USING OXYGEN AND BIOPRESERVATION AS HURDLES TO IMPROVE SAFETY OF COOKED FOOD DURING STORAGE AT REFRIGERATION TEMPERATURES

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of NYDIA MUNOZ find it satisfactory and recommend that it be accepted.

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USING OXYGEN AND BIOPRESERVATION AS HURDLES TO IMPROVE SAFETY OF COOKED FOOD DURING STORAGE AT REFRIGERATION TEMPERATURES

Abstract

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Cook-chill food products are of concern because they can support the microbial growth of pathogens when subjecting to time/temperature abuses during processing and distribution. The maximum recommended storage temperatures for such products is 5 to 8 °C. In retail and domestic storage, product temperature violations are often 2 to 4 °C higher than those recommended. During consumer transport, temperature violations may rise to 13 °C higher than those recommended. Since temperature alone cannot be regarded as an adequate barrier to control the growth of pathogens in cook-chill food products, additional barriers should be considered to ensure food safety. This study examined two strategies for protecting low-acid cook-chill food products in terms of temperature violations. These strategies include the incorporation of oxygen or a protective culture as a secondary barrier, along with low temperature storage.

The first strategy investigated the use of headspace oxygen in different model foods to prevent the growth of anaerobic pathogenic bacteria in in-pack pasteurized chilled food at various storage temperatures. Results showed that the structure and properties of the food significantly affected the mobility of the oxygen within it. Oxygen diffusion from the pouch headspace was limited to the food surface. Oxygen did not reach the center and bottom portions of the food during storage time so that oxygen levels which favor *C. botulinum* growth were maintained. Findings suggest that the air/oxygen present in the package headspace may not be considered as a food safety hurdle in the production of pasteurized packaged food.

In the second strategy, the antagonistic activity of *Lactobacillus rhamnosus* GG on five pathogens and its heat sensitivity in soup products were observed. Then *Lactobacillus rhamnosus* GG effectiveness as a protective culture on *Listeria* and *Salmonella* growth at conditions simulating the application of cook-chill processing was determined. Overall, *Lactobacillus rhamnosus* GG showed an inhibitory effect against all pathogens tested. Heat resistance of *Lactobacillus rhamnosus* GG depends on temperature and soup properties. *Lactobacillus rhamnosus* GG had a bactericidal effect on *Listeria* and *Salmonella* at 15 °C and a bacteriostatic effect on *Listeria* at 10 °C during storage for 21 days.

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Dedication

This dissertation is dedicated to my daughter Tanaydí Cosme and my son Nyathan Cosme

CHAPTER 1 INTRODUCTION

1. Background

Cook-chill foods products were introduced in the 1970s as a more convenient food option than frozen food (Juneja and Snyder, 2008). Convenience has always been the main driver for cook-chill food purchases (Del Torre et al., 2004; Van der Horst et al., 2011; Wolfson et al., 2016), and these foods have gained global popularity. Consumers are increasingly concerned about nutritional value and food safety. This motivates an increasing demand for convenient food of better nutritional quality and less preservatives. Cook-chill food products are offering what consumers are looking for in a home-style meals presentation. The global market of ready to eat frozen/refrigerated food is expected to growth by 8% during the forecast period 2016-2026 (Newswire Association, 2016).

Cook-chill food products are also known as REPFEDs (Refrigerated Processed Foods of Extended Durability) (Daelman et al., 2013). They are a varied group of food products owing to the diversity of ingredients, processing conditions and packaging systems used in their production process. Cook-chill food includes a vast range of food products including sauces and gravies, soups, casseroles, desserts and ready-to-eat meals. Since such foods are made up of common ingredients including meat, fish, potatoes, rice, pasta, and vegetables, most of the cook-chill foods have low acidity and high water activity (a_w). Foods with a pH ≥ 4.6 and a_w >0.85 are classified by the US Food and Drug Administration (FDA) as low-acid foods. Cook-chill foods usually fall into this category and can be compromised by many pathogens and toxins. Therefore, they are closely monitored by regulatory agencies.

Cook-chill food products are prepared for consumption by using mild temperatures between 65 to 95 °C during the cooking process (Lovdal et al., 2011). The food is prepared and cooked just as consumers would cook it at home. As a result, nutritional quality is enhanced, and natural flavors are retained. Therefore, additional salt and fat are not needed to please consumers. Cook-chill food products offer to the consumer most of the nutritional benefits of each ingredient with lower levels of sodium and fat. In contrast, most shelf-stable canned or dried food products require artificial preservatives and chemical additives to achieve a shelf life of one year or more.

Despite the benefits that cook-chill food products offer to the consumers such products are often associated with foodborne illnesses, which represent a significant public health concern in the United States (Marder et al., 2017). The three main microbial hazards in cook-chill food products are non-proteolytic *Clostridium botulinum*, *Bacillus cereus* and *Listeria monocytogenes* (Daelman et al., 2013). Those psychrotrophic pathogens can grow in low-acid foods during extended shelf life at refrigeration temperatures. The minimum growth temperatures for non-proteolytic *Clostridium botulinum*, *Bacillus cereus,* and *Listeria monocytogenes* are 3.3 °C, 4 °C, and -0.4 °C, respectively (ECFF, 2006).

The food industry primarily controls the microbial safety of cook-chill food products using heat treatment, and cold storage (< 5 °C). Normally, two safe harbors heat treatments are applied to the production of cook-chill food products based on a defined number of decimal reductions (D-values) of a specific target organism (Daelman et al., 2013). The first heat treatment achieves 70 °C for no less than 2 minutes in the core of the food (or equivalent treatment for a 6D reduction of *Listeria monocytogenes*). The second one achieves 90 °C for 10 min in the core of the food (or equivalent treatment for a 6D reduction of non-proteolytic *Clostridium botulinum* spores). This heat treatment eliminates all vegetative stages of any pathogen present and causes enough damage

to inactivate spores of non-proteolytic *C. botulinum*. However, heat-resistant spores of *B. cereus, proteolytic C. botulinum*, and other spore-forming microorganisms can survive this treatment. Proteolytic *Clostridium botulinum*, a gram-positive spore former bacterium that produces botulinum neurotoxin, is a major concern for the food industry due to the severe health effects of foodborne botulism. Refrigeration has proven to be an effective control measure to control the germination and growth of the spore stage of this pathogen, which has a minimum growth temperature of 10 °C.

The post-cooking/pasteurization operations for cook-chill food products, such as portioning and packaging, are a potential source of recontamination by *Listeria monocytogenes*. Listeriosis is a rare human disease with serious health effects for certain groups, including children, the elderly, immunocompromised individuals and pregnant women (Linaous and Sofos, 2007). The case fatality of listeriosis is 20 to 30%, which is the second highest among bacterial diseases (Murphy et al., 2005; Linaous and Sofos, 2007). *L. monocytogenes* growth is significantly retarded at the refrigeration temperatures that are used for storing cook-chill food products. However, controlling *L. monocytogenes* growth during storage can be notoriously difficult due to its psychrotrophic nature.

Although *C. botulinum*, *L. monocytogenes* and mesophilic pathogens growth can be controlled through a combination of cooking and storage temperatures, temperature abuse of low-acid food products is a major factor in pathogens outbreaks (Golden et al., 2017). Certainly, temperature control and maintenance of an adequate cold chain conditions are critical to food safety. However, since certain cold chain steps are especially weak, many studies have focused on these steps (Derens-Bertheau et al., 2015). In retail displays, product temperature can often rise above the manufacturer’s recommendation, as well as during consumer transport and in domestic storage. In

retail and domestic storage, product temperature violations are often 2 °C to 4 °C higher than those recommended. During consumer transport, temperature violations may rise up to 13 °C higher than those recommended.

Since temperature alone cannot be regarded as an adequate barrier to control the growth of foodborne pathogens in cook-chill food products, additional barriers should be considered to ensure food safety. One barrier may be the addition of oxygen since bacteria vary widely in their ability to use and tolerate oxygen (Prescott, Harley and Klein, 2002). *Clostridium botulinum* is an anaerobic obligate bacterium since it does not use oxygen for growth and eventually dies in the presence of O_2. Oxygen can be applied to the food/packaging system, either directly by gas mixture, sealing without applying vacuum or indirectly through packaging food in films with a higher oxygen transmission rate (OTR). The justification to add this safety barrier is that oxygen present in the package headspace may delay/inhibit toxin production by *C. botulinum* or promote the development of spoilage microorganisms resulting in spoilage prior to toxigenesis (Dufrense et al, 2000). For oxygen to suppress the growth of *C. botulinum* in food, it should be able to dissolve on the surface of food and diffuse through the food.

Oxygen solubility and diffusion phenomena have been studied for different food matrices (Penicaud et al., 2010; Chaix et al., 2016). Once headspace oxygen dissolves at the food surface, it diffuses slowly in solid food matrices and quickly in liquid food matrices. In both cases, an oxygen gradient through the food is generated. This may influence the growth of *C. botulinum* at different locations within the food, favoring the growth at locations with lower oxygen concentrations. However, there is little or no research on the influence of an oxygen concentration gradient on *C. botulinum* growth within various foods. Therefore, research is needed on oxygen

diffusion and microbial growth in foods at different storage temperatures. This will help determine the effectiveness oxygen as a control barrier during product temperature violations.

The application of protective cultures (PCs) represents another barrier. The effectiveness of protective cultures in controlling the growth of pathogens has been documented in the literature. These pathogens include *C. botulinum*, *L. monocytogenes*, *E. coli* O157:H7, *B. cereus* and *Salmonella* spp. in food substrates, including fruit and vegetables, commercial cook-chill soups, meats (fresh and cooked), seafood, and tofu (Bredholt et al., 1999; Schillinger et al., 2001; Rodgers et al., 2003; Vermeiren et al., 2004; Chahad et al., 2012; Gaggia et al., 2015). However, the use of PCs at food industry level is limited. Research is needed on the survival of PCs in the pasteurization process, as applied to cook-chill foods.

2. Hypothesis and Objectives

This study examined two strategies for protecting low acid cook-chill food products in terms of temperature violations. These strategies include the incorporation of oxygen or protective cultures as a secondary barrier, along with low temperature storage to ensure food safety. We developed one hypothesis for each applied strategy on the applications' limitations for food products at the industrial level.

2.1 Oxygen based strategy

Clostridium botulinum is an anaerobic bacterium that does not use oxygen for growth and eventually dies in the presence of oxygen. This is because anaerobic bacteria do not have the elaborate system of defenses that aerobic bacteria have. The system relies on a series of special enzymes in large quantities, including super dismutase, catalase, and peroxidase, which can

scavenge the toxic compounds formed in an oxygen-rich atmosphere. Anaerobic bacteria produce these enzymes in very small amounts, or not at all. Thus, the variability in oxygen tolerance of obligate anaerobes is thought to be influenced by the amount of those enzymes that they can produce. For oxygen to suppress the growth of *C. botulinum* in food, it should be able to dissolve on the surface of food and diffuse throughout the product. Our central hypothesis of this strategy was that if oxygen diffuses through the food fast enough to reach the bottom, then it may control the germination and growth of *C. botulinum* spores during temperature abuse. The objectives of this study-based strategy are:

1. To study oxygen diffusion on model foods with different viscosities at 8 °C, 12 °C and 20 °C.
2. To monitor the growth of *Clostridium sporogenes* PA 3679 as a surrogate of *Clostridium botulinum* within the food (top, center, and bottom layers).

2.2 Protective culture based strategy

Lactic acid bacteria (LAB) are the most common microorganisms used as protective cultures due to their antagonistic properties. When LAB compete for nutrients and space in food systems, their metabolites often include active antimicrobials, such as organic acids, hydrogen peroxide, and peptide bacteriocins. Due to LAB's activity against certain foodborne pathogens, as well as their ability to grow when temperatures exceed the recommended limit for a product, they are considered useful in the biopreservation of cook-chill foods. Our hypothesis for this strategy was that if the protective culture population can survive the temperature used during the portioning and packaging operations, then it may control pathogen growth during temperature abuse by competitive exclusion. The objectives of this study-based strategy are:

1. To determine in-vitro whether *Lactobacillus rhamnosus GG* is effective in inhibiting foodborne pathogens.
2. To determine *Lactobacillus rhamnosus* GG heat sensitivity on soup products.
3. Moreover, evaluate its effectiveness as the protective culture at conditions simulating the commercial application of cook-chill processing throughout storage at 5 °C, 10 °C, and 15° C on *L. monocytogenes* and *S.* Typhimurium growth.

3. Dissertation outline

This dissertation is organized into five chapters. The first provides background information, hypothesis, and objectives of this research. Chapter 2 reviews the methods largely employed in the production of cook-chill food, including hot-fill and sous vide. The description of production methods elucidates the microbial hazards associated with such products. Chapter 2 also reviews the importance of temperature control in the product cold chain. It also examines the application of oxygen and bio-preservation as a hurdle to improve the safety design of cook-chill food products. Chapter 3 investigates the application of oxygen as a hurdle in in-pack pasteurized food products during storage at different temperatures in order to control the germination and growth of proteolytic *C. botulinum* spores. Chapter 4 evaluates the performance of *Lactobacillus rhamnosus* GG as a protective culture on a cook-chill food product to control the growth of *Listeria monocytogenes* and *Salmonella* Typhimurium at low temperature storage. Chapter 5 compiles major findings from this research and provides recommendations for future research.

Chapter 3 and 4 are prepared based on manuscripts published or submitted to following journals. The format of these Chapters follows the requirements of each different journal with minor changes of the Figure/Tables and References numbering.

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CHAPTER 2 LITERATURE REVIEW

1. Introduction

Cook-chill food products are minimally processed food, processed by moderate heat treatment followed by chilling storage and distribution. Those products are gaining popularity since its introduction in the 1970s. As people time get more limited, consumers are turning to them more and more as a regular meal. It became all about convenience. Consumers are also demanding for food with enhanced taste, better nutritional quality, and less preservatives. These demands have led to growth in the application of hot-fill and *sous vide* methods to produce cook-chill food products. With cook-chill foods becoming so much more of a fixture in homes, the safety of the products becomes even more important. This Chapter first describes the methods largely employed in the production of cook-chill food, including hot-fill and *sous vide* in Section 2. Section 3 and Section 4 focus on the temperature control in the product cold chain, and microbial hazards associated with cook-chill food, respectively. Finally, Section 5 reviews the application of oxygen and bio-preservation as a hurdle to improve the safety design of cook-chill food products.

2. Cook-chill production methods

Cook-chill food products can be produced by hot fill or *sous vide* ("under vacuum") methods, depending on the recipe (Shakila et al, 2011). **Figure 2.1** displays a flow process diagram for both methods. Any food recipe of pumpable consistency (foods with chunks smaller than about 1-inch diameter) is cooked in volume in kettles to the desired end point at temperatures < 100 °C (Xie, 2000). Thereafter, the food is filled while hot into a 1 to 1 and ½ gallon casing or bucket, form-film pouch or any suitable container. The packaged food is cooled in 0 °C water tumble chiller, a blast chiller, or a circulating cold-water bath (Chater, 2002; Poumeyrol et al., 2012). The food is

then stored under controlled low temperature conditions above the freezing point. Hot-fill processing preserves the food products for up to five days, including the production day.

The *sous vide* method is applied to food recipes containing solid ingredients of more than 1-inch diameter, as well as large cuts of fish and meats. It works by packaging the raw food recipe into heat-stable vacuumed-sealed pouches or trays for slow cooking in a cook/chill tank (Baldwin 2012; Sebastiá et al., 2010). The food is normally cooked at a temperature below 80 °C for longer times (up to 48h) (Oz & Zikiro, 2015). After cooking, the food is chilled and stored at a low temperature above the freezing point.

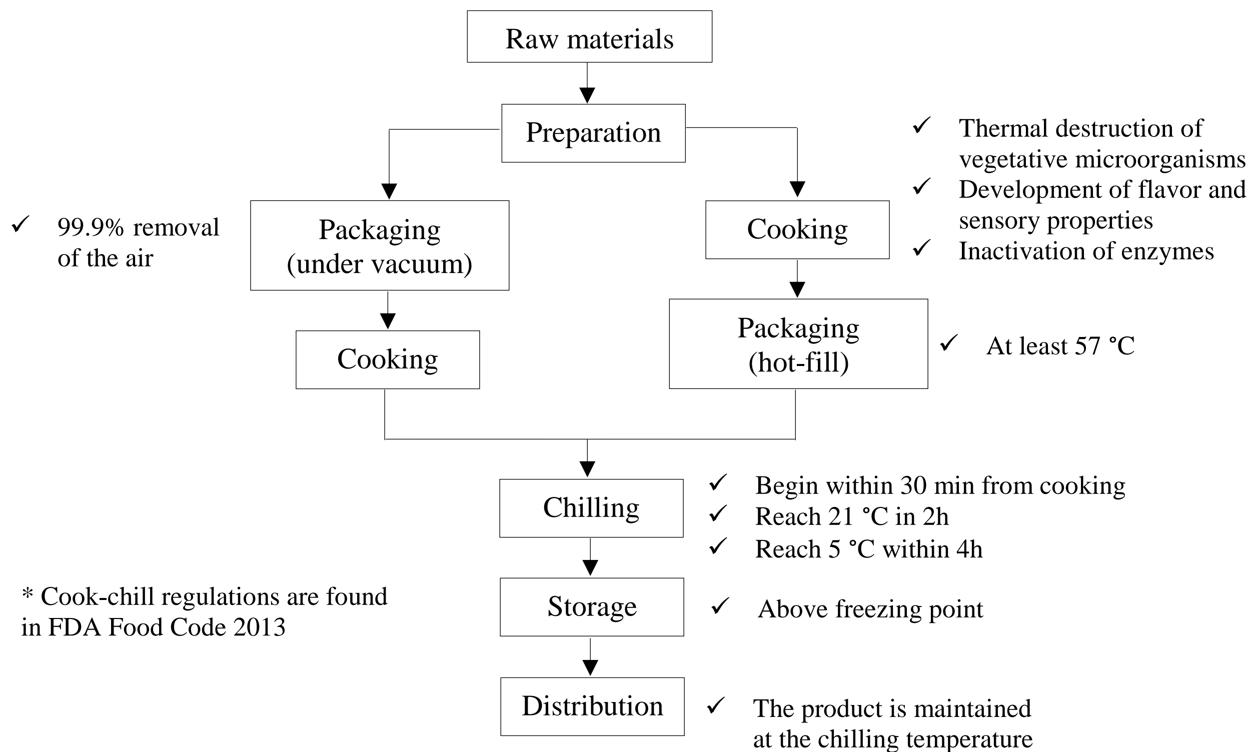

Figure 2.1 Cook-chill food product production methods

In both methods, the aim of the cooking/pasteurization process is to ensure destruction of vegetative stages of any pathogenic microorganism present. Two safe harbor heat treatments are commonly used in the production of cook-chill food, based on a defined number of decimal reductions (D-values) of a specific target organism (Daelman et al., 2013a). The first heat treatment is to achieve 70 °C for no less than 2 minutes in the core of the food, or an equivalent treatment, to cause a 6D reduction of *Listeria monocytogenes*. This is generally accepted as the most heat resistant vegetative pathogen. Therefore, if the heat treatment is completed successfully, all vegetative stages of any pathogen present will be destroyed. However, spores of *Bacillus cereus* and *Clostridium botulinum* (proteolytic and non-proteolytic strains) can survive this treatment.

