (1993) observed that in artificially contaminated eggs, the inoculum increased 10-fold during the first 24 h postinoculation, as confirmed by Gast and Holt (2000b).

The initial growth phase may involve the bacterium using its iron reserves, which appear to be sufficient to support about four generations. When the iron reserves are exhausted, cells enter a lag phase, where, in the majority of eggs, there is little or no change in the numbers of *Salmonella* organisms. It has been postulated that there may be leakage of nutrients from the yolk, leading to a bacterial attraction towards the yolk, some weeks after storage. So the bacteria will then (after weeks of storage) attach to and penetrate through the vitelline membrane and gain access to the yolk contents in order to grow (Baron et al., 1997).

Post-laying internal *Salmonella* contamination of eggs from environmental sources occurs through the shell membrane penetration (De Reu et al., 2006). Miyamoto et al. (1998b) explored the potential of *Salmonella* to penetrate egg shells by immersing the eggs in SE and ST solutions at varying times post-laying. The highest incidence of internal *Salmonella* contamination occurred when eggs were between 15 min and 3 h post-laying (the shortest time period reported) and stored at 25°C (compared with 3.25 h to 6 h, 1 day and 7 days post-laying). Refrigerating eggs at 4°C for 15 min prior to *Salmonella* exposure significantly decreased penetration of the eggshell. It was suggested that this was due to reduced growth at the lower temperature. This indicates that refrigeration of eggs at collection may be a useful tool for minimizing internal *Salmonella* contamination. However, realistically this is difficult to implement as it will not prevent any contamination that occurs in the housing prior to collection. The ability of SE and ST to penetrate eggshells was not significantly different. The age of the hen and eggshell characteristics such as area, shell thickness and number of pores does not significantly influence the eggshell penetration by SE (De Reu et al., 2006).

STRATEGIES TO REDUCE *SALMONELLA* CONTAMINATION IN EGGS

An outbreak in Sweden is an illustrative example of possible *Salmonella* control measures. The Swedish policy of *Salmonella* control in poultry is based on the principles of a comprehensive control throughout the farm to fork, for example whenever *Salmonella* is

found in poultry feed, or in poultry, the *Salmonella* contaminated feedstuffs and/or infected birds are removed from the food-chain (Wierup et al., 1995).

Considerable public and private resources have been invested throughout the world in attempting to control the egg-borne transmission of *Salmonella*. A risk assessment study performed in the USA recommended intervention at multiple steps in the farm-to-fork continuum, as the most productive overall strategy (Hope et al., 2002). Three key interventions to prevent the contamination of shell eggs by SE have been the development of egg quality–assurance programs on farms, the rapid and sustained refrigeration of eggs from farm to consumer, and the education of consumers and food workers about the risks associated with pooling, handling, and consuming raw or undercooked eggs. Refrigerated storage and pasteurization of eggs are highly effective post-production options for risk mitigation. In most egg quality–assurance programs, repeated isolation of SE from a farm environment results in a diversion of eggs from that farm to egg-processing plants, where all egg products (i.e., pasteurized liquid egg or powdered egg products) are heat treated to reduce bacterial contamination (Braden 2006).

Education of food workers and of consumers of the importance of limiting pooling of eggs, and of handling and cooking eggs safely, has also helped to control SE infection. Physicians can play a role in SE control by educating patients of the risks associated with consumption of raw or undercooked eggs, the need to avoid contamination of other foods with raw eggs, and the importance of hand washing after handling eggs. Medical directors of hospitals and long-term care facilities that care for debilitated and immunocompromised patients (who are especially prone to severe *Salmonella* infection) should ensure that only pasteurized egg products be served to patients. In addition, physicians caring for patients with possible *Salmonella* infection should follow the guidelines on submission of diagnostic specimens, including stool specimens (Braden 2006),

Nevertheless, the preponderance of effort and expenditure has been devoted to controlling SE infections in laying flocks. In the early years after SE was first identified as a significant public health problem, most control plans focused on trace-back testing and eradication efforts. In recent years, assortment of microbial quality-assurance programmes for commercial laying flocks have been proposed and implemented by government agencies and by the poultry industry (Hogue et al., 1997b; US Food and Drug Administration, 2004). These programmes have represented a more proactive, and thereby far more effective, alternative to trace-back eradication. Most of these combine a battery of risk-reduction practices for egg producers, with a testing component designed to identify problem flocks for further attention (sometimes including regulatory intervention). The testing part of these programmes also serves as a means of assessing the ongoing efficacy of the risk-reduction practices to ensure that the commitment of resources to quality assurance programmes is cost-effective. In the most common approach to testing, environmental samples are collected and tested to screen for flock infection, and egg samples are subsequently tested to determine whether an ongoing threat to public health exists. Eggs from flocks that test positive must generally be diverted for pasteurization (Hogue et al., 1997b; US Food and Drug Administration, 2004).

Risk-reduction practices that are common to most quality assurance schemes include using chicks from flocks that are certified as uninfected by breeder-flock testing protocols, such as those of the National Poultry Improvement Plan in the USA (Rhorer 1999), implementing effective procedures for controlling rodents and other pests, heightened

biosecurity measures for poultry facilities, thorough cleaning and disinfection of facilities between flocks and refrigeration of eggs as soon as possible after collection. This type of approach has been associated with significant reductions in the incidence of SE infections in both egg-laying flocks and humans in several states in the USA (White et al., 1997; Mumma et al., 2004).

Another important tool for combating SE and ST infection in poultry is vaccination. The vaccines are targeted for these most often reported serovars of human infections. However, vaccination is not, at the moment, a control option for many other serovars which can be present on poultry farms. Infection of poultry by serovars other than *S. Gallinarum* (biovars *Gallinarum* and *Pullorum*) does not generally induce clinical signs except in young birds (EFSA 2004).

Vaccination of pullets or hens with either killed or live preparations has reduced (but not entirely prevented) fecal shedding, organ invasion and egg contamination, following challenge with SE (Gast et al., 1992, 1993; Zhang- Barber et al., 1999). This protection can be particularly significant for highly susceptible hens undergoing an induced molt (Holt et al., 2003; Nakamura et al., 2004). However, vaccination does not construct an impenetrable barrier to SE infection, since protective immunity induced by vaccines has been overcome occasionally by high challenge doses. Poor vaccine performance has sometimes been tied to severe rodent or sanitation problems in laying houses (Davies and Breslin 2003b). Nevertheless, even when vaccination has not completely prevented SE infection in commercial flocks, it has generally been able to accomplish meaningful reductions in egg contamination (Davies and Breslin 2004). In the UK, a declining prevalence of SE infections in humans was observed to follow the initiation of widespread vaccination of laying hens (Cogan and Humphrey 2003). Vaccination may be most valuable as an adjunct to other risk-reduction practices, especially when applied to highly susceptible flocks or flocks exposed to severe challenges from environmental sources (Gast 2005).

The *Salmonella* vaccines currently authorized for use in poultry in the Member states in the EU have been authorized on the basis of the mutual recognition procedure. Both live and inactivated *Salmonella* vaccines are available. At the moment, the extent of vaccination of breeders and laying hens in different Member States differs considerably (EFSA 2004). Vaccination programmes against SE reducing the shedding and contamination of eggs have been applied at least during rearing to all laying hens at the latest from 1 January 2008 on in Member States as long as they did not demonstrate a prevalence below 10% (Kyprianou 2006). When vaccination is used in *Salmonella* control programmes, possible interferences with standard *Salmonella* bacteriological and serological detection methods may be a disadvantage. In addition, there is concern over the use of antimicrobial resistance markers in some vaccines. One possible disadvantage of the use of live vaccines would be the spread of the strain to environment or to humans. Experience based on widespread use of existing *Salmonella* vaccines over several years and the results of monitoring, indicates that the vaccine strains of concern have not been disseminated in the environment or to humans. Use of inactivated vaccines against SE may also interfere with surveillance and control programmes for *S. Pullorum/Gallinarum* (EFSA 2004).

Other strategies to control *Salmonella* infection in laying hens aim at preventing intestinal colonization, based on the use of prebiotics, probiotics, synbiotics and other feed additives (De Buck et al., 2004). On the other hand, the administration of *Salmonella* specific

bacteriophage (Borie et al., 2011) or antibodies (Gurtler et al., 2004) can have beneficial effect in *Salmonella* control.

The most promising option for achieving sustainable reductions in the prevalence of contaminated eggs appears to be the patient and persistent application of risk-reduction programmes of verified efficacy. However, one potential area of vulnerability in microbial quality-assurance schemes for shell eggs is created by the possibility that *Salmonella* serotypes other than Enteritidis might become significant sources of egg-transmitted human disease. Although the epidemiological association between SE and eggs has been strong and unique, other paratyphoid serotypes (including ST, *S. Heidelberg* and *S. Thompson*) have also been reported to be capable of colonizing reproductive organs of chickens and thereby causing egg contamination (Snoeyenbos et al., 1969; Cox et al., 1973; Keller et al., 1997; Okamura et al., 2001b; Gast et al., 2004). Recently, the Centers for Disease Control and Prevention in the USA have implicated eggs and egg-containing foods as the principal sources of human *S. Heidelberg* infections (Hennessy et al., 2004). Nevertheless, several pivotal aspects of current risk-reduction efforts, such as testing and vaccination, focus almost exclusively on identifying or controlling SE and are not intended to address the possible presence of other *Salmonella* serotypes in eggs. Although targeting control measures to specific disease agents is crucial for mounting rapid responses to public health emergencies, risk-reduction practices that are not inherently agent-specific (such as biosecurity, rodent control, cleaning and disinfection and egg refrigeration) may be of even greater long-term importance because of their ability to minimize the opportunities for another pathogen to emerge and cause a new egg-borne disease crisis (Gast 2005).

CONCLUSION

Eggs are extremely nutritious. They are loaded with high-quality proteins, vitamins, minerals, good fats and various trace nutrients. However, the risk of getting sick if consuming eggs contaminated with *Salmonella* is situated on the first place. Eggs are considered as being potentially contaminated with salmonellas. That is why, their handling, storage, industrial processing and cooking in order to be consumed must respect the established rules so as to prevent any risks. Contaminated eggs are a source of contamination for other food they come in direct contact with, especially with the directly consumable food (which is not thermally processed). On the other hand, the egg has an impressive arsenal of antimicrobial protective mechanisms, including both nonspecific physical barriers and highly efficacious microbiocidal molecules.

Although it is possible to infect eggs with various bacterial serovars under the artificial conditions of a laboratory experimental setup, under natural conditions, this is a rare event. When it occurs, it usually causes so much damage that the egg will be easily identified as being infected. SE is unique in the way that it can pass into the egg and multiply inside it without inducing noticeable changes. Combining this exceptional trait with the pathogenicity for the human intestinal tract allowed this serotype of *Salmonella* cause foodborne disease.

Contamination of the eggs is not only by penetration through the shell, but also by passage from the hen's intestinal tract to the reproductive tract and from there incorporation into the forming egg on the vitelline membrane, in the egg white or the shell membranes. It

turns out that many different *Salmonella* serotypes can pass from the intestine of the chicken into its blood stream. Even passage from the blood stream into the hen's reproductive tract is not a unique characteristic of SE. However, this serovar, has the capacity to survive the attacks by antimicrobial molecules during the formation of the egg in the hen's oviduct.

While vaccination programme against SE and ST is a very valuable prevention practice to reduce hen infection and human disease, continued improvement and implementation of detection and environmental controls will also be effective in reducing egg contamination and therefore improving public health.

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Chapter 6

IMPACTS OF ALLOCHTHONOUS AND AUTOCHTHONOUS YEAST STARTERS: CASE STUDIES IN FRUIT WINE FERMENTATIONS

On-ong Chanprasartsuk¹ and Cheunjit Prakitchaiwattana^{2,}*

¹Department of Food Science, Faculty of Science, Burapha University, Saensook,
Mueang, Chonburi, Thailand

²Department of Food Technology, Faculty of Science,
Chulalongkorn University, Phyathai Road, Pathumwan, Bangkok, Thailand

ABSTRACT

Wine is a plant-derived alcoholic beverage, produced from raw materials that contain sugars or compounds that can be hydrolyzed to sugars. Fruits are increasingly used for wine making because of the diversity of raw materials, their uniqueness and health benefits. Many fruits which possess attractive color, aroma and taste, including temperate (i.e., apple, strawberry, pear, peach, cherry, raspberry) and tropical fruits (i.e., pineapple, orange, mango, banana) have been used for wine making. Quality and specific characteristics of the fruit wines depend mainly upon types of raw materials, wine maker's techniques, and particularly microbial strains and fermentation processes. Fleshy fruits such as berry, pepo, hesperidium, drupe and pome can all be used for wine making. However, process of making fruit wine is different from grape wine based on their specific physiological property, texture of fruit pulp and chemical composition of the juice. Unlike grape, fruits especially tropical fruits are usually low in sugars and other nutrients, yet high in acidity, which are not suitable for wine fermentation process. Therefore, adjusting sugar content, adding water to dilute the acidity and enhancing selected minerals to fruit juice prior to fermentation have generally been practiced. Consequently, these modifications affect the yeasts associated with fermentations. For fruit wines, the specific art or technology used to improve the wine quality is still at the initial development stage. The use of commercial strains of *S. cerevisiae* as an allochthonous starter culture at ambient temperature is a common practice in fruit wine production. Nevertheless, many winemakers still prefer wines produced by spontaneous

* Corresponding author: Telephone: +66 2 218 5531. Fax: +66 2 254 4314, Email: Cheunjit.p@chula.ac.th
Additional email: pcheunjit@yahoo.com.

fermentation because spontaneous/autochthonous yeasts, wild yeasts, provide unique flavors and exceptional quality of traditionally produced fruit wines. Local yeasts selected from spontaneous fermentation system are likely to be of superior quality compared to selected foreign yeasts and can be developed to be an autochthonous starter. The selected local yeasts should be able to adapt to the conditions of fruit juice, effectively dominate the fermentation, and become the most significant group of microbes in wine-making process. Selection of the suitable local yeasts can ensure the retaining of typical sensory properties of fruit wines produced in any given region. This chapter proposes case studies of yeasts in fruit wine fermentations to reflect impacts of allochthonous and autochthonous starters on wine quality. The work appears to have important implications for developing fruit wine and can contribute to an important advancement in any fermentation products.

Keywords: wine, fruits, fermentation, allochthonous yeast, autochthonous yeast, yeast starters

INTRODUCTION

Even though grapes are the main raw material for wine making, many researchers continue to investigate the suitability of fruits other than grapes. Increasingly, fruit becomes popular ingredient for wine production due to its diversity, uniqueness and health benefits. Quality and specific characteristics of the fruit wines depend mainly upon types of raw materials and wine maker's techniques. Generally, wine qualities are determined by flavor and aroma, which mainly generate during the fermentation.

Scientific data and advance technologies are well explored for grape wine fermentation. For wine fermentation, yeasts have profound impact on wine quality and value. Yeasts are in charge of the alcoholic fermentation on grape juice, thereby contributing to the basic structure and individuality of wine flavor and aroma. The major volatile products of yeast metabolism are ethanol and CO₂. They are relatively small amount compared to other components yet fundamentally contribute to wine flavor. The main groups of compounds forming fermentation bouquet are organic acids, higher alcohols and esters as well as, to a lesser extent, aldehydes (Lambrechts and Pretorius 2000). The compounds formed during alcoholic fermentation have a crucial influence on the volatile composition of wine. The dimensions of this contribution vary with species and strains of yeast. The alcoholic fermentation may be spontaneously occurred by indigenous yeasts or conducted by specific strains of inoculated yeasts to produce quality wine. Moreover, species specific starter cultures and/or a mix of these starter cultures can be an alternative for improving flavor quality.

S. cerevisiae as a starter culture together with ambient temperature fermentation are also used for the fruit wine production (Akubor 1996). To improve fruit wine fermentation, many researchers over the last few decades have tried to conduct a systematic investigation of yeasts associated with fruit juice fermentation. Natural yeast ecology from the spontaneous fermentation of fruit juices has been studied in order to select and develop species-specific starter cultures as autochthonous yeast starters for high quality wine production. In addition, fruit wine fermentation by starter culture inoculation of *Saccharomyces* and/or non-*Saccharomyces* as allochthonous yeast starters are also explored and developed as well as the optimization of fermentation condition.

PINEAPPLE WINE

Pineapple (*Ananas comosus* L. Merr.) is a fruit widely grown in tropical and sub-tropical countries. Pineapple juice is considerably varied in sugar and acidity. Major carbohydrate constituents are sucrose, glucose and fructose while major acids are citric acid and malic acid (Bartolomé et al., 1995; Bartholomew et al., 2003). There are a significant amount of amino acids such as glycine, alanine, methionine and leucine in ripe pineapple juice, while the others such as lysine, proline, histidine and arginine are present at relatively low levels (Gortner et al., 1967). The pineapple juices have been used successfully for alcoholic fermentation and also wine production (Alain et al., 1987; Ezeronye 2004; Chuaychusri et al., 2005).

In development of pineapple wine making process, many allochthonous yeast strains were tested and utilized. Alain and others (1987) investigated fermentation performances of seven *Saccharomyces cerevisiae* strains and *Candida utilis* CBS 5609 in sterile pineapple juices, cultivar "Cayenne Lisse" under different conditions. It was found that the natural pH (3.9) and acidity of the juice did not affect the growth of yeasts or the alcohol generated. Interestingly, the *Saccharomyces* strains isolated from tropical climate (Africa) performed better fermentation activity in pineapple juice compared to the strains obtained from temperate zone (i.e., France and USA). The tropical strains generated the highest amount of alcohol at 35°C. In contrast, *Candida utilis* fermented moderately well in this juice. The result is in line with the report of Ayogu (1999) which found that *S. cerevisiae* from the tropical origin (Africa) performed better alcoholic fermentation of pineapple juice (produced 10.2% (v/v) alcohol) compared to a commercial wine yeast (produced 7.4% (v/v) alcohol). Ruengrongpanya (1996) further reported that some *S. cerevisiae* strains could generate alcohol in pineapple juices up to 13.42% (v/v). However, in terms of organoleptic properties, pineapple wine containing lower alcohol content was more acceptable. This shows the importance of the yeast growth and the metabolic activity generated.

In addition to the primary metabolites such as ethanol, a number of the secondary metabolites also significantly impact the basic chemical composition and individuality of the pineapple wine's flavor and aroma. Pino and Queris (2010) demonstrated that the major constituents of volatile compounds in pineapple wine (cultivar Red Spanish) fermented with *S. cerevisiae* (bakery yeast, Fermipan Lefersa, La Habana) at 26°C, were ethyl octanoate, ethyl acetate, 3-methyl-1-butanol, and ethyl decanoate. Potentially, the most important volatile compounds of pineapple wine consisted of ethyl octanoate, ethyl acetate, and ethyl 2-methylpropanoate (Table 1).

In development of autochthonous starters, yeasts associated with the spontaneous fermentation in pineapple juices are investigated. The result shows many species of yeasts such as *Pichia anomala*, *Pichia guilliermondii*, *P. sydowiorum*, *Torulaspota delbrueckii*, *Candida versatilis*, and *Candida apicola* are generally found in tropical fruits including pineapple. However, the spontaneous fermentation of pineapple juices seems to be responsible by only a few yeast species (Rale and Vakil 1984; Tokouka et al., 1985; Warnasuriya et al., 1985). Chanprasartsuk et al. (2010a) demonstrated that only two main yeast species, *Hanseniaspora uvarum* and *P. guilliermondii*, were observed in the spontaneous fermentation system of pineapple juices cultivar Smooth Cayenne from Thailand and Australia (Figure 1). Additional yeast species were rarely found in this ecosystem. Apart from the two main species, *Candida tropicalis*, *Candida* sp., *Erythrobasisidium hasegawianum*

and *Saccharomyces ludwigii* were found in a few occasions. Similarly, Di Cagno et al. (2010) also reported that *P. guilliermondii* was the dominant species found during natural fermentation of Italian pineapple juice. The study indicated that the occurrence of *H. uvarum* and *P. guilliermondii* in freshly crushed juice did not depend on climate or region.

