Heat, Acid and Osmotic Stress Tolerance of Leuconostoc mesenteroides as Influenced by Prior Exposure to Various Mild Stress Conditions

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HEAT, ACID AND OSMOTIC STRESS TOLERANCE OF
LEUCONOSTOC MESENTEROIDES AS INFLUENCED BY PRIOR
EXPOSURE TO VARIOUS MILD STRESS CONDITIONS

A Thesis
Submitted to the Graduate Faculty of the
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by
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ABSTRACT

The importance of Leuconostoc mesenteroides is recognized for its contribution to taste in cultured dairy products. It has been cited for its potential role as a probiotic. Consumer demand exists for new dairy products with health benefits. In the manufacture of probiotic-products survival of Leuconostoc mesenteroides would depend on its ability to tolerate challenging processing and storage conditions. Improved viability by prior exposure to mild stresses can increase stress tolerance toward a more severe stress. Furthermore, it can result in cross-protection due to connection of several proteins in response to stresses. Objective was to evaluate the effect of prior exposure to various mild stress conditions on the survival of Leuconostoc mesenteroides in challenging conditions. Leuconostoc mesenteroides spp. cremoris Vivolac Cremosa CIT/FPC Series cells were subjected to four mild stresses (acid, heat, ethanol and oxidative). Each mild stress had three levels of intensity; low, medium and high. Then culture was subjected to challenging acid, heat or osmotic conditions. MRS Agar was used for plating. Plates were incubated aerobically (30°C 48 h). The experiments were repeated three times with duplicate readings. Data were analyzed as a RBD using the Glimmix procedure and Tukey mean separation with a level of significance of 0.05. Heat tolerance of Leuconostoc mesenteroides at 60 °C was significantly enhanced by subjecting the bacteria to acid mild treatments of pH 5.0, 4.5 and 4.0 and heat mild treatments of 30, 35 and 45 °C (P<0.05). Acid tolerance of Leuconostoc mesenteroides was not enhanced by application of any of the mild stresses studied when compared to their respective controls (P>0.05). However good survivability was achieved with the application of the acid mild stress having cell counts ranging from 9.36 -8.77 log CFU/mL. Leuconostoc mesenteroides survivability was not affected with the exposure to osmotic challenge (P>0.05). Furthermore, osmotic conditions promoted the growth Leuconostoc mesenteroides when compared to their initial
cell counts (P<0.05). The enhancement of its heat tolerance and the robustness displayed to the acid and osmotic challenging conditions has possible applications of *Leuconostoc mesenteroides* spp. *cremoris* in food products in which such challenging conditions are encountered.
ABBREVIATIONS

- ACC: Acid challenging condition
- AEMS: after exposure to mild stress
- AT: acid tolerance
- ATR: acid tolerance response
- GIT: gastro intestinal tract
- HCC: Heat challenging condition
- HSP’s: heat shock proteins
- LAB: lactic acid bacteria
- NFDM: non-fat dry milk
- OCC: Osmotic challenging condition
- pH\textsubscript{i}: internal pH
- ROS: Reactive Oxygen Species
CHAPTER 1: OVERALL INTRODUCTION

1.1 Leuconostoc mesenteroides ssp. cremoris

Leuconostoc mesenteroides ssp. cremoris is a Gram-positive lactic acid bacteria that belongs to the phylum Firmicutes (de Paula et al., 2014). These bacteria are non-motile, facultative anaerobe, non-spore forming, vancomycin-resistant, and they usually have a spherical shape and occur usually in pairs or chains (Vedamuthu, 1994).

In the dairy industry, the importance of Leuconostoc mesenteroides is widely recognized for their contribution to flavor and aroma in cultured dairy products (Hemme and Foucaud-Scheunemann, 2004). It is usually used in combination with Lactococcus (mixed strains) for increase the production of flavor compounds and improvement of the texture by gas production (Cogan and Jordan, 1994). Leuconostoc mesenteroides has a great economic importance in the dairy industry due to its capability of producing CO₂ from carbohydrates, flavor compounds (diacetyl, acetate and ethanol) in many cultured dairy products (Vedamuthu, 1994). Diacetyl is the primary source of aroma and flavor in cultured dairy products like sour cream, cultured buttermilk, creamery butter, dressed cottage cheese (Pack et al., 1967).