The second heat treatment is to achieve 90 °C for 10 min in the core of the food, or equivalent treatment, to cause a 6D reduction of non-proteolytic *Clostridium botulinum* spores. This heat treatment eliminates all vegetative stages of any pathogen present and causes enough damage to inactivate spores of non-proteolytic *C. botulinum*. However, heat-resistant spores of *B. cereus, proteolytic C. botulinum*, and other spore-forming microorganisms can survive this treatment.

When the cooking/pasteurization process is performed out of the package, as in the hot-fill production method, it introduces the risk of post-process contamination with *L. monocytogenes* and other enteric pathogens (e.g. *Salmonella sp*.). Such pathogens can be present in the production environment, or in ingredients added after cooking but prior to the packaging operation. However, cooking the food inside the package, as in the sous vide production method, prevents recontamination (Daelman, 2013b; Mason, 1990).

The cook-chill food products need to be cooled immediately after cooking to ensure food safety. The final temperature of the product should be below 5 °C (FDA, 2013). The cooking

processes used in the production of cook-chill food does not achieve commercial sterilization, and food products must be refrigerated to stabilize them. Disease-causing microorganisms can grow and multiply at temperatures between 5 °C and 57 °C (41 °F to 135 °F), which is why this range is called the temperature danger zone (FDA, 2013; NRA, 2017). Within the zone, microorganisms grow quickly, between 21 °C and 49 °C (70 °F to 120 °F). Every time food is held in the temperature danger zone, it is being abused. Therefore, it is critical to move foods through the zone quickly in order to reduce this growth. The current FDA Food Code recommendations call for a two-stage cooling process. It should start within 30 minutes from cooking. First, cooked food must be cooled from 57 °C to 21 °C (135 °F to 70 °F) within two hours. Then it should be cooled from 21 °C to 5 °C (70 °F to 41 °F) or lower in the next four hours. This cooling process ensures that food passes through the temperature danger zone quickly. This prevents bacterial growth and keeps food safe.

Non-protelytic *C. botulinum* can grow in foods at 3.3 °C (38 °F) and *L. monocytogenes* can grow at 1 °C (34 °F). Consequently, limits have been stablished to the refrigerated storage shelf-life of cook-chill processed foods that use refrigeration as the only barrier to pathogens growth. The US 2013 Food code limits the shelf life of food products held at 5 °C for no more than 7days, including the preparation day. When the food products are cooled to 1 °C within 48 hours of reaching 5°C and held at that the shelf life is extended to 30 days after the day of preparation.

3. Cold chain overview

Once the cook-chill food is produced, it should be stored and distributed at a controlled low temperature. Temperature control and maintenance of adequate cold chain conditions are critical to food safety. There is a sequence of refrigeration steps along the supply chain to keep perishable

food in the safe temperature range. This is referred to as the cold chain (Mercier et al., 2017). **Figure 2.2** illustrates an outline of the steps in a normal cold chain. The cold chain normally begins immediately after the production process for cook-chill food. The cold chain ends when the food is reheated and consumed by consumers. In the middle, based on the market demand, the food may travel through several storage and distribution centers before being dispatched to retailers. At the retail point, the food is placed in display cabinets for sale.

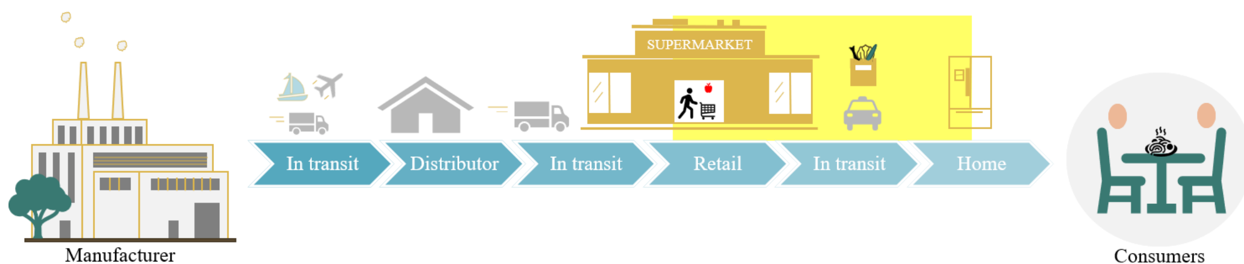

Figure 2.2 Overview of the main steps in a typical cold chain

Today, it is widely known that some cold chain steps are particularly weak. Therefore, these points have undergone considerable study (Derens-Bertheau et al., 2015). Laguerre et al. (2013) presented the results of two surveys performed between 2006 and 2009 on the temperature evolution of food products throughout the cold chain. For these surveys, a small temperature recorder was placed inside the food product at the end of the production line, but before shipping the product along the supply chain. Consumers who found recorders were asked to send them back to the laboratory. One survey employed 480 recorders to collect time/temperature data on three types of refrigerated products, including fresh meat, a meat product, and yogurt. Around 65% of the recorders found were returned, which allowed the observation of the time/temperature data of 314 product items. Results showed that temperature control is critical in the last three steps in the cold chain. The average product temperature was 2 °C higher than the recommended value for

30% of products in a display cabinet, 70% during transit after shopping, and 40% in domestic refrigerators. The other survey monitored the time/temperature history of 200 smoked salmon products, which again indicated that temperature abuse occurred during the last three steps.

Lunden et al. (2014) investigated the extent and magnitude of noncompliance in the temperature of food products in 32 retail food stores in Finland. The temperature of four food categories was monitored, namely fresh fish, minced meat, vacuum packed ready-to-eat processed fish and other ready-to-eat products. The temperature in 50 % of the products was found to be in violation, and 17.9% of the products exceeded the temperature limit by over 3 °C for more than 30 min. Fresh fish and vacuum-packed as well as processed fish products were the products most often in noncompliance.

Baldera et al. (2016) measured the temperature in several display refrigerators and at the surface of eight refrigerated food categories to verify whether the cold chain was respected at the retail point. The food categories include fresh meat, meat preparations, meat products, dairy products, fishery products, ready-to-eat (RTE) food, refrigerated vegetables and mixed (different kinds of foods within the refrigerator). The temperature control requirements (TCR) for the cooling equipment range between 3 °C and 8 °C depending on the food category. Results show that the display temperature of the cooling equipment exceeded the TCR for all food categories, except meat preparations and fishery products. The percentage of noncompliance in cooling equipment tested for groups of meat products, refrigerated vegetables, and RTE food was significantly higher, from 40 to 80%. When the temperature was measured at the surface of the food located at the top shelf of the cooling equipment, 100 % of the food for groups of ready-to-eat and vegetable was in not compliance during the summer season. The TCR for such categories is 4 or 5 °C, and the temperature measured was 4 °C higher than that recommended.

Gonzales et al. (2013) determined the average temperature of ready-to-eat seafood product at the retail display. Results showed that all the products tested had a higher temperature than the maximum 4 °C recommended by the manufacturers. In the case of smoked salmon, despite the open refrigerated displays showed temperatures ranging from 1.5 °C and 5 °C, the temperature of the product was on average 8 °C (ranging from 5 °C to 14 °C). In addition, the temperature distribution within the refrigerated displays was not homogeneous, especially in the largest ones.

Retail stores widely use open refrigerated cabinets as a primary means of keeping chilled food at the required temperature while allowing the customer almost unrestricted access to the food (Evans et al., 2007). However, these cabinets are not energy-efficient (Kuo et al., 2015). Often, they fail to provide the temperature necessary for proper storage of chilled food. Most high-temperature abuse occurs in the front of the display cases (Atilio de Frias et al., 2015).

Laws and regulations are applied in the food chain to mitigate risk and enhance food safety from the primary producer to retail. However, as soon as the product is purchased by the consumer, there is little knowledge of how the product is treated. Derens-Bertheau et al. (2015) followed the time/history of 83 chilled ham products, and found for the transport after purchase by the consumer, the mean product temperature was 6.5 °C, with a minimum of 1.1 °C and a maximum of 20.4 °C. These high temperatures are due to the lack of refrigeration equipment at this step.

The domestic refrigerator is a common, if not universal, household device throughout much of the world (James et al., 2017). James et al. (2008) reported that in domestic storage, mean refrigerator temperatures range from 8-10 °C. The results of nine studies conducted in United Kingdom, France, Ireland and Greece (Koutsoumanis and Gougouli, 2015) with a total of 1171 consumer refrigerators tested, showed that 64.1% of the refrigerators were operating at temperatures above 5 °C. Many other studies (Roccato et al., 2017) confirm that domestic

refrigerators throughout the world operate above the recommended temperatures and are not appropriately maintained (Masson et al., 2017). It is important that the process of maintaining a cold chain does not conclude with the retailer (Ovac and Jevšnik, 2009). Maintaining a cold chain should continue up to and within a consumer's home.

4. Microbiological hazards

Food produced by hot-fill and *sous vide* technology offer convenience, extended shelf life and deliver home-made style meals. However, for cook-chill food, there are several microbiological safety concerns (Juneja and Snyder, 2007). First, these products are normally formulated with little or no preservatives and have low acid and high moisture content. Second, they are subjected to minimal thermal processing, are not commercially sterile and must be refrigerated. Third, vacuum packaging creates a favorable environment for anaerobic and facultative pathogens such as *Clostridium botulinum* and *Bacillus cereus* to grow and produce toxins. Finally, there is a high probability of temperature abuse during distribution and storage.

Cook-chill food is a heterogeneous group of food products (Daelman et al., 2013a). Such products are made up of normal ingredients, including meat, fish, potatoes, rice, pasta, and vegetables. That is why the majority of cook-chill food has low acidity and high water activity (a_w). The pH value is used to refer to the degree of acidity and is an important factor that affects the survival and growth of microorganisms. In general, the optimum pH range for bacteria is 6.0-8.0, and most cannot growth below 4.6 (Juneja et al., 2012). Water activity refers to water in food that is available to support microbial growth and chemical reactions. Typically, bacteria need $a_w > 0.90$ to grow (Driscoll, 2014). *Staphylococcus aureus* is the most a_w–tolerant pathogen. It can grow and produce toxin in aerobic conditions at a_w 0.85 and in anaerobic at a_w 0.91 (Drisvroll et al.,

2014). Foods with a pH ≥ 4.6 and a_w >0.85 are classified by the US Food and Drug Administration (FDA) as low-acid food. Cook-chill food usually falls into this food category and is closely monitored by regulatory agencies because they can support the outgrowth of many pathogens and toxin development due to intrinsic properties.

As mentioned previously, the cooking process of cook-chill food uses temperatures below 100 °C. Typical temperature ranges from 65 to 95 °C (Rodger, 2004). These temperatures are too low to destroy the spore stages of pathogens bacteria belonging to the *Clostridium* and *Bacillus* genus. Consequently, the microbial safety of cook-chill food relies primarily upon refrigerated storage. Chilling involves reducing the food temperature with the purpose of decreasing the rate of enzyme activities, which slows or stops microbial growth and activity in the food. Microbial growth can take place in a wide range of temperatures, and the cardinal growth temperatures (minimum, optimum and maximum) are used to describe microorganisms' ability to grow in a particular range. The optimum growth temperature represents the temperature at which, biochemical processes leading to the growth of a specific microorganism are operating most efficiently (Walker, 1992; James and James, 2014). As the temperature increase up above the optimum, the growth rate diminish until the maximum growth temperature is reached. The minimum growth temperature (MGT) represents the lowest temperature at which growth can occur for a particular microorganism. This is the temperature of most concern for cook-chill food.

At MGT, the lag phase before growth is significantly extended and the growth rate is minimized (Burnett et al., 2005). As the temperature approaches the minimum for growth, cell division ceases. Since, the reaction rate of enzyme-catalyzed reactions and the fluidity of the cytoplasmic membrane decrease, leading the disruption of the cell transport mechanism and subsequent decreased growth rates. When a microorganism is stored below MGT gradual death

can occur, but often microorganisms survive and restart growth if the temperature subsequently rises.

Relatively small increases in the storage temperature can cause a significant increase in the growth rate of several pathogens. **Table 2.1** lists the pathogenic microorganisms of concern, along with their principal growth boundaries related to cook-chill products. *L. monocytogenes*, non-proteolytic *C. botulinum* and *B. cereus* are of particular relevance for cook-chill foods. *L. monocytogenes* is the most resistant non-spore-forming cold growing bacteria. Non-proteolytic *C. botulinum* and *B. cereus* are the spore-forming bacteria with lower minimum growth temperatures. Based on those minimum growth temperatures and the ability of non-proteolytic *C. botulinum* and *B. cereus* spore to germinate and produce toxins, the storage temperature, and distribution for cook-chill food has been recommended < 5 C (ECFF, 2006).

When the recommended temperature is not retained throughout the cold chain, it may rise above 10 °C for a prolonged period. In this case, proteolytic *C. botulinum* and *C. perfringens* are also of concern. In addition, the handling necessary to package food after processing and during hot-fill production method may lead to recontamination of the treated products with pathogens such as *L. monocytogenes*, *Escherichia coli* O157: H7, *Yersinia enterocolitica,* and *Salmonella* spp. In any case, the absence of a competitive microflora may for more proliferation of pathogens already present in the foods.

Table 2.1 Commonly accepted growth boundaries of selected pathogenic microorganisms

Microorganism	Growth boundaries			
	Min temp (°C)	**Min pH**	**Min a_w**	**Aerobic/ anaerobic**
Listeria monocytogenes	-0.4	4.3	0.92	Facultative
Bacillus cereus	4	4.5	0.93	Facultative
Campylobacter jejuni	32	4.9	0.99	Microaerophilic
proteolytic Clostridium botulinum	10-12	4.6	0.93	Anaerobic
non-proteolytic C. botulinum	3.3	5	0.97	Anaerobic
Clostridium perfringens	12	5.5-5.8	0.935	Anaerobic
Escherichia coli	7-8	4.4	0.95	Facultative
Escherichia coli O157:H7	6.5	4.5	0.95	Facultative
Salmonella	6	4.0	0.94	Facultative
Staphylococcus aureus	5.2	4.5	0.86	Facultative
Vibrio cholerae	10	5	0.97	Facultative
Vibrio parahaemolyticus	5	4.8	0.94	Facultative
Yersenia enterocolitica	-1.3	4.2	0.96	Facultative

*Adapted from ECFF, 2006

5. Strategies to improve food safety

In order to reduce the risk posed by foodborne pathogens in cook-chill foods, governmental and regulatory agencies have focused on the implementation of effective systems, such as Hazard Analysis and Critical Control Points (HACCP), within the food production chain (Poumeyrol et al., 2014). A key criterion for cook-chill foods, as well as for all food products, is that they must be safe at the point of consumption. Unfortunately, as noted in previous sections, the lack of temperature control through distribution, retail and domestic storage of cook-chill foods can often counteract much of the effort made in enhancing and maintaining food safety at the early stage of the production chain. When temperature abuse occurs, low-acid cook-chill food is unprotected.

Therefore, it is desirable to base the food preservation system on more than one controlling factor during the cold chain.

Recipe modification is one approach to prevent microbial growth, by increasing the salt or acid content and decreasing the water activity of foods. The Advisory Committee on Microbiological Food Safety (ACMFS) has recommended the use of the following factors to be modified in recipes:

1. A pH ≤ 5 throughout the food and throughout all components of complex foods.
2. A minimum salt level of 3.5% in the aqueous phase throughout the food and throughout all components of complex foods.
3. A water activity ≤ 0.97 throughout the food and throughout all components of complex foods.

These controlling factors should be used alone or in combination to prevent growth and toxin production by non-proteolytic *C. botulinum* in chilled foods with a shelf life of more than ten days and storage temperatures of 3 to 8 °C. Studies have assessed the effectiveness of adding salt and acid to food recipes to control the growth of foodborne pathogens (Christopher and Wallace, 2014; Khanipour et al., 2016; Peck and Stringer, 2005; Taormina, 2010). For instance, it is generally accepted that the growth of *C. botulinum* is prevented at pH 4.6 or below, that 10% NaCl will inhibit growth, and that the minimum water activity allowing growth is 0.93 and 0.96 with glycerol and NaCl, respectively (Peck and Stringer, 2005). Although this approach can be followed at low cost, it can affect the sensory characteristics of the foods and the nutritional content (Rybka-Rodgers, 2001; Mitchel et al., 2012). In addition, consumers are demanding minimally processed foods with less chemical preservatives. Thus, chemical methods are limited to natural antimicrobial substances, such as the use of organic acids, spices, and salts. These are limited further to the products in which such ingredients can be incorporated. For example, lactate was

recognized as a universal preservative, however commercial trials demonstrated that it increases saltiness, influences color and flavor. (Rodgers, 2004). Producers are looking for methods that decrease chemical preservatives without increasing microbial risks to the consumer. In that context, the use of oxygen and protective cultures represent a viable alternative to reduce chemical preservatives in cook-chill foods (Gaggia et al, 2011; Rodgers, 2008).

5.1 Oxygen as a preservative

The atmospheric air is composed of several gases but two make up most of the composition with the others being in trace amounts. Nitrogen and oxygen are the largest components at about 78 % and 20.9 %, respectively. Nitrogen gas is inert and is not much of a concern towards foods. Indeed, Nitrogen is used to protect food by providing an inert filler gas during modified atmosphere packaging (Oliviera et al., 2015). However, oxygen plays an important role in many aspects of food science. Oxygen is involved in many detrimental chemical reactions that reduce the quality of foods such the oxidation of lipids, proteins, vitamins, and pigments (Sardarodiyan and Sani, 2016; Van Bree et al., 2012; Wirth et al., 2010; Zakrys-Waliwander et al., 2012). Oxygen is also important to determine the ability of outgrowth of microorganisms. Oxygen is needed for respiration of many fruit and vegetables.

In most instances, oxygen contact with foods needs to be minimized to prevent deteriorative reactions from occurring and reducing quality and shelf life. However, the production of an anaerobic environment can lead to serious food safety concerns if other conditions are not insured to prevent the growth of important pathogens. Then in some cases, oxygen presence is desired for safety reasons. For instance, low levels of oxygen in surroundings some food products can be

beneficial to ensure safety by preventing the growth of the anaerobic pathogenic bacterium *Clostridium botulinum*.