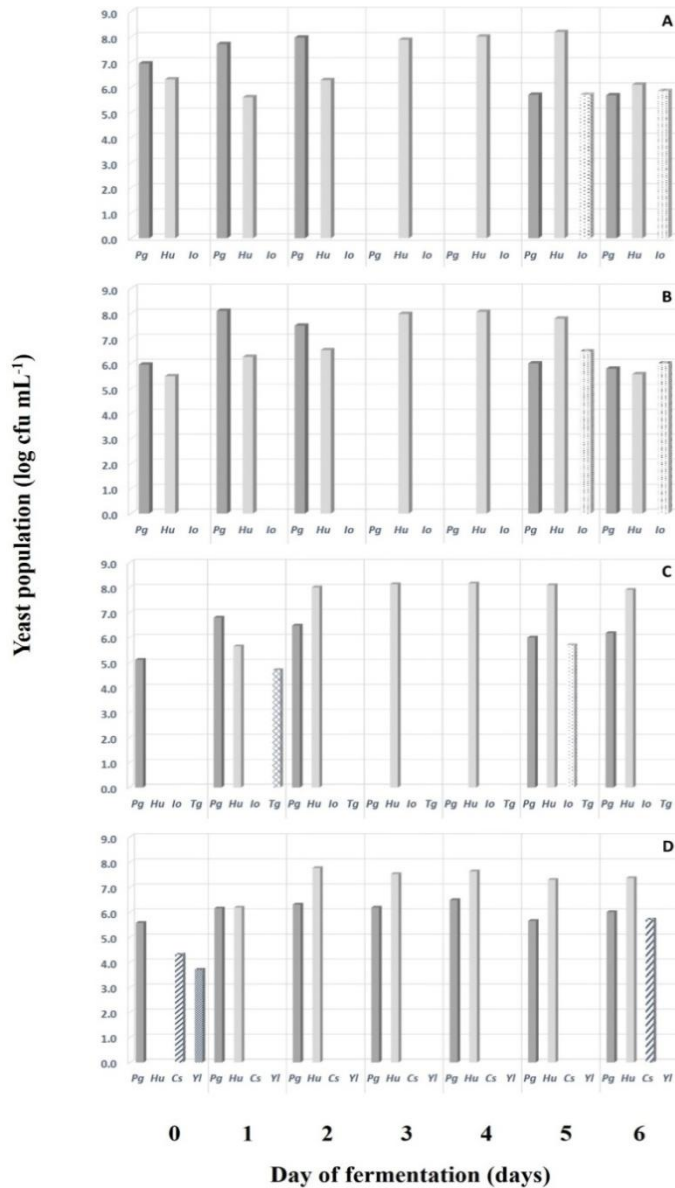


Figure 1. Spontaneous fermentation system of pineapple juices cultivar Smooth Cayenne from Thailand (TH) and Australia (AUS); A: TH region 1, B: TH region 2, C: AUS region 1, D: AUS region 2; Pg: *P. guilliermondii*, Hu: *H. uvarum*, Io: *Issatchenkia occidentalis*, Tg: *Tremella globispora*, Cs: *Candida* sp., Yl: *Yarrowia lipolytica* (modified from Chanprasartsuk et al., 2010b).

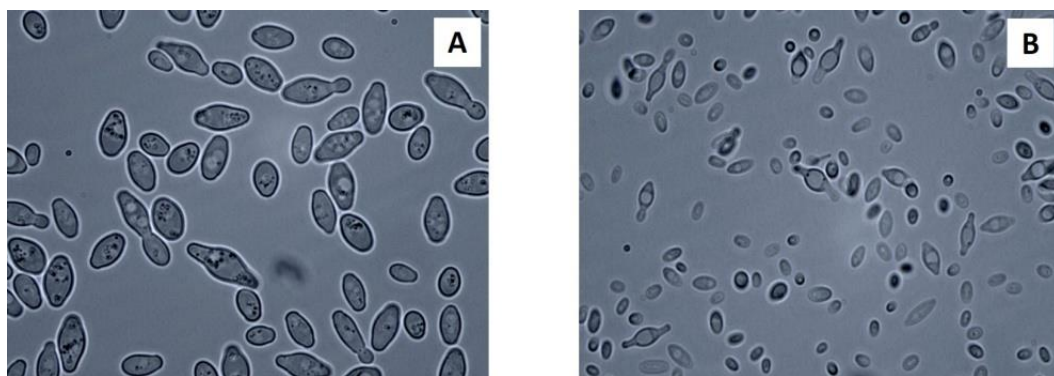


Figure 2. Morphological cells of autochthonous yeasts isolated during natural fermentation of pineapple juice; A: *S'codes ludwigii*, B: *H. uvarum* (Photos by: On-ong Chanprasartsuk).

Instead, the condition of juices seemed to be selective for the yeasts. According to Chanprasartsuk et al. (2010a), *P. guilliermondii* was present as dominant species during the early stage of the fermentation, while *H. uvarum* became more prevalent until the final day of fermentation. Their populations increased from the initial level of approximately $5 \log \text{cfu ml}^{-1}$ to approximately $8 \log \text{cfu ml}^{-1}$ at the end of fermentation (Figure 1). Ethanol generated in the system of these natural fermentations was varied between 1 to 4% (v/v). All organic acid remained constant throughout the fermentation. Interestingly, *S. cerevisiae* was not observed at any stage of the pineapple juice fermentation. The absence of *S. cerevisiae* may result from the influence of bromelain, a specific type of protease, which could theoretically inhibit many types of yeast, especially wine yeast.

The result from using the mixed starter of *S'codes ludwigii* and *H. uvarum* isolates from pineapple origin in wine produced from chaptalized Smooth Cayenne pineapple must showed potential in making high quality pineapple wine (Chanprasartsuk et al., 2010b). Chanprasartsuk et al. (2010b) also found that *S'codes ludwigii* could play a major role in alcoholic fermentation and help prolong the viability of *H. uvarum* during the initial stage of the fermentation (the morphological properties of these two yeasts are displayed in Figure 2). Since *H. uvarum* could generate 2-phenylethyl acetate in pineapple juice and provide rose and flower aroma attributes, this might enhance the complexity of volatile compound in pineapple wine. Apart from ethanol, major volatile compounds identified in the obtained pineapple wine were ethyl decanoate, ethyl dodecanoate, isoamyl alcohol, ethyl acetate and 2-phenylethyl acetate. Compared to the work done by Pino and Queris (2010), ethyl acetate was also identified as potentially most important compounds to pineapple wine.

In 2012, Chanprasartsuk et al. also applied these two yeasts (*S'codes ludwigii* and *H. uvarum*) for Queen pineapple juice fermentation. The juice of this cultivar generally contains higher sugar content but lower acidity than that of cultivar of Smooth Cayenne. After fermentation, the mixed cultures of *S'codes ludwigii* and *H. uvarum* produced the highest alcohol content of 14.0% (v/v) in the final day of fermentation. In contrast, the interaction between *S'codes ludwigii* and *H. uvarum* in Smooth Cayenne pineapple juices was not present in the fermentation system of the Queen juice. Yeast origin is one of criteria in selecting fruit wine strains, especially pineapple wine since the fruit cultivar could likely impact fermentation activity of autochthonous yeast isolates.

ORANGE WINES

Oranges (*Citrus sinensis*) is widely cultivated in both tropical and subtropical regions (Selli, et al., 2008) Orange juice has long been used as a raw material for wine production (Selli et al., 2002, 2003, 2004 and 2008; Gupta et al., 2009; Kelebek et al., 2009). In orange juice, major form of carbohydrate is sucrose while citric acid is the main acid (Kelebek et al., 2009). This condition is suitable for wine making since it contains high levels of sugar and a low pH range (3-4). Such conditions are optimal for yeast growth to perform an alcoholic fermentation.

In contrast to a few yeast species responsible for the spontaneous fermentation of pineapple juices, diverse indigenous yeast strains in fresh squeeze juice and in natural fermentation ecosystem of orange juice have been observed in many reports. In addition, cultivar and cultivation climate seem to have significant impact on yeasts associated with these ecosystems. Arias et al. (2002) found that *Hanseniaspora occidentalis* and *H. uvarum* represented up to 73% of total yeast found in fresh squeezed orange juices from the USA. Furthermore, additional yeasts present in this system were *Clavispora lusitaniae*, *Geotrichum citri-aurantii*, *H. occidentalis*, *Hanseniaspora vineae*, *Pichia fermentans*, and *Saccharomycopsis crataegensis*. In contrast, the report of Las Heras-Vázquez et al. (2003) showed that diverse yeast species such as *C. tropicalis*, *Cl. lusitaniae*, *H. uvarum*, *Pichia anomala*, *P. fermentans*, *Rhodotorula mucilaginosa*, *S. cerevisiae*, *Saccharomyces unisporus*, and *Trichosporon asahii* were found in freshly crushed orange juices from Spain. Soka and Susanto (2010) also demonstrated that yeasts isolated from fermented juices of different cultivars of Indonesian oranges were different. For instance, *Pichia veronae* was found in only fermented Indonesian Pontianak orange juice, while *Cryptococcus albidosimilis* and *I. orientalis* were found in Sunkist orange and Indonesian Medan orange, respectively. However, the functions of the spontaneous yeast during orange juice fermentation were not reported in these studies.

For orange wine making, production of alcohol and volatile compounds during fermentation of orange juice is greatly depended on yeast strains selected as a starter. Ndip et al. (2001) studied the fermentative ability of yeast strains, isolated from fruits (orange, grapefruit and pineapple), in orange wine production in comparison with commercial *S. cerevisiae* (baker's yeast). The highest (10.47% v/v) and the lowest (7.68% v/v) of alcohol content, with corresponded residual sugar concentration of 1.88% w/v and 7.7% w/v were found in fermentation of chaptalized orange must with allochthonous yeast starter, *S. cerevisiae*, and autochthonous yeast starter, *Saccharomyces ellipsoideus*, respectively. Furthermore, autochthonous yeast of *S. ellipsoideus* was the most suitable yeast strains for orange wine production since it received the highest acceptability score.

In addition, it was also found that the allochthonous *S. cerevisiae* strains generated only about 3-7% w/v alcohol when orange juice is fermented without chaptalization. *S. ellipsoideus* originated from orange juices had slower rates of sugar utilization and ethanol production compared to the strains isolated from the other sources. The wine fermented by this yeast contained the highest ethanol and the lowest methanol contents (Okunowo et al. 2005; 2007). The result reflected an appropriate performance of autochthonous strains in orange juice fermentation. *S. ellipsoideus* adapted well in the juice and performed a complete ethanolic fermentation with low methanolic generation.

Moreover, Mingorance-Cazorla et al. (2003) found that *S. cerevisiae* from orange origin lowered the presence of higher alcohol in natural orange must. The low concentration of higher alcohol might be due to insufficient amino acid content in the orange juice. The other yeasts such as *P. fermentans*, *R. mucilaginosa*, and *H. uvarum* strains originated from orange juice produced quality orange wine with low alcohol content. However, *P. fermentans* increased the amount of higher alcohols and ethyl acetate in orange musts. Additionally, Fan et al. (2009) investigated the volatile compounds of orange wine produced by spontaneous fermentation comparing with inoculated fermentation (commercial *S. cerevisiae*, at 20°C). Greater number and quantity of volatile compounds were identified in spontaneously fermented wine. Alcohols, esters and terpenes were the most abundant volatile compounds in both types of the orange wines. Selli et al. (2008) also confirmed that in spontaneously fermented orange wine (at 19°C), alcohols, terpenes and esters were the most abundant odor-active compounds. According to the studies of orange wine production as demonstrated above, autochthonous/indigenous yeast isolates are potential starters specific for quality orange wine production.

MANGO WINES

Mango (*Mangifera indica* L.) is one of the most popular dessert fruits of the tropics. It has rich, luscious, aromatic flavor and delicious taste in which sweetness and acidity are delightfully blended. Ripe mango fruits contain 70% to 80% of water by weight and ripened mango juice is normally used as a raw material for wine production (Reddy and Reddy 2005 and 2009). Juice from ripened mango is rich in sugar (16-18% w/v), of which sucrose, glucose and fructose are as the major composition while malic acid is the main acid (Giri et al. 1953; Anon 1962). The juice also contains antioxidants, particularly, carotenoids. The total soluble solids of mango juice are between 14.2% and 20.5% with pH between 3.8 and 4.5 (Reddy and Reddy 2005). The chemical composition of mango juice is suitable for wine production (Czyhrinciw 1966). In wine making process, mango juice can be prepared by mechanical or enzymatic extraction of mango pulp. The slurry or treated pulp is squeezed or filtrated through cheesecloth to obtain the juice. The suitability of mango juice for wine making is usually investigated by following the regular process of fruit wine making (Kulkarni et al. 1980; Onkarayya and Singh 1984; Akubor 1996; Reddy and Reddy 2005 and 2009).

To develop mango wine, the inoculation of must with different strains of selected yeasts in order to minimize the influence of wild yeast on wine quality have been widely studied. In contrast, the contribution of wild/indigenous yeasts to alcoholic fermentation and the impact of metabolites on organoleptic properties are rarely researched. In mango fruit ecosystems, *P. fermentans* was frequently isolated from mango fruits (Kunkee and Goswell 1977; Vacek et al. 1979; Las Heras-Vazquez et al. 2003). When the fruit were overripe or rotten, species such as *Metschnikowia pulcherrima*, *Debaryomyces hansenii*, *I. orientalis*, *Hypho-pichia burtonii*, *Trichosporon cutaneum*, and others were frequently found. The fermentation on rotting fruit was caused by *H. uvarum*, *H. valbyensis*, *P. guilliermondii*, *P. fermentans*, *Pichia kluyveri*, and, to a lesser extent, *S. cerevisiae*. The diverse yeast species were also found in fresh, fermenting and fermented juice of mango such as *M. pulcherrima*, *T. cutaneum*, *Kloeckera*

apiculata, *Torulopsis/Candida glabrata*, *Torulopsis apicola*, *C. tropicalis*, *Candida krusei*, *Candida sorbosa*, *Candida diversa*, *Pichia terricola*, *Pichia membranaefaciens*, *Hy. burtonii*, *Rhodotorula graminis* and *Aureobasidium pullulans* (Suresh et al., 1982). Only the report of Warnasuriya et al. (1985) demonstrated the presence of *Saccharomyces* yeasts (*Saccharomyces italicus*) in mango juices.

As stated above, only the inoculation of must with selected yeasts, particularly *S. cerevisiae* from different origins, have been widely evaluated. Ezeronye (2004) reported the nutrient utilization pattern of *S. cerevisiae* from palm wine origin in chaptalized mango must fermentation compared with the other wine yeasts. It was found that nutrient utilization profile of the *S. cerevisiae* isolate showed high degree of adaptability compared to the other yeasts in both nutrient utilization and growth performance. The obtained mango wine had 11% alcohol. However, in natural mango juices (without chaptalization) wine yeasts had better fermentation activity than palm wine isolates. Reddy and Reddy (2005) selected three yeast strains from different sources and evaluated their fermentation performances in mango juice without chaptalization under different temperature and pH conditions. Among three stains, *S. cerevisiae* CFTRI 101 (wine strains) showed promising results under all conditions followed by the yeast strain isolated from palm wine and baker's yeast (PWY1). The wine strain adapted well to the conditions of mango must, started ethanol production within 4 hours of inoculation, and completed the fermentation within 70 to 76 hours. The baker's yeast did not well utilize sugar from the mango must and had a slow fermentation rate. The fermentation rate of yeast from palm wine was relatively close to wine yeast but it had lower alcohol tolerance. Wine yeast produced larger amounts of esters compared to the other two. The mango wines contained 7–8.5% (w/v) ethanol and were well-accepted.

Santiago-Urbina et al. (2011) also evaluated the alcohol production of yeasts isolated from palm wine ("taberna" indigenous wine of Mexico) in mango juice comparing to reference strains of *S. cerevisiae* NRRL-Y-2034. It was found that the alcohol production of yeast isolates was not as high as the reference strains. Furthermore, Ameyapoh et al. (2010) reported the alcoholic fermentation performance of *S. cerevisiae* L2056 (France) in pasteurized mango juice. Twenty-four hours after the beginning of alcoholic fermentation, the viable cells were over 90%. Under this condition, yeast produced 2.24% (w/v) ethanol in 72 h. In addition, Reddy and Reddy (2009 and 2010) reported that fermentation conditions i.e., temperature, pH, SO₂, and aeration influenced yeast growth and composition of mango wine. An increase in temperature during fermentation resulted in increased amount of yeast biomass and volatile compounds. The sum of all the secondary metabolites was also increased as fermentation temperature was raised from 15°C to 35°C. Optimum pH was a very important factor in producing good quality mango wines. Although high concentration of SO₂ led to a decrease in yeast growth, change in SO₂ concentration only affected wine quality slightly. Presence of dissolved O₂ increased both ethanol content and yeast biomass. Formation and concentration of the volatile compounds found in mango wine depended on mango cultivar and *S. cerevisiae* strain used. However, higher alcohols and esters were found as abundant volatile compounds in the wine. Yeast strains selected as starter culture and enzymatic treatment for juice extractions also significantly influenced chemical composition of mango wines, which consequently influenced sensory characteristics.

Li et al. (2011) studied the fermentation properties of three different strains of *S. cerevisiae* i.e., *S. cerevisiae* var. bayanus EC1118, *S. cerevisiae* var. chevalieri CICC1028, and *S. cerevisiae* var. *cerevisiae* MERIT.ferm in mango must without chaptalization. It was

found that all yeast strains produced wines containing about 8% (v/v) ethanol. Sugars such as glucose, fructose, and sucrose were mostly used at the end of fermentation. Malic acid was significantly decreased whereas the concentration change in citric, succinic, and tartaric acids were insignificant. Various types of volatile compounds mainly fatty acids, alcohols, and esters were produced. The kinetic changes of volatiles were similar among the three yeasts but the concentrations of some volatiles varied with yeast. This demonstrates that different *S. cerevisiae* strains produce mango wines with distinct characteristics.

Apart from strain selection, application of yeast immobilization systems for mango wine fermentation was also studied. Srirakul et al. (2007) and VarakuMar et al. (2012) evaluated the suitability of immobilized *S. cerevisiae* cells for mango wine fermentation. The immobilized cells performed proper alcoholic fermentation and also produced acceptable amount of volatile compounds including ethyl acetate, higher alcohol and others. Consequently, the mango wines produced by this procedure had potentially better aroma than those obtained by free cell fermentation.

Recently, Li et al. (2012a) proposed the application of non-*Saccharomyces* yeasts, *Williopsis saturnus* strains, in improvement of flavor quality of mango wine (strain details are provided in Table 1). It was found that these yeasts retained certain amounts of monoterpenes in mango juice and produced terpenols and their corresponding esters. This helped mango wine to retain the aromatic hints of fresh mango. The fermentation performances of mixed-culture of *S. cerevisiae* and *W. saturnus* in mango juices from different varieties were also evaluated. Both yeasts grew well in all juices from different mango varieties but their fermentation profiles were different upon mango varieties. Fructose, glucose and sucrose were mostly utilized in all juices. While changes in citric, tartaric, malic, acetic and succinic acids depended on mango varieties, the changes of major volatiles were similar in all varieties. The amount of volatiles particularly most of the terpenes was significantly decreased and new volatiles such as β -citronellol were present. In the development of mango wine fermentation as reviewed in this chapter, the contribution of indigenous yeasts to mango juice fermentation or the use of yeasts from mango origin for making mango wine have not been widely reported.