1.1.1 Aroma Production

The major characteristic related to the use of Leuconostoc is the production of diacetyl, acetate, and ethanol (Vedamuthu, 1994). The level of diacetyl to give the wanted aroma ranges from 1.6 to 4 ppm (Parker and Elliker, 1953). Aroma production can be considered possible when cell concentrations are about 5 × 10⁶ (Hemme and Foucaud-Scheunemann, 2004). Early studies have demonstrated that in order to metabolize citrate a low pH environment is required (Pack et al., 1967). Research shows that the enzyme (citrate permease) that facilitate the uptake of citrate is only active at pH below 6.0 (Harvey and Collins, 1962). That is true for those milk systems that
contain mixed cultures of *Leuconostoc* and *Lactococcus*. For milk systems containing pure *Leuconostoc* an optimal pH between 4.1 and 4.4 has been reported (Lundstedt and Corbin, 1983). Lundstedt and Corbin (1983) found that at pH below 5.2 up to 86% of the citrate present in the milk was consumed. *Leuconostoc mesenteroides* ssp. *cremoris* has shown to develop high aromatic products at pH adjusted below 4.24 (Vedamuthu, 1994). Citrate permease is an inducible enzyme, which works with the concentration of citrate in the solution. The natural concentration of citrate in milk is around 0.2%. The Code of Federal Regulation (CRF) allows the addition of 0.15% of citrate (Vedamuthu, 1994). Diacetyl can further be reduced by diacetyl reductase to acetoin and 2,3-butanediol if the level of citrate in milk decreased from its threshold (0.5%) (Pack *et al.*, 1967). Pack *et al.* (1967) also stated that cooling the cultured product below 7°C delays the activity of the diacetyl reductase.

### 1.1.2 Openness

*Leuconostoc* has been known for its activity in the openness of certain ripened-veined cheeses like; Blue Cheese and Stilton (Pedro Nieto-Arribas *et al.*, 2010). *Leuconostoc mesenteroides* creates small intracurd openings through the production of CO₂ which enhances the colonization of other specific microorganisms in these types of cheeses (Pedro Nieto-Arribas *et al.*, 2010). In pressed ripened cheeses like Edam and Gouda small opening on the surface are due to the CO₂ formation by *Leuconostoc* rather than by mechanical press (Vedamuthu, 1994). In order to achieve this effect, the cell concentration must range from $5 \times 10^6$ to $5 \times 10^7$ (Hemme and Foucaud-Scheunemann, 2004)

### 1.1.3 Probiotic Characteristics

Recently *Leuconostoc mesenteroides* has received attention for its potential role as a probiotic in dairy food products (Hemme and Foucaud-Scheunemann, 2004, de Paula *et al.*, 2014). To be
considered a probiotic culture, the microorganism must survive the gastrointestinal conditions of acid, bile and steep oxygen gradients present in the gastrointestinal tract (Capozzi et al., 2016). It must be able to colonize and adhere to the intestinal cells and it must present therapeutic benefits upon its consumption (Fontana et al., 2013). Also, probiotic cultures must possess the technological suitability to withstand the storage conditions and the ability to be produced in large scale (de Paula et al., 2014).

Few in vivo human studies have been made with Leuconostoc in comparison to those of Lactobacillus (de Paula, 2015). Leuconostoc mesenteroides ssp. cremoris has proven to be an extremely potent cytokine producer-10, IFN-γ, IL-12 (Riina et al., 2008). The anti-inflammatory effects these cytokines could aid in the treatment of inflammatory conditions such as ulcerative colitis, pouchitis, and irritable bowel syndrome (Riina et al., 2008). Giving evidence that it has a better clinical efficiency to enhance the response to allergies as well as in the protection against respiratory infections than the most common genera used for their probiotic properties (Lactobacillus, Lactococcus, Bifidobacterium, Propionibacterium and Streptococcus).