The presence or absence of oxygen determines what types of microorganism will be able to grow in or on a food product. Microorganisms have different respiratory and metabolic needs, and can be grouped based on their need for oxygen (O_2) to grow as a follow (Prescott, Harley and Klein, 2002):

1. Aerobe. Strictly aerobic microorganisms have an absolute requirement for oxygen since they use it as a final electron acceptor in the transport chain during respiration. They require molecular oxygen to grow at near atmospheric concentration (20.96%).
2. Microaerophilic. In this group, microorganisms grow best in low oxygen concentration compared with normal atmospheric concentration. They require an oxygen concentration of between 2 and 10%.
3. Anaerobe. Strictly anaerobic microorganisms do not need oxygen to grow, as they do not use it as a final electron acceptor in respiration. Instead, they use sulfate, nitrate, iron, manganese, mercury, and carbon monoxide. In the presence of normal atmospheric concentration, they can be killed.
4. Aerotolerant. They do not need oxygen to grow since they make use of fermentation or anaerobic respiration to generate energy. However, unlike anaerobes, they simply ignore oxygen when it is present, and grow equally well regardless of whether oxygen is present.
5. Facultative. These are the most versatile microorganisms since they grow with or without oxygen. They can metabolize energy both aerobically and anaerobically. However, when oxygen is present, they grow better.

Oxygen may be present in several forms in food products including triplet oxygen and as reactive oxygen species (ROS). Atmospheric triplet oxygen is the most abundant and stable form of oxygen (Johnson and Decker, 2015). In contrast, ROS are highly unstable ions and molecules derived from the partial reduction of oxygen and can be toxic in different degrees to the microorganisms. Singlet oxygen, superoxide, peroxide, and hydroxyl radical, are all examples of ROS (Abdulsalam et al., 2016; Decamps et al., 2014). ROS can damage virtually any macromolecule (eg. lipids, protein and DNA of cells) or structure with which they come in contact. Therefore, microorganisms need some way to decompound some the ROS that may form from atmospheric oxygen.

Three main enzymes decompound those toxic byproducts: superoxide dismutase, peroxide, and catalase (Rolfe et al., 1978). Microorganisms that can grow in the presence of oxygen such as aerobic bacteria are equipped with a defense system containing these enzymes that makes aerobic life possible (Hentges, 1996). Obligate anaerobic bacteria usually lack all three enzymes. The study conducted by Rolfe et al. (1978) showed that the most oxygen-sensitive anaerobes, as a rule, contained little or no superoxide dismutase. Also, the study showed that very sensitive anaerobes, which reduced comparatively large amounts of oxygen and exhibited no superoxide dismutase activity, were killed after short exposure to oxygen. Then the presence of oxygen in the food/packaging system may be able to kill or inhibit the growth of anaerobic pathogenic bacteria such as *Clostridium botulinum* in cook-chill food during storage.

5.1.1 Oxygen in the food/packaging system

The preparation of cook-chill food products by the *sous vide* method involves a vacuum packaging operation before cooking. The operation consists of sucking out the air from the

food/package system. Next, it is sealed tightly, usually by joining (Bilska, 2011). This encloses the food products in a reduced oxygen environment.

Anaerobic and facultative pathogens are of concern in the *sous vide* cook-chill food products, since the packaging conditions used during food production selects for them. *Clostridium botulinum* (type E), *Bacillus cereus* and *Listeria monocytogenes* are the three main hazards of cook-chill foods, due to their ability to grow under anaerobic conditions during refrigerated storage. Potential toxin production by *C. botulinum* in vacuum packaged cook-chill food products during refrigerated storage has been reported (Lindstrom et al., 2006; Tansey et al., 2010). In the case of temperature abuse, the reduced oxygen environment and the absence of a competitive microflora can increase the probability of *C. botulinum* spore germination, and eventually toxin production.

Oxygen can be applied to the food/package system, either directly by gas mixture, sealing without applying vacuum, or indirectly through packaging food in films of higher oxygen transmission rate (OTR) (Dufresne et al, 2000). The rationale for this additional safety barrier is that oxygen in the package headspace may delay/inhibit toxin production by *C. botulinum* or enhance the growth of spoilage microorganisms, resulting in spoilage prior to toxigenesis (Dufresne et al, 2000).

There are two types of oxygen in the food/packaging system: residual oxygen in the headspace of the package, and dissolved oxygen in the food. When food is pasteurized, there is a temperature increase in the food/package system. During heating, the dissolved oxygen is expelled out from the food to the package headspace. This creates two distinguished regions in the top layer of the food. The region over the top layer of the food is comprised of the headspace and it has a high oxygen concentration. The rest of the food is the low oxygen concentration region.

5.1.2 Oxygen diffusion

The oxygen mobility in the food/package system is important. The oxygen present in the headspace dissolves at the surface of the food and diffuses through it. This generates an oxygen concentration gradient. The rate of oxygen diffusion through the food determines its potential to control anaerobic growth in the whole food. The kinetics of this gas within the food strongly depends on the value of diffusivity (D_{O2}) and geometry of the food (Chaix et al., 2016). Knowledge of overall mass transfers in food/package system and their mathematical modeling is necessary to predict gas concentrations in the headspace, as well as the gas gradients within the food. This elucidates microbial growth during storage.

Methodologies to estimate D_{O2} in food follow three main approaches (Bhunia et al., 2016). One approach, known as the manometry method is to monitor the pressure change as a function of time in the headspace of a closed-chamber containing the test food. Another approach is using the "Time-lag" method. The method employs a permeation system, in which a constant O_2 concentration generated at each side of the food is maintained at either a low-oxygen or an oxygen-free condition. Then the change in O_2 concentration or partial pressure at sink-side is monitored as a function of time. A third approach, known as '*Sorption kinetics'*, is where O_2 ingress in the food is monitored with time and the data is fitted to the analytical solution of Fick's second law.

5.1.3 Oxygen quantification techniques

Techniques available to measure oxygen in food include gravimetry, the Winkler test, manometry, gas chromatography (GC), electronic paramagnetic resonance (EPR), the Clark electrode, and luminescence sensors (Pénicaud et al., 2012; Banerjee et al., 2016). The technical characteristics of these techniques are summarized in **Table 2.2**. Gravimetry and manometry

techniques are limited to measurements in the global system, excluding local measurements within a food product, which can be a drawback. Chemical techniques such as Winkler titration enable direct measurement of dissolved oxygen concentrations. However, colorimetric tests can only be applied to liquid samples, and their success critically depends upon the manner in which the sample is handled. At all stages, precautions must be taken to ensure that oxygen is neither introduced to nor lost from the sample.

In spite of its numerous capacities for oxygen measurements, EPR has not yet been fully mastered. Firstly, observed radicals cannot yet be completely identified. Therefore, EPR must be coupled with another analytical method, such as high-performance liquid chromatography (HPLC) and/or mass spectrometry (MS), raising the cost of analysis (Pénicaud et al., 2012). Second, some technical problems are encountered in EPR oximetry, including non-resonant absorption at high frequencies. This results in unwanted heating of aqueous samples, long acquisition times, poor signal-to-noise ratio, and motion artifacts. Therefore, the spectra obtained from one measurement to another differ widely, even for the same sample. This renders the numerical treatment of data complex and time-consuming. These limitations explain why the quantification of dissolved oxygen and its radicals from data obtained by EPR is currently arduous.

The Clark electrode and gas chromatography (GC) techniques are regularly used to detect oxygen in foods. The main drawbacks of such techniques are consumption of oxygen during the measurements. The Clark electrode has an advantage over GC since it can be used to measure the oxygen content in both the food and surroundings. Recently, the use of a luminescence sensor technique to measure oxygen in food/package systems is becoming popular. Since its development in 1968, many improvements have been made. Nowadays, efficient miniaturized sensors are commercially available (Pénicaud et al., 2012). This technique allows non-destructive monitoring

of oxygen of the enclosed food or the environment inside the pack and provides data on the current status of the food in such packs (Benerjee et al., 2016). In addition, oxygen molecules are not consumed by the sensor during measurements.

The luminescence sensor technique is based upon the effect of dynamic luminescence quenching by molecular oxygen (Santoro et al., 2016). **Figure 2.3** shows the quenching mechanism. Quenching involves dynamic collision between triplet oxygen and the excited electronic state of the oxygen -sensitive probe, and leads to a reduction of its intensity and decay time. The relationship between intensity and the concentration of oxygen is given by the Stern-Volmer equation (Santoro et al., 2016; Benerjee et al., 2016; Pénicaud et al., 2012):

$$\frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + k_q \tau_0 [O_2] = 1 + k_{sv}[O_2]$$

Where I_0 and I, τ and τ_0 are the luminescence intensities and the lifetimes of the luminophore in the presence and in the absence of the quencher, respectively; $[O_2]$ is the oxygen concentration and k_q is the bimolecular quenching constant, which gives a measure of the quenching efficiency or accessibility of the chromophore to O_2 (Benerjee et al., 2016). k_q is directly proportional to the permeability of the matrix containing the molecular probes. k_{sv} is the Sterne-Volmer constant.

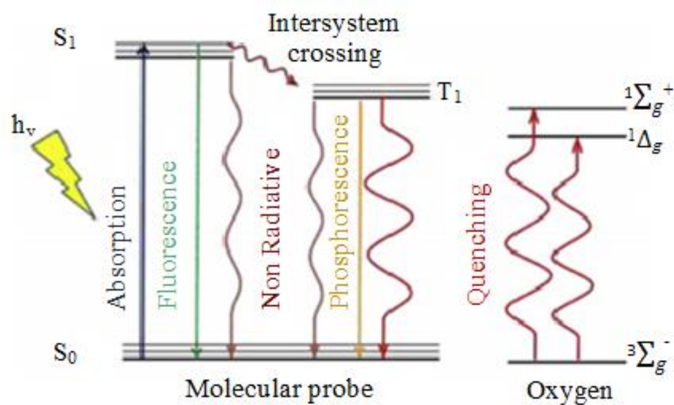

Figure 2.3 Mechanism of dynamic quenching (taken from Santoro et al., 2016). The excitation energy of the oxygen-sensitive probe is quenches by triplet oxygen (quencher). It is then excited to singlet state oxygen, by means of non-radiative energy transfer from the luminophore to the ground state triplet oxygen. In this case, the oxygen-sensitive probe returns to its ground state without the emission of light.

Table 2.2 Technical characteristics of oxygen measurement techniques.

		Gravity	Winkler test	Manometry	*EPR	Clark electrode	*GC	Luminescence
Measurement medium								
	Gaseous			✓	✓	✓	✓	✓
	Liquid	✓	✓		✓	✓		✓
	Solid	✓			✓	✓		✓
Measured parameter		Mass	Mol	Total pressure	Pick width or area	Current intensity	Peak area	Intensity or life time or phase shift
Calibration necessary to obtain dissolve [O_2]				✓	✓	✓	✓	✓
O_2 consumption			✓			✓	✓	
Sample destruction			✓				✓	
Invasive probe design						✓		✓
Measurement time		hours	minutes	hours	minutes	seconds	minutes	seconds

*EPR:electronic paragmatic resonance; GC:gas chromatography. Adapted from Penicaud et al., 2012.

5.2 Bio-preservation by means of protectives cultures

Bio-preservation is not new but garnered attention since it implies a natural means of preservation that fits consumer demand for less chemical preservatives (Grande et a., 2007). This approach involves using microorganisms and/ or their metabolites to inhibit other microorganism populations, which may spoil foods or pose a risk of transmission to humans during food consumption (Castellano et al, 2008). This is intended to extend shelf life and to enhance food safety and stability. The most frequent applications are the use of protective cultures (PCs) as controlled microflora and bacteriocins as antimicrobials.

5.2.1 Protective culture approach

Preparations containing live cells of microorganism that can protect the food during storage are called protective cultures (PCs) (Gaggia et al., 2011). The principle behind the bio-preservation using protective cultures is competitive exclusion. It states that two populations competing for the same resource cannot coexist at constant population values if other ecological factors remain constant. Therefore, one microbial population will display other microbial population due to its higher ability to use available substrates and space or to produce and release antimicrobial substance, which antagonizes the growth of its competitors. PCs inhibit undesirable flora through the production of low molecular weight antimicrobials including lactic acid, hydrogen peroxide (H_2O_2) and bacteriocins (Rodgers, 2003). The generation of such antimicrobial substances is not instantaneous instead it is gradual. Therefore, the safe design of food systems should consider both the rate of production of the antibacterial substances by the PCs and the time required to reach sufficient inhibiting concentrations in the food. For instance, it should be faster than the rate of a target pathogen growth or toxin production, in the case of an exotoxin producer (i.e. *C. botulinum*).

In that way, the target pathogen is expected to be inhibited before its population reaches the established tolerance limit or its toxin effective concentration. Also, temperature is an important factor to consider since it can affect not only the growth rate of both the pathogens and PCs but also the sensitivity of pathogens and activity of bacteriocins.

5.2.2 Requirements of protective cultures

Preparations of live microorganism used as PCs in food biopreservation are expected to meet several criteria and requirements. In general, the most relevant prerequisites of biopreservative agents can be summarized according to **Fig. 2.4** (Ghanbari et al., 2013). Primarily, consumer protection is the most important aspect. Especially, when the PCs are applied to food products which are not cooked before consumptions (such as ready to eat food), but also for other types of food categories since cross-contamination, both at the retail and consumer level, are possible. Then the absence of pathogenic traits should be demonstrated for cultures intended for use in food products (Gaggia et al., 2011). Besides, the extent to which such PCs or their metabolism products (i.e. acid production) may affect the chemical, physical, and sensory attributes of the food products should be considered. Hence, PCs should not cause any unfavorable effects on the target food. The capacity of PCs to produce and release active antagonistic metabolites against a broad spectrum of relevant foodborne pathogenic and spoilage microorganisms (bacteria and fungi) is another significant prerequisite. Additionally, the competence of surviving adverse conditions encountered in the different operation units employed during food production and maintaining inhibitory activities during storage is of great significance.

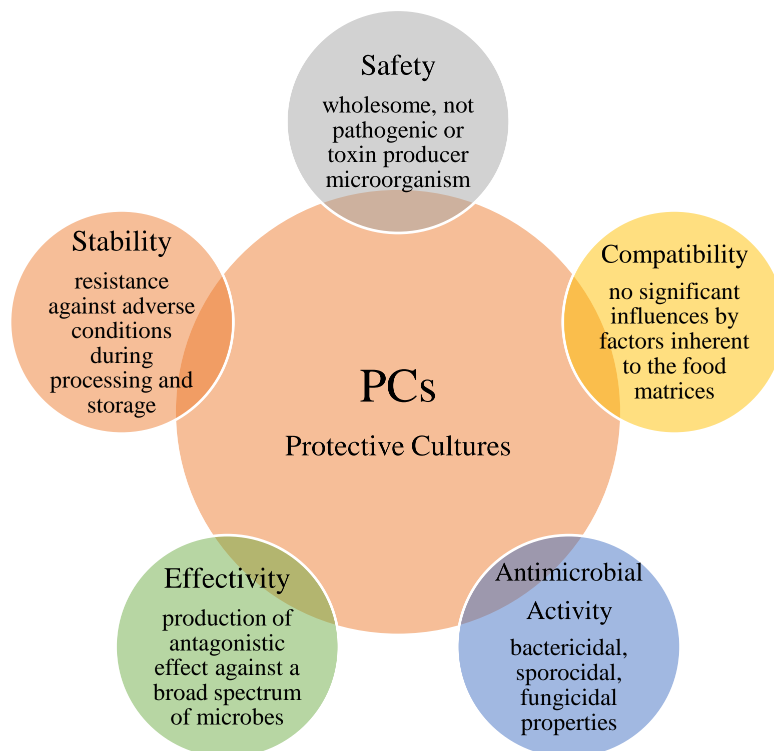

Figure 2.4 Protective cultures requirements for biopreservation application in food systems. (Adapted from Ghanbari et al., 2013)

5.2.3 Lactic acid bacteria as protective cultures

Protective cultures should in the first instance be considered as an additional safety factor, with the potential of improving the microbiological safety of food (Holzapfel et al., 1995). Their application should support good manufacturing practices, thus reducing risks of growth and survival of pathogens and spoilage microorganisms. Also, under abuse conditions of temperature and handling, their metabolic activities may serve as an indicator of microbial risk.

In most cases, lactic acid bacteria (LAB) are applied as PCs. They are naturally present in fermented food products, have a long history of safe use and form part of the gut microflora of humans (Maragkoudakis et al., 2009). All this has procured many LAB the GRAS (generally recognized as safe) status by the US Food and Drug Administration. In addition, LAB strains

produce a range of compounds that have antimicrobial activity against pathogenic and spoilage microorganism in food products, which enhance microbiological safety and shelf life. Other characteristics that make these bacteria suitable candidates as PCs in different food preservation scenario are; their ability to grow at refrigeration temperature, to tolerate atmosphere packaging, low pH, high salt concentrations, and the presence of additives such as ethanol (Calo-Mata et al., 208).

LAB are a heterogeneous group of Gram-positive fermentative bacteria belonging to six Families, in particular (Felis et al., 2016):

1. *Aerococcaceae*, with genera *Abiotrophia*, *Aerococcus*, *Dolosicoccus*, *Eremococcus*, *Facklamia*, *Globicatella*, and *Ignavigranum.*
2. *Carnobacteriaceae*, with genera *Alkalibacterium*, *Allofustis*, *Alloiococcus*, *Atopobacter*, *Atopococcus*, *Atopostipes*, *Carnobacterium*, *Desemzia*, *Dolosigranulum*, *Granulicatella*, *Isobaculum*, *Lacticigenium*, *Marinilactibacillus*, *Pisciglobus*, and *Trichococcus*.
3. *Lactobacillaceae*, with genera *Lactobacillus* and *Pediococcus*.
4. *Leuconostocaceae*, with genera Leuconostoc, *Fructobacillus*, *Oenococcus*, and *Weissella*.
5. *Streptococcaceae*, with genera *Lactococcus*, *Lactovum*, and *Streptococcus*.
6. *Enterococcaceae*, with genera *Bavariicoccus*, *Catellicoccus*, *Enterococcus*, *Melissococcus*, *Pilibacter*, *Tetragenococcus*, and *Vagococcus*.

LAB can exert a bioprotective or inhibitory effect against other microorganisms during their growth in food products by better competing for nutrients and space (Hugas, 1998; Castello et al., 2008). Also, the preservative capacity of LAB is attributed to the production of antagonistic compounds such as organic acids, bacteriocin, hydrogen peroxide, and enzymes (**Table 2.3)** (Holzapfel et al., 1995; Calo-Mata et al., 2008). In accordance with the product and processing

condition, one or more of these compounds may constitute a basis for the selection of a particular LAB for protective culture application.