BANANA WINE

Bananas (*Musa* species) are an important food crop planted extensively in tropical and subtropical regions. They have a unique flavor and also contain high sugar levels, low acid content, as well as high levels of mineral and vitamin. Bananas contain ~78% of water, ~23% of carbohydrate, ~1% of proteins and 1% of mineral with pH of ~5 (Stover and Simmonds 1987; Robinson 1996; Food and Agriculture Organization of the United Nations (FAO), 2003). *Musa* species at common ripe stage contain carbohydrate ranged from 21.0 g to 24.0 g per 100 g which is suitable for alcoholic beverage fermentation (Oyenuga 1968; Kundu et al., 1976). Sugar, fructose and glucose, are mostly found while malic acid and citric acid are the principal acids in ripe banana (Hotsommer 2001; Judprasong et al. 2011). Generally, quality banana wine is obtained from over-ripe fruit (Kotecha et al., 1994; Akingbala et al., 1992; Byarugaba-Bazirake 2008). In East African countries, bananas have been used for making traditional alcoholic beverage called banana beer. The beer is made by naturally fermenting

banana juice blended with sorghum flour. This represents the indigenous banana juice fermentation.

According to Wilson et al. (2012), the growth of lactic acid bacteria, yeast, molds, and aerobic mesophilic bacteria were observed in banana juice during the 72 hours of spontaneous fermentation system. The initial sugar level in banana juice was decreased from 19°Brix to 7°Brix. Titratable acidity increased from 0.18% lactic acid to 0.90% lactic acid, pH decreased from 4.8 to 4.0, while alcohol concentration increased to 7% (v/v) after 72 h of fermentation. Unlike usual wine fermentation, the fermentation was initiated by lactic acid bacteria followed by alcoholic fermentation by yeasts. Under this condition, lactic acid bacteria created an acid environment which was favorable to the proliferation of yeasts to conduct alcoholic fermentation (Holzapfel 2002). Although banana is known to be inhabited by mixed yeast flora of *P. anomala*, *P. guilliermondii*, *P. sydowiorum*, *Tor. delbrueckii*, *C. versatilis*, and *C. apicola* which are generally found in tropical fruits (Rale and Vakil 1984; Tokouka et al., 1985; Warnasuriya et al., 1985), the role of yeasts in banana pulp fermentation and/or yeasts associated with spontaneous fermentation of juice and/or wine have not been well studied. Only Idise et al. (2011) demonstrated the characteristics of naturally fermented banana wine compared to the wine fermented with baker's yeast. The fermentation profiles of natural flora and inoculated yeast in all conditions as well as wine characteristics and organoleptic properties were relatively similar. The produced wines contained ~1.3% (v/v) alcohol and titratable acidity varied from ~0.012 to 1.3% with pH of ~3-4. Malolactic fermentation was evident after 48 h in all fermentation batches.

In contrast to the study on spontaneous fermentation in banana wine, Onwuka and Awam (2001) and Akubor et al. (2003) investigated fermentation behavior of *S. cerevisiae* (baker's yeast) in chaptalized banana juice from different *Musa* species. In this must, the yeasts utilized sugar at high rate and rapidly produced metabolites allowing pH to drop drastically at the first three to five days. The final alcoholic concentration in banana wine ranged from ~5 to ~11% (v/v) with pH of 3-4. Moreover, Raquel Mendonca et al. (2011) evaluated fermentation performance of selected *S. cerevisiae* strains (UNICAMP-V1, UFMG-A905, UFMG-A1007 and UFMG-A1240) in banana pulp compared to commercial yeast (baker's yeast). One of the selected strains, UNICAMP-V1, produced higher amount of ethanol (94.06%) compared to the baker's yeast (83.07%), whereas the alcohol production of the other selected strains was less efficient. Although fermentation performance of the *S. cerevisiae* strains UNICAMP-V1 and the commercial yeast was better in terms of high alcohol level and low methanol content, they did not produce higher alcohols or any significant aroma compounds. Therefore these two were recommended for the production of distilled spirits made of banana rather than for wine making. The variation in alcohol content might be influenced by elastic pulp present in the juices. The amount of the pulp present in the juices was depended upon extraction mean and/or banana cultivars (Byarugaba-Bazirake 2008). In addition, Jitjaroen (2007) produced banana wine from mashed banana with two commercial Brewer's yeast strains (*S. cerevisiae* Siha 3 and G 74). The main organic acids in the banana wine were malic acid (1.97 g L⁻¹) and citric acid (1.42 g L⁻¹). The major aroma components produced were acetic acid ethylester, acetic acid 2 and 3-methylbutylester, 2 and 3-methyl-1-butanol and phenethyl alcohol.

APPLE CIDER AND WINE

Apple (*Malus domestica*) fruit is used to make mild alcoholic beverages (Bhutani et al., 1989; Gasteineau et al., 1970; Joshi and Thakur 1994). Apple is associated with cider more than any other alcoholic beverages (Amerine et al., 1967; Joshi 1995; Sandhu and Joshi 1994). Cider is a low alcoholic drink produced by fermentation of apple juice known by different names such as cidre (France), sidre (Italy), sidra (Spain) and apfel wein (Germany and Switzerland). Like grape wine, cider have been produced and consumed worldwide. Cider can be sweet or dry depending upon the alcohol content. Cider is categorized into soft cider (1-5% alcohol) or hard cider (6-7% alcohol) (Downing 1989; Joshi 1995).

The microorganisms associated with cider fermentation have been extensively studied and well described. Cider with acceptable level of alcohol and acidity generally obtains from the fermentation of apple juice with *S. cerevisiae* along with *Schizosaccharomyces pombe* (O'Reilly and Scot 1993). The optimum temperature for cider fermentation ranges from 15 to 18°C (Beach 1957). Nevertheless, the study on cider produced by spontaneous fermentation is still needed. Therefore, research on yeasts in spontaneous fermentation system for cider making has been carried out. Similar to grape wine fermentation, non-*Saccharomyces* yeasts, such as *Kloeckera*, *Candida*, *Pichia*, *Hansenula*, *Hanseniaspora* and *Metschnikowia* mainly grow during the first few stages of the process, and *Saccharomyces* later dominates during ethanolic fermentation in natural fermentation system (Michel et al., 1988; Cabranes 1994). However, the diversity of the yeasts is influenced by several factors such as geographic location, climatic conditions, apple varieties as well as the cider making technology i.e., apple juice extraction methods (Poulard et al., 1985; Cabranes et al., 1990; Mangas et al., 1994; del Campo et al., 2003).

From the report of Valles et al. (2007), yeasts associated with the fermentation systems of apple juices from different extraction methods were significantly different. The musts obtained by pneumatic pressing were dominated by only non-*Saccharomyces* yeasts (*Hanseniaspora* genus and *M. pulcherrima*) whereas in the apple juices obtained by traditional pressing, both *Saccharomyces* together with non-*Saccharomyces* were always found. The *Saccharomyces* species present were *S. cerevisiae* and *S. bayanus*. These two *Saccharomyces* were found as dominant yeasts in natural fermentation of cider in every report whereas the presence of non-*Saccharomyces* yeasts seemed to be varied depended on production region (Morrissey et al., 2004; Coton et al., 2006; Valles et al., 2007).

As cider, apple wine is another product made from apple juice or concentrate by alcoholic fermentation but it has higher alcohol content of 11-14%. In apple wine making, amelioration with sugar or juice concentrate is required along with acidification (Joshi et al., 2000) in order to create the suitable condition for yeasts to completely conduct alcoholic fermentation. Ammonium salts are generally added to the juice to reduce the higher alcohol generation caused by non-degradation of amino acids in the must (Beech 1972).

For the development of apple wine fermentation, selection and/or improvement of yeasts strains for enhancing quality flavor and obtaining appropriate amount of phenolic compounds associated with antioxidant properties are usually studied. Satora et al. (2009) found that volatile composition and other wine properties were mainly influenced by strains of yeasts and type of fermentation. *Saccharomyces cerevisiae*, generally used for apple wine fermentation, could utilize sugar up to 95%. Furthermore, *S. cerevisiae* (Johannisberg-

Riesling) could utilize malic acid during alcoholic fermentation which helped lowering acidity whereas *S. cerevisiae* (Steinberg) did not have this property. Additionally, cell immobilization positively affected the ethanol content, but decreased the antioxidant activity of the wines (Satora et al., 2008; 2009).

Kunicka-Styczyńska and Pogorzeiski (2009; 2012) also compared the fermentation performance of industrial wine yeasts, *Saccharomyces bayanus* and two interspecies hybrids (*S. cerevisiae* x *S. bayanus*), in apple must containing different malic acid contents. Fermentation profiles of wines from three yeasts were different, but similar organic acid profiles of malic, pyruvic and citric acids were also dominantly found. Yeast strains responded individually to different acidities of the fermentation environment. The study further showed that the performance of yeast strains was not significantly influenced by chemical composition of apple musts. In contrast, malic acid is one of the principal factors in the adaptive evolution of the strains. Strains improved by interspecific hybrids tended to change their fermentation performances more drastically than the intraspecific in apple must.

The research on spontaneous fermentation process and the use of autochthonous isolate starter have not been extensively reported so far. Spontaneous yeasts might not be appropriate for apple wine making as a result of the low resistance to the increase of ethanol concentration during fermentation. Therefore, the wine made by this mean generally contains high amount of unfermented sugar. In addition, the apple wines fermented spontaneously were characterized by more esters and methanol but fewer higher alcohols compared to the inoculated (Satora et al., 2009).

STRAWBERRY WINE

Strawberry (*Fragaria x ananassa*) is an important commercial fruit consumed worldwide (Sharma et al., 2009). Strawberry composition consists of fructose, glucose and sucrose as well as organic acids i.e., citric acid, malic acid, ascorbic acid and succinic acid. Moreover, it is a source of vitamin C, folate and a variety of non-nutritive phytochemicals such as anthocyanins, ellagitannins, flavonols and flavanols. Most of these polyphenolic compounds express antioxidant capacities and promising health effects (Wang and Lin 2000; Proteggente et al., 2002; Hannum, S.M., 2004; Scalzo et al., 2005a; Scalzo et al., 2005b; Tulipani et al., 2008, 2009; Aaby et al., 2012; Giampieri et al., 2012).

Recently, strawberry fermented products i.e., wine and vinegar have been introduced as health food. The research in development of strawberry wines, particularly yeasts associated with fermentation, are extensive compared to the other fruit wines. In spontaneous alcoholic fermentation of strawberry, the factors such as strawberry cultivars and cultivation locations mainly affected variety of yeast strains associated with the fermentation ecosystems. Hidalgo et al. (2013) reported that during spontaneous alcoholic fermentation of strawberry pulp of *Fragaria ananassa*, Camarosa variety in southern Spain, the yeast strains present were not diverse. Only three yeasts of *Issatchenkia terricola*, *S. cerevisiae* and *H. uvarum* were found. However, the amount of these yeasts was sufficient to complete the spontaneous fermentation. Autochthonous *S. cerevisiae* was the main species present throughout the process. On the other hand, yeasts associated with the spontaneous fermentation of Mediterranean strawberry fruits (*Arbutus unedo* L.) in southern region of Portugal, were

obviously different in term of diversity and the strains present (Santo et al., 2012). *A. pullulans*, *Dothichiza pithyophila*, *Dioszegia zsolttii*, *H. uvarum* and yeasts belonging to the genera *Metschnikowia*, *Cryptococcus* and *Rhodotorula* were found at the early stage of Mediterranean strawberry fermentation. Then, *Metschnikowia* and *Hanseniaspora* were more prevalent at the end of the initial stage of fermentation. Furthermore, *I. orientalis*, *Lachancea thermotolerans* and *Candida zemplinina* were additionally observed in the first stage and in the beginning of the second stage of the spontaneous fermentation. *S. cerevisiae* was rarely present in this fermentation ecosystem even though this yeast was present at low level on the fruit surfaces.

The low diversity of *S. cerevisiae* strains during the spontaneous of fermentation of *Fragaria x ananassa* was also reported by Hidalgo et al. (2013). In this *Fragaria x ananassa* spontaneous ecosystem, *P. membranaefaciens* was additionally present concurrently with *S. cerevisiae*. The research shows that the strawberry pulp itself could be a substrate for autochthonous *S. cerevisiae* to complete alcoholic fermentation (Hidalgo et al., 2013; Santo et al., 2012) which similar to the spontaneous fermentation of grape and apple musts (Sabate et al., 2002; Valles et al., 2007). However, in spontaneous fermentation of strawberry wine, yeasts produced less alcohol and the must was more easily to be contaminated (Ma et al., 2003).

There were several studies focused on application of autochthonous *S. cerevisiae* isolates for strawberry wine making. The autochthonous isolates were successfully tested as a potential starter for quality wine fermentation. The fermentation performances of the autochthonous isolates selected from strawberry juices were found significantly better than allochthonous/commercial *S. cerevisiae* strains as evaluated by Pan et al. (2005a), Hidalgo et al. (2013) and Wang et al. (2010). The wines fermented with appropriate autochthonous isolates exhibited clear and bright color, pure taste and classical flavor of strawberry wine. This type of wines composed of isobutyl alcohol, isoamyl alcohol, ethyl lactate and β -phenethyl alcohol (Wang et al., 2010).

In contrast, the use of allochthonous/commercial yeasts in strawberry wine production resulted in good fermentation at rapid rate was also reported (Yang et al., 1991; Pan et al., 2007; Gao et al., 2008; Sharma et al., 2009). For instance, Gao et al. (2008) used commercial fruit wine yeast, *S. cerevisiae* XEC19, to complete strawberry wine fermentation within 7 days under 21°C. The final alcohol and sugar contents of the obtained strawberry wine were 10% and 4.5%, respectively. The wine had full-bodied flavor, dark red color, high quality and good clarification level. Sharma et al. (2009) studied the fermentation process and physicochemical change during the fermentation of three strawberry cultivars namely, Camarosa, Chandler, and Douglas with active dry yeast. The obtained wine had pH of 3.18-3.26, sugar content of 8.1-9.7 °Brix with 0.124-0.135% reducing sugar, 9.2-11.5% (v/v) alcohol and 0.65-0.73% titratable acidity.

From commercial aspects, frozen strawberry was mainly used for wine fermentation studies (Han et al., 2007; Zhou et al., 2008). As a result, comparison of wine quality produced from fresh and frozen strawberries was studied. Also, the fermentation conditions affected quality of strawberry wine i.e., material pretreatment, fermentation strains and inoculation, fermentation temperature, sugar content, $K_2S_2O_5$ content, and fermentation cycle were investigated. The result showed that the optimum condition was achieved when the fermentation was conducted at 22°C for 5 days with 4% inoculation of yeast, 20% sugar content and 140 mg kg⁻¹ $K_2S_2O_5$, after frozen strawberry was pretreated by

thermovinification. Meanwhile, Zhou et al. (2008) also produced the strawberry wine by fermenting strawberry primarily kept at -10°C with different strains of yeast at room temperature. Quality of the fruit wine was assessed by sensory evaluation and the analysis of volatile compound, polyphenol, and acid contents. The optimal conditions were 250 mg kg^{-1} sulfur dioxide, 30% sugar content (by fruit weight), multistarter cultures fermentation by *S. cerevisiae* strain 1450 and R92 at a ratio of 1:1, water added at the twice amount of the frozen juice, and fermentation temperature at $10\text{-}15^{\circ}\text{C}$ for 12 days. The result demonstrated that pre-storage of strawberry improved the quality of final product due to the decrease of fruit temperature which was subsequently favorable for low temperature fermentation.

RASPBERRY WINE

The red raspberry (*Rubus idaeus*) is a commercial fruit widely grown in Russian Federation, Poland, USA, Serbia and Ukraine (Statistical Division of the Food and Agriculture Organization of the United Nations (FAOSTAT), 2012). Raspberry has a desirable color, firm texture, and good sensory attributes including aroma, sweetness, and acidity (Malowicki et al., 2008). Also, this fruit is a good source of polyphenolic phytochemicals particularly anthocyanin pigment, which is responsible for its characteristic red color (Darte et al., 2010a; Darte et al., 2010b; Gonzalez et al., 2011) and other phenolic acids, such as p-coumaric, caffeic, ferulic and ellagic acids (Häkkinen et al., 1999). The raspberry pulp consists of succinic acid and malic acid as main specific organic acid which differs from the other berries. The main sugars are sucrose, fructose and glucose (Daurte et al., 2010a). Its pH, total soluble solid and reducing sugar content were 3.2-3.7, 10.0-15.0°Brix and 4.17 g/100 g of pulp, respectively (Rommel et al., 1990; Daurte et al., 2010a; Darte et al., 2010b; González et al., 2011; Djordjević et al., 2015) with approximately 90% moisture content, 0.9 g of total protein/100g pulp and 0.4 g of ash/100g of pulp (González et al., 2011). Based on these chemical and physical properties, raspberry pulp and juice could be used as a substrate for yeast to conduct alcoholic fermentation. In addition, the attractive color, aroma and taste also increase the potential of using raspberry as raw material for fruit wine production.

In various scientific studies of spontaneous fermentation of raspberry, only González et al. (2011) demonstrated the fermentation properties of natural yeast flora in solid-state fermentation (SSF) system of ripe raspberry pulp. The spontaneous fermentation commenced and alcohol was slowly generated during the first 20 h, and rapidly increased to approximately 0.26 g/100 g of pulp at 60 h. Although the microbes associated with this autochthonous fermentation were not characterized, it was noted that yeasts were the main organism responsible for the fermentation. Unlike grape, fermentative microbe i.e., *Saccharomyces cerevisiae*, in particular, did not dominate the system to complete alcoholic fermentation at that alcohol level even though sugar still remained after 60 h. The result reflects low diversity of yeast strains in microbiota of fresh raspberry pulps.

When the raspberry pulps were, however, subjected to fermentation by inoculation with alcohol producing yeasts *S. cerevisiae*, the yeast completed alcoholic fermentation process by producing alcohol after 20 h of fermentation then rapidly produced up to 0.45-0.46 g alcohol/100 g of pulp within 60 h. Sugars in the pulp were utilized approximately 94%

indicating that they were mainly converted to alcohol. In contrast, indigenous flora had more potential to produce compounds, such as glycerol contributed to sensory quality, comparing to the alcoholic production yeast. However, further research of indigenous yeasts and their roles in raspberry pulp or juice fermentation has not been carried out. The other researches are mainly focused on evaluation of selected allochthonous starter for quality raspberry wine making.

Daurte et al. (2010a) reported the fermentation properties of 15 strains of *S. cerevisiae* and one strain of *S. bayanus* in raspberry wine fermentation (strain details are provided in Table 1). These yeasts were isolated from ecosystems of fruit juice and cassava fermentations. Raspberry juices with sugar content adjusted to 16 °Brix were fermented at 22°C. The resulted raspberry wines fermented with several strains of *S. cerevisiae* and *S. bayanus* isolated from fruit origins contained high contents of total volatile compounds ranged from 67.57 to 87.43 mg L⁻¹. Even though some strains from fruit origin produced high amount of acetates and higher alcohols, they unfortunately produced acetic acid which was negative factor for raspberry wine making. Thus, the yeasts strains chosen as the most suitable for raspberry wine were the strains producing low concentrations of volatile acids and high concentrations of acetates, higher alcohols and ethyl esters during alcoholic fermentation.

In the same year, Daurte et al. (2010b) further evaluated the profile of metabolites produced by selected strains in raspberry wines. All selected yeasts produced similar profile of compounds determining wine flavors but the quantity of each compound from each strain were significantly different. When these selected strains were used to produce wine and sensory evaluation was conducted, the *S. cerevisiae* strain UFLA FW in which generated the lowest concentration of volatile sulphur but the highest concentrations of acetates and total volatile compounds was the yeast that produced a raspberry wine with a good result on both chemical and sensory quality.