Epidemiological studies made on Leuconostoc mesenteroides strains show an effect in the reduction of acute diarrhea in children that consumed yogurt containing Leuconostoc compared to the product without it (Bhasin, 2002). The probiotic effects of Leuconostoc mesenteroides have also been studied in animals. After 4 days of feeding Leuconostoc mesenteroides, at high cell concentration($1 \times 10^8$ CFU/ml) to lactose-intolerant induced rats, diarrhea disappeared when compared to control in which rats continue to show diarrhea (de Paula, 2015).

Leuconostoc mesenteroides possess the ability to produce mannitol from fructose fermentation. This sugar is metabolized independently from insulin and it has its application in diabetic food products (von Weymarn et al., 2002).
Probiotic microorganisms are important for balancing the microbiota present in the GIT. They promote the integrity of the epithelial barrier and the development of mucosal integrity (de Paula et al., 2014). \textit{Leuconostoc mesenteroides} has shown to have antimicrobial properties against various pathogenic bacteria like \textit{Salmonella}, \textit{Shigella}, \textit{Vibrio}, \textit{E.coli} (Shona, 2008). Multiple antimicrobial compounds can be produced by \textit{Leuconostoc mesenteroides} such as carbon dioxide, ethanol and acetic acid (Hemme and Foucaud-Scheunemann, 2004). Furthermore, the bacteriocins produced by \textit{Leuconostoc mesenteroides} can be used as bio preservatives in food products (de Paula et al., 2014). Studies have shown a strong activity against \textit{Listeria monocytogenes} and other psychotropic bacteria. Multiple bacteriocins have been isolated and studied such as Leucocin and Mesentericin (de Paula, 2015).

1.2 Probiotic Products

The survival of probiotic bacteria during the processing of food products and the passage through the GIT depends on a wide range of variables such as; culture condition, food matrix’s physical and chemical characteristics, and processing conditions (de Paula et al., 2014). As being part of the food industry probiotics are commonly grown in high densities in synthetic media, they are dried by different means, added to the desired product and stored until human consumption (Corcoran et al., 2008).

The industry of probiotic dairy products has been growing during the last years due to the increasing consumption of these products and the availability of various strains with probiotic properties (de Paula, 2015). Some cheeses that have successfully worked as carriers for probiotics are brined-white cheese, Feta, Gouda, Emmental, Mozzarella, and Cheddar (Tamime, 2008). The production of probiotic dairy products represents a challenge for the microorganism itself. Since it is expected to survive and remain viable in the dairy product to represent some therapeutic value...
to the consumer (McMahon and Broadbent, 2008). Among the challenges a probiotic microorganism encounters in the manufacture of probiotic products the presence of chemical and physical agents, the co-existence and relationship with other organism, and as mentioned before the extended storage that can be for over 3 months (Tamime, 2008). These factors can represent physical and chemical challenges in the environment that can influence the probiotic viability and expected counts \((1 \times 10^6 \text{ CFU/ml})\). Moreover, the probiotic chosen should not produce metabolites that represent damage to the quality and standard of identity of the product it is used in (Muir, 2010). Nevertheless, some benefits have been found in the incorporation of probiotic in cheeses, in comparison to fermented milks like yogurt (McMahon and Broadbent, 2008). The complex matrix of protein and fat serves as protection for the probiotic microorganism to enhance its survival through harsh environments as well as the lower acidity levels. McMahon and Broadbent (2008) stated that low-fat cheeses would be a better delivery food for probiotics than yogurt because it allows the cells to better withstand the low pH conditions found in the stomach.