Table 2.3 Antagonistic compounds produced by lactic acid bacteria

Metabolite	Spectrum of inhibition	References
Organics acids		
lactic acid acetic acid propionic acid	Putrefactive and Gram-negative bacteria, Gram-positive bacteria and fungi	Holzapfel et al., 1995; Lind et al., 2005; Dalié et al., 2010
Bacteriocins		
nisin lacticin plantaricin pediocin enterocin sakacin carnobaceriocin	Gram-positive bacteria and their spores	Rodriguez et al., 2005; Deegan et al., 2006; Trinetta et al., 2012; Zhu et al., 2014
Hydrogen peroxide	Pathogens and spoilage organisms, especially in protein-rich foods	Holzapfel et al., 1995
Enzymes		
lactoperoxidase system with H_2O_2	Pathogens and spoilage bacteria (milk and dairy products)	Holzapfel et al., 1995
lysozyme	Gram-positive bacteria	Mastromatteo et al., 2010
Low-molecular metabolites		
reuterin (3-hydroxypropionaldehyde)	Wide spectrum of bacteria, molds, yeasts, and protozoa	Liu and Yu, 2015
diacetyl	Gram-negative bacteria	Holzapfel et al., 1995

Acid production is a common feature among LAB, although not all LAB can produce antimicrobial peptides during growth. LAB produce lactic acid as the major metabolic end-product

of carbohydrate fermentation (Calo-Mata et al., 2008). It lowers the pH of the food and directly inhibits the growth of many microorganisms. Organic acids in their undissociated form penetrate bacteria membranes, lowering intracellular pH and interfering with metabolic processes such as oxidative phosphorylation. As mentioned before LAB are distinguished by their capacity to tolerate low pH, which allow them to grow in environments where other bacteria are unable to grow thus ensuring food safety.

Regarding bacteriocins, these compounds are proteins and polypeptides with bactericidal activity against a narrow spectrum of closely related bacteria. Bacteriocins affect membranes by dissipating the membrane potential and thus causing a collapse of proton motive force (Holzapfel et al., 1995). Also, bacteriocins interfere with the DNA and protein synthesis (Calo-Mata et al., 2008). The activity spectrum of bacteriocins is restricted to Gram-positive bacteria. Gram-negative bacteria are not sensitive to bacteriocins from LAB apparently because of their outer membrane protecting them by excluding the bacteriocins (Holzapfel et al., 1995). Member of the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Enterococcus*, *Streptococcus*, and *Pediococcus*, are known to produce and secret bacteriocins (Bennik et al., 1999; Settanni and Corsetti, 2005; Ustyugova et al., 2012; Heredia-Castro et al., 2015).

Hydrogen peroxide (H_2O_2) is produced by many LAB strains in the presence of oxygen, which acts as a strong oxidizing agent in the membrane lipids and cell proteins to destroy bacterial activity (Schnürer and Magnusson, 2005). Additionally, when H_2O_2 is coupled with the naturally present lactoperoxidase-thiocyanate enzyme system in milk, hypothiocyanite and other products form and prevent the growth of unwanted spoilage microorganisms. Low molecular weight carbonyls formed during lactic fermentations, like diacetyl, which is notorious for its desirable flavor characteristics, is also antimicrobial but with less preservative potential. It acts by interacting with

arginine-binding proteins at low pH (Calo-Mata et al., 2008). However, the amounts of diacetyl needed to exert antimicrobial activity (close to 200 mM) dramatically alter both the taste and aroma of the product. Reuterin, or 3- hydroxypropionaldehyde (3-HPA), is a product from glycerol fermentation produce by several LAB strains especially *Lactobacillus reuteri* (Dalié et al., 2010; Holzapfel et al., 1995). It shows broad-spectrum antimicrobial activity toward a wide range of food pathogens and spoilage microorganisms including Gram-negative and Gram-positive bacteria, yeast, mold, and protozoa (Liu and Yu, 2015). 3-HPA modifies thiol groups within proteins and small molecules, which induce oxidative stress and eventually lead to bacterial cell death.

5.2.4 Application of protective cultures in biopreservation of refrigerated food

To prevent the growth of pathogenic bacteria during refrigerated storage, protective cultures have been tested in a variety of food systems by different authors. This section will review examples where LAB have successfully been employed, at laboratory scale, as 'protective cultures'.

Iglesias et al. (2017) demonstrated the ability of *Lactobacillus rhamnosus* GG to control the growth of *L. monocytogenes* and *Salmonella* in minimally processed pears during refrigerated storage at 10 °C. Alegre et al. (2011) tested *Lactobacillus rhamnosus* GG to preserve minimally processed apples during storage at 5 °C and 10 °C. These authors found that the presence of *Lactobacillus rhamnosus* GG reduces *L. monocytogenes* population by 1-log unit. The effectiveness of *Pseudomonas graminis* (strain CPA-7) to control *Salmonella* and *L. monocytogenes* growth in cut melon was tested by Abadias et al. (2014). *Pseudmonas graminis* effectively reduce by >3.5-log unit the population of both pathogens at 10 °C. *Lactobacillus*

paracasei controlled the development of *L. monocytogenes*, *Salmonella enterica* and *E. coli* in an artichokes-base ready to eat food product during storage at 4 °C (Valerio et al., 2013).

Melero et al. (2013) to control safety during storage, applied the hurdle technology concept (MAP and protective culture) to extend the shelf-life of both fresh chicken meat burgers and chicken legs. The strategy involves the application of *Leuconostonoc pseudomesenteroides* and *Bifidobacterium longum* as protective cultures to reduce the level of *L. monocytogenes* and *Campylobacter jejuni*, respectively. *Lc. pseudomesenteroides* reduced the population of *L. monocytogenes* by 1.22 log CFU/g in MAP chicken burger meat and *B. longum* reduced by 1.16 log CFU/g the population of *C. jejuni*. The authors reported a shelf-life product extension from 10 d to 17 to 24 d upon packaging under modified atmosphere for chicken meat burgers. Likewise, the shelf-life of fresh chicken legs was extended from 3 d (air-packed) to 6 to 9 d (MAP-packaged). The successful application of *Leuconostoc carnosus* 4010 as a protective culture to a vacuum-packed, cooked sausage was demonstrated by Budde et al. (2003). The bacteriocin-producer *Lc. carnosus* 4010 immediately decreased the population of the spiked *L. monocytogenes* to level below the detection level (<10 CFU/g) and no increase in the population of the pathogen was observed during storage at 5 °C for 21 days.

Rodgers in 2002 demonstrated that *Lactococcus lactis* and *Pediococcus pentosaceus* effectively controlled non-proteolytic *C. botulinum* growth on selected commercial hot-fill meals during refrigerated storage at 10 °C. The addition of the PCs singularly and as a mixture reduce the non-proteolytic *C. botulinum* populations to undetectable levels, prevented toxigenesis, and gas formation in all the product tested (chicken casserole, vegetable curry, beef stroganoff, lamb hot pot, veal casserole, and chicken satay). Later Rodgers et al. (2003) proved that *Lactococcus lactis* and *Pediococcus pentosaceus* effectively controlled non-proteolytic *C. botulinum* growth on

commercial soups during refrigerated storage at 10 °C without affecting the sensory quality of the products. Berdholt, Nebakken, and Holck (1999) showed the inhibitory effect of several LAB on *E. coli* O157: H7 and *L. monocytogenes* on cooked, sliced and vacuum packaged ham at 8 °C with acceptable sensory quality. In 2001, Schillinger and collaborators studied the efficacy of nisin in combination with PCs against *L. monocytogenes* in tofu. They reported the complete suppression of *L. monocytogenes* using *Enterococcus feacium*, and *Lactococcus lactis* in combination with nisin at 10 °C for 1 week.

The application of pure bacteriocins in food systems has various drawbacks, the major of which is the decreased efficacy during storage determined by the binding to food components (fat or protein particles) and food additives (Gaggia et al., 2011). In this regard, protective cultures can provide a constant amount of viable bacteriocin and other antimicrobial compounds in response to temperature changes. However, PCs are sensitive to heat. Protection of the PCs is necessary during short pasteurization or during the hot-fill steps of food products to guarantee high populations of the cultures in the final product. This may limit their use during processing of cook-chill food products. In general, PCs are incorporated after thermal processing and their microbiological grade and aseptic/hygienic handling are critical to prevent contamination of the final product. Encapsulation technology has allowed the availability of high concentrate commercial preparation of LAB. However, most the scientific references related to the improvement in LAB resistance are focus on control release and survival through the gastrointestinal route and not to the processing conditions. There is lack of data regarding the D and z values of the encapsulated LAB. This information is vital to the application of PCs at an early stage during preparation of cook-chill food products and determined its survival to heat conditions (e.g. pasteurization processes) to enhance food safety and quality.

6. Final remarks

Cook-chill food is varied group food products designed to meet the consumer demand for convenience food products. However, there is a risk of pathogen growth to unsafe levels on cook-chill products in the face of the unsuitable temperatures observed in open refrigerated displays at retail and the usual consumer practices regarding transport and storage of chilled products. Then consumer exposure to pathogenic bacteria due to consumption of cook-chill food products is influenced not only by the industrial production process but also by the consumer behavior.

When temperature abuse occurs, low-acid cook-chill food is unprotected. Therefore, it is desirable to base the food preservation system on more than one controlling factor during the cold chain. Use of chemical preservatives is not compatible with the increasing consumer awareness and desire for natural products and processes. The latest trend is the emphasis on the discovery of natural alternatives to traditional techniques, thus avoiding the use of chemical additives for the preservation of food products. The use of natural agents such O_2 and LAB as protective cultures may well provide at least part of the solution. Nevertheless, the implementation of both strategies to food products should be thoughtful only as an additional hurdle to good manufacturing processing and distribution practices.

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CHAPTER 3 HEADSPACE OXYGEN AS A HURDLE TO IMPROVE THE SAFETY OF IN-PACK PASTEURIZED CHILLED FOOD DURING STORAGE AT DIFFERENT TEMPERATURES

Abstract

This study investigated the use of headspace oxygen in a model food system to prevent the growth of anaerobic pathogenic bacteria in in-pack pasteurized food at various storage temperatures. Three model food formulations prepared with tryptic soy broth and three agar concentrations (0.1, 0.4 and 1.0%) were sealed without removing the air from the package in high oxygen barrier pouches (OTR = 0.3 $cm^3/m^2 \cdot day \cdot atm$). Important properties influencing bacterial growth, including pH and water activity (a_w) were determined. The oxygen sorption kinetics of each model food were obtained at three different storage temperatures (8, 12, and 20 °C) using an OxySense Gen III 300 system. An analytical solution of Fick's second law was used to determine the O_2 diffusion coefficient. Growth challenge studies at 12 and 20 °C were conducted at three selected locations (top, center, and bottom layers) in model foods containing 1% agar. Model foods were inoculated with *Clostridium sporogenes* PA 3679 (300 spores/mL), and were classified as low-acid (pH > 4.5, aw > 0.85). When the storage temperature decreased from 20 to 8 °C, the oxygen diffusion decreased from 0.82×10^{-9} m^2/s to 0.68×10^{-9} m^2/s. As the agar concentration was increased from 0.1 to 1.0%, the effective oxygen permeability decreased significantly ($p = 0.007$) from 0.88×10^{-9} m^2/s to 0.65×10^{-9} m^2/s. When the inoculated model foods were stored at 12 °C for 14 days, *C. sporogenes* PA 3679 was unable to grow. As the storage temperature was increased to 20 °C, significant bacterial growth was observed with storage time ($p < 0.0001$), and the *C. sporogenes* PA 3679 population increased by around 6 log CFU/g. However, the location

of the food did not influence the growth distribution of *C. sporogenes* PA 3679. These results demonstrate that oxygen diffusion from the pouch headspace was primarily limited to the food surface. Findings suggest that the air/oxygen present in the package headspace may not be considered as a food safety hurdle in the production of pasteurized packaged food.

Keywords: *Clostridium*, food safety, pasteurization, oxygen diffusion

1. Introduction

Consumers today prefer food that requires minimal preparation time compared to conventional meals. They prefer high-quality foods that are nutritious, low levels of preservatives, and minimally processed (Peck and Stringer, 2005; Rajkovic et al., 2010). Consumer preference has led to the development of in-package pasteurized foods. These foods are also known as refrigerated processed foods of extended durability (REPFEDs), cook-chill, ready-to-eat, and sous-vide foods (Choma et al., 2000; Daelman et al., 2013; Peck and Stringer, 2005). These types of products are gaining popularity due to the aforementioned consumer preferences (Brunner et al., 2010; Rodgers et al., 2003). For example, the total UK prepared chilled food market increased by 33% from November 2008 to January 2016 (Kantar WorldPanel, 2016).

REPFEDs are a heterogeneous group of food products typified by a variety of ingredients, processing conditions and packaging systems used in their production process. Based on production conditions, REPFED products can be categorized into three groups (Daelman et al., 2013):

1. Products pasteurized in-pack at 90 °C for at least 10 min or equivalent to achieve a 6D reduction of non-proteolytic psychotropic *Clostridium botulinum* spores.
2. Products pasteurized in-package at 70 °C for at least 2 min or equivalent to achieve a 6D

reduction of *Listeria monocytogenes*.

3. Products pasteurized out of the pack and then packed. These products are not defined by a specific P_{value}, and either the P_{90} or P_{70} pasteurization treatments are possible.

For REPFED products, there are a few microbiological safety concerns (Juneja and Snyder, 2008). First, these products are generally formulated with little or no preservatives and have low acid and high moisture content. Second, they undergo minimal thermal processing, are not commercially sterile and must be refrigerated. Third, vacuum packaging provides a favorable environment for anaerobic and facultative pathogens such as *Clostridium botulinum* and *Bacillus cereus* to grow and produce toxins. Finally, there is a high probability of temperature abuse during distribution and storage.

The storage temperature of chilled foods may vary greatly during manufacturing, distribution, retail, and in-home storage. According to Bruckner et al. (2012), temperatures in trucks during poultry and milk distribution range from 3 °C to 15 °C and 3.6 °C to 10.9 °C, respectively. Additional temperature abuse may occur during retail display. Temperatures above 7 °C are often common in refrigerated display cabinets of convenience stores (Dodds, 1995; Koutsoumanis and Gougouli, 2015; Rybka-Rodgers, 2001; Walker, 1992). Before the stores and homes, there is little or no temperature control after products are purchased. Tamagnini et al. (2008) mentioned that Marklinder et al. (2004) found that 5–20% of foods, in general, were stored at temperatures above 10 °C in home refrigerators, with maximum temperatures from 11 °C to 18 °C. Koutsoumanis and Gougouli (2015) combined the results of nine surveys conducted in the UK, France, Ireland and Greece, finding that out of over 1000 consumer refrigerators, 64.1% were operating above 5 °C. Therefore, chilled foods may undergo temperature abuse conditions in the cold chain, and low-acid cook-chill foods are unprotected under these circumstances. Since we cannot rely exclusively

on the maintenance of refrigerated conditions to assure safety, it is essential to address these challenges.

Non-proteolytic *C. botulinum* and *B. cereus* are spore-forming bacteria with the lowest minimum growth temperatures at 3.3 °C and 4 °C, respectively (ECFF, 2006). Based on this, as along with the ability of non-proteolytic *C. botulinum* and *B. cereus* spores to germinate and produce toxins, the recommended storage and distribution temperatures for cook-chill foods is under 5 °C (ECFF, 2006). If food products are subjected to temperatures above 10 °C for a prolonged time during the cold chain, proteolytic *C. botulinum* and *Clostridium perfringens* are also of concern. In addition, the absence of a competitive microbiota can increase proliferation of these pathogens.

However, the use of oxygen provides an alternative for in-package pasteurized food. Bacteria vary widely in their ability to use and tolerate oxygen (Prescott et al., 2002). *Clostridium botulinum* is an anaerobic bacterium that does not use oxygen for growth and eventually dies in the presence of oxygen (Prescott et al., 2002). Anaerobic bacteria do not have the elaborate system of defenses that aerobic bacteria have since the system relies on a series of special enzymes in large quantities. These include super dismutase, catalase, and peroxidase, which can scavenge toxic compounds that form in an oxygen-rich atmosphere (Jasso-Chávez et al., 2015; Johnson, 2009). Anaerobic bacteria produce these enzymes in very small amounts, or not at all. Thus, the variability in oxygen tolerance of obligate anaerobes may be influenced by the amount of those enzymes that they can produce.

In order for oxygen to suppress the growth of *C. botulinum* in food, it should be able to dissolve in the food surface and diffuse throughout the product. In this study, we addressed these challenges by assessing the use of oxygen to improve the food safety design of cook-chill foods during

temperature abuse. Oxygen diffusion in food model/packaging systems was observed at 8, 12 and 20 °C. Three food models with different matrices were compared. The growth of *Clostridium sporogenes* PA 3679 as a surrogate of *Clostridium botulinum* was monitored within the food (top, center, and bottom layers).

2. Materials and Methods

2.1 Food model preparation and properties measurements

Tryptic soy broth (TSB) culture medium was used as a model food. The medium was prepared according to the manufacturer's instructions (Bacto, BD™) and was supplemented with different agar concentrations (0.1, 0.4, and 1.0% w/v). The resulting model foods were in liquid, semisolid and solid at temperatures below 45 °C. pH and water activity (a_w) were determined with a potentiometer (Mettler Toledo, EL 20) and a vapor sorption analyzer (Decagon Devices, Inc. VSA1042), respectively.

2.2 Food/packaging system set up

For oxygen sorption kinetics, light-sensitive oxygen sensors (OxyDot) were adhered at selected locations in the pouches to monitor O_2 concentration at the headspace, as well as within the food during storage time (**Fig. 3.1**). The adhesive used to glue the OxyDots was RTV108-12C (Momentive, MIL-A-46106B Compliant) high performance silicone sealant. A small amount was applied to the inside of the pouch at selected locations. Using a vacuum pen, the OxyDots were picked up with the coating side in contact with the pen, and then gently pressed to the adhesive inside the pouches. The adhesive was allowed to dry completely for 8 h, and then the model foods were dispensed for further analysis.

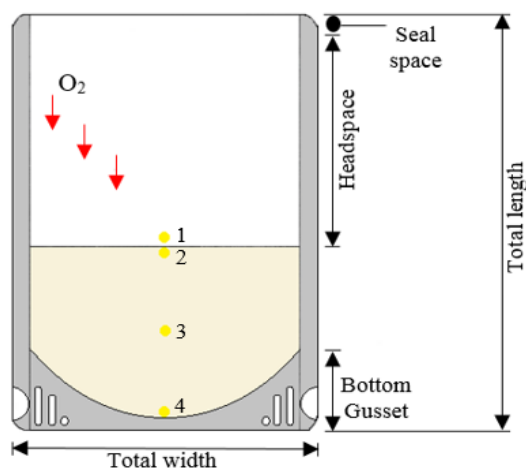

Figure 3.1 Pouch dimension and set up. Pouch dimension: W x L x BG (13cm × 18.5cm × 3.5cm). OxyDot 1 is located just above the food model surface, OxyDot 2 is located just below the food model surface, OxyDot 3 is located at the center of the food model column, OxyDot 4 is located at the food bottom layer. Distance between OxyDots 2-3 and 3-4 is 3cm. Sealed space was approximately 0.8cm. Total height of food column is 7.5 cm.