Recently, Djordjević et al. (2015) evaluated the performance of immobilized commercial *S. cerevisiae* EC1118 and RC 212 starters in raspberry wine fermentation comparing to suspended cells. The raspberry juice contained 11.7 °Brix of sugar with pH of 3.0 and was fermented at 15°C. Under this condition, the immobilized cells had a shorter lag phase and faster sugar utilization and ethanol production compared to those fermented with suspended cells. In comparison with strain EC1118, strain RC212 performed significantly slower fermentation rate and produced much higher amount of acetic acid but lower amount of glycerol. This observation clearly reflects an impact of yeast strains on raspberry juice fermentation and wine quality.

In addition, black raspberry (*Rubus coreanus* Miquel), belonging to the Rosaceae family is cultivated in various regions of Asia, including Korea, China, and Japan. Black raspberry wine is one of the important products derived from this fruit in the regions (Hager, Howard, Prior, and Brownmiller 2008). Apart from color, the chemical and physical properties of the black raspberry are similar to the red raspberry which are rich in phenolic compounds and other antioxidant agents (Kim 1996; Ku and Mun 2008; Lim et al., 2012). A large number of researchers are interested in the medicinal properties of the raspberry wine involving the changes of phenolic compounds and bioactive agents during fermentation. The alcoholic fermentation process seems to increase the medicinal properties of raspberry juices. Lim et al. (2012) fermented black raspberry juice with active dry yeast *S. cerevisiae* and found that polyphenol contents and anti-inflammatory activities were significantly increased. These

desirable medicinal properties obtained through alcoholic fermentation mechanism of the yeast under 25°C for 15 days. This finding on the medicinal properties was also supported by Nogueira et al. (2008) who demonstrated that the level of some phytochemical compounds in black raspberry wines evidently increased during alcoholic fermentation. This *might be due to the function of yeasts* in breaking down plant cell walls causing the release of phenolic compounds as similarly found during the fermentation of cider. The study also proposed the development of black raspberry wine in term of flavor quality; however, only the use of commercial wine strains has been evaluated.

Latterly, Kim et al. (2015) investigated the performances of 12 commercial wine yeasts in black raspberry wine fermentation to develop a wine with high flavor quality. This study indicated that yeast strains that generated high amount of esters and terpenes contributed to floral and fruity aromas of the wines would be a potential strain to be developed for quality raspberry wine. Ethyl esters, particularly ethyl hexanoate (apple, fruity and sweet aroma characters), ethyl benzoate (fruity and floral aroma characters) and ethyl cinnamate (white flower aroma character), were reported as the most important groups of aromatic compounds in black raspberry wines because of their extremely low odor thresholds (0.001–0.05 mg L⁻¹). Their concentrations depended on the yeast strain, fermentation temperature, aeration and sugar content.

CHERRY WINE

Cherry, belonged to genus *Prunus*, is one of the most popular temperate fruits with unique flavor and color. It is mainly cultivated in temperate regions of Europe, Asia, and North America. Cherry is rich in anthocyanins (Kirakosyan et al., 2009), and phenolic acids (Jakobek et al., 2009). Cherry has been used in food industry for the production of several kinds of food and beverages including wines (Sun et al., 2013). Cherry wine is a processed cherry product with specific aroma and taste. This wine has become well renowned in China. As a result, a large number of researches have been carried out by the Chinese over the past decade. The sour cherry (*Prunus cerasus* L.) is normally used for wine making studies since its pH is suitable for wine fermentation. Furthermore, sweet cherry (*Prunus avium* L.) and Chinese cherry (*Prunus pseudocerasus* L.) are also reported as raw material for cherry wine fermentation (Xiao et al., 2015).

Aroma components are the most important and outstanding characteristic of cherry wine. It is derived from hundreds of volatile compounds from cherry berry, from wine-making by yeasts, and aging process (Weldegergis et al., 2007; Sun et al., 2011; Xiao et al., 2015). Most of the odorous compounds, more than 1,000 volatiles, which are produced during fermentation has been confirmed in the wines including alcohols, esters, organic acids, aldehydes, ketones, phenols, terpenes and furans, etc. (Castro et al., 2004; Xiao et al., 2014). Cherry variety is one of the important factors influencing flavor and aroma of the wine, but the yeast strains used for fermentation also significantly determine the sensory characteristics. Thus, the selection of a proper yeast strain is crucial for development of the desired cherry wine characters (Patel and Shibamoto 2002). Nowadays, a wide range of wine yeast strains (allochthonous yeasts) are commercially available offering a wine maker an opportunity to

explore one or more suitable yeasts for reliable, consistent and predictable cherry wine quality.

Sun et al. (2011) investigated the fermentation of tart variety 'Early Richmond' cherry with different commercial strains of *S. cerevisiae* including BM4_4, RA17, RC212, D254, D21 and GRE at 20°C. These commercial yeasts generated ethanol in cherry wines ranged from 11 to 12% (v/v) with small variation of pH values (4.07–4.22). All strains produced relatively different amount of volatile acidity. *S. cerevisiae* strains D254 and GRE generated the highest concentrations of volatile acid mainly acetic acid, while *S. cerevisiae* strains RC212 and RA17 generated this volatile acid lower than that of the others. The levels of titratable acidity of the wines from all strains were ranged from 5.79 to 6.71 g L⁻¹ of malic acid. In addition, the strains of *S. cerevisiae* were capable of metabolizing malic acid. Hence, they could be further used during de-acidification of cherry wine. With respect to the ability to produce volatile compounds associated with wine aroma, all strains produced relatively similar aromatic profiles. The major volatile compounds generated were five alcohols including 1-propanol, 2-methylpropanol, 3-methylbutanol, benzyl alcohol, and 2-phenylethyl alcohol at the concentration of ≥10 mg L⁻¹. Moreover, these yeasts also generated 14 esters including ethyl lactate, which was the most important volatile ester, and ethyl acetate, which was one of the major esters in cherry wines. Although the development of yeast strains by selection from indigenous strains has been broadly researched, the report on the ecology of both *Saccharomyces* and non-*Saccharomyces* (autochthonous yeasts) associated with cherry fruits or spontaneous cherry wine fermentation has not yet published.

Moreover, the application of non-*Saccharomyces* has been increasingly investigated. The fermentation profiles of commercial non-*Saccharomyces* and *S. cerevisiae* in fresh crushed cherry fruits of tart variety "May Duck" has been studied (Sun et al., 2014). The strains subjected to study were *S. cerevisiae* strains Lalvin EC1118 and D254, *Tor. delbrueckii* ZYMAFLORE Alpha (TD n. Sacch) and *M. pulcherrima* JS22. The fermentation was conducted with single, mixed and sequential inoculations at 25°C. The mixed *M. pulcherrima* JS22 and *S. cerevisiae* fermentation showed a completely different yeast-yeast interaction. *S. cerevisiae* demonstrated a rapid fermentation rate, while the number of *M. pulcherrima* JS22 dropped dramatically during the fermentation, and was not detected after 7 days. For volatile compounds, multistarter cultures noticeably enhanced the production of acids, alcohols, aldehydes ketones and esters compared to the single culture. Thus, the combined use of non-*Saccharomyces* along with *S. cerevisiae* in cherry wine fermentation could give the complexity of aromas and sensory quality of cherry wine products. Also, Comitini et al. (2011) and Sadoudi et al. (2012) found that the yeast characters, roles and interaction during multistarter fermentations of non-*Saccharomyces* with *S. cerevisiae* in cherry wine were relatively similar to their characters and roles during grape wine fermentation. As a result, the combined use of non-*Saccharomyces* along with *S. cerevisiae* in cherry wine fermentation gave complex aroma and enhanced sensory quality of cherry wine.

It is well known that cherry fruits are rich in polyphenol compounds which display a broad spectrum of health benefits (King and Youez 1996; Stoner et al., 2008; Yao et al., 2004). Therefore, various researches are focused on polyphenol compounds in cherry wine which might associate with yeast activities during alcoholic fermentation. Sun et al. (2011) also investigated the influence of the above mentioned six different commercial strains of *S. cerevisiae* on the increase of polyphenols during the fermentation. Five phenolic acids and four anthocyanins were identified during the study including chlorogenic acid,

neochlorogenic acids, cyanidin 3-glucosylrutinoside, and cyanidin 3-rutinoside as the major polyphenols of all cherry wine. When principle component analysis was used to classify cherry wine, the wine can be categorized into three different groups. The first group, able to produce higher amount of chlorogenic acid, neochlorogenic acid, p-coumaric acid, and caffeic acid, was the wine samples fermented with *S. cerevisiae* strains BM4_4 and D21. The second group characterized by a higher content of cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-glucosylrutinoside and peonidin 3-rutinoside was the wines from *S. cerevisiae* strains RC212 and D254. The last group with relatively lower concentration of polyphenols was the wines from *S. cerevisiae* strains RA17 and GRE. This study demonstrated that the concentration of phenolic compounds in cherry wines was closely related to compounds generated from yeasts fermentation which was similar to the result found in raspberry wine as previously described.

Furthermore, Xiao et al. (2015) demonstrated that various phenolic compounds in cherry wines were also closely related to cherry cultivar and variety. However, cultivar had more influence on phenolic compounds of cherry wines than variety. In addition, researches on wine making from indigenous cherry cultivars such as Oblačinska (sour cherry), *Prunus cerasus*, and Cornelian cherry, *Cornus mas* L., showed thorough chemical properties of the wine, especially characterizing phenolic compounds and their medicinal properties (Rakonjac et al., 2010; Pantelić et al. 2014; Tešević et al. 2009).

EXOTIC FRUIT WINES

Wines produced from commercial fruits have been extensively researched and developed as reviewed above. Many other fruits including indigenous fruits in various regions have also been used for wine making. In addition, the studies of the fruits, with attractive color, aroma and taste, as well as potential for making attractive aromatic wine, such as Lychee (*Litchi chinensis* Sonn) and Papaya or Pawpaw (*Carica papaya*), are relatively informative. Information on selected strains of commercial *Saccharomyces* and non-*Saccharomyces* as well as their roles in aromatic compound generation including inoculation protocols were also reported by several research groups such as Alves et al. (2010), Zeng et al. (2008), Chen et al., (2015), Ezeronye et al., (2004) and Lee et al. (2010a, 2010b, 2012), etc.

Unfortunately, there was only limited information about the spontaneous fermentation used on exotic fruits compared to those about commercial *Saccharomyces* yeasts. Cashew apple (*Anacardium occidentale* L.) is one of the exotic fruits that information on yeasts associated with spontaneous fermentation is detailed. Through this natural fermentation system, the alcohol content reached ~7% v/v but sensory quality was still lower than that of the wine produced from commercial *Saccharomyces* yeasts (Joseph 2010). The other work reported performance of spontaneous isolates in exotic fruit wine is from Gabiroba fruit (*Campomanesia pubescens*) fermentation. Although, the alcohol production was not complete (~4% w/v alcohol) in this spontaneous fermentation, the complex aromatic compounds were generated. However, when spontaneous non-*Saccharomyces* isolates were used in a form of single starter, they generated lesser concentration of higher alcohols relative to the commercial *S. cerevisiae* UFLA CA 1162 (Duarte et al., 2009; 2010a).

Table 1. Autochthonous and allochthonous yeasts in fruit wine fermentations

Fruits	Yeasts in fruits and spontaneous fermentation	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
Pineapples (<i>Ananas comosus</i> L. Merr.)	Main yeasts: <i>P. guilliermondii</i> , <i>H. uvarum</i> (similarly present in natural fermented juices from different regions) (Di Cagno et al. 2010; Chanprasartsuk et al. 2010b)	Autochthonous isolates: <i>H. uvarum</i> , <i>S'codes ludwigii</i> (25°C) (Chanprasartsuk et al. 2010a)	12% (v/v) alcohol major volatile compounds: ethyl decanoate, ethyl dodecanoate, isoamyl alcohol, ethyl acetate and 2-phenylethyl acetate
	Minor yeasts: <i>P. anomala</i> , <i>P. sydowiorum</i> , <i>Tor. delbrueckii</i> , <i>C. versatilis</i> , <i>C. apicola</i> , <i>C. tropicalis</i> , <i>Candida</i> sp., <i>E. hasegawianum</i> and <i>S'codes ludwigii</i> (associated to fruits and fermented juices) (Rale and Wakil 1984; Tokouka et al. 1985; Warnasuriya et al., 1985; Chanprasartsuk et al. 2010b)	Allochthonous strains: <i>C. utilis</i> CBS 5609 <i>S. cerevisiae</i> isolates (ambient) (Alain et al., 1987; Ayogu, 1999; Ruengrongpanya 1996) <i>S. cerevisiae</i> (baker's yeast, Fermipan Lefersa, La Habana) (26°C) (Pino and Queris 2010)	7 - 13% (v/v) alcohol major volatile compounds: ethyl octanoate, ethyl acetate, 3-methyl-1-butanol and ethyl decanoate
Oranges (<i>Citrus sinensis</i>)	Main yeasts: <i>P. veronae</i> (in fermented Pontianak orange juice), <i>C. albidosimilis</i> (in fermented Sunkist orange), <i>I. orientalis</i> (in fermented Indonesian Medan orange) (Soka and Susanto 2010) Minor yeasts: <i>H. occidentalis</i> , <i>H. uvarum</i> , <i>Cl. lusitaniae</i> , <i>G. citri-aurantii</i> , <i>H. occidentalis</i> , <i>H. vineae</i> , <i>P. fermentans</i> , <i>S'copsis crataegensis</i> (in freshly squeezed orange juices from USA) (Arias et al., 2002) <i>C. tropicalis</i> , <i>Cl. lusitaniae</i> , <i>H. uvarum</i> , <i>P. anomala</i> , <i>P. fermentans</i> , <i>R. mucilaginosa</i> , <i>S. cerevisiae</i> , <i>S. unisporus</i> , <i>T. asahii</i> (in the freshly crushed orange juices from Spain) (Las Heras-Vázquez et al., 2003)	Autochthonous isolates: <i>S. ellipsoideus</i> (ambient) (Ndip et al. 2001) <i>P. fermentans</i> , <i>R. mucilaginosa</i> , <i>H. uvarum</i> (Mingorance-Cazorla et al. 2003) Spontaneous fermentation (19 - 29°C) (Selli et al., 2008; Fan et al., 2009) Allochthonous strains: <i>S. cerevisiae</i> (baker's yeast) (Ndip et al. 2001) Commercial <i>S. cerevisiae</i> , (20°C) (Fan et al. 2009)	~8% (v/v) alcohol with lower methanol volatile compounds; higher alcohols and ethyl acetate increased by <i>P. fermentans</i> major volatile compounds: alcohols, terpenes and esters ~10% (v/v) alcohol with higher methanol major volatile compounds: alcohols, esters and terpenes

Table 1. (Continued)

Fruits	Yeasts in fruits and spontaneous fermentation	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
Mango (<i>Mangifera indica</i> L.)	Main yeasts: <i>P. fermentans</i> (in mango fruits) (Kunkee and Goswell 1977; Vacek et al. 1979; Las Heras-Vazquez et al., 2003) Minor yeasts: <i>M. pulcherrima</i> , <i>T. cutaneum</i> , <i>K. apiculata</i> , <i>To./C. glabrata</i> , <i>To. apicola</i> , <i>C. tropicalis</i> , <i>C. krusei</i> , <i>C. sorbosa</i> , <i>C. diversa</i> , <i>P. terricola</i> , <i>P. membranaefaciens</i> , <i>Hy. burtonii</i> , <i>R. graminis</i> , <i>A. pullulans</i> (in fresh, fermenting and fermented juice of mango) (Suresh et al., 1982)	Allochthonous strains: <i>S. cerevisiae</i> isolated from palm wine, commercial <i>S. cerevisiae</i> CFTRI 101, baker's yeast (PWY1) juices (Reddy and Reddy 2005) <i>S. cerevisiae</i> L2056 (France) without chaptalized (Ameyapoh et al., 2011) <i>S. cerevisiae</i> var. bayanus EC1118, <i>S. cerevisiae</i> var. chevalieri CICC1028 and <i>S. cerevisiae</i> var. cerevisiae MERIT.ferm (20°C) without chaptalized (Li et al., 2011) Mixed culture of <i>S. cerevisiae</i> MERIT.ferm and <i>W. saturnus</i> var. mrakii NCYC500 (20°C) (fermented various mango cultivars) (Li et al., 2012)	7 - 8.5% (w/v) alcohol 2.24% (w/v) alcohol in 72 h ~8% (v/v) alcohol major volatile compounds: fatty acids, alcohols and esters; MERIT.ferm: higher alcohols, isoamyl and 2-phenylethyl acetates increased; CICC1028: medium-chain fatty acids and ethyl esters of decanoate and dodecanoate increased fermentation profile varied upon mango varieties, similar changes of major volatile compounds, terpenes of the mango juices decreased, and new volatiles β-citronellol produced
Bananas (<i>Musa</i> species)	Main yeasts: <i>P. anomala</i> , <i>P. guilliermondii</i> , <i>P. sydowiorum</i> , <i>Tor. delbrueckii</i> , <i>C. versatilis</i> , <i>C. apicola</i> (on banana fruits) (Rale and Vakil 1984; Tokouka et al., 1985; Warnasuriya et al., 1985) Spontaneous fermentation: initiated by lactic acid bacteria followed by alcoholic fermentation by yeasts (Holzapfel 2002)	Spontaneous fermentation (Idise et al., 2011) Allochthonous strains: <i>S. cerevisiae</i> (baker's yeast) (Onwuka and Awam 2001; Akubor et al., 2003) Bakers' yeast without chaptalized (Idise et al., 2011) Commercial <i>S. cerevisiae</i> SIHA, <i>S. cerevisiae</i> G74 (Jitjaroen 2007)	~1.3% (v/v) alcohol, titratable acidity ~0.012 - 1.3%, pH ~3-4.12 ~5 to ~11% (v/v) with pH 3 - 4 (in different banana cultivars) similar to spontaneous fermentation standard alcohol level with major volatile compounds: 15 esters, 4 alcohols, 3 acids,

Fruits	Yeasts in fruits and spontaneous fermentation	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
			4 phenols, 1 ketone; prominent aroma components: acetic acid ethylester, acetic acid 2 and 3-methylbutylester, 2 and 3-methyl-1-butanol, and phenethyl alcohol
Apple (<i>Malus domestica</i>)	Main yeasts: <i>Kloeckera</i> , <i>Candida</i> , <i>Pichia</i> , <i>Hansenula</i> , <i>Hanseniopsis</i> and <i>Metschnikowia</i> , <i>S. cerevisiae</i> and <i>S. bayanus</i> (in spontaneous fermentation system for cider making) (Michel et al., 1988; Cabranes 1994; Morrissey et al., 2004; Coton et al., 2006; Valles et al., 2007)	Spontaneous fermentation (Satora et al., 2009) Allochthonous strains: <i>S. cerevisiae</i> Johannisberg-Riesling <i>S. cerevisiae</i> Steinberg, (tested in wine making) (Satora et al., 2008; 2009) <i>S. cerevisiae</i> , <i>S. bayanus</i> and interspecies hybrids (<i>S. cerevisiae</i> x <i>S. bayanus</i>) (immobilized form, tested in wine making) (Kunicka-Styczyska and Pogorzeiski 2009; 2012) Mixed culture of <i>S. cerevisiae</i> and <i>Schiz. pombe</i> (O'Reilly and Scot 1993; Joshi et al., 2000)	contains high amounts of unfermented sugars with more esters and methanol and fewer higher alcohols standard alcohol level (~11 - 14% v/v) with lower acid standard alcohol level with higher acid standard alcohol level with similar acid profile mainly composed of malic, pyruvic and citric acids (each strains conducted different fermentation profiles) 11 - 14% (v/v) alcohol
Strawberry (<i>Fragaria x ananassa</i>)	Main yeasts: <i>I. terricola</i> , <i>S. cerevisiae</i> and <i>H. uvarum</i> (in spontaneous alcoholic fermentation of strawberry pulp of <i>Fragaria ananassa</i> , Camarosa variety in southern Spain) (Hidalgo et al., 2013) Main yeasts: <i>A. pullulans</i> , <i>D. pithyophila</i> , <i>Dio. zsoitii</i> , <i>H. uvarum</i> , <i>Metschnikowia</i> , <i>Cryptococcus</i> , <i>Rhodotorula</i> Minor yeasts: <i>I. orientalis</i> , <i>L. thermotolerans</i> and <i>C. zemplinina</i> (in spontaneous fermentation of Mediterranean strawberry)	Spontaneous fermentation (Ma et al. 2003) Autochthonous isolates: <i>S. cerevisiae</i> isolates (21 ~ 23°C) (Pan et al., 2005; Hidalgo et al., 2013; Wang et al., 2010) Allochthonous strains: commercial <i>S. cerevisiae</i> XEC19 (21°C) (Gao et al., 2008)	less alcohol level standard alcohol level (~13% with clear and bright color with best strains); major volatile compounds: isobutyl alcohol, isoamyl alcohol, ethyl lactate and β -phenethyl alcohol 10% alcohol and sugar 4.5% with full-bodied flavor, dark red color