### 1.3 Stress Response Overview

During the food production process, bacteria may be exposed to different stresses such as cold, heat, and acid, bile, osmotic and oxidative among other stresses (Serrazanetti et al., 2013). Like all living organisms, bacteria have developed different defense mechanisms that enable their survival to these stresses (Corcoran et al., 2008). Stress may be defined as any alteration in the permissive environmental parameters that leads to a response by biological organisms (Panoff et al., 1998). LAB have developed defense mechanisms against stresses, enabling them to survive under deleterious growth conditions or sudden environmental changes. Understanding these mechanisms could be a mean to improve the robustness of strains to diverse stress conditions (D'Angelo et al., 2017). Some of the *Leuconostoc* species can survive for long periods of time in unfavorable
environments as diverse as sugar, oil or dairy products. They can remain viable for many years in contact surfaces (Hemme and Foucaud-Scheunemann, 2004).

1.4 Stress Cross-Protection

Some of the stress-induced genes seem to be strictly specific to a certain stress, while others can be induced by a variety of stresses and are thought to be general stress response genes, which are part of the “cross-protection” mechanisms (Drauzio, 2010). In this sense, the adaptive changes caused by one stress may make the organism more fit to resist the adverse effects of another type of stressor. Improved viability by exposure to mild stresses has been proved to increase stress tolerance and it may result in cross-protection due to the connection of several stress-induced proteins in the response to various stresses (van de Guchte et al., 2002). This is industrially used to increase survival and activity of starter cultures during and after manufacture (Parente and Cogan, 2004). Cross-protected organisms respond better to novel stressors at different levels (Hartke et al., 1995). The stress response pathways extensively overlap and are induced to various extents by the same environmental stresses. Bacterial cultures exposed to one stress may develop cross-protection against other stresses. This mechanism has received several names including: environmental adaptation, stationary phase protection, or cross-protection. Moreover, there is limited amount of information regarding the cross-protection response of Leuconostoc mesenteroides.
CHAPTER 2: EVALUATION OF *LEUCONOSTOC MESENTEROIDES*’S TOLERANCE TO HEAT CHALLENGING CONDITIONS

2.1 Review of Literature

2.1.1 Heat Tolerance

Research has demonstrated that bacteria possess the ability to resist and adapt to unfavorable environments and that survival through adverse conditions is regularly enhanced by the induction of a stress response (Kang *et al.*, 2015). One of the most studied responses is the one toward heat shock. It is characterized by the transient inductions of proteins and physiological changes that render the bacteria more fit to withstand more severe stress conditions.

Living cells respond to a abrupt increase in temperature by rapid induction of genes resulting in elevated levels of heat-shock proteins (HSPs), as a defense mechanism to safeguard survival (Carper *et al.*, 1987). The major HSPs, include chaperones DnaK, GroEL, and GroES, as well as the Clp family of proteins, are a main factors in protein quality control in both stressed and unstressed bacteria (Salotra *et al.*, 1995). Heat resistance to the induction general stress responses. HSP have various roles in cell physiology such as ribosome stability, temperature sensing, and control of ribosomal function (De Angelis and Gobbetti, 2004).

Other forms of environmental stress can induce a heat shock-like response in *Leuconostoc mesenteroides* (Salotra *et al.*, 1995). When bacteria were subjected to 10°C a response like the one observed on heat shock at 40°C was obtained. Exposure of *Leuconostoc mesenteroides* to 10°C led to a strong induction of GroEL and DnaK (Salotra *et al.*, 1995). The inductions of HSP by various stress indicate the reliance on chaperones as part of the general stress response in *L. mesenteroides*. (Salotra *et al.*, 1995).
2.1.2 Acid Tolerance

When lactic acid bacteria are subjected to acid stress one of the first mechanisms used by the bacteria to defend against the detrimental effects of acid is to induce the heat shock chaperones (De Angelis et al., 2001). A linking between acid stress and heat shock chaperones has been proved in several other Gram-positive bacteria (Cotter and Hill, 2003). Acid stress induced 33 proteins, among which DnaK, GroEL, and UV inducible proteins, were present in *L. lactis* ssp. *lactis* (Jayaraman et al., 1997). DnaK and GroEL are also induced following acid adaptation of *Lactobacillus delbrueckii* (Fernandez et al., 2008). Moreover