One batch of each formulation was dispensed in triplicate in 250 mL volumes in 13 cm × 18.5 cm × 3.5 cm stand up multilayered plastic pouches (Kurarister™ CF, Kuraray Company of America, Inc.). Next, the open edge of the pouches was sealed without a vacuum, using a manual impulse heat sealer (Hang bag sealer MP-12, Midwest Pacific®, Rocky Mount, MO, USA). This provided a water-tight and air-tight seal for an effective subsequent sterilization procedure by means of retorting. This thermal process allowed for preparation of the model foods without background microbiota, which could disturb the measurement of oxygen concentration in the different food/packaging systems. The oxygen transmission rate (OTR) of the film used in the pouch was 0.3 $cm^3/m^2 \cdot day \cdot atm$. The pouches had a laminated structure with three layers of

polyethylene terephthalate (12 μm thickness), biaxial oriented polyamide (15 μm) and cast polypropylene (50 μm) films.

2.3 Storage conditions and oxygen measurements

After sterilization, the pouches were stored at 8, 12, and 20 °C ± 0.5 °C for 2 days in a vertical position. The dissolved oxygen concentration at selected locations was monitored using the OxyDot-OxySense system (OxySense® 310, OxySense, Inc., Dallas, TX, USA). During the oxygen readings in pouches the incubator was opened several times and during these measurements, only the incubator temperature fluctuated by ± 2 °C. This technique relied on a noninvasive, light sensitive sensor (OxyDot-O2xyDot®) that was placed on the inside of the pouches before filling and sealing (Al-Qadiri et al., 2015). Next, the oxygen concentration was monitored with a fiber optic reader pen through the outside of the pouch. The pen reader device was connected to a computer that was equipped with OxySense's Gen-III software for data acquisition. The oxygen level was reported in parts per billion (ppb).

2.4 Oxygen sorption kinetics modeling and analytical solution

The transient oxygen transport process in a bulk medium was described using Higbie's (1935) penetration theory, which assumes that equilibrium exists at the gas-medium interface. The oxygen concentration at the gas-medium interface was determined by Henry's law (Eq. (1)) (Chaix et al., 2015).

$$C_{O_2,F} = S_{O_2} P_{O_2,HS} \quad (1)$$

where $C_{O_2,F}$ (mol/Kg) is the oxygen solubility in the model food, $P_{O_2,HS}$ (Pa) is the partial pressure of O_2 in the package headspace, and S_{O_2}(mol/Kg-Pa) is the solubility coefficient of O_2. Secondly, the transient oxygen diffusion within the medium was assumed to obey Fick's second law (Eq. (2))

$$\frac{\partial C_{O_2,F}}{\partial t} = D_{O_2} \frac{\partial^2 C_{O_2,F}}{\partial x^2} \qquad (2)$$

where, $\partial C_{O_2,F}$ (mol/Kg) is the change in local oxygen concentration in food at any time t (s), and x is the distance from the gas-medium interface along the height of the medium (m). $D_{O_2}$is the diffusion coefficient of oxygen (m^2/s) and is assumed to be constant in the model food, which was considered to be homogenous and isotropic. The diffusion process was assumed to be one-dimensional along the height of the food sample. The medium was assumed to be semi-infinite, with the following initial and boundary conditions:

$$C_{O_2,F} = C_0, \ x \geq 0, \ t = 0 \qquad (3)$$

$$C_{O_2,F} = C_s, \ \ x = 0, \ t \geq 0 \qquad (4)$$

$$C_{O_2,F} = C_0, \ x \to \infty, \ t > 0 \qquad (5)$$

where C_s is the oxygen concentration at gas-medium interface, in equilibrium with partial pressure of oxygen at the package headspace. During measurement, the oxygen ingress from the bottom and both sides of the medium can be neglected, as it was packaged with high oxygen-barrier pouches. Therefore, the system was considered to be insulated from both sides and bottom during the study.

The analytical solution of Fick's second law with aforementioned boundary conditions was given by Crank (1975):

$$\frac{C_{O_2,F}-C_0}{C_s-C_0} = erfc\left(\frac{x}{2\sqrt{D_{O_2}t}}\right) \tag{6}$$

The Eq. (6) was solved in Matlab 2013a (MathWorks Inc., Natick, MA, USA). The experimental data obtained from the OxyDot located at 1.35 ± 0.15 cm below the surface was used for the computational process. The estimated D_{O_2} was used to predict the oxygen concentration at time *t*. The errors between experimental and predicted concentrations were minimized by using root mean square errors (RMSE) as follows:

$$RMSE = \sqrt{\frac{1}{p}\sum_{t=1}^{p}\left(C_{O_2,F_{et}} - C_{O_2,F_{pt}}\right)^2} \tag{7}$$

where *p* is the number of observations; $C_{O_2,F_{et}}$ and $C_{O_2,F_{pt}}$ are the experimental and predicted oxygen concentrations at time *t*, respectively.

2.5 Microbial growth study

The model food was microbiologically analyzed at two temperatures (12 and 20 °C) during a maximum period of 14 days to observe the behavior of *C. sporogenes* PA 3679 under temperature abuse. Since the minimum growth temperature for proteolytic *C. botulinum* is between 10 and 12 °C, the growth study was not carried out at 8 °C. The food/ packaging system was prepared as described in **Section 2.2**. The model food containing 1.0% agar was selected for the study. This

formulation allowed us to monitor microbial growth during storage time within the food due to its solid state, which facilitated slicing of the food to collect samples from the three selected locations (top, center, and bottom layers) for microbial counts.

2.5.1 Microorganism and spore's suspension preparation

The stock culture of *C. sporogenes* PA 3679 spores was obtained from the Center for Technical Assistance of the former National Food Processors Association (NFPA, Dublin, CA, USA). At the present, this strain is available from the American Type Culture Collection (ATCC 7955; ATCC, Manassas, VA, USA). The spore suspension was kept in a refrigerator at 4 °C until use (Mah et al., 2009). To prepare a working culture, a multi-step stage inoculation procedure was employed (Mah et al., 2009). Ten milliliters of tryptone-peptone-glucose-yeast extract broth (TPGY) was inoculated with 10-µL stock spore suspension and incubated for 2 days at 32 °C in a GasPak™ 150 Anaerobic System anaerobic jar (BD Diagnostic Systems, Sparks, MD, USA). The anaerobic atmosphere inside the jar was generated using a BD GasPak EZ anaerobic container system sachet with an indicator, Ref 260001 (BD). The TPGY broth medium consisted of 50 g of tryptone, 20 g of yeast extract, 5 g of peptone, 4 g dextrose, and 1 g sodium thioglycolate (all from Difco, Becton Dickinson, Sparks, Md., U.S.A) in 1 L of distilled water. Subsequently, 100 ml of TPGY broth was inoculated with 1mL of the previous incubated culture, followed by 2 days incubation under the same conditions. Then the vegetative cell culture was transferred into 1 L of TPGY broth and the flask was incubated anaerobically for 2 days, as described previously. The culture was then washed three times by centrifugation with a Beckman J2-21 centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA) with a Fiberlite F14B rotor (Fiberlite Centrifuge Inc., Santa Clara, CA, USA) at 15 000 g for 10 min at 4 °C. Washing was performed with sterile M/15 Sørensen's phosphate

buffer, pH 7.0, consisting of 5.675 g sodium phosphate, dibasic (Na_2HPO_4); 3.63 g potassium phosphate, monobasic (KH_2PO_4); and 1 L deionized water; pH adjusted to 7.0 then sterilized by autoclaving.

To induce sporulation, the vegetative cell pellet was resuspended, first in 100 mL of a sporulation medium and then in 900 mL of the same medium, giving a total volume of 1 L. The sporulation medium consisted of 60 g tryptone, 1 g dextrose, 1 g sodium thioglycolate (all from Difco, Becton Dickinson, Sparks, Md., USA) in 1 L of distilled water. The medium pH was adjusted to 5.0 with 1 M HCl, and 5 g calcium carbonate (J.T. Baker Chemical Co., Phillipsburg, NJ, USA) was added, followed by sterilization by autoclaving. After 10 days of anaerobic incubation at 32 °C, the spore crop obtained was washed three times by centrifugation as aforementioned for vegetative cells and resuspended in M/15 Sørensen's phosphate buffer. The spore suspension prepared was found to contain around $9.3 \pm 0.14 \times 10^6$ spores/mL, and was stored at 4 °C under aerobic conditions prior to use (Mah et al., 2009).

2.5.2 Inoculation of the model food in pouches

After sterilization, the model food in pouches was inoculated with a spore suspension of *C. sporogenes* PA 3679 previously heat shocked at 80 °C for 20 minutes. During inoculation, the temperature of the pouch content was kept at 48 °C. To determine how much spore suspension (inoculum) was needed to get an inoculation dose of around 300 spores per ml of food in the pouches, the following calculations were conducted. First, the desired spore dose value in the food (300 spores/ml) was multiplied by the total volume of the food in the pouches (250 mL). Then the product was divided by the spore crop concentration ($9.3 \pm 0.14 \times 10^6$ spores/mL). It was found that when using a spore crop with $9.3 \pm 0.14 \times 10^6$ spores/mL, the amount of inoculum needed for

the desired spore dose was too small to dispense accurately. Therefore, the original spore suspension was diluted with peptone water to obtain a final concentration of around 10^4 spores/mL. The selected volume of spore inoculum was injected into the pouches under aseptic conditions using a manual syringe-septum technique (septum no. 940301, grey, 15 mm; Mocon, Inc., Brooklyn Park, MN) (Al-qadiri et al., 2015). For this, septa were placed outside the pouches at the three selected locations. Then the total volume of the inoculum was injected in three portions, each corresponding to a location in the pouch (top, center or bottom). The pouches were gently shaken to evenly distribute the inoculum in the food while avoiding aeration.

After inoculation, the pouches were stored at 12 °C and 20 °C for 14 days. The pouches were sampled in duplicate daily for those stored at 20 °C, and every 2 days for those stored at 12 °C. These sampling intervals were selected because bacteria were expected to grow faster at 20 °C. Food was allowed to equilibrate to the storage temperature for one hour. Next, microbial counts were conducted and were taken as the 0-day condition. Four pouches, two inoculated and two non-inoculated were stored at 12 °C and 20 °C. In these pouches, the oxygen concentration was monitored in the selected food locations and reported as a percentage. This growth experiment included two independent repetitions.

2.5.3 Microbial counts

The media used included Shahidi-Ferguson Perfringens (SFP) agar, phosphate-peptone water, and peptone water. The model foods were cut in half (**Fig. 3.2**), and then 10g of each layer (top, center or bottom) was suspended in a stomacher bag, containing 90 ml of phosphate-peptone water. The food layer suspensions were homogenized for 45 seconds at 200 rpm in a Seward Circulator 400 stomacher (Seward, London, UK) and 10-fold serially diluted in sterile 0.1% peptone water.

From each dilution, 1ml by duplicate was pour-plated into Petri dishes using SFP agar. The SFP medium was autoclaved at 121 °C for 15 min and held in a 48 °C water bath prior to use. A negative control, consisting of 1 ml of 0.1% sterile peptone water pour-plated into Petri dishes using SFP agar, was also prepared in duplicate. Next, the plates were incubated anaerobically for 3 days at 32 °C. After incubation, dilutions yielding 25-250 colony forming units (CFU) were counted and CFU/g was calculated.

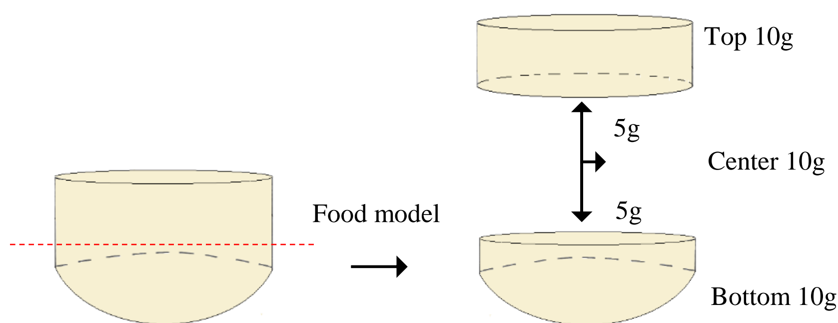

Figure 3.2 Food model sample cutting and sub sample collection for *C. sporogenes* (PA 3679) was measured at three different locations (top, center, and bottom)

3. Statistical Analysis

The oxygen mass transfer data were analyzed with the Statistical Analysis System (SAS 9.2, SAS Inst. Inc., Cary, NC, USA). The GLM procedure was used to conduct a two-way ANOVA test to observe the effects of agar content in the model food at different storage temperatures on oxygen diffusion and solubility coefficients. The same command was used to observe the effect of food location in *Clostridium sporogenes* (PA 3679) growth during storage time. Multiple comparisons were performed as needed by calculating the least square difference between the

means (LSMeans). A significance level of 0.05 was applied in all tests. Bar charts for bacterial growth were generated with Microsoft Excel 2010 (Microsoft Corp, Redmond, WA).

4. Results and Discussion

The pH values of the model foods ranged from 7.32 ± 0.01 to 7.34 ± 0.01, while a_w ranged from 0.994 ± 0.001 to 0.995 ± 0.001. These values allowed us to categorize the model foods as low-acid foods.

4.1 Mathematical modeling of oxygen solubility and diffusivity

The mean values of oxygen diffusion (D_{O_2}) and solubility (S_{O_2}) coefficients of 0.1, 0.4, and 1.0 agar percentage for 20, 12, and 8 °C are listed in **Table 3.1**. Statistical analysis showed that the interaction p-value was not significant, either for D_{O_2} (p = 0.412) or S_{O_2} (p = 0.061) coefficients. Therefore, how D_{O_2} and S_{O_2} change with temperature does not depend on the agar %, and vice versa. The ANOVA test was then re-run without the interaction (Ott & Longnecker, 2010), and the two main effects of storage temperature and agar % on D_{O_2} and S_{O_2} in the model foods were examined.

Table 3.1 Oxygen mass transfer parameters for all model food and different storage temperatures

Agar (%)	Temperature (˚C)	$D_{O2\ value}$ (10^{-9} m^2/s)	$S_{O2\ value}$ (10^{-8} mol/kg/Pa)
	20	1.03(0.11)	1.03(0.03)
0.1	12	0.87(0.02)	1.56(0.04)
	8	0.73(0.22)	1.61(0.03)
	20	0.75(0.05)	0.97(0.05)
0.4	12	0.78(0.13)	1.60(0.02)
	8	0.70(0.05)	1.48(0.05)
	20	0.70(0.05)	0.83(0.13)
1.0	12	0.74(0.08)	1.53(0.01)
	8	0.59(0.09)	1.50(0.01)

Data are presented as mean (standard deviation)

4.1.1 Main effects on oxygen solubility

Results show that each main effect p-value of temperature ($p < 0.0001$) and agar % ($p = 0.004$) was statistically significant in the oxygen solubility. The marginal means for both effects are plotted in **Fig. 3**. By increasing storage temperature from 8 to 20 °C, oxygen solubility decreased from 1.53×10^{-8} mol/kg/Pa to 0.95×10^{-8} mol/kg/Pa. When the agar content in the model food increased from 0.1 to 1.0%, the solubility decreased from 1.40×10^{-8} mol/kg/Pa to 1.28×10^{-8} mol/kg/Pa. The decrease in O2 solubility due to temperature increase was 0.58×10^{-8} mol/kg/Pa, while that due to agar % increase was 0.12×10^{-8} mol/kg/Pa. Thus, it can be concluded that temperature had a higher impact on the O_2 solubility than agar %. In addition, the highest O2 solubility in the model food was observed at 8 °C with 0.1% agar in the formulation.

The solubility of gases is not constant in all conditions, and the solubility of oxygen has been shown to be temperature-dependent. An increase in temperature results in a decrease of gas solubility in water and aqueous media. Chaix et al. (2014) compiled findings from several studies on oxygen solubility in water and some foods at different temperatures. They found that the oxygen

solubility in water decreased from 2.3×10^{-8} mol/kg/Pa to 9.8×10^{-9} mol/kg/Pa as temperature increased from 0 to 50 °C. An increase in agar has been found to increase the dry matter in model foods. The addition of dry matter has been reported to decrease oxygen solubility (Pénicaud et al., 2012).

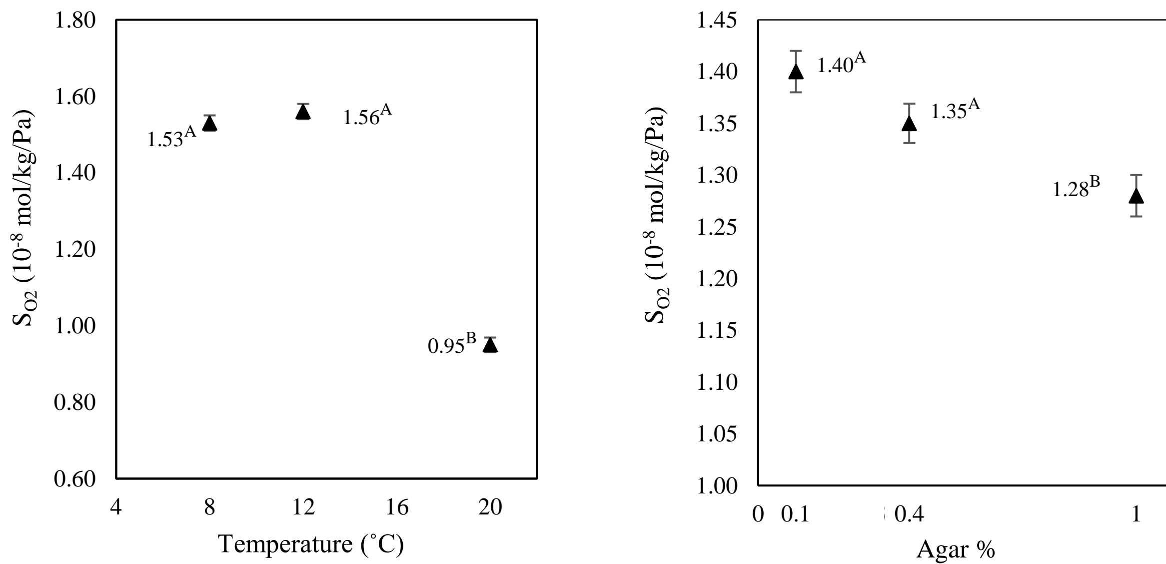

Figure 3.3 Plot of oxygen solubility coefficient marginal means for temperature and agar % factors. Different letters for mean values represent significant differences. Standard error bars of the means.