Table 1. (Continued)

Fruits	Yeasts in fruits and spontaneous fermentation	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
	fruits (<i>Arbutus unedo</i> L.) in southern region of Portugal) (Santo et al., 2012)	Active dry yeasts <i>S. cerevisiae</i> (fermented strawberry cultivars, Camarosa, Chandler and Douglas) (Sharma et al., 2009) Multistarter of <i>S. cerevisiae</i> strain 1450 and R92 (fermented frozen juice and fresh juice as raw material) (10 ~ 15°C) (Zhou et al., 2008)	9.2 - 11.5% (v/v) alcohol with 0.65 - 0.73% titratable acidity standard alcohol level and strawberry wine quality
Red raspberry (<i>Rubus idaeus</i>)	Spontaneous fermentation: in solid state fermentation (SSF) of ripe pulp the spontaneous microbes commenced the fermentation within 20 h (González et al., 2011)	Spontaneous fermentation (SSF on pulp, 60 h) (González et al., 2011) Allochthonous strains: <i>S. cerevisiae</i> IFI83 (SSF on pulp, 60 h) (González et al., 2011) <i>S. cerevisiae</i> UFLA FW 15 isolated from fruit, <i>S. cerevisiae</i> CAT-1, <i>S. cerevisiae</i> VR-1 and <i>S. bayanus</i> CBS 1505 (22°C) (Daurte et al., 2010c) <i>S. cerevisiae</i> EC1118 and RC 212 (15°C) (Djordjević et al., 2015)	~0.26 g alcohol/100 g of pulp ~0.45 - 0.46 g alcohol/100 g of pulp standard alcohol level with low concentrations of volatile acids and high concentrations of acetates, higher alcohols, ethyl esters, and volatile fatty acids EC1118 performed faster fermentation rate giving standard alcohol level with higher amount of glycerol and RC 212 giving standard alcohol level with higher amount of acetic acid
Black raspberry (<i>Rubus coreanus</i> Miquel)	no report	Allochthonous strains: active dry <i>S. cerevisiae</i> yeast (25°C) (Lim et al., 2012)	standard wine with increases/decreases of some phytochemical compounds: rutin, 3,4-dihydroxybenzoic acid, caffeic acid, gallic acid, and p-coumaric acid were increased by 27 - 188%, while catechin, epicatechin, malvidin-3-glucoside, myricetin, and quercetin were decreased by 5 - 30%

Fruits	Yeasts in fruits and spontaneous fermentation	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
		<i>S. cerevisiae</i> M1 and Enoferm CSM (Kim et al., 2015)	standard alcohol level with high amount of esters and terpenes; key aromatic compounds, ethyl esters, particularly, ethyl hexanoate, ethyl benzoate and ethyl cinnamate
Cherry genus <i>Prunus</i>	no report	<p>Allochthonous strains: commercial <i>S. cerevisiae</i> BM4_4, RA17, RC212, D254, D21 and GRE (fermented tart variety 'Early Richmond'; 20°C) (Sun et al., 2011)</p> <p>Commercial <i>S. cerevisiae</i> strain Lalvin EC1118 and D254, and <i>Tor. delbrueckii</i> ZYMAFLORE Alpha^{TD n. Sacch} and <i>M. pulcherrima</i> JS22 (fermented tart variety "May Duck"; 25°C) (Sun et al., 2014)</p>	<p>~11 to 12% (v/v) alcohol with pH 4.07 - 4.22, with lower acetic acid (RC212 and RA17), with higher acetic acid (D254 and GRE) major volatile compounds: five alcohols including 1-propanol, 2-methylpropanol, 3-methylbutanol, benzyl alcohol, and 2-phenylethyl alcohol and 14 esters including ethyl lactate, the most important volatile ester, and ethyl acetate</p> <p>standard wine character, best quality obtained from multi-starter of <i>M. pulcherrima</i> JS22 and <i>S. cerevisiae</i> that enhanced the production of acids, alcohols, aldehydes, ketones, and esters</p>

Table 2. Exotic fruit wine and yeasts evaluated in fermentations

Fruits	Region of Fruits	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
Cacao (<i>Theobroma cacao</i> L.)	Native to the tropical regions of Central and South America and widely cultivated in many regions of tropical regions	<p>Allochthonous strains: <i>S. cerevisiae</i> strains CA116, CA1162, CA1183) (Dias et al., 2007)</p> <p><i>S. cerevisiae</i> UFLA CA 1162 (20°C) (Duarte et al., 2010a)</p>	<p>standard wine character; level of higher alcohols, methanol, esters and acetaldehyde was in accordance with the standards established for table wine; CA1183 performed best and produced higher ethanol</p> <p>~4% w/v alcohol; major acids: succinic, malic, acetic; major volatile compounds: acetaldehyde, 1,1-diethoxyethane, ethyl acetate, methanol, 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol</p>
Cashew apple (<i>Anacardium occidentale</i> L.)	Originally native to northeastern Brazil, widely grown in tropical regions, India, Nigeria, Pakistan and Southeast Asia	<p>Spontaneous fermentation: <i>S. cerevisiae</i>, <i>S. cerevisiae</i> var. ellipsoideus, <i>Rhodotorula</i> sp., <i>Candida</i> sp., <i>Lactobacillus brevis</i>, <i>Gluconobacter oxydans</i> and <i>Klebsiella aerogenes</i> found during fermentation (28°C) (Joseph 2010)</p> <p>Allochthonous strains: <i>S. cerevisiae</i> var. bayanus (Mohanty et al., 2006), <i>S. cerevisiae</i> isolated from palm wine (Ezeronye 2004), <i>S. cerevisiae</i> var ellipsoidius (28°C) (Attri 2009; Joseph 2010), commercial baker's yeast (28 °C) (Ogunjobi and Ogunwolu 2010)</p>	<p>~7% (v/v) alcohol with pH 3.9 (lower sensoria quality than <i>S. cerevisiae</i> var ellipsoidius inoculation)</p> <p>6-12% (v/v) alcohol with pH 3.6-4.1; slightly yellowish; acidic taste</p>

Fruits	Region of Fruits	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
Cupuassu (<i>Theobroma grandiflorum</i> Schum.)	Tropical fruits native to Brazil	Allochthonous strains: <i>S. cerevisiae</i> UFLA CA 1162 (20°C) (Duarte et al., 2010a)	~4% (w/v) alcohol; major acids: succinic, malic, acetic; major volatile compounds: acetaldehyde, 1,1-diethoxyethane, ethyl acetate, methanol, 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol
Gabioba (<i>Campomanesia pubescens</i>)	Tropical fruits native to Brazil	Spontaneous fermentation: <i>Candida quercitrusa</i> and <i>I. terricola</i> found during fermentation (Duarte et al., 2009) Spontaneous fermentation (Duarte et al., 2010a)	lower amount of alcohol and lesser concentration of higher alcohols relative to <i>S.cerevisiae</i> UFLA CA 1162 ~4% (w/v) alcohol; major acids: succinic, malic, acetic; major volatile compounds: acetaldehyde, 1,1-diethoxyethane, ethyl acetate, methanol, 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenylethanol, and 2-phenoxyethanol
Lychee (<i>Litchi chinensis</i> Sonn)	Tropical and subtropical fruit tree native to China, India and wildy cultivated in Southeast Asia, the Indian subcontinent and South Africa	Spontaneous fermentation (20°C) (Alves et al., 2010) Allochthonous strains: <i>S. cerevisiae</i> UFLA CA116, UFLA CA1183 and UFLA CA1174) (20°C) (Alves et al., 2010)	major volatile compounds: alcohol and esters ethanol, ethyl octanoate, ethyl decanoate, 3-methyl-1-butyl acetate (isoamyl acetate), ethyl hexanoate, diethyl succinate, and 2-phenylethanol major volatile compounds: similar to spontaneous fermentation; UFLA CA1183 produced the most complex aroma; UFLA CA1174 produced higher amount of ethyl acetate

Table 2. (Continued)

Fruits	Region of Fruits	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
		<p>Activated industrial yeast <i>S. cerevisiae</i> (R2, Mauri Co., Australia) (10-15°C) (Zeng et al., 2008)</p> <p><i>Tor. delbrueckii</i> PRELUDE (Chen et al., 2015)</p> <p><i>W. saturnus</i> NCYC22 (Chen et al., 2015)</p> <p><i>Kluyveromyces lactis</i> KL71 (Chen et al., 2015)</p>	<p>~9% (w/v) alcohol; major acids: citric, malic lactic; major volatile compounds: ethyl octanoate, ethyl decylate, acetic ester, ethyl hexoate, acetic isopentyl ester, and citronel ethyl acetate</p> <p>~8% (v/v) alcohol; performed fastest growth rate and high sugar consumption with aroma character-impact terpenes and terpenoids, generated high levels of ethanol, isoamyl alcohol, 2-phenylethyl alcohol, ethyl octanoate, and ethyl decanoate and retained high OAVs* of lychee aroma-character compounds cis-rose oxide, and linalool character-impact terpenes and terpenoids</p> <p>~1% (v/v) alcohol, consumed the highest amount of nitrogen, with aroma character-impact terpenes and terpenoids, over-produced ethyl acetate</p> <p>~3% (v/v) alcohol with aroma character-impact terpenes and terpenoids with higher OAVs* of geraniol and citronellol, over-produced ethyl acetate</p>

Fruits	Region of Fruits	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
Papaya or Pawpaw (<i>Carica papaya</i>)	tropical fruit native to southern America, Africa and Southeast Asia	<p>Allochthonous strains: baker's yeast (Ezeronye 2004)</p> <p><i>S. cerevisiae</i> var. bayanus R2, <i>W. saturnus</i> var. mrakii NCYC2251 mixed and sequential inoculation (20°C) (Lee et al., 2010; 2012a)</p> <p><i>W. saturnus</i> var. mrakii NCYC2251, <i>W. saturnus</i> var. saturnus NCYC22 and <i>W. saturnus</i> var. sargentensis NCYC2727 (20°C) (Lee et al., 2010)</p>	<p>~12% (v/v) alcohol at pH 3.2; major volatile compounds: isobutyl alcohol, isoamyl alcohol and 2-phenylethanol</p> <p>mixed inoculated: ~5% (v/v) alcohol; negative sequential inoculated: ~5% (v/v) alcohol with similar volatile profile to mixed culture; positive sequential inoculated: ~2% (v/v) alcohol, with lower content of volatile compounds</p> <p>utilized glucose over fructose and partially degrading malic acid; major volatile compounds: esters, acids, and alcohols; minor volatile compounds: benzyl isothiocyanate, butyric acid, 2-ethylhexanol, benzaldehyde, and β-damascenone</p>
Guava (<i>Psidium guajava</i> L.)	Tropical and subtropical regions, native to Mexico, Central America, and northern South America	<p>Allochthonous strains: baker's yeast (Pino and Queris 2011)</p> <p><i>S. cerevisiae</i> NCIM 3095 and NCIM3287 (Sevda and Rodrigues 2011)</p>	<p>10.8% (v/v) alcohol with pH 3.2; major odor-active volatiles: (<i>E</i>)-β-damascenone, ethyl octanoate, ethyl 3-phenylpropanoate, ethyl hexanoate, 3-methylbutyl acetate and 2-methyltetrahydrothiophen-3-one</p> <p>NCIM 3095 performed much better fermentation</p>

Table 2. (Continued)

Fruits	Region of Fruits	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
Jaboticaba (<i>Myrciaria jaboticaba</i> Berg)	Tropical fruits native to Brazil	Allochthonous strains: <i>S. cerevisiae</i> UFLA CA 1162 (20°C) (Duarte et al., 2010a)	~4% (w/v) alcohol; major acids: succinic, malic, acetic; major volatile compounds: acetaldehyde, 1,1-diethoxyethane, ethyl acetate, methanol, 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenylethanol, and 3-ethoxy-1-propanol
Longan (<i>Dimocarpus longan</i>)	Tropical fruits native to Southern Asia	Allochthonous strains: <i>S. cerevisiae</i> HK-4, <i>S. bayanus</i> EC 1118 (fermented Thai Longan with varied must conditions) (Chomsri et al., 2003) <i>S. bayanus</i> EC 1118, <i>S. carlbergensis</i> TISTR 5345 (immobilized) (fermented Thai Longan) (Kongruang and Wonganu 2007)	~ 6 -11% (v/v) alcohol pH 3.3 - 3.9; acidity as citric acid were 6.30 - 10.60; EC 1118 performed faster alcohol production rate ~9% (w/v) alcohol
Umbu (<i>Spondias tuberosa</i> L.)	Tropical fruits in Brazil	Allochthonous strains: <i>S. cerevisiae</i> UFLA CA 1162 (Duarte et al., 2010a)	~5% (w/v) alcohol; contained malic, succinic and acetic acid; major volatile compounds: 3-methyl-1-butanol, 2-methyl-1-propanol, ethyl acetate, and 2-methyl-1-butanol
Yellow mombin (<i>Spondias mombin</i> L.)	Native to the tropical Americas, including the West Indies. The tree has been naturalized in parts of Brazil, Africa, India, Sri Lanka and Indonesia	Allochthonous strains: <i>S. cerevisiae</i> (Flashmman, São Paulo, Brazil) (20°C) (Severo Jr et al., 2007)	~ 11% (v/v) alcohol and all physico-chemical characteristics were within the norms specified by the Brazilian legislation

Fruits	Region of Fruits	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
Mangosteen (<i>Garcinia mangostana</i>)	Tropical rainforest of some Southeast Asian nations such as Indonesia, Malaysia, Sri Lanka, Philippines, and Thailand	<p>Allochthonous strains: baker's yeast, <i>S. cerevisiae</i>, <i>S. sake</i>, <i>S. cerevisiae</i> var. <i>montachae</i> and <i>S. cerevisiae</i> var. <i>burgundy</i> (Lauhkitikul and Wasikadilok 1999; Lauhkitikul et al., 2008)</p> <p><i>S. cerevisiae</i> (Pasteur Institute, HCM City, Vietnam) (varied sugar and yeasts ratios) (Minh 2014)</p>	<p>~11 - 12% (v/v) alcohol with pH 3.5; wine from <i>S. cerevisiae</i> var. <i>burgundy</i> had best sensorial quality</p> <p>~10 - 13% (v/v) alcohol</p>
Jambal fruit (<i>Synzygium cumini</i>)	Tropical fruits native to Bangladesh, India, Nepal, Pakistan, Sri Lanka, Malaysia, the Philippines, and Indonesia	<p>Allochthonous strains: <i>S. cerevisiae</i> var. <i>bayanus</i> (Chowdhury and Ray 2007)</p> <p><i>S. cerevisiae</i> (Shrotri et al., 1963; Khurdiya and Roy 2001)</p> <p><i>S. cerevisiae</i> (Patil et al., 2012)</p>	<p>~6% (v/v) alcohol with acidic taste; high tannin (1.7 mg/100ml)</p> <p>pH 3.50; 11.23% (v/v) alcohol; 0.37% total acidity (citric acid); 0.036% volatiles acidity (acetic acid)</p> <p>varied TTS from ~8 - 11%; yeast inoculum from ~5 - 20% yielding alcohol level from ~7 - 10 (v/v)</p>
Mulberry (<i>Morus alba</i> L.)	Native to northern China, and widely cultivated	<p>Spontaneous fermentation 24 °Brix and pH 3.5 (18 - 20°C) 96 h (Duyen et al., 2013)</p> <p>Allochthonous strains: commercial <i>S. bayanus</i> (Srisamatthakarn et al., 2001)</p> <p><i>S. cerevisiae</i>, Montrachet and Burgundy (Chernchujitt et al., 2002; Pongsayarm 2004)</p>	<p>5% (v/v) alcohol with pH 3.5</p> <p>10.4 - 11.2% (v/v) alcohol with pH 3.3</p> <p>~14.0% (v/v) alcohol with phenolic compounds (208 to 299) mg L⁻¹, and anthocyanins (2,099 to 2,269 mg L⁻¹)</p>

Table 2. (Continued)

Fruits	Region of Fruits	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
		<i>S. cerevisiae</i> V1, E1, G1 and <i>T. delbruekii</i> (20°C), mixed and sequential (collection of the Section of Microbiology and Biochemistry, Geisenheim Research Center, Germany) (Chomsri, et al., 2012)	
Tamarind (<i>Tamarindus indica</i>)	Indigenous to tropical Africa widely distributed throughout to South Asia, Northern Australia, and throughout Oceania, Southeast Asia, Taiwan and China	Allochthonous strains: <i>S. cerevisiae</i> WPYCW, UWPYCW and brewer's yeast (DSY) (Phunjumpa et al., 2005) <i>S. cerevisiae</i> Sweden (Tanamool et al., 2005)	~9 - 14% (v/v) alcohol with pH 2.9 - 3.5; WPYCW and <i>S. cerevisiae</i> Sweden produced the highest alcohol level up to 14% (v/v)
Santol (<i>Sandoricum koetjape</i> Merr.)	Tropical fruit wildly grown in India and Southeast Asia	Allochthonous strains: <i>S. cerevisiae</i> SIHA3 and <i>S. cerevisiae</i> G74 (Jitjaroen et al., 2009)	~12 - 13% (v/v) alcohol with pH 3.3-3.6; contained citric, malic, and acetic acids
Peach (<i>Prunus persica</i>)	Native to Northwest China	Allochthonous strains: <i>S. cerevisiae</i> (Joshi et al., 2005; Davidović et al. 2013)	~4% alcohol with pH 3.3; contained higher amount of total phenolics; total flavonoid content relative to white wines with acceptable sensorial quality
Persimmon (<i>Diospyros kaki</i> L.f.)	Wildly cultivated in Eastern Asia	Allochthonous strains: <i>S. cerevisiae</i> IFFI 1346, IFFI 1363, CICC 31482, D254 and CGMCC2.346 (Zhu et al., 2014)	Standard wine character ; OAVs* including ethyl hexanoate, ethyl octanoate, methyl decanoate, linalool, and geraniol; IFFI 1363 and D254 strongly correlated with persimmon, aroma harmony, fruity, fusel, taste balanced, and fullness
Pomegranate (<i>Punica granatum</i> L.)	Widely cultivated throughout the Middle East and Caucasus region, northern Africa and tropical Africa, the Indian subcontinent, Central Asia, and the drier parts of southeast Asia	Allochthonous strains: <i>S. cerevisiae</i> var. bayanus AWRI R2 (Mauri Yeast Australia) (22°C) (fermented varied Pomegranate cultivars) (Mena et al., 2012)	~8 - 9% (v/v) alcohol with TTA from ~5 - 20 g L ⁻¹ citric acid; volatile acidity from ~0.3-0.4 g L ⁻¹

Fruits	Region of Fruits	Yeasts in wine fermentation	Fermentation properties and/or Wine compositions
Cagaita (<i>Eugenia dysenterica</i> DC)	Tropical fruits native to Brazil	Allochthonous strains: <i>S. cerevisiae</i> UFLA CA11 and CAT-1 (free and immobilized, 20°C) (Oliveira et al., 2011)	~87 - 95 g L ⁻¹ alcohol with higher alcohols from ~37 - 82 mg L ⁻¹ , and ethyl ester from 1.5 - 2.8 mg L ⁻¹ , UFLA CA11 performed faster alcohol and produced higher level of ethyl ester; CAT-1 produced greater amount of higher alcohol level

OAVs* = higher odor activity values

There are many other types of fruits that have been evaluated for wine making such as economically important fruits such as Cacao (*Theobroma cacao* L.), Peach (*Prunus persica*); fruits native to particular region such as Gabiroba (*Campomanesia pubescens*), Cupuassu (*Theobroma grandiflorum* Schum.), Jaboticaba (*Myrciaria jaboticaba* Berg), Umbu (*Spondias tuberosa* L.), Jambal fruit (*Synzygium cumini*), Mulberry (*Morus alba* L.), Cagaita (*Eugenia dysenterica* DC); and fruits cultivated in particular regions such as Guava (*Psidium guajava* L.), Longan (*Dimocarpus longan*), Yellow mombin (*Spondias mombin* L), Mangosteen (*Garcinia mangostana*), Tamarind (*Tamarindus indica*), Santol (*Sandoricum koetjape* Merr.), Persimmon (*Diospyros kaki* L.f.), Pomegranate (*Punica granatum* L.). However, the intention of researchers conducted these work is towards the selection of commercial *Saccharomyces* yeasts and/or *Saccharomyces* isolates as well as optimization of fermentation conditions to produce an acceptable wine. The details of strains evaluated and their fermentation properties in each fruit are provided in Table 2.