4.1.2 Main effects on oxygen diffusivity

The main effect of agar percentage yielded an F ratio of F (2, 15) = 6.94, p = 0.007. As the agar percentage increased from 0.1 to 1.0% in the model food, oxygen diffusion decreased significantly from 0.88×10^{-9} m^2/s to 0.65×10^{-9} m^2/s (**Fig. 3.4**). Storage temperature did not affect significantly oxygen diffusion, at F (2, 15) = 3.25, p = 0.067. In general, when storage temperature

decreased from 20 to 8 °C, oxygen diffusion decreased from 0.82×10^{-9} m^2/s to 0.68×10^{-9} m^2/s (**Fig. 3.4**). Overall, highest D_{O_2} were obtained at 20 °C and 0.1 agar %.

Oxygen diffusion in the air and in water at 20 °C has been reported as 2.03×10^{-5} m^2/s and 2.10×10^{-9} m^2/s, respectively (Denny, 1993). In water, the diffusion coefficient of oxygen is 10,000 times smaller than in air. One reason is that the viscosity of water is 1.002 centipoise, while the viscosity of air is 0.018 centipoise at 20 °C (Abulencia and Theodore, 2009). Oxygen diffusion values obtained for the model foods used here are smaller than those reported for water in the literature.

In our study, water was used to prepare the model foods, and when the media culture powder and agar was added, the viscosity increased. This could reduce the mobility of oxygen throughout the food. It is also important to mention that the agar used here is a mixture of agarose and agaropectin molecules. The agarose molecules are responsible for gel structure formation (Labropoulos et al., 2001) while there are less agaropectin molecules which could form micro-aggregates that do not induce gelation. In our study, as the agar content increased, the model food showed a more solid-like behavior due to the development of a stable network structure. This structure traps or holds water in the food. It appears to be strong enough to increase the tortuosity and change the O_2 diffusion pathway, thus reducing oxygen mobility, since diffusion must go around the obstacles.

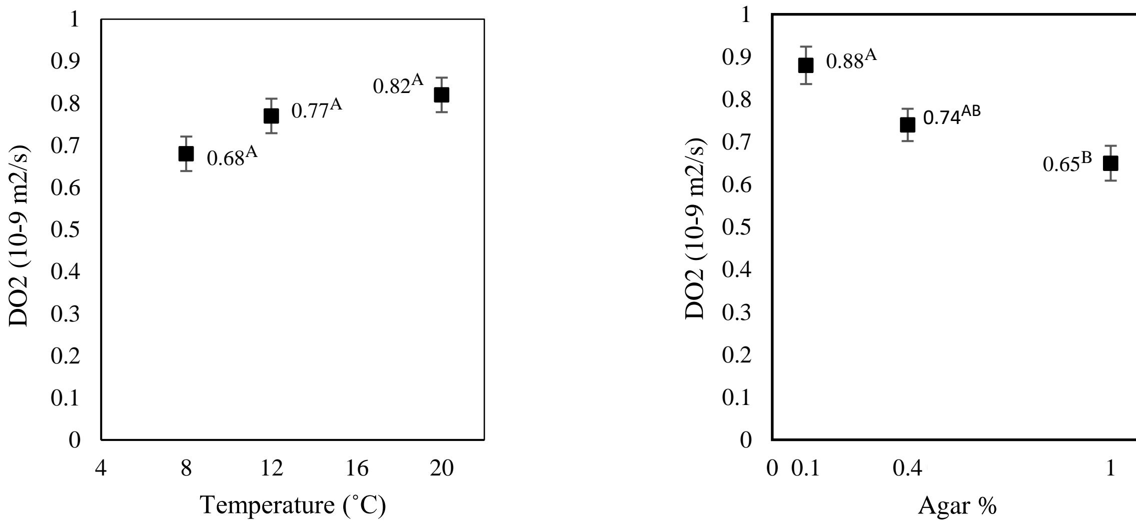

Figure 3.4 Plot of oxygen diffusion coefficient marginal means for temperature and agar % factors. Different letters for mean values represent significant differences. Standard error bars of the means.

4.2 Microbial counts

In this study, we determined the growth behavior of *C. sporogenes* within the food at 12 and 20 °C during storage time (**Fig. 3.5**). When food was stored at 12 °C, bacteria did not grow. Slight changes were observed in bacteria counts and a logarithmic increment was not observed during storage. On the other hand, rapid growth was observed at 20 °C, and bacteria grew significantly ($p < 0.0001$) at around 6 log units from its initial point. Bacteria counts at 20 °C could not be recorded after four days of storage due to food spoilage. By day 5, bacterial growth caused significant changes in the food structure, and the food could not be cut into discrete layers. Therefore, local counts for the top, center, and bottom layers were not possible for the remainder of the storage time. The analysis of variance showed that the growth of *C. sporogenes* PA3679 was not significantly influenced by the food location ($p = 0.296$).

The growth of bacteria depends on external factors and the internal properties of food, such as pH and water activity. Food matrix properties were suitable for microbial growth in terms of pH, a_w, and dextrose in the food could be used as a carbon source. No growth occurred at 12 °C because minimum growth temperature for the bacteria to grow ranges between 12 to 15 °C (Hong et al., 2016). At this temperature, growth is delayed significantly, and it could not be detected during the storage time used in this study. The minimum growth temperature of proteolytic *C. botulinum* is often reported to range from 10 to 12 °C (Gungvig et al., 2013; Lindstrom et al., 2006; Peck, 2009; Peck et al., 2011). In some studies, a higher range is given, from 12.8 to 16.5 °C (Hinderink et al., 2009). In either case, our *C. sporogenes* PA 3679 minimum growth temperature results were in accordance with the minimum growth temperature ranges for proteolytic *C. botulinum*. However, comparing the growth kinetics of both bacteria is recommended before using *C. sporogenes* PA3679 as a surrogate to study the growth of proteolytic *C. botulinum* in food (Hong et al., 2016).

Although the inoculated model food stored at 20 °C, *C. sporogenes* PA 3679 grew well, the oxygen concentration gradient created within the food did not influence bacterial growth (**Table 3.2**). Headspace oxygen concentration during storage time did not dip below 21% (data not shown). At the beginning of storage time, oxygen concentration ranged from 6.58 to 9.37% in the top layer of the food. In the middle layer, it ranged from 1.16 to 1.65%, approximating zero at the bottom layer. There was an increase in O_2 concentration at the top layer with storage time. However, there was no significant increase in subsequent layers. O_2 concentration at the top layer was higher at 12 °C than at 20 °C storage temperature. This was likely because oxygen is more soluble in food at lower temperatures. Results show that the O_2 amount present in the top layer did not prevent the growth of *C. sporogenes*. According to Meyer (1929), the oxygen threshold reported for *C. sporogenes* is 21%. Oxygen concentrations at the three locations were below 21%,

supporting *C. sporogenes* PA 3679 growth. In addition, Meyer (1929) reported that there is a wide variability in oxygen tolerance among the *Clostridium* genus. The threshold of *C. botulinum* types A, B, and C is 6.75%, 8.4%, and 2.7%, respectively. Thus, growth results within the food obtained in this study using *C. sporogenes* should not be used as a reference for *C. botulinum* growth behavior under air, since the threshold for both bacteria differs. However, our results indicate that oxygen diffusion within the model food is very slow. In addition, oxygen levels in the center and bottom layers did not increase at 12 °C or 20 °C during storage time. In these layers, O_2 levels ranged from 0 to 1.6%, conditions that could support the growth of *C. botulinum* at 20 °C, and for a longer term at 12 °C. Therefore, our findings suggest that air/oxygen in the package headspace, which results from packaging food without a vacuum, may not be considered as a hurdle in the food safety design of cook-chill products.

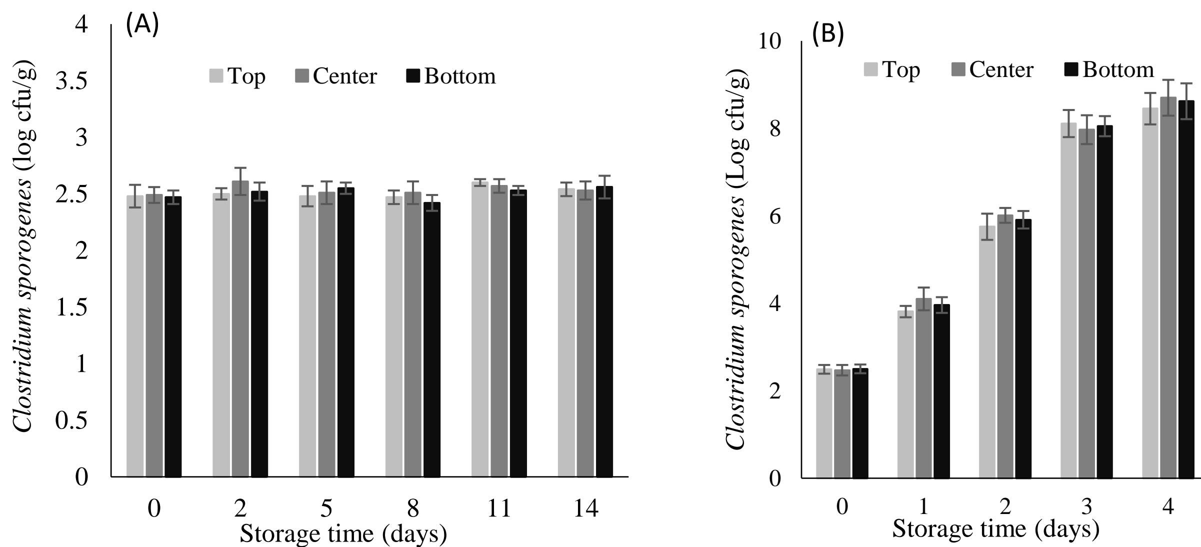

Figure 3.5 *Clostridium sporogenes* (PA 3679) growth evolution within food (top, center and bottom layers) during storage time at 12 °C (A) and 20 °C (B).

Table 3.2 Oxygen concentration gradient within the model food at selected locations during storage time at two different temperatures. Oxygen concentration is expressed as a percentage (%).

Temperature (°C)	Time (days)	Un-inoculated food			Inoculated food		
		Top	Center	Bottom	Top	Center	Bottom
12	0	8.00(0.30)	1.16(0.13)	0.63(0.15)	9.37(1.67)	1.18(0.05)	0.71(0.23)
	2	17.36(3.64)	0.54(0.05)	0.27(0.02)	19.77(0.61)	0.25(0.14)	0(0)
	5	11.55(3.25)	0.56(0.10)	0(0)	18.99(1.12)	0.16(0.06)	0(0)
	8	18.92(1.34)	0.90(0.27)	0(0)	19.76(0.12)	0.38(0.16)	0.09(0.01)
	11	17.22(2.46)	0.33(0.06)	0(0)	17.14(0.13)	0.57(0.06)	0(0)
	14	17.64(1.33)	0.77(0.14)	0(0)	17.96(1.47)	0.18(0.03)	0(0)
20	0	6.58(1.12)	1.65(0.10)	0.36(0.15)	7.97(1.67)	1.58(0.11)	0.51(0.23)
	1	9.63(3.64)	0.73(0.13)	0.09(0.03)	10.10(2.36)	0.65(0.16)	0(0)
	2	11.26(2.3)	1.26(0.10)	0(0)	12.60(2.10)	1.45(0.12)	0(0)
	3	12.11(2.9)	1.21(0.27)	0(0)	13.96(1.85)	1.38(0.16)	0.10(0.01)
	4	12.65(2.3)	1.28(0.13)	0(0)	14.08(1.32)	1.56(0.11)	0.09(0.03)

Data are presented as mean (standard deviation)

5. Conclusions

In this study, we investigated the effects of using oxygen in the package headspace to prevent the growth of *C. botulinum* in pasteurized chilled food during temperature abuse. *Clostridium sporogenes* PA 3679 was used as a model organism. Since this is a surrogate for proteolytic *C. botulinum*, the results are not necessarily 100% representative for *C. botulinum*. Therefore, the results should be considered as conceptual, with no firm conclusions for *C. botulinum*.

Storage temperature is an important factor in the margin of safety for pasteurized chilled food products. Storing food for 14 days at 12 °C has been found to prevent the growth of *C. sporogenes* PA 3679. During mild temperature abuse, food products may still be safe to consume. Bacterial growth increases rapidly when storage temperature increases to 20 °C. Therefore, exposing food

products to severe temperature abuse may promote faster proliferation of proteolytic *C. botulinum*, compromising food safety. In this study, we found no beneficial effects of air/oxygen in the package headspace to prevent bacterial growth. Oxygen diffusion from the pouch headspace was primarily limited to the food surface layer. The structure and properties of the food significantly affected the mobility of the O_2 within it. Therefore, O_2 did not reach the center and bottom portions of the food during storage time, so that oxygen levels which favor *C. botulinum* growth were maintained. Our findings suggest that air/oxygen present in package headspace may not be considered as a hurdle in the food safety design of cook-chill products with a solid jelly-like structure and high water content.

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CHAPTER 4 REFRIGERATED SOUP BIOPRESERVATION BY *LACTOBACILLUS RHAMNOSUS GG*

Abstract

This study explored the antagonistic activity of *Lactobacillus rhamnosus* GG (*Lactobacillus rhamnosus* GG) on foodborne pathogens and its heat sensitivity in soup products. We also evaluated its effectiveness as a protective culture on *Listeria monocytogenes* and *Salmonella* Typhimurium growth at conditions simulating the commercial application of cook-chill processing. We screened the isolates of *Lactobacillus rhamnosus* GG for their inhibitory effect on *Staphylococcus aureus*, *Escherichia coli* O157; H7, *Escherichia coli* O104; H4, *L. monocytogenes* and *S.* Typhimurium using the spot agar test and the well-diffusion method. We determined the thermal resistance of *Lactobacillus rhamnosus* GG in cream of potato (CP) and vegetable beef (VB) soups between 55-62.5 °C using the replicate-sampling technique in disk-shaped cells. Soup samples were inoculated with a suspension containing *S.* Typhimurium, *L. monocytogenes* and/or the probiotic strain *Lactobacillus rhamnosus* GG, packaged and stored at 5, 10 and 15 °C for 21 days. Microorganism was periodically enumerated. Overall, *Lactobacillus rhamnosus* GG showed an inhibitory effect against all pathogens tested on spot agar test. Inhibition zones ranged in diameter from 2.0 to 3.0 cm. *E. coli* O157: H7 was the most sensitive, whereas *S.* Typhimurium was the least sensitive to the inhibitory effect of *Lactobacillus rhamnosus* GG. *D*-values obtained in CP soup exceeded those obtained in VB soup at the temperatures tested. $D_{55\text{-}62.5}$ values of *Lactobacillus rhamnosus* GG ranged between 0.7-24.2 min and 0.5-15 min in CP and VG soup, respectively. The z-value for CP and VB soup was around 5 °C. *S.* Typhimurium and *L. monocytogenes* populations were more affected by co-inoculation with *Lactobacillus rhamnosus*

GG at 15 °C, which reduced the populations to undetectable levels by the end of storage period. The probiotic population increased about 1-log unit at 15 °C. At 10 and 5 °C, it was maintained throughout the experiment at around 10^8 CFU g^{-1}. Results demonstrated the potential of *Lactobacillus rhamnosus* GG as a protective culture in controlling *S.* Typhimurium and *L. monocytogenes* growth in soup products.

Key words: Bio-preservation, Cook-chill, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Lactobacillus rhamnosus*, Protective culture

1. Introduction

Episodes of foodborne illness caused by contamination from *Listeria monocytogenes* or *Salmonella* spp. are frequent (Cabedo et al., 2008; Marder et al, 2017: Scallan et al., 2011). The FoodNet database identified 127 cases of listeriosis and 7,554 cases of salmonellosis in 2016 in United State (Marder et al, 2017). The largest number was reported for *Salmonella* spp. However, *Listeria monocytogenes* was identified as the pathogen with the highest hospitalization (97%) and mortality (13.4%) rates. Listeriosis causes mild flu-like symptoms, but rarely causes gastroenteritis in healthy adults. In contrast, it may have serious clinical manifestations for susceptible population groups. In children, elderly and immunocompromised persons, it may manifest as severe infection (Cabedo et al., 2008). Infection in pregnant women may lead to abortion, stillbirth or perinatal morbidity (Pochop et al., 2012). Although the incidence of listeriosis is low compared to salmonellosis, the severity of the illness to susceptible population groups cause 20-30% of the deaths associated with foodborne pathogens in the United States (Aymerich et al., 2005; Gombas et al., 2003; Lianou and Sofos, 2007; Scallan et al., 2011).

Refrigerated, ready-to-eat (RTE) food products have been linked to numerous outbreaks of listeriosis and salmonellosis (NACMCF, 2005; Pouillot et al., 2010). Most concerning are products

that are not subjected to a listericidal process, as well as those susceptible to post-processing contamination with an extended refrigerated shelf life to allow pathogen growth (Lianou and Sofos, 2007). However, the demands of modern life and modern eating habits have favored the development and availability of such products.

Among refrigerated RTE foods, cook-chill products are of particular concern. Most such foods are formulated with low sodium levels and are also low-acid. This supports the growth of many foodborne pathogens. During the cook-chill process, food is cooked in volume to an exact degree of doneness and then chilled very quickly to safe holding temperatures (≤ 5 °C). Two main variations of the process exist one for liquid, pumpable foods and another for whole muscle meats or solid foods. Once cooked, the liquid food is hot filled into portion-controlled bags. A cold-water bath then rapidly chills the packaged food. Food recipes containing large cuts of meats and solid foods are vacuum-sealed in plastic bags with seasoning and then cooked and chilled. Regardless of the process used, these foods must be chilled within 30 minutes after they are cooked. In the case of liquid food, while hot (at least 135 °F/57 °C) portioning and packaging operations are carried out in those 30 minutes. Regulations for cook-chill processing in retail or food service operations are specified in the 2013 FDA Food Code in Chapter 3-401, 3-501 and 3-502. Additional guidelines are found in Annex 3, Chapter 3 and Annex 6, Chapter 2 of the Food Code.

Handling and packaging after cooking provide an opportunity for re-contamination with pathogen bacteria such as *Listeria monocytogenes* and *Salmonella* spp., due to the persistence of these pathogens in food processing environments (Abai et al., 2017; Brasileiro et al., 2016; Yana et al, 2016). The ability of *L. monocytogenes* to form biofilms on all of the surfaces make it a formidable contaminant in food processing areas (Muriana et al., 2002). *Salmonella* spp. is a ubiquitous pathogen, which may be introduced into food processing zones through direct contact

with infected humans or faecally contaminated environments (Montiel et al., 2015). If those pathogens contaminate the food and survive the hot-filling step, they can become a hazard during extended shelf-life at refrigeration temperatures.

Several studies have identified microbial contamination by these microorganisms in cook-chill foods, which is characterized by low prevalence and low concentrations. The microbial risk of these foods is small when stored at the correct temperature and within proper time periods (Daelman et al., 2013). However, temperature abuse may occur in the cold chain, and some steps are especially weak, including retail display, transport after purchase and domestic refrigerators (Derens-Bertheau et al., 2015; Røssvoll et al., 2014). These steps are more problematic in terms of temperature control. Many studies demonstrate that the temperature of retail display and domestic refrigerators often fluctuate exceeding 5 ˚C (James et al., 2017; Kou et al., 2015; Koutsoumanis and Gougouli, 2015). In addition, there is little or no control on temperature after the products are purchased and transported to homes. *L. monocytogenes* is a psychrotrophic bacteria that grow slowly at refrigeration temperatures. *Salmonella* spp. is a mesophilic pathogen that can survive under refrigeration and grow during temperature abuse. It is of particular concern when the cold chain is not respected, especially for low-acid foods, which often go unprotected in such circumstances.

Given the risk of temperature abuse, it is crucial to identify alternative hurdles to maintain safety during the extended shelf-life (5 days up to 4 weeks) of cook-chill foods. Bio-preservation is used to control the growth of spoilage and pathogenic bacteria. This preservation method enhances the safety and stability of food products without modifying their sensory qualities by using certain non-pathogenic microorganisms, their metabolites, or both (Melero et al., 2013). Various methods have been used to apply bio-preservation on food systems. These include adding

a pure culture of the viable protective culture, adding purified or semi-purified antimicrobial substances or adding a mesophilic protective culture as a fail-safe protection against temperature abuse (Ananou et al., 2010; Carlin et al., 2016; El Bassi et al., 2009; Gálvez et al., 2007; García et al., 2010; Hugas, 1998; Iglesias et al., 2017; Jacobsen et al., 2003; Khan et al., 2010; Tomé et al., 2008).

Lactic acid bacteria (LAB) are the most common microorganisms used as protective cultures, due to their antagonistic properties. They are generally recognized as safe (GRAS) for consumption (Angiolillo et al.,2014; Melero et al., 2013; Settani and Corsetti, 2008). When LAB compete for nutrients and space in food systems, their metabolites often include active antimicrobials, such as organic acids, hydrogen peroxide, and peptide bacteriocins (El Malti and Amarouch, 2008; Favaro et al., 2015; Gómez-Sala et al., 2016; Nath et al., 2014). Due to LAB's activity against certain foodborne pathogens and their resistance to high temperatures, as well as their ability to grow when temperatures exceed the recommended limit for a particular product, they are considered useful in the biopreservation of cook-chill foods.

Therefore, this study evaluated the effectiveness of in-vitro *Lactobacillus rhamnosus GG* (*Lactobacillus rhamnosus* GG) for inhibiting foodborne pathogens and determined its heat sensitivity on soup products. We also evaluated its effectiveness as a protective culture on *L. monocytogenes* and *S.* Typhimurium growth at conditions simulating the commercial application of cook-chill processing during storage at 5, 10, and 15 °C.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

Tryptic soy broth (TSB), nutrient broth (NB), brain-heart infusion broth (BHI), agar and xylose lysine deoxycholate (XLD) agar were obtained from Becton, Becton, Dickinson, and Company.

Buffered peptone water (BPW), peptone and Man, Rogosa, and Sharpe (MRS) both broth and agar were obtained from Thermo Fisher Scientific, Inc. Muller Hinton agar and broth were obtained from Oxoid. Listeria oxford agar was supplied by Sigma-Aldrich (758050 Oxford formulation medium, with a 75805-selective supplement).

A dietary supplement (Culturelle®, Digestive health probiotic; 30 capsules per package) containing 100% *Lactobacillus rhamnosus* GG (ATCC® 53103™) as a probiotic source was obtained from a local pharmacy. Each capsule is purported by the manufacturer to contain 10 billion live cells, microcrystalline cellulose, and milk protein as a carrier. An accurate count is important to verify the manufacturer's claim and determine the amount of capsule content needed for food inoculation. Therefore, five individual capsules were tested, and the average of the cell populations was reported as the actual count. Then, 0.1 g of the culture in each capsule was mixed in 9.9 ml of 0.1% sterile peptone water. This was allowed to dissolve for 20 minutes and was followed by serial dilution. Aliquots of 1ml of the last three dilutions were pour-plated in duplicate into the MRS agar. The inoculated plates were incubated under aerobic conditions for 72 h at 37 °C. Cell colonies were enumerated using a Leica Quebec Darkfield Colony Counter (Leica, Buffalo, N.Y., U.S.A.).

For the screening assay, *Lactobacillus rhamnosus* GG was propagated in MRS broth aerobically at 37 °C for 48h. Indicator pathogenic strains used include *Listeria monocytogenes* (ATCC® 19112™), *Staphylococcus aureus* (ATCC® 29213 ™), *Escherichia coli* O157: H7 (ATCC® 43888 ™), *Escherichia coli* O104: H4 (ATCC® BAA-2326 ™) and *Salmonella* Typhimurium (ATCC® 13311 ™). All pathogens were obtained from Microbiologics Inc. (Saint Cloud, Minn, USA). *S. aureus* and *E. coli* O157: H7 were propagated in TSB; *E. coli* O104: H4 and *S.* Typhimurium in NB; and *L. monocytogenes* in BHI. The strains were incubated in aerobic

condition at 37 °C for 24h. Cultures were maintained at -80 °C in the correspond broth supplemented with 20% (v/v) glycerol. Cultures were activated by successive transfer in the respective media and aerobic incubation at 37 °C.

2.2 Screening assay for possible antimicrobial activity

A spot-on lawn test and well-agar diffusion assay were used to determine antagonistic activity. The spot-on lawn test was carried out according to Oliviera et al., 2008. Briefly, after 18 h incubation, *Lactobacillus rhamnosus* GG active culture was spotted three times (20 µm drops) on the surface of MRS agar plates. Then, the MRS plates were incubated aerobically for 24 h at 37 °C to allow colonies to develop. Overnight cultures of pathogens were mixed 1:100 (1%, v/v) with TSB, NB or BHI soft agar (containing 0.7% agar, w/v) and poured over the MRS plates containing the developed colonies of *Lactobacillus rhamnosus* GG. Plates were incubated aerobically for 24 h at 37 °C. At the end of the incubation, the inhibition zones surrounding the spotted isolates were measured. The isolates with an inhibition zone over 1 mm were determined to have antimicrobial activity.

For the well-agar diffusion assay, after 18 h incubation, *Lactobacillus rhamnosus GG* active culture was centrifuged at 6000 rpm for 20 min at 4 °C (Sorvall®, RT6000B refrigerated centrifuge). The cell-free supernatant (CFS) was collected and pH adjusted to 6-7 with 1 M NaOH to rule out any inhibitory effect of organic acids. This was then sterilized by filtration through a 0.20 µm membrane filter (Pall Corporation, Ann Arbor, MI, USA). Soft agar was prepared by supplementing Muller Hinton broth with 0.7% agar (w/v). The pathogens in the overnight cultures were inoculated (1%, v/v) into the soft agar and poured over MH agar plates. After solidification, 3 wells of 8mm diameter were punched on each plate and filled with 100 µL of CFS. The plates

were incubated for 2 h at 4 °C to allow initial CFS diffusion. Then plates were incubated aerobically for 24 h at 37 °C and the inhibition zones around the wells were measured.

2.3 Thermal inactivation and bacterial enumeration

Thermal inactivation studies were conducted to determine *D* and *z*-values of *Lactobacillus rhamnosus* GG at four temperatures; 55, 57.5, 60 and 62.5 °C. The food matrices tested were cream of potato (CP) and vegetable beef (VB) soups. Six-time intervals were employed between 15 s to 6 min, depending on the treatment temperature. Aluminum disk-shaped cells were used as test containers to hold the soup samples inoculated with *Lactobacillus rhamnosus* GG during the inactivation studies (**Fig. 4.1**). These cells have a base with a screw-on cap to allow easy loading and unloading of the test samples. The overall dimensions of the loading cavity with a 1 g sample size capacity are; 18 mm diameter, and 4 mm height (Jin et al., 2008). A rubber O-ring band is placed between the base and the cap to provide a hermetic seal. All inactivation studies were carried out with three independent samples per each interval time.

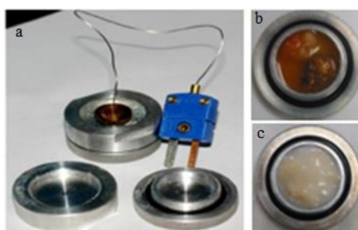

Figure 4.1 Test disk pictures; a) test disks with and without thermocouple integrated on the cap; b) test disk with vegetable beef soup; c) test disk with cream of potato.

Soup samples were prepared by aseptically combining equal portions of commercial condensed soup with the recommended diluent, as recommended by manufacturer's instructions

(Campbell® Soups Co.). The condensed soups, water or whole milk were separately autoclaved at 121 °C for 15 min at 15psi. *Lactobacillus rhamnosus* GG was introduced to the food matrix via water or milk, depending on the suggested diluent for a given soup at a concentration of 10^6 cells per gram of soup. To prepare the soup samples, CP and VB soups were mixed 1:1 with previously inoculated milk and water, respectively. Next, homogenization was conducted by vortex for 30 seconds. Aluminum test cells were filled with soup samples and immersed in an IsoTemp 215 water bath (Fisher Scientific, Waltham, MA, USA) at the selected temperatures. Come-up-time was verified using a non-inoculated sample inside a test cell, with a K-type thermocouple located at the center of the test cell and connected to a Memory HiLogger LR8400-92 (Hioki corp., Nagano, Japan) (**Figure 4.1**). The come-up-time (~120-134 s) for the sample core to reach within 0.5 °C of the target temperature was used as time zero for the heat treatment. Test cells containing soup samples were subsequently removed at uniform time intervals, starting with the time zero samples. Once they were removed from the water bath, the test cells were immediately placed in an ice-water bath to stop the thermal inactivation. Soup samples were taken from the test cells and serially diluted in buffered peptone water. Volumes of 1ml from the last 3 dilutions were pour-plated in duplicate into MRS agar and incubated aerobically for 48 h at 37 °C. Plates with 25-250 colonies were enumerated using a colony counter (American Optical Quebec, Darkfield colony counter 3325, Buffalo, NY, USA).

D-values (i.e., the time required for 1-log reduction in microbial load at a given temperature) were calculated by plotting the log number of survivors against time for each heating temperature, using Excel software (Microsoft Corp, Redmond, WA). The line of best fit for survivor plots was determined by linear regression analysis, in which the *D*-value is the negative reciprocal of the slope of the best straight line. A linear regression was computed from log *D*-values versus

temperature, and an estimate of the *z*-value (i.e., the temperature required for a 10-fold reduction of *D*-value) was obtained from the absolute value of the inverse of the slope.

2.4 Bio-preservation of soup by *Lactobacillus rhamnosus* GG

Cream of potato (CP) soup batches was prepared in a safety cabinet hood, in which an experimental cook-chill production line was assembled and sanitized. The production line includes three main stations; cooking, bagging and cooling stations. The soup recipe consisted of two ingredients, as described in **Section 2.3**. After ingredients were mixed, the soup was heated in the cooking station until the core reached 70 °C, and held for 2 min in a large beaker covered with aluminum foil using a hot plate stirrer. Next, the soup temperature was equilibrated and held at 57 °C ± 0.5. For inoculation studies, three trials were prepared: 1) soup with *Lactobacillus rhamnosus* GG (10^9 CFU g-1); 2) soup with *L. monocytogenes* (10^3 - 10^4 CFU g-1) + *S.* Typhimurium (10^3 - 10^4 CFU g-1); 3) soup with *Lactobacillus rhamnosus* GG + *L. monocytogenes* + *S*. Typhimurium. Aliquots of 50 ml of the milk used in each batch were reserved and used as a carrier during the inoculation step. The bacteria inoculums were mixed with the milk aliquots at room temperature. The mixture was then added and distributed into the soups by stirring for 2 minutes.

In the packaging process, 30 g of each soup batch were hot-filled into multi-layered plastic pouches (Kurarister^{TM} CF, Kuraray Company of America, Inc.), for a total of 48 pouches per soup trial. Next, the open edge of the pouches was sealed using a manual impulse heat sealer (Hang bag sealer MP-12, Midwest Pacific®, Rocky Mount, MO, USA) operated with the timer knob at 6. This provided a water-tight and air-tight seal, preventing contamination during subsequent chill processing at 3 °C in the cooling station. Samples were cooled by submerging the pouches in ice-water and stored at 5, 10 or 15 °C for 21 days.

Measurements of pH and water activity (a_w), as well as *Lactobacillus rhamnosus* GG, *L. monocytogenes,* and *S.* Typhimurium counts, were obtained just before storage at the predesignated temperatures. Thereafter, microbial counts determinations were carried out every 3 days during storage time for a total of 8 screening points. All trials were carried out with two independent samples per screening point. Measurements of the pH and a_w of the soup were determined with a potentiometer (Mettler Toledo, EL 20) and an Aqua lab CX-2 water activity meter (Decagon Devices, Pullman, WA), respectively. Aliquots of 10g of each soup sample were homogenized in sterile stomacher bags for 60 s at 100 rpm, with 90 ml of buffered peptone water in a Seward Circulator 400 stomacher (Seward, London, UK). The resulting suspensions were subjected to serial dilution with 0.1% peptone water. Counts of *Lactobacillus rhamnosus* GG was obtained by pour-plating techniques on MRS agar and aerobic incubation for 48 h at 37 °C. Counts of *L. monocytogenes* and *S.* Typhimurium were obtained by spread-plating on Oxford agar containing listeria selective supplement or XLD and aerobic incubation for 24 h at 37 °C.

3. Statistical Analysis

Statistical analyses were performed with InfoStat software (student version). An analysis of variance ($\alpha = 0.05$) and Tukey test were performed to identify significant differences between the treatments. Scatter charts for bacterial growth were generated with Microsoft Excel 2010 (Microsoft Corp, Redmond, WA).

4. Results and Discussion

Results show that the actual count of *Lactobacillus rhamnosus* GG in the probiotic powder from the commercial capsules was 10^{11} CFU per gram of powder. Plate cultures showed that the

bacteria isolated from probiotic powder formed large, white colonies with an average diameter of 1.8 mm. Further microscopic analysis also showed that the isolates were gram-positive uniform rods, coinciding with the basic characteristic of the lactic acid bacteria labeled by the manufacturer. Identification by API biochemical test and 16 SrRNA gene analysis were not carried out since research shows that the identified microorganism in Culturelle is a 99% match with the strain labeled by the manufacturer (Cruz-Guerrero et al., 2014). The capsules were used before the expiration date to ensure maximum cell viability.

The average water activity of CP and VB soup was 0.993 and 0.999, respectively, and the pH was 6.28 and 4.90 (**Table 4.1**). Low-acid food refers to any food (other than alcoholic beverages) with a finished equilibrium pH above 4.6 and a water activity above 0.85. This excludes tomatoes and tomato products, which have a finished equilibrium pH of less than 4.7. According to that definition, the soup products in this study are low-acid foods and are expected to support the growth of the pathogenic microorganisms encountered in cook-chill foods.

Table 4.1 Heat resistance of *Lactobacillus rhamnosus* GG in cream of potato or vegetable beef soup.

Soup	*D* values (min)				*z* values	pH	a_w
	55 ˚C	57.5 ˚C	60 ˚C	62.5 ˚C	(˚C)		
CP	24.2(0.2)^{ax}	10.2(0.4)^{bx}	3.7(0.2)^{cx}	0.7(0.2)^{dx}	5.0	6.28(0.02)	0.993(0.002)
VB	15.0(0.7)^{ay}	5.1(0.3)^{by}	0.9(0.1)^{cy}	0.5(0.1)^{cx}	4.8	4.90(0.01)	0.999(0.004)

Values are means (standard deviation). Values in the same row (ab) and column (xy) followed by different letters are significantly different ($p<0.05$). CP: Cream of potato soup. VB: Vegetable beef.

4.1 Antimicrobial activity screening

Lactic acid bacteria can produce antimicrobial substances including organic acids, hydrogen peroxide, and bacteriocins. Therefore, it is a prerequisite to identify whether the antimicrobial

activity of isolates is caused by any these antimicrobial substances. **Fig. 4.2** shows the growth inhibitory effect of *Lactobacillus rhamnosus* GG against *S. aureus* on the spot agar test. Similarly, the growth inhibitory effect of *Lactobacillus rhamnosus* GG against the other foodborne pathogens tested was observed and the size of the inhibition zone is summarized in **Table 4.2**.

Overall, *Lactobacillus rhamnosus* GG inhibitory effect it appears to be broad, inhibiting gram-negative bacteria (*E. coli* O154: H4, *E. coli* O157: H7 and *S.* Typhimurium) as well as gram-positive bacteria (*L. monocytogenes* and *S. aureus*). The halo of inhibition ranged in diameter from 2.0 to 3.0 cm (20-30 mm). Based on the results, *E. coli* O157: H7 was the most sensitive, whereas *S.* Typhimurium was the least sensitive to the inhibitory effect of *Lactobacillus rhamnosus* GG. The CFS did not show antagonistic activity against the pathogens tested with the well-diffusion method. The results suggest that the pH adjustment results in the loss of the antimicrobial properties of the supernatant. Consequently, a further test of the CFS such treating it with proteases or catalase to determine if bacteriocins or peroxides contribute to inhibition was not carried out. Then the inhibitory effect observed on the pathogens tested by *Lactobacillus rhamnosus* GG may be attributed to the production of organic acids, presumably lactic acid.

The production of lactic and acetic acid on members belonging to the *Lactobacillus* genus has been reported (Neal-McKinney et al., 2012). These short-chain organics acids are known to exhibit high antimicrobial activity against microorganism due to the easy diffusion of the non-dissociated form through the cell membranes of pathogens (Poppi et al., 2015). The organic acids in the antimicrobial CFS of *Lactobacillus rhamnosus* GG have been identified using HPLC analysis and its inhibitory effect examined (De Keersmaecker et al., 2006). The acids include acetic acid, pyroglutamic acid, formic acid and lactic acid. The latter was identified as the main antimicrobial compound produced by *Lactobacillus rhamnosus* GG.

Table 4.2 Inhibitory effect of *Lactobacillus rhamnosus* GG against selected pathogens.