CONCLUSION

In the development of fruit wine productions, the selection of yeast strains for fermentation is one of the most important criteria for the success of every fruit wine making. Spontaneous fermentation has been reported as the methodology to improve the complex aromatic compounds in various types of fruit wines (i.e., strawberry, orange and banana). However, in spontaneous fermentation of fruit wines, must is easily contaminated and yeasts produce less alcohol. In addition, spontaneous fermentation system might not be appropriate for making some fruit wines i.e., apple, pineapple. The reason may be due to the lower resistance of the spontaneous yeasts to the increase of ethanol concentration during alcoholic fermentation. The autochthonous isolates as starters for making wine are therefore used in some fruit wine makings. Nonetheless, the fruit wines, i.e., pineapple orange and strawberry, fermented with appropriate autochthonous isolates exhibit good taste and classical flavor of the wines which are significantly better than those fermented with the commercial starter. However, commercial yeasts and/or isolates which are mainly allochthonous *Saccharomyces* yeasts are mainly studied in fruit wine development by using various yeast strains, inoculation protocol, immobilized starters, the use of hybrid-yeast strains, and/or different fermenting conditions. As a result, the fermentation efficiency can be improved and the resulting wine are accordingly met the standard wine characters.

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Chapter 7

TRADITIONAL CHEESES PRODUCED IN BRAZIL: CHARACTERISATION, PRODUCTION TECHNOLOGIES AND HEALTH IMPLICATIONS

*Beatriz Martins Borelli¹, Inayara Cristina Alves Lacerda²,
Fernanda Corrêa Leal Penido² and Carlos Augusto Rosa^{1,*}*

¹Departamento de Microbiologia, Instituto de Ciências Biológicas,
Universidade Federal de Minas Gerais, Belo Horizonte,
Minas Gerais, Brasil

²Departamento de Alimentos, Faculdade de Farmácia,
Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brasil

ABSTRACT

The beginning of cheese-making processes dates back more than 8,000 years ago. Currently, there are more than 1,000 varieties of cheese throughout the world, each one possessing unique characteristics regarding its form and flavour. The history of cheese making in Brazil started with the Portuguese colonisation, which was responsible for introducing the first cattle herds and for initiating the manufacturing of cheeses similar to the cheese “Serra da Estrela” that had only been produced in Portugal. The traditional Minas cheese is one of the oldest cheeses produced in Brazil and is responsible for generating income for a great number of small farmers. This traditional cheese is produced in the regions of Minas Gerais, Southeastern Brazil. Among other typical Brazilian cheeses, the Coalho cheese and the Butter cheese are strictly produced in the Northeast, while the Serrano cheese and the Colonial cheese are restricted to the South. The traditional production of cheese in Brazil involves the fermentation of raw cow's milk by lactic acid bacteria, which occurs naturally via the indigenous microbiota from milk and by the addition of commercial rennet. The microbial diversity contributes to the great differences in the organoleptic characteristics found in these traditional cheeses, and the main genera of lactic acid bacteria involved in the production processes are

* Corresponding author at: Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, 31270-901, Brasil. Tel +553134092751. Email address: carlrosa@icb.ufmg.br (C.A. Rosa).

Lactococcus, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Enterococcus*. The raw-milk cheeses are of considerable importance in public health, given their particular conditions of production, and may become an important route of transmission of numerous pathogens, such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., *Shigella* spp. and *Listeria monocytogenes*, among others. Given the economic, social and cultural importance of artisanal cheeses produced in Brazil, together with the poor information available on the microbiological characteristics of these products, the understanding of the microbiota involved in the manufacturing processes, including the traditional technologies, as well as of the role played by the contaminant microorganisms are of great value. In this chapter, an overview about the traditional cheeses produced in Brazil is highlighted, focusing on the production technologies, the microbiota involved in the processes and the implications for public health.

1. INTRODUCTION

Cheese is one of the oldest processed foods ever recorded in human history, and it has contributed significantly to the development of civilisations (Kosikowsky 1970). The natural process of the coagulation of milk from goats and ewes, performed by man and subsequently transformed by him, led to the development of a food that, according to historical documents, was produced by the first civilisations (Cichoscki et al. 2002). The history of cheese goes back to antiquity, although many experts consider the Middle Ages as the starting point of its production. The oldest known evidence of cheese making is a series of cave paintings done approximately 5,000 to 6,000 years before Christ (BC) that were discovered in Libya. Additional evidence is on the murals in Sumerian tombs dating back to 3,500 years BC, which have writings about the production of butter and cheese (Netto 2014). It is likely that cheese first appeared with the domestication of animals and the use of their products (Cichoscki et al. 2002). Apparently, goats and ewes were the first herds; however, cattle has become the dominant species in dairy production in most of the world (McSweeney and Fox 2004).

The consumption of "solidified milk" was reported dating back to 8,000 years BC, and archaeological findings have revealed the existence of cheeses made from cow's and goat's milk as far back as 6,000 years BC. Murals in Egyptian tombs from 2,000 BC depict cheese-making scenes in ancient Egypt, and the Bible cites this product in more than one passage from the Old Testament. Back then, the Christian monks transformed cheese making into a real art, introducing many of the varieties consumed today. During the Renaissance, cheese lost some of its popularity because it was considered unhealthy; however, cheese regained its popularity soon afterwards (Perry 2004).

In Brazil, the origins of dairy products date back to 1581, when the Portuguese colonisers imported cattle and transmitted cheese-manufacturing techniques to the native population. Together with the Portuguese luggage came the recipe for the cheese, which is now known as *Serra da Estrela* cheese and was the precursor of *Serro* cheese produced in the region of "Vila do Príncipe," known today as the "Serro" region, Minas Gerais State, Brazil (Perry 2004). The cheese recipe was modified and adapted to the environmental conditions of each region in Brazil (Borelli et al., 2006; Lima et al. 2009).

In the late 17th century, characterised by the beginning of the gold cycle, its manufacture began to be recognised as an economic activity, and it was developed in Minas Gerais and

other regions in the country (Dias 2010). Brazilian industrial production started in 1888 thanks to the Portuguese immigrant Dr. Carlos Pereira de Sá Fortes, who brought two cheesemaker masters from Holland, establishing the first adapted production of Dutch cheese in the region of Mantiqueira, in the Minas Gerais state. In the 1930s, industrial large-scale development was established (Abreu 2005). Currently, fermented milk products, such as cheese, can be either artisanal or industrial, while its manufacturing technique remains a complex procedure that combines science and tradition (Chaves-López et al. 2014).

2. CHEESE PRODUCTION

Cheese is the common denomination for a solid product obtained from the fermentation and coagulation of milk, and it is produced throughout the world in a wide range of flavours, textures and forms (Fox and Mc Sweeney 2004). According to the General Standard for Cheese (CODEX STAN 283-1978), cheese is “the ripened or unripened soft, semi-hard, hard, or extra-hard product, which may be coated, and in which the whey protein/casein ratio does not exceed that of milk, obtained by: (a) coagulating wholly or partly the protein of milk, skimmed milk, partly skimmed milk, cream, whey cream or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulating agents, and by partially draining the whey resulting from the coagulation, while respecting the principle that cheese-making results in a concentration of milk protein (in particular, the casein portion), and that consequently, the protein content of the cheese will be distinctly higher than the protein level of the blend of the above milk materials from which the cheese was made; and/or (b) processing techniques involving coagulation of the protein of milk and/or products obtained from milk which give an end-product with similar physical, chemical and organoleptic characteristics as the product defined under (a).” In Brazil, according to the “Portaria” nº 146 published in 1996, which approves technical regulations for the identification and quality of milk products (Brazil 1996), cheese is “the fresh or ripened product obtained by partial separation of milk whey or reconstituted milk (whole, partially or totally skimmed) or milk whey physically coagulated by rennet, the action of specific enzymes from specific bacteria, organic acids, isolated or in combination, all suitable for food use, with or without addition of edible substances and / or spices and / or condiments, specifically indicated additives, flavouring substances and dyestuffs.”

The four basic ingredients considered essential to the production of most cheeses are milk, rennet, salt, and microorganisms. These ingredients are used through the different stages of cheese making: acidification, coagulation, syneresis and ripening (Fox et al. 2000; Fox and Cogan 2004). The most crucial stage in this process includes the conversion of the liquid milk into a semi-solid gel. Later on, syneresis, the expulsion or extraction of whey from this gel, results in the formation of cheese curd. The step of coagulation involves the aggregation of the casein and is normally achieved by the addition of a coagulant, usually rennet, to the milk, although it can also be accomplished by a pH reduction through acidification (Bennet 2004).

Dehydration essentially characterises the cheese-making process, in which the fat and casein in milk are concentrated between 6- and 12-fold, depending on the cheese variety.

The main factors responsible for the regulation and control of the biochemical transformation that occurs during ripening are the moisture content, level of salt, pH and cheese microbiota.

These factors determine the flavour, aroma and texture of the finished cheese. Therefore, the nature and quality of the finished product are largely determined by the processing steps. Nevertheless, the characteristic flavour and texture of the individual cheese varieties are developed during the ripening phase (Fox and McSweeney 2004). During the historical process, cheese gained a huge typological variety, largely artisanal features, maintained by tradition, which seeks to preserve regional identity and to respect the population's food tastes.

In South America, Brazil is the largest producer of cheese and, since 2002, is the sixth largest world producer.

In 2011, the country produced 867,000 tons of cheese (SEBRAE 2014); however, it is important to emphasise that the Brazilian production of artisanal cheeses, as well as its consumption, is highly significant but is not always included in official statistics (Netto et al. 2008). The state of Minas Gerais is the largest cheese producer in Brazil and, in 2006, produced approximately 215,000 tons.

The main feature of Minas Gerais cheese production is the artisanal manufacturing, which represents a significant portion of all the cheese produced in the state. This activity represents an income source for many family farmers and takes place in the six traditional micro-regions of the state, with approximately 30,000 artisan cheese producers. The amount of cheese annually produced in Minas Gerais corresponds to 70,000 tons. Of this total, 33,570 tons are produced in the six traditional micro-regions, generating approximately 26,870 direct jobs (Menezes 2011).

Because of its historical and cultural environment, artisanal cheeses have great social importance in Brazil (Pinto et al. 2009b). In this country, the cheeses that can be considered artisanal are the *Minas* cheese produced in Minas Gerais; *Coalho* cheese and *Butter* cheese produced in the Northeast; and *Serrano* cheese and *Colonial* cheese produced in the Southern region (IMA, 2009; Arcuri et al. 2013) (Table 1).

The state of Minas Gerais is recognised as the biggest cheese producer in Brazil. Among the varieties produced, traditional cheese stands out for being a secular product. Artisanal *Minas* cheese has been manufactured in Minas Gerais since the 18th century, and its consumption is a habit that goes beyond the boundaries of the state territory (Netto 2014). Traditional Minas cheese is produced with raw cow's milk, and since 2002, it has a specific legislation that regulates its production process in the Minas Gerais state (Minas Gerais 2002).

Later, in 2008, artisanal cheese production in Minas Gerais was recognised as immaterial cultural heritage by IPHAN (National Institute of Historical and Cultural Heritage) in recognition of the importance of this tradition as a cultural value.

These cheeses are traditionally made from raw milk, employing a natural whey culture as a starter and calf quimosin as coagulant, and the issues surrounding their safety, quality and provenance in order to protect both producers and consumers is always of concern.

In several countries, many varieties of cows', goats' and ewes' milk cheese are produced in farms following traditional techniques, without the intentional addition of commercial starter cultures. These cheeses are generally referred to as "artisanal" or "traditional."

During different steps of cheese-making, technological parameters, such as renneting, acidification, heating, whey drainage, salting and ripening, greatly influence the

characteristics of the final product and play a major role in its microbial composition, being responsible for the biodiversity enhancement (Randazzo et al. 2009).

Table 1. Brazilian traditional cheeses named after the region of origin and production characteristics

Name	States	Number of producers	Annual production (tons)
Serrano	RS	3,000	7,300
Coalho	PE, RN, CE, SE	2,000	32,120
Serro	MG	881	3,106
Alto do Paranaíba (Cerrado)	MG	6,112	17,357
Canastra	MG	1,529	5,787
Araxá	MG	943	2,755

CE = Ceará, MG = Minas Gerais, PE = Pernambuco, RN = Rio Grande do Norte, RS = Rio Grande do Sul, SE = Sergipe (Dias 2010).

3. TRADITIONAL CHEESE PRODUCTION TECHNOLOGIES IN BRAZIL

Cheeses are produced all over the world and may differ in type, size, colour and flavour. Cheese making in different locations was adapted to climatic conditions, availability of herds, soil and pasture characteristics, as well as the cultural and social levels of civilisations (Netto 2014). The art of making cheese is a relatively simple process that has been performed in the same manner for millennia. Cheese is defined as a food product obtained from curdled milk and separated from whey that can be eaten fresh or ripened for a variable time; however, for the development of a differentiated product, special procedures are required for each type of cheese made.

Cheese comprises a complex ecosystem in a continuous flow in terms of extrinsic factors, such as manufacturing process and ripening conditions, and intrinsic factors, such as physicochemical parameters and interactions among different microbial communities. The wide variety of biochemical reactions and microbial interactions that occur during the maturation process are essential for the development of cheese characteristics, such as pleasant aroma and taste, the prevention of deterioration, the inhibition of the development of pathogens and, more recently, the regulation of health (Ahola et al. 2002). Cheeses made from raw milk have a higher microbial diversity than cheeses made from pasteurised milk; consequently, there are differences in the sensory quality of these two types of cheeses (Grappin and Beuvier 1997; Beresford et al. 2001).

Traditional Minas cheese is “cheese made according to historical and cultural tradition of the state of Minas Gerais in Brazil where it is produced from whole, fresh and raw cow’s milk, removed and processed at the property of origin; and firm in consistency, colour and flavour characteristic, uniform mass, free from dyes and preservatives, with or without mechanical holes” (IMA, 2002). The ripening period ranges from 17 to 60 days (IMA 2013). Brazilian laws allow the commercialisation of cheeses made from raw milk, which are

regulated by the Federal Inspection Service, if the products undergo the minimum period of 60 days of maturation (MAPA 2000). This long maturation time prevents the commercialisation of the product. Therefore, the majority of the Minas cheese production made with raw milk is sold illegally. These traditional cheeses are made from raw milk on farms without any technological monitoring, resulting in the achievement of a product with varied composition and quite different characteristics of aroma, flavour, body and texture, which can compromise the final quality (Siqueira et al. 1986). The place where the cheese is produced is usually characterised by simple masonry construction situated near the corral. For cheese making, the fresh raw milk is strained through a filter, a strainer (which can be made of metal, stainless steel or aluminium) or a cloth directly into the manufacturing tank. Then, a commercial rennet (liquid or powder) is added. In many Brazilian regions, the addition of a fermented whey, known as "pingo," is a characteristic of this process. The "pingo," used as a starter culture, is a natural lactic culture obtained from the whey drained from the cheese covered with coarse salt. The "pingo" is gathered in a container placed just below a hole in the cheese production counter, which has a slight slope to facilitate the flow of whey. The mixture (milk, rennet and "pingo") is allowed to rest for a period of approximately one hour. The amount of "pingo" added depends on the amount of milk used, the time of the year and the cheese manufacturing process.

After the resting period, the curd is broken with the aid of a polyethylene or wood shovel, and grains of irregular sizes submerged in a generally milky white or yellowish whey are obtained. The curd is decanted for 10 to 15 minutes for whey drainage. The mass is collected on sieves and pressed manually in plastic moulds. In some regions of Brazil, the mass is pressed within cloths before the cheese is shaped. For dry salting, cheeses are covered with coarse salt and allowed to rest for 5 to 6 hours. After this initial rest, the coarse salt is removed from the surface of the cheeses, which are then turned, coated again with salt and left in the same position until the next day. In this step, the whey called "pingo" is collected.

The salt is then removed, and the cheeses are kept in moulds where they dry for 24 hours. The cheeses are removed from the moulds and lightly scraped with a little metallic grater to make the rind very fine and uniform. The cheeses are placed on wooden shelves and turned to allow the maturation process to happen. The ripening time varies for each region. According to consumer demand, cheeses may be sold when they are still fresh (IMA, 1999). Because there is no standardisation in the manufacturing process regarding coagulation time, type of rennet used, pressing, salting and moisture content of the final product, it is possible to find a great variety of artisanal cheeses that do not follow any identity or quality standard on the market (Borelli et al. 2006). The steps involved in the production of traditional cheeses in Brazil (Figure 1 and 2) can vary considerably from one region to another. This variation characterises the different types of artisanal cheeses produced in the country.

Traditional cheese manufacturing, diversity in herd management, and cheese-making and cheese-ripening practices, often in small-scale production, have generated the diversity of characteristics in ripened cheeses that still exists in traditional cheeses currently. By contrast, industrial cheese production processes, often applied on large scale, are designed to standardise the ripened cheese to reduce the variability at the end of the chain. This is done by a reduction in variability of milk matrix characteristics and cheese-making practices. Pasteurisation is one method commonly used to standardise the microbial composition and improve the microbial safety of milk by reducing its microbial load and biodiversity. Thus,

the adoption of new practices throughout the production chain might be necessary (Bachmann et al. 2011; Montel et al., 2014).