ATCC #	Microorganism	IZ* (cm)
43888	*Escherichia coli* O157:H7	3.0(0.15)^{a}
BAA-2326	*Escherichia coli* O104:H4	2.8(0.25)^{a}
19112	*Listeria monocytogenes*	2.7(0.25)^{a}
29213	*Staphylococcus aureus*	2.1(0.21)^{b}
13311	*Salmonella typhimurium*	2.0(0.06)^{b}

*IZ-inhibition zone

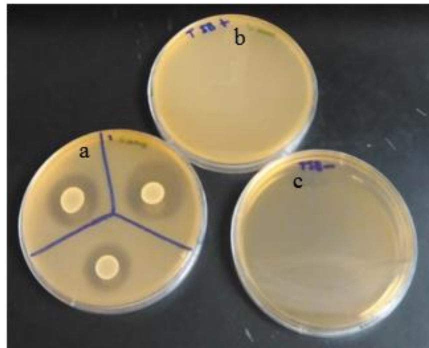

Figure 4.2 Antimicrobial activity of *Lactobacillus rhamnosus* GG against *S. aureus* as demonstrated by inhibition zone produce with the spot-on lawn test (a), positive control inoculated with *S. aureus* (b) and negative control non-inoculated (c).

4.2 Thermal inactivation studies

The initial population of *Lactobacillus rhamnosus* GG in cream of potato and vegetable beef soup analyzed immediately after inoculation were approximately 6.73 ± 0.21 and 6.65 ± 0.19 log CFU/g, respectively. It took the disk-shaped cells in the soup samples' core between 106 to 122 s to reach within 0.5 °C of the water temperature (come-up-time). Reduction in initial levels during come-up-time (55-62.5 °C) ranged from 0.08 to 0.52 log CFU/g in VB, and from 0.13 to 0.43 log

CFU/g in CP soup. Figure 3 shows log-linear thermal death curves with no significant shouldering or tailing for both CP and VB soup. Overall, the *D*-values in the two soups decreased significantly ($p<0.05$) as temperature increased from 55 to 62.5 °C. The D_{55}, $D_{57.5}$, D_{60}, $D_{62.5}$ values of *Lb. rham GG* in CP soup were 24.2, 10.2, 3.7, 0.7 min, respectively, and 15, 5.1, 0.9, 0.5 min in VB soup (**Table 4.1**).

It appears that the thermal resistance of *Lactobacillus rhamnosus GG* in CP soup was higher than that of VB soup. At 55, 57.5 and 60 °C, the *D*-values in VB soup were significantly smaller than those in CP soup. However, the value at 62.5 °C was statistically similar (*p*-value = 0.2739). This is probably due to the soups' different pH value. Although the heat resistance of microorganisms is typically influenced by the time and temperature of treatment, intrinsic factors of the heating medium (e.g. food) such as pH, a_w, and composition of the medium may drastically influence heat resistance, even during heat treatment. According to published literature, many authors have revealed that acidification of the heating medium decreases the heat resistance of any given microorganism (Collado et al., 2003; Samapundo et al., 2011).

In this study, the combined effect of heat treatment and pH appear to be responsible for inactivation of *Lactobacillus rhamnosus GG* at temperature 55-60 °C. At temperatures above 60 °C, inactivation is due mostly to heat treatment. No differences were found in *z*-values obtained in the two soups. Thus, the differences in heat resistance related to the heating medium were retained for all temperatures examined.

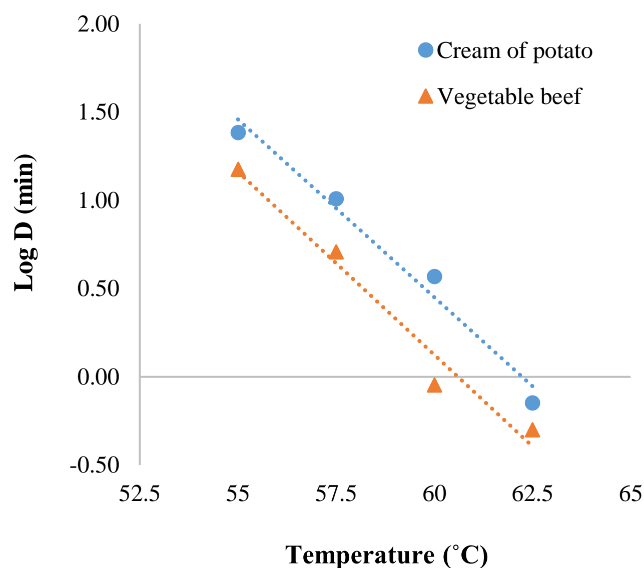

Figure 4.3 *D*-value as a function of temperature for *Lactobacillus rhamnosus* GG in two different soup matrices.

4.3 Bio-preservation of soup by *Lactobacillus rhamnosus* GG

Adding the protective culture (PC) after the pasteurization/cooking step is recommended to guarantee high populations of the cultures in the final product (Rodgers et al., 2003). Exposure of the *Lactobacillus rhamnosus* GG population to temperatures above 65 °C during such steps would kill the cells. As previously noted, the heat resistance of *Lactobacillus rhamnosus* GG in CP soup was higher than that in VB soup. Thus, the CP soup is more suitable for bio-preservation. We selected this as a food system to test the performance of *Lactobacillus rhamnosus* GG as a protective culture against *L. monocytogenes* and *S.* Typhimurium in the case of post-processing contamination during packaging operation. The benefit of using CP soup is that less probiotic powder needs to be added to guarantee high concentration of *Lactobacillus rhamnosus* GG. This is attractive in terms of cost. In addition, CP soup is a thick, milk-based soup with a rich, buttery

finish and an ivory color. The appearance of CP soup can disguise the addition of probiotic powder. In contrast, the appearance of vegetable soup may be affected, like any other clear soup.

In cook-chill processing, the chilling process must start at least 30 minutes after cooking. This leaves time for hot portioning and packaging if the food is being pasteurized/cooked out of the package. In this study, it took around 20 minutes to complete the packaging process. **Table 4.3** summarized the log reduction expected if *Lactobacillus rhamnosus* GG is exposed at temperatures ranging from 55 to 62.5 °C for 20 minutes during the packaging step. We chose to cool down the soup after cooking at 70 °C for 2 min to 57.5 °C for the inoculation and packaging steps. At 60 or 62.5 °C, the expected log reduction is 5.4 or 28.6, respectively. At 55 °C, the expected log reduction is 0.8, which has the least impact on the PC population. However, the packaging step should not be carried out at that temperature, since the minimum temperature allowed is 57 °C (135 °F). Thus, we used 57.5 °C in this study as the best alternative in term of cost, temperature and impact *Lactobacillus rhamnosus* GG population.

Table 4.3 Expected log reduction on *Lactobacillus rhamnosus* GG population expose at different temperatures for 20 minutes.

Temperature (˚C)	D-value (min)	Log reduction
55	24.2	0.8
57.5	10.2	2.0
60	3.7	5.4
62.5	0.7	28.6

Fig. 4.4 shows populations of *L. monocytogenes* in CP soup that was co-inoculated with *Lactobacillus rhamnosus* GG along 21 days of storage at 5, 10 and 15 °C, compared to the soup

that was not co-inoculated. Initial counts of *L. monocytogenes* with and without *Lactobacillus rhamnosus* GG were in average 1.89 ± 0.1 and 1.82 ± 0.1 log_{10} CFU g^{-1}, respectively. Growth in the *L. monocytogenes* population inoculated without *Lactobacillus rhamnosus* GG was not noticeable until 9 and 15 days of storage at 15 and 10 °C. Then growth was observed up to 5.0 and 3.6 log_{10} CFU g^{-1}, correspondingly (**Fig. 4.4a**). When the pathogen was co-inoculated with *Lactobacillus rhamnosus* GG and stored at 15 °C, its population decreased 0.2-log units after 18 days, when the counts were below detection levels (**Fig. 4.4b**). A different trend was observed in co-inoculation at 10 °C, where the pathogen population increased slightly after 9 days but decreased after 15 days. Pathogen population growth at 5 °C was not noticeable throughout storage period, whether it was inoculated alone or in combination with *Lactobacillus rhamnosus* GG.

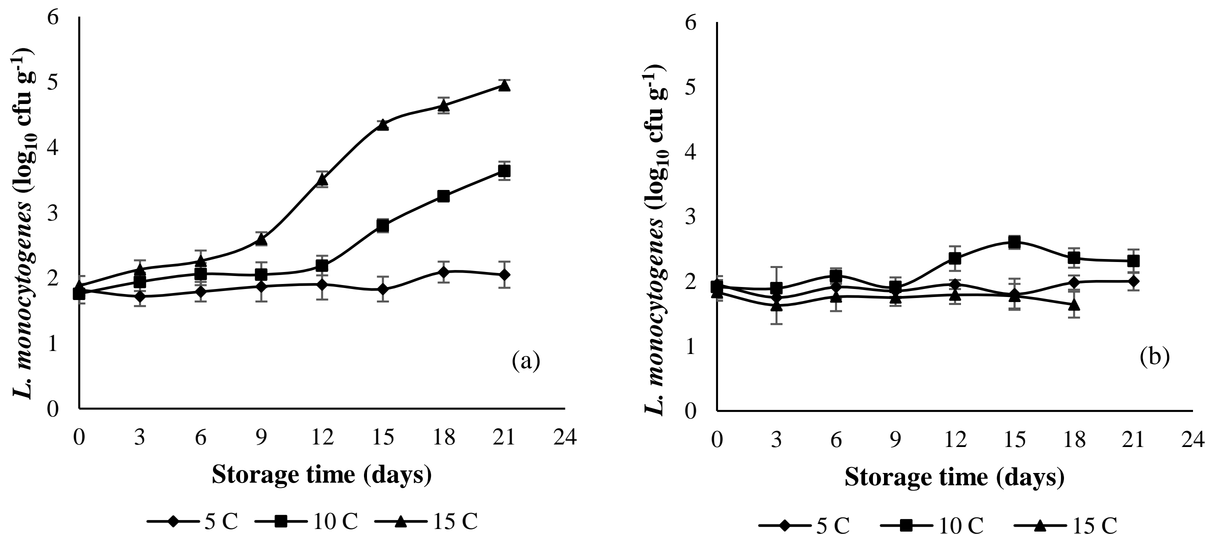

Figure 4.4 Growth of *L. monocytogenes* inoculated alone (a) or co-inoculated (b) with *Lactobacillus rhamnosus* GG in cream of potato soup stored at 5, 10, and 15 °C for 3 wk.

After inoculation with or without *Lactobacillus rhamnosus* GG, the *S.* Typhimurium population was not detected at 5 or 10 °C during the storage period. It appears that the 20-minute

exposition at 57.5 °C during inoculation and packaging reduced the pathogen population below detection limits. At 15 °C, when the pathogen was inoculated alone its population was able to increase from initially undetectable levels at 3 days. Then it had a progressive increase during the rest of the storage period up to 4.4 ± 0.1 $\log_{10}$ CFU g^{-1} (**Fig. 4.5a**). In the case of co-inoculation at 15 °C, the pathogen population remain undetectable (data not shown).

For each temperature, results of *Lactobacillus rhamnosus* GG population were pooled for CP soup inoculated alone or co-inoculated with the pathogens. There were no significant differences in lactobacilli population when means were compared by Tukey test ($p < 0.05$) (**Fig. 4.5b**). Results show that the population at 5 °C did not grow. At 10 and 15 °C, the lactobacilli population increased slightly, by 0.4 and 1-log unit, respectively. The growth at 10 °C was lower than 0.5-log units and was not considered to be relevant.

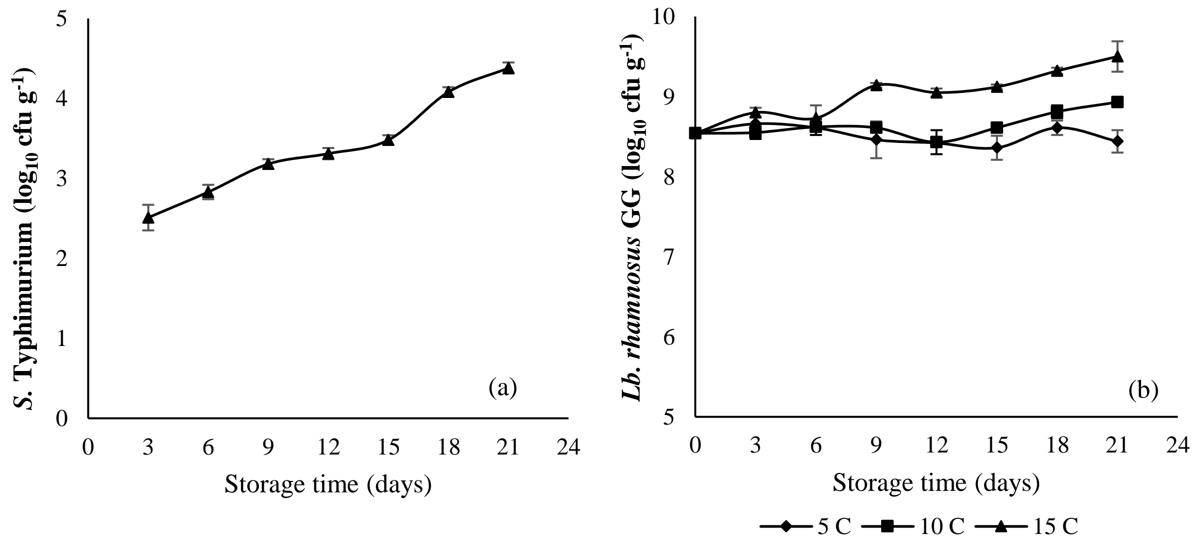

Figure 4.5 Growth of *S.* Typhimurium in cream of potato soup stored at 15 ˚C (a) and growth of *Lactobacillus rhamnosus* GG in cream of potato soup stored at 5, 10, and 15 ˚C for 3 wk (b).

5. Conclusion

In summary, findings indicate that *Lactobacillus rhamnosus* GG shows promise as a protective culture. Antimicrobial activity screening tests displayed a broad antimicrobial spectrum, which included gram-negative and gram-positive foodborne pathogens. Such activity was mainly attributed to organic acids production. To protect the *Lactobacillus rhamnosus* GG population during cook-chill processing, we selected the appropriate product and designed a process with a time /temperature combination with less impact on survival. This guaranteed a high concentration for effectiveness. We controlled *L. monocytogenes* and *S.* Typhimurium growth during extended shelf-life by simulating cook-chill processing. Findings show that co-incubation with PC had a bactericidal effect on *L. monocytogenes* and *S.* Typhimurium at 15 °C, and had a bacteriostatic effect on *L. monocytogenes* at 10 °C. Although the use of *Lactobacillus rhamnosus* GG did not alleviate all technical and practical food safety issues associated with temperature abuse that many cook-chill products face, this study identified an alternative to controlling the growth of foodborne pathogens to protect against temperature abuse.

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CHAPTER 5 CONCLUSIONS, CONTRIBUTION TO KNOWLEDGE AND RECOMMENDATIONS

1. Major conclusions

Control of temperature is an important factor in the margin of safety for cook-chill food products during production and distribution. Refrigeration storage is the most widely used barrier for the preservation of such foods. It is vital to respect temperature through the cold chain to maintain the safety and to reduce the risk of diseases transmitted by the food. However, the product temperature at retail, during transport to home and at domestic storage is often reported to be in violation. Thus, the present research was focused on the application of oxygen and protective cultures as additional barriers to ensure the safety of cook-chill food products in the case of temperature abuse. The major findings can be summarized as a following:

1. It was found that storing food for 14 days at 12˚C prevented the growth of *C. sporogenes*. During mild temperature abuse, food products may still be safe to consume. However, bacteria growth increased rapidly when storage temperature increased to 20˚C. Therefore, exposing food products to severe temperature abuse may promote faster proliferation of proteolytic *C. botulinum*, thus compromising food safety.
2. Oxygen diffusion from the pouch headspace was primarily limited to the food surface layer. The structure and properties of the food significantly affected the mobility of the O_2 within it. Oxygen did not reach the center and bottom portions of the food during storage time so that oxygen levels which favor *C. botulinum* growth were maintained.
3. Air/oxygen present in package headspace may not be considered as a hurdle in the food safety design of cook-chill products with a solid jelly-like structure and high water content.

4. *Lactobacillus rhamnosus* GG showed to have a good potential to be used as a protective culture. It can inhibit the growth of potential pathogens that may be present in cook-chill food products and it is able to survive low storage temperatures and growth at a temperature above 5℃.
5. Heat resistance of *Lactobacillus rhamnosus* GG depends on temperature and food properties. Protection to the heat of *Lactobacillus rhamnosus* GG population was achieved by selecting the right product and inoculation temperature.
6. Co-incubation with *Lactobacillus rhamnosus GG* had a bactericidal effect on *L. monocytogenes* and *S.* Typhimurium at 15 ˚C, and a bacteriostatic effect on *L. monocytogenes* at 10 ˚C. *Lactobacillus rhamnosus* GG may offer an alternative chemical preservatives to control foodborne pathogens growth as a fail-safe protection against temperature abuse.

2. Contribution to knowledge

This was the first study investigating the influence of the oxygen concentration gradient generated in the food/packaging system on the growth of anaerobic bacteria within the food. In addition, the present research provided heat resistance data on commercial soups of the microencapsulated probiotic *Lactobacillus rhamnosus* GG that was not available in the literature and is relevant for its application as a protective culture in such foods. Lastly, the research proved that protective culture can survive the hot-fill production process used in cook-chill foods preparation and performed successfully in the case of product temperature violation.

3. Recommendation for future research

In this research, the inhibitory effect of *Lactobacillus rhamnosus* GG was observed in gram-positive and gram-negative non-spore former bacteria. Spore former bacteria such as *Clostridium botulinum* and *Bacillus cereus* can survive the cooking/ pasteurization temperature. If the product temperature is violated the spore stage of such pathogens may germinate and growth leading to food toxigenesis. Therefore, the inhibitory effect of *Lactobacillus rhamnosus* GG on the spores' stages of *Clostridium botulinum* and *Bacillus cereus* should be investigated

In addition, the heat resistance data collected about *Lactobacillus rhamnosus* GG was limited to two food matrices. Food processors do not have room in their facilities to grow fresh preparation of protective cultures. Instead, a better approach is using microencapsulated preparations, which offer consistency, less handling and are easy to add to the food. However, heat resistance data of microencapsulated preparation are scarce. Thus, studies focus on heat resistance data collection need to be conducted.

Also, this research did not investigate the effect of the incorporation of *Lactobacillus rhamnosus GG* or its metabolites in the chemical, physical, and sensory attributes of cream of potato soup. Then before considering *Lactobacillus rhamnosus* GG for biopreservation application in such product the absence of any detrimental effect on the quality and sensory attributes should be demonstrated.