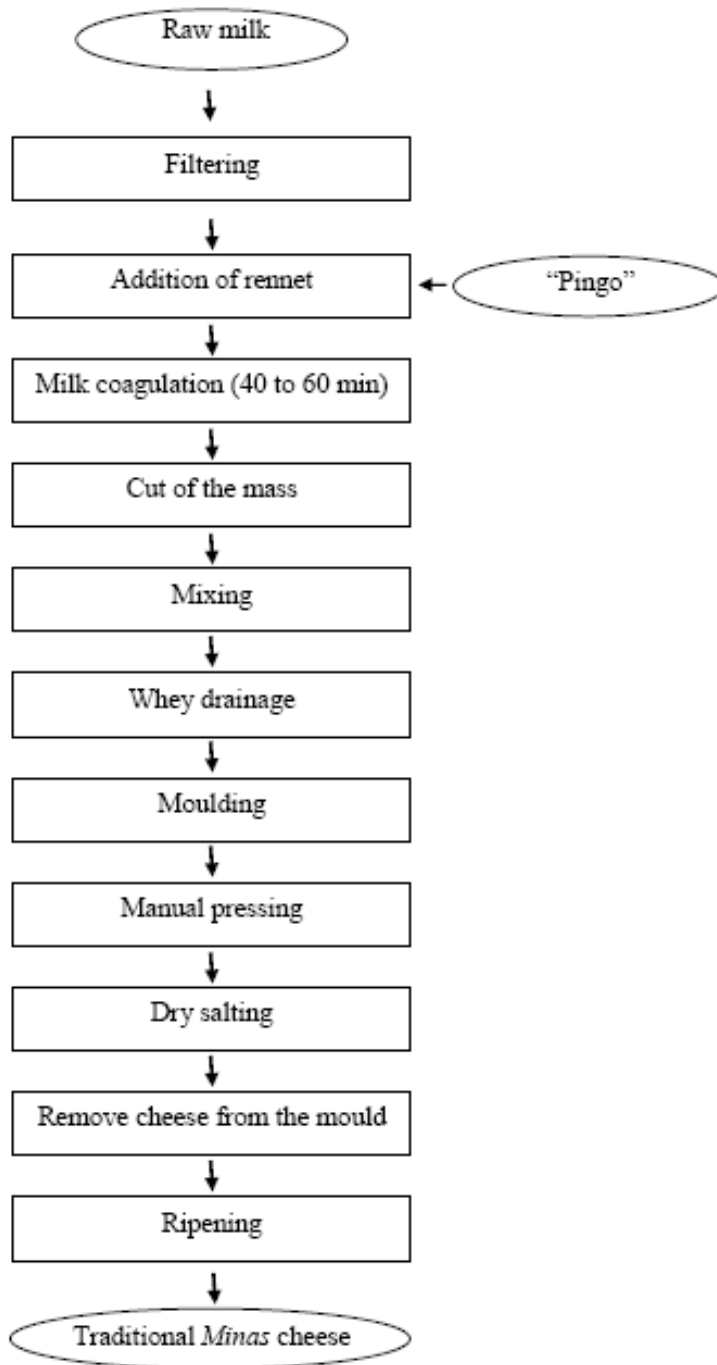


Figure 1. Flow chart of Traditional *Minas* cheese production.

4. MICROBIOTA INVOLVED IN THE PRODUCTION OF TRADITIONAL CHEESES

Cheese is characterised by the presence and succession of various microorganisms, including bacteria, yeasts and moulds, that interact to play a major role during the cheese-making process and maturation (Ndoye et al. 2011). These microorganisms depend on factors that influence microbial growth and survival, such as the original microbiota of raw milk, the acidification process and storage conditions (Beresford et al.; 2001; Beresford and Williams; 2004; Irlinger and Mounier 2009). There are three types of microbiota: primary starter, secondary cultures and non-starter bacteria (Fox et al. 2000). Cheese starter cultures are involved in acid production during the cheese-making process and in the maturation process to various extents in combination with ripening cultures. Non-starter lactic acid bacteria, other bacteria, yeasts and moulds that originated from milk or its processing environment also play a significant role during maturation (Ndoye et al. 2011).

The raw milk microorganisms are an important part of the microbiota of many traditional cheeses. The milk from the upper part of the udder of a healthy lactating female is frequently considered to be sterile. Milk microbiota composition depends directly on the composition of the microbiota from sources directly in contact with the milk, such as the animal's teat and the dairy equipment (i.e., milking machine, milk line and tank). Moreover, it depends on the composition of the microbiota from indirect sources, such as the litter, feed, drinking and washing water, milker, cowshed and milking parlour air, which may act as direct sources (Michel et al. 2001; Julien et al. 2008; Verdier-Metz et al. 2009; Tormo et al. 2011; Mallet et al. 2012). Some of the strains present in vat milk, either indigenous or starter, can grow, survive and even become predominant during cheese making.

The accidental microbiota of raw milk is normally heterogeneous, and more than 100 genera and 400 microbial species have already been detected (Fox and McSweeney 2004; Montel et al. 2014). This microbiota is mainly composed of Gram-negative bacteria (≥ 90 species), Gram-positive and catalase positive bacteria (≥ 90 species), lactic acid bacteria (LAB) (≥ 60 species), yeasts (≥ 70 species), and moulds (≥ 40 species). LAB is often regarded as the main bacteria in raw milk for historical and technological reasons. Raw milk microbiota is very rich, containing, for instance, a wide variety of halophilic and/or alcalophilic Gram-positive or Gram-negative bacteria and yeasts (Callon et al. 2007; Saubusse et al. 2007; Fricker et al. 2011). Psychotrophic bacteria are naturally present in raw milk, which is often conserved at refrigeration temperature before cheese making, and they can reach counts of up to 10^5 CFU/mL. The most commonly occurring psychotrophs in raw milk are *Pseudomonas* spp., *Acinetobacter* spp. and Enterobacteriaceae, such as *Hafnia alvei*. Psychotrophic bacteria are recognised as a cause of milk spoilage, which may be due to their enzymes with proteolytic and lipolytic activities (Martins et al. 2006; Hantsis-Zacharov and Halpern 2007; Ercolini et al. 2009). During all steps of milk processing, faeces may be indirect sources of many microorganisms, such as Enterobacteriaceae, spoilage bacteria (butyric acid bacteria spores), yeasts, *Bifidobacterium* and various LAB (*Aerococcus viridans*, *Enterococcus hirae*, *Lactobacillus mucosae*, *Lactobacillus brevis*, and *Lactobacillus plantarum*) (Baroiller and Schmidt 1990; Rasmussen et al. 1991; Beerens et al. 2000; Gelsomino et al. 2001; Kagkli et al. 2007; Montel et al. 2014). Some of these

microorganisms, especially LAB, may be beneficial. LAB cultures have been mostly used for acidification (Fox and McSweeney 2004).

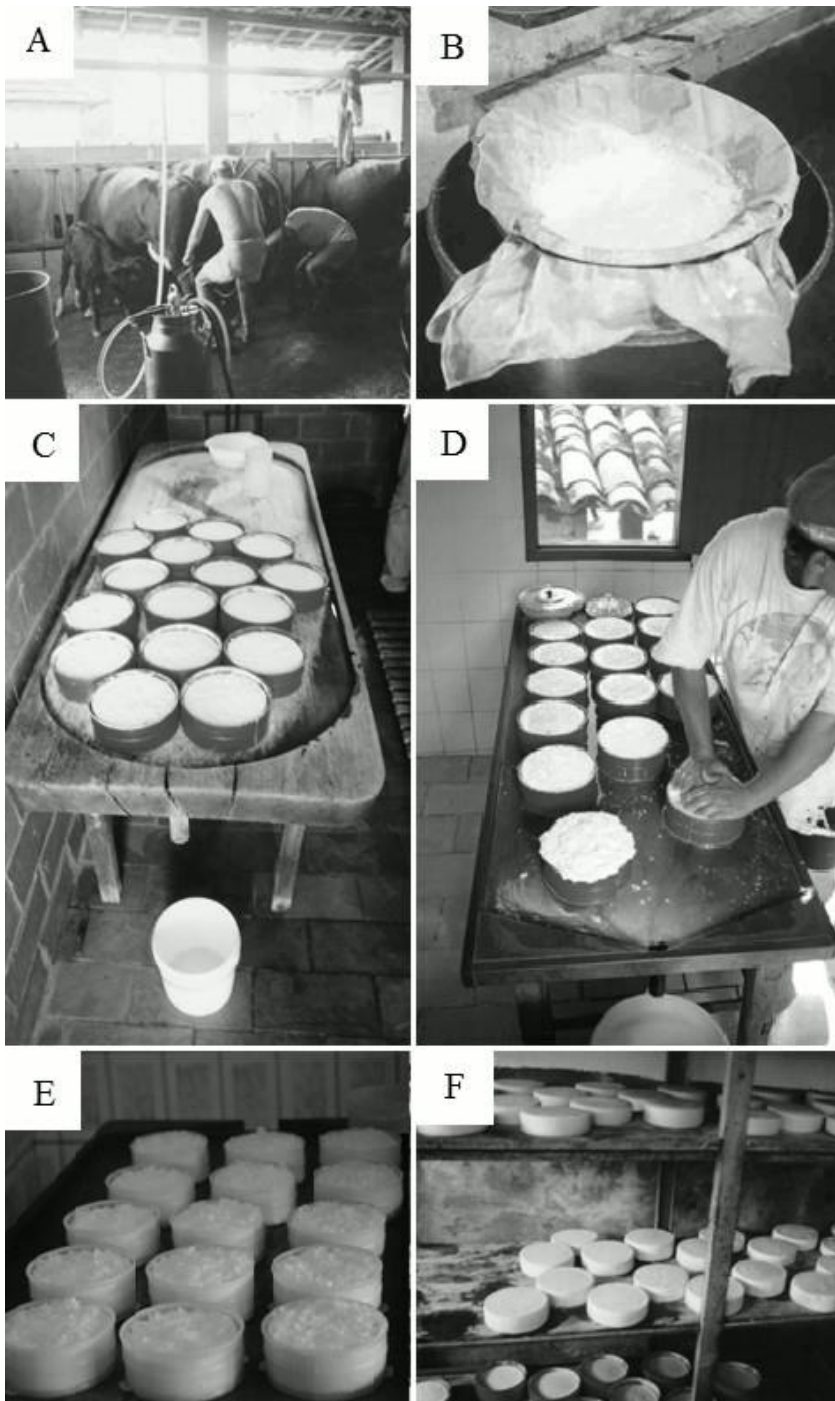


Figure 2. Production steps of the traditional *Minas* cheese (A = Milking; B = Filtering; C = “Pingo” obtainment; D = Manual pressing; E = Dry salting; F = Ripening).

In Brazil, there are few studies on the role of microorganisms on the surface of the equipment and cheese ripening shelves as contaminants in the microbiota involved in the manufacturing process and ripening of this food (Galinari et al. 2014). Licitra et al. (2007) and Didienne et al. (2012) demonstrated that the wooden surfaces of vats used to produce PDO Salers and Ragusano cheeses are a source of microorganisms, especially active acidifying LAB. These microorganisms produce a biofilm that is stable over several seasons once it has become established on a vat surface. Wooden vats can increase microbial levels in milk compared to those in milk before pouring into the vat (Lortal et al. 2009; Didienne et al. 2012; Settanni et al. 2012). The microbial biofilms, which do not change with season or shelf age, are a possible source of microorganisms of surface microbiota for smear cheeses which are not often deliberately inoculated with surface microorganisms (Montel et al. 2014). Galinari et al. (2014) evaluated the microbial composition of biofilms formed on wooden forms, tables and shelves used in the production of traditional cheeses in Brazil. The authors determined the counts of *Staphylococcus aureus*, *Escherichia coli*, coliforms, yeasts, presumptive mesophilic *Lactobacillus* spp. and *Lactococcus* spp. in these biofilms. They observed that the wooden shelves had the highest populations of microorganisms among the substrates studied, with the exception of coliforms, and that *E. coli* was found in higher counts in the tables used in the manufacture of cheeses.

Cheese microbiota is further enriched by the particular practices involved in cheese making. For some artisanal cheese production in Brazil, the natural starter employed, known as “pingo,” is produced by the addition of whey from an older cheese covered with salt. This whey is routinely used to culture LAB before their inoculation into raw milk, being responsible for the acidification step. LAB are generally employed because of their essential contribution to the flavour, texture and nutritional value of food products, in addition to their natural antimicrobial properties that extend shelf life (Favaro et al. 2014).

Yeasts are associated with the secondary microbiota of a wide variety of cheeses (Fleet 2006; Gardini et al. 2006; Capece and Romano 2009; Lavoie et al. 2012). These microorganisms are involved in the ripening process, have high proteolytic and lipolytic activities, and contribute to texture alterations and biosynthesis of aromatic compounds (Fleet and Mian 1987; Roostita and Fleet 1996; Ferreira and Viljoen 2003). Investigations of yeast composition in cheese revealed a large diversity of species belonging mainly to the following genera: *Candida*, *Cryptococcus*, *Debaryomyces*, *Geotrichum*, *Kluyveromyces*, *Kodamaea*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Torulaspora*, *Trichosporon*, *Yarrowia* and *Zygosaccharomyces* (Cardoso, et al. 2015). Although the prevalence of different yeast species depends on the type of the cheese under consideration, *Debaryomyces hansenii* is one of the most common isolated species (Büchl and Seiler 2011). The occurrence of this species and other yeasts with high counts in cheese is attributable to their tolerance of low pH, reduced water activity and high salt concentrations, as well as to their ability to grow at low storage temperatures that characterise the ripening environment (Ferreira and Viljoen 2003).

Cardoso et al. (2015) determined the frequency and seasonal diversity of the yeasts during the ripening of *Serro* cheese and observed that the most prevalent species isolated were *D. hansenii*, *Kodamaea ohmeri* and *Kluyveromyces marxianus*. Borelli et al. (2006) showed that *K. ohmeri* was the fourth most prevalent species in artisanal *Canastra Minas* cheese produced in Minas Gerais, Brazil. The presence of *K. ohmeri* in two different artisanal cheeses produced in the state of Minas Gerais (*Serro* and *Canastra Minas* cheeses) indicates that this species is active in the yeast community responsible for the production of these

traditional cheeses (Cardoso et al. 2015). Wanderley et al. (2013) reported the occurrence and pathogenicity of *Candida* spp. in artisanal *Colonial* cheese produced in Santa Catarina, Brazil. The authors analysed 45 cheese samples and isolated 251 strains of *Candida*, with growth capacity at 37 °C. Of these, 2.4% were identified as *C. albicans* and 97.6% as *Candida non-albicans*, which were identified as 79.3% *C. krusei*, 12.3% *C. glabrata* and 6.0% *C. tropicalis*. These species are considered opportunistic yeasts.

Galinari et al. (2014) evaluated the presence of yeast in the moulds, counters and shelves used in the manufacture of artisanal cheeses and *Serro da Canastra* and observed that the largest populations of yeast were present in the maturing shelves (3.25 log CFU/cm²). In all cheese samples analysed, the yeast counts ranged from 5.0 to 5.2 log CFU/g in *Serro* and *Canastra* cheeses, respectively. Yeasts associated with traditional cheeses can act in two ways. First, these yeasts may cause spoilage of the product, resulting in an undesirable flavour, discoloration, gas production and changes in texture (Fleet 1990). However, the yeasts may exert beneficial effects through proteolytic and lipolytic activities, leading to the formation of flavour during ripening (De Freitas et al. 2009; Fadda et al. 2004).

Some studies have been conducted on the bacterial diversity of traditional Minas cheese (Borelli et al. 2006; Lacerda et al. 2011; Lima et al. 2009; Nóbrega 2007). These studies have shown that *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus brevis*, *Lactobacillus rhamnosus*, *Enterococcus faecalis*, *Pediococcus acidilactii* and *Weissella paramesenteroides* were the most frequent species associated with this food. The bacterial communities associated with ripening *Minas* cheese were determined by clone library-based 16S rRNA gene sequence analysis, a cultivation-independent method (Lacerda et al. 2011). The results showed that *Lactococcus lactis* was the most prevalent species after seven days of ripening. *Streptococcus salivarius* was the most prevalent species after 60 days of ripening. *Lactobacillus arizonensis* was the second most prevalent species after 60 days of ripening, representing 24.1% of the total clones. *Lactobacillus plantarum* and *L. lactis* *L. brevis*, *Sphingomonas* sp. and an uncultured *Streptococcus* sp. were also found. These results showed that the bacterial community associated with traditional *Minas* cheese is diverse and mainly composed of lactic acid bacteria. The diversity of the sensory characteristics of cheeses results from the balance among the aromatic compounds arising from many metabolic pathways (i.e., sugars, citrate, lactate, amino acid and fatty acid catabolism, casein breakdown and lipolysis). Oxidative pathways occur on the surface, whereas fermentative pathways occur in the core (Smit et al. 2005). Microbial interactions in the cheese ecosystem are governed by intrinsic (e.g., availability of substrates and co-factors, presence of inhibitor/activator compounds, pH and redox potential) and extrinsic factors (e.g., oxygen availability, temperature, relative humidity) (Montel et al. 2014).

Raw milk cheeses ripen faster and acquire richer, more intense flavours than pasteurised or microfiltered milk cheeses (Grappin and Beuvier 1997; Beuvier and Buchin 2004; Bachmann et al. 2011; Van Hekken 2012). Raw milk cheeses generally have larger amounts of most aromatic compounds (i.e., acids, aldehydes, alcohols, esters and sulphur compounds), with the exception of some ketones. The effects of raw milk microflora on texture differ according to cheese variety and processing conditions (Beuvier and Buchin 2004). Pasteurisation denatures milk enzymes, such as proteases or lipases (Hayes et al. 2001; Hickey et al. 2007), and dramatically reduces the levels of natural milk microbiota and associated enzymes, all of which are involved in the formation of raw milk cheese characteristics (Beuvier et al. 1997).

5. BRAZILIAN TRADITIONAL CHEESES

According to Guerrero et al. (2009), a traditional food product is defined as “a product frequently consumed or associated with specific celebrations and/or seasons, normally transmitted from one generation to another, made accurately in a specific way according to the gastronomic heritage, with little or no processing/manipulation, distinguished and known because of its sensorial properties and associated with a certain local area, region or country.” Traditional products are part of the socio-cultural and gastronomic identity of people, constituting a patrimony that is worth protecting. Types of cheeses are produced traditionally in many regions of the world and have a great variety of physicochemical, sensorial and microbiological characteristics. There is a growing interest in traditional dairy products, such as cheeses, manufactured in an artisanal way due to the uniqueness of such products and the difficulties in imitating them on an industrial scale (Rosa et al. 2008). Traditional cheeses, when produced from milk obtained hygienically from healthy cows kept on natural pastures, may be safe for consumers; however, there is no standardisation of the manufacturing process, especially in relation to the time of coagulation, natural starter culture used, salt and humidity of the final product (Borelli et al. 2006).

The history of cheese making in Brazil started with the Portuguese colonisation. The Portuguese introduced the first cattle herds and brought the recipe of *Serra da Estrela* cheese, which is the most famous Portuguese semi-soft cheese produced with raw ovine milk. Despite the fact that *Serra da Estrela* cheese requires a maturation time of 45 days, it is usually not sold before 60 days (Macedo and Malcata 1997). Its recipe was modified and adapted to the environmental conditions of each region of Brazil (Borelli et al. 2006) and served as a reference for other Brazilian traditional cheeses, such as *Minas*, *Coalho*, *Butter*, *Serrano* and *Colonial* cheeses.

5.1. Traditional *Minas* Cheeses

Traditional *Minas* cheese is one of the most ancient and traditional cheeses produced in Brazil. In the Minas Gerais state, there are six traditional micro-regions that are producers of artisanal cheeses: Araxá, Campo das Vertentes, Canastra, Cerrado, Serro and Triângulo Mineiro, including 73 municipalities of a total of 853 in the state. The traditional *Minas* cheeses are basically produced with the same technology in the Minas Gerais state, as previously mentioned; however, the final characteristics of the cheese vary according to the state region where it is produced due to the type of soil, pasture, climate, altitude, water and pluviometric index. Studies show that the basic difference between cheeses produced in the Araxá, Canastra, Cerrado and Serro regions is that, in the Serro region, manual pressing is done without the aid of cloths, which is different from the other three regions (Araújo 2004; Emater 2004; Pinto 2004; Martins 2006; Dores 2007). The differences in the curd pressing process may lead to the production of cheeses with different humidity levels and mass compression.

In addition to the rennet and salt, the use of the endogenous microbiota (“pingo”), which could be specific for each region, as a co-adjutant for cheese making during the manufacturing process gives each cheese its differentiated sensory characteristics. Currently,

Minas Gerais state laws establish guidelines for the production of traditional Minas and define the cheese maturation period as between 17 and 22 days (IMA, 2013).

Due to differences in the production technologies, the endogenous starter culture employed, soil characteristics and the climate of the two regions and the ripening period of *Canastra* and *Serro* Minas cheeses, both cheeses have different physicochemical and sensory properties.

The traditional *Minas* cheese from Serra da Canastra has the following characteristics: semi-hard consistency with a tendency to be soft with a "buttery" nature; compact texture; yellowish-white colour; yellow, thin rind without cracks; cylindrical; height between 4.0 and 6.0 cm; diameter of 15.0 to 17.0 cm; weight from 1.0 to 1.2 kg; and a slightly acidic, not spicy and pleasant flavour (EMATER 2004). *Serro* cheese has a cylindrical shape, approximately 14 cm in diameter and height ranging from 4 to 6 cm. In some places, it is produced with a slightly larger diameter and is somewhat lower in height. Its rind is usually whitish, tending to become a thin yellowish crust when cured for a few days. Internally, the mass is white and tough and, sometimes, slightly brittle. It has small mechanical openings and often diverse and small irregular holes (Furtado 1980).

5.2. Butter Cheese

Butter cheese, also known as "Sertão cheese," "the cream cheese of the North" and "the cream cheese of the Northeast," is produced and widely consumed in the North and Northeast Brazilian regions, mainly in the states of Rio Grande do Norte, Paraíba and Pernambuco (Cavalcante and Costa 2005; Cruz et al. 2009; Nassu et al. 2009). Butter cheese is a typical product of northeast cooking.

This cheese is produced by a high number of traditional cheese factories located in this region (Mesquita et al. 2010).

Butter cheese production is completely empirical and occurs mostly in traditional processing units and in some small industries. Therefore, the lack of standardisation during its manufacture, storage and distribution leads to wide variation in its chemical composition and sensory characteristics (Cavalcante and Costa 2005; Nassu et al. 2009). The technology of its production has great variability but has in common the steps of skimmed milk coagulation, removal of the whey, mass acidification, mass washing with water and/or milk, salting, mass fusion with bottled butter fat and moulding (Ventura 1987).

According to the "Instrução Normativa" n° 30 published in 2001 (BRASIL 2001), which approves the technical regulation of identity and quality of bottled butter fat, Coalho cheese and Butter cheese are products obtained by the coagulation of milk using food-grade organic acids, removal of the whey and melting of the curd with the addition of local artisan-type butter (Brasil 2001).

This cheese has medium to high humidity and 25 to 55% of the fat content in total solids. It has a soft consistency, tending to greasiness, and is close-grained and semi-friable, with small holes containing liquid fat inside. The flavour is slightly sharp, resembling butter, slightly acidic and may be salted; the colour is straw-yellowish; and it has a little pronounced odour resembling butter and a thin rind without cracks (Nassu et al. 2009; Mesquita et al. 2010).

5.3. *Coalho* cheese

The semi-arid climate and the vegetation of the Northeast region of Brazil directly influence the production of one of the best-known Northeastern foods: *Coalho* cheese. *Coalho* cheese is a typical Brazilian food that has been produced from raw milk in the Northeastern region for over 150 years. This product possesses a high commercial value due to the simple technology applied during its manufacture, the high yield and the good acceptance by consumers (Silva et al. 2010). The name “*Coalho*” is derived from the use of natural rennet in its manufacture. *Coalho* cheese has been produced primarily in the Northeastern States of Brazil: Pernambuco, Ceará, Rio Grande do Norte and Paraíba, where it is among the main types of traditional cheeses. Fully incorporated into the regional culture, this cheese is becoming more popular in the Southeast region. This cheese has considerable input in the economy, being significant in the income of many milk suppliers, especially those who do not have access to milk processing plants (Silva et al. 2012).

Currently, *Coalho* cheese is produced on an industrial scale, with the use of the milk pasteurisation process aiming to ensure consumer safety. Nevertheless, only the production facilities under inspection by the Brazilian Inspection System use milk heat treatment. In 85% of the cases, the milk used in the preparation of *Coalho* cheese is not pasteurised (Nassu et al. 2001). In the search for a suitable lactic starter culture for the production of *Coalho* cheese from pasteurised milk, it is important to know the composition and the technological properties of the lactic bacteria microbiota associated with this food. Most *Coalho* cheese making is still traditional, because the cheese is made with raw milk, industrialised rennet and salt, without the addition of starter cultures. The main features of this cheese are its slightly salty and acidic flavour and its resistance to heat without melting. With this cheese, it is possible to prepare the popular "roast cheese" (Silva et al. 2012). The cheese is made with semi-cooked mass and is traditionally eaten fresh or matured (Nassu et al. 2001). Most *Coalho* cheese is produced in small farms and/or in urban or rural dairies through a process often with inadequate hygienic conditions. As a result, the product usually has a large number of microorganisms that can cause deterioration and/or a reduction in the shelf life. Many of these microorganisms are indicators of poor sanitary conditions of the place as well as the improper handling of raw materials, utensils and equipment (Peixoto et al. 2007). Given its large consumption in several Northeast states, there is specific legislation only for this product. This regulation defines *Coalho* cheese as the cheese obtained by the coagulating of milk with rennet or other suitable coagulating enzymes; the coagulation may or may not be supplemented by the action of selected lactic bacteria, commonly commercialised at a maximum of ten (10) days after its production (Brasil 2001; Dantas 2012). *Coalho* cheese is classified as a medium-to-high humidity cheese and has between 35% and 60% fat in dry matter. It has semi-hard and elastic consistency, a compact texture (without holes) or opened texture with mechanical holes, a uniform yellowish white colour, a mild flavour that is slightly acidic and salty, a slightly acidic odour characteristic of fresh curd and a thin rind that is not very well defined. Despite the established legal requirements, *Coalho* cheese is processed in different ways, resulting in different types of cheese because of the particular changes that each manufacturer makes in the steps of the manufacturing process and the different milk treatments (Dantas 2012). Three stages directly influence the characteristics of this cheese: the use of raw milk, cooking the mass (the cooking temperature varies from producer to producer), and salting the mass directly (Bruno and Carvalho 2009).

Milk reception is usually performed on the production site and filtered on a cotton cloth or fine mesh made of a plastic material. The mass baking process is conducted by incorporating the whey, which had been previously removed and heated to a temperature between 85°C and 100°C. If this separation has not occurred previously, the mass baking process is performed with hot water or steam, directly over the curd, to obtain a pre-cooked mass at 45°C and baked between 45°C and 60°C. The salting occurs with the addition of sodium chloride to avoid bloating of the mass, which is caused by the presence of faecal bacteria, one of the main contaminants found in this cheese (Queiroz et al. 2008).

5.4. Colonial Cheese

Colonial cheese is a typical Brazilian product with a mildly spicy flavour, ripening of 30 days and shelf life of 120 days; no specific legislation determines its identity and quality standards or its labelling, nutritional and consumer information (Fava et al. 2012). It is produced in the Fourth Colony of Italian Immigration, a region close to the municipality of Santa Maria in the state of Rio Grande do Sul, and it is very similar to *Minas* fresh cheese (Uliana and Rosa 2009).

5.5. Serrano Cheese

Serrano cheese is traditionally manufactured by family farmers in the south of Brazil, in the highlands of the Rio Grande do Sul state, and more specifically, from the Caxias do Sul region, which consists of 49 municipalities that are part of the Coredes Serra, Hortências and Campos de Cima da Serra regions. The Caxias do Sul region is located at an altitude of 950 m above sea level with average temperatures ranging from 4°C to 13°C in the winter and from 18°C to 26°C in the summer (Rosa et al. 2008). The tradition of producing *Serrano* cheese in the Campos de Cima da Serra region dates back more than 200 years (Cruz et al. 2014). Although it is a small-scale artisanal product, it has a high acceptance among consumers (Rosa et al. 2008).

Serrano cheese is produced with raw milk from free-range cows raised on large ranches and fed on the native pastures, using wooden tools and containers, without the addition of starter cultures (Souza et al. 2003; Cruz et al. 2014). In general, it is consumed after a short period of ripening, usually from 15 to 30 days, although the present Brazilian regulation on food safety for cheeses made from raw milk states a minimum ripening period of 60 days prior to consumption (Rosa et al. 2008; Cruz et al. 2014). Souza et al. (2003) showed the importance of ripening for more than 30 days to allow for microbiological stabilisation of *Serrano* cheese.

Serrano cheese is characterised as a low-moisture cheese, semi-skimmed, with fat content between 25% and 44% of total solids content (Rio Grande do Sul 2010). It is mainly characterised as an uncooked, semi-hard mass product of intense taste with a thin, uniform, smooth and soft straw-yellowish rind and a whitish cheese bulk with irregularly distributed holes (Rosa et al. 2008, Cruz et al. 2014). Originally, it was produced in a round shape, with an average weight of 3.0 kg. Currently, due to a larger demand, it has been produced in a rectangular shape, with a weight that ranges from 1.8 to 2.0 kg.

Because *Serrano* cheese is manufactured with raw milk and no commercial starters are used, fermentation occurs naturally, and the ripening process depends entirely on natural microbiota (Rosa et al. 2008). Lactic acid bacteria comprise the main microbial group present (89% of total isolates) during ripening, primarily lactobacilli followed by enterococci and lactococci. Lactobacilli prevail throughout the manufacturing and ripening processes, and their abundance suggests that these microorganisms play an important role in the production of this traditional product (Souza et al. 2003). During its ripening, pH decreases, probably due to the production of lactic acid and, then, increases, suggesting metabolic activity of the moulds and yeasts, which use the lactic acid that is produced as a source of energy (Rosa et al. 2008).

Cheeses with short ripening periods, such as *Serrano* cheese, are characterised by proteolytic activity greater than lipolytic activity as a common feature. Rosa et al. (2008) found that most of the enzyme activity found in the water extracts of *Serrano* cheese would have their origin in lactobacillus and enterococci, suggesting autolysis of these microorganisms. The authors also found an increase in free amino acids during ripening, a reliable indicator of a successful cheese maturation process.

6. HEALTH IMPLICATIONS

Cheeses are fermented foods produced from milk that, due to the fermentation process, have quite diverse microbiota, which may be formed of desirable and undesirable microorganisms. Cheese is considered a versatile and available food with characteristics that offer many opportunities for strategic marketing (Wilkinson et al. 2001); for example, the possibility of being a carrier of probiotic bacteria can be highlighted (Cruz et al. 2011). The pH of the cheese, its high water activity, and its solid matrix, with a relatively high concentration of lipids, are characteristics that can help maintain the viability of probiotic microorganisms, as well as provide protection to these microorganisms during their passage through the human gastrointestinal tract. Other characteristics, such as low salt concentration and the absence of preservatives (Buriti et al. 2005), offer excellent conditions for the survival and multiplication of probiotic strains. Based on this observation, several authors have suggested that the cheese is a product that is more suitable as a vehicle for probiotics than fermented milks and yogurts (Gardiner et al. 1998; Stanton et al. 1998; Daigle et al. 1999; Corbo et al. 2001; Heller et al. 2003; Boylston et al. 2004; Bergamini et al. 2005). Several microorganisms, especially LAB with antimicrobial properties, have been commonly associated with food. The use of their strains as probiotic and bioprotective cultures in fermented food has also been widely studied (Reis et al. 2012).

Furthermore, a LAB growth in cheese is often followed by the production of texturing compounds, such as exopolysaccharides, that improve the rheological properties of fermented milk products, such as viscosity and texture. In addition, LAB strains can produce antimicrobial compounds, such as bacteriocins, with applications in food preservation and various health-promoting compounds, such as vitamins, antioxidants and bioactive peptides (Favaro et al., 2015). Certain probiotic LAB present the ability to resist acidic conditions and the presence of bile salts in the gastrointestinal tract and, additionally, are able to produce bacteriocins that are active against food pathogens and spoilage microorganisms. Therefore,

LAB contribute to the improvement of the fermentation process, enhancing the quality and safety of food products (Stiles and Holzapfel 1997; Leroy and De Vuyst 2004; Zhang et al. 2010). Special attention has been given to studies focusing on the prevention of foodborne pathogens and spoilage bacteria in fermented foods (De Vuyst and Vandamme 1994; Garcia, et al. 2010). Pinto et al. (2011) evaluate the survival of *Staphylococcus aureus* in *Serro Minas* cheese manufactured with raw milk, containing different concentrations of nisin (a bacteriocin), during 60 days of ripening. These authors observed that nisin was effective in reducing *S. aureus* counts in *Serro* cheese, a reduction of 1.2 and 2.0 log cycles after the 7th day of ripening for cheese containing 100 IU mL⁻¹ and 500 IU mL⁻¹ of nisin, respectively, compared with the control sample.

There is extensive literature describing the strains of LAB from traditional dairy products able to inhibit the most important cheese pathogens, such as *Listeria monocytogenes*, *S. aureus*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (Diop et al. 2007; Dal Bello et al. 2010; Ortolani et al. 2010). Some of these authors show that LAB strains have the genetic potential to produce bacteriocins or that they produce them *in vitro*. Single strains of *Lactococcus garvieae* and *Lc. lactis* inhibited *S. aureus* early in a cheese matrix (Alomar et al. 2008; Cretenet et al. 2011). *Hafnia alvei* inhibited shigatoxin-producing *Escherichia coli* O26:H11 in the core of uncooked pressed cheeses and microbial consortia may enhance this inhibition (Delbès-Paus et al. 2012). Pinto et al. (2009a) reported that only a bacteriostatic effect against *Listeria innocua* was observed with the application of LAB as starter cultures in the production of traditional *Serro Minas* cheese to during the ripening period. These bacteria beneficially affect human health by improving the balance of the intestinal microbiota and the mucosal defences against pathogens (Boylston et al. 2004).

Many studies have revealed the presence and/or survival of pathogenic bacteria in traditional cheese manufactured with raw milk in different countries (Psoni et al. 2003; Aygun et al. 2005; Rogga et al. 2005; Borelli et al. 2006; Carvalho et al. 2007; De Buyser et al. 2001; Hamana et al. 2002; Alegría et al. 2009; Pinto et al. 2009), including Brazilian cheeses (Carmo et al. 2002; Veras et al. 2008). Cheeses are ready-to-eat food products that do not undergo any further treatment to ensure their safety before consumption. The contamination of cheese with foodborne pathogens may occur at several stages (Kousta et al. 2010). The microbial contamination of cheese may originate from various sources, such as the natural starter culture, brine, floor, cheese vat, curd cutting, cold room, production room air (Temelli, et al. 2006) and food handlers (Callon et al. 2008). Tondo et al. (2000) studied a dairy product processing plant for 2.5 years to examine the presence of *S. aureus* and to identify potential sources of contamination. Ten samples out of 3,200 dairy products were positive for *S. aureus*, 90.4% (19/21) samples of raw milk were contaminated with *S. aureus* and 35.2% (19/51) of food handlers were asymptomatic carriers of *S. aureus*. No *S. aureus* contamination was found on the machinery.

Among the pathogens found in traditional cheeses, due to the absence of GMP (Good Manufacturing Practices), there are coliform bacteria, *Escherichia coli*, *Salmonella* spp., *S. aureus* and *L. monocytogenes* (Santana et al. 2008, Sobrinho et al. 2012). The contamination of curd *Coalho* cheese with enterotoxigenic coagulase-positive and negative *Staphylococcus* is a public health problem because of the risk of causing food poisoning (Borges et al. 2008). The incidence of coagulase-positive *Staphylococcus*, mainly *S. aureus* in *Coalho* cheese, is high and has been reported in several studies (Borges et al. 2003; Feitosa et al. 2003; Lima

2005). Borges et al. (2003) evaluated 43 samples of *Coalho* cheese produced in Ceará and found that 91% of the samples were contaminated with coagulase-positive *Staphylococcus*. Feitosa et al. (2003) reported that 72.7% of *Coalho* cheese samples produced in different micro-regions of Rio Grande do Norte had coagulase-positive *Staphylococcus* counts. Bruno et al. (2005) compared the microbiological quality of traditional *Coalho* cheese samples, made from raw milk with the same amount of industrialised cheese produced with pasteurised milk. All *Coalho* cheese and industrialised cheese samples had *E. coli*; however, *Salmonella* was detected only in the *Coalho* cheese. Three *Coalho* cheese samples and industrialised cheese presented coagulase-positive *Staphylococcus* at counts above 10^7 CFU/g. Santana et al. (2008) analysed *Coalho* cheese samples commercialised in the municipality of Aracaju, Northeast Brazil, and classified them all as unfit for human consumption. The authors found positive samples for *Salmonella* spp. (26.7%), coagulase-positive *Staphylococcus* (46.7%), total coliforms (93.3%) and faecal coliforms (80.0%). The presence of *L. monocytogenes* was detected in *Coalho* cheese samples by Borges et al. (2003), while Branco et al. (2003) observed the presence of the same pathogen in industrialised *Coalho* cheeses.

In research conducted with *Colonial* cheeses in Santa Catarina, Rossi et al. (2007) found that only 8.57% of the samples were fit for human consumption. Almost 90% of the samples analysed had coagulase-positive *Staphylococcus* at counts above 10^5 CFU/g, representing a potential risk to consumer health. The authors also found positive samples for *Salmonella* spp. (6%) and *L. monocytogenes* (6%).

Samples of milk, whey culture and *Canastra Minas* cheese, both fresh and ripened, collected by Pereira et al. (2008) on farms in the Serra da *Canastra* region were evaluated for the presence of coliforms. Twenty-five per cent of all evaluated milk samples had high scores for total and faecal coliforms. The contamination of whey culture by total coliforms was detected in 25% of the samples. Counts above 10^4 MPN/g of coliforms were recorded in 28.6% of fresh cheese samples, while the ripened cheese had low levels of total and faecal coliforms. Outbreaks of food poisoning caused by *Staphylococcus* toxin were previously associated with the consumption of traditional cheeses in Brazil (Carmo et al. 2002), resulting from the ingestion of low levels of staphylococcal enterotoxins; however, Borelli et al. (2011) investigated the population dynamics of *Staphylococcus* spp. during the 60-d ripening of *Canastra Minas* cheese at three farms located in the state of Minas Gerais, Brazil. Cheese samples that were matured for 0, 7, 15, 30, and 45 days presented staphylococci counts from 10^3 to 10^8 CFU/g. Enterotoxins A, C, and D were not detected in any of the cheese samples.

The microbiological quality of artisanal *Minas* cheese produced in the city of Serro (Minas Gerais) was evaluated by Brant (2003). Of the 40 samples analysed, 37 (92.5%) were unfit for human consumption, and coagulase-positive *Staphylococcus* was the main cause of condemnation. None of the samples showed contamination by *Salmonella* spp. or *L. monocytogenes*. Pinto et al. (2008) evaluated the survival of *L. innocua* in *Serro Minas* cheese during ripening for 60 days and observed that the reduction of *L. innocua* among the ripening was not only related to the decrease of pH as a result of organic acid production but also to the production of bacteriocins by LAB. The results found in this work show that these conditions were not enough to eliminate the bacteria in *Serro Minas* cheese after 60 days of ripening at 30°C.

Studies on the quality of traditional cheeses have shown that the prevalence of foodborne pathogens in raw milk cheese is influenced by numerous factors, such as failures in hygiene, poor quality of raw milk, farm management practices, geographical location, and season, and

this set of factors compromise the quality of these products. Therefore, the development of dairy products containing probiotic bacteria is a major focus of the food industry. These foods are classified as functional foods, and their products are being increasingly accepted by consumers worldwide. Cheese is considered an important matrix that can be used as a vehicle for these probiotics, which imply benefits to human health. Furthermore, these bacteria are able to produce antimicrobial substances that control the populations of pathogens during the production process, providing more safety to the final product.

CONCLUSION

Traditional cheeses, produced from raw milk, may pose risks to human health when the procedures that guarantee food safety are not adopted along the food chain. At the farm level, to prevent raw milk contamination, it is essential to implement good farm practices and a mastitis control program. The standardisation of the manufacturing process, as well as the definition of its ripening period, may generate data to support its origin certification, valuing it as a traditional, safe and good-quality product, which is essential to ensure the sustainability of producers dedicated to this activity.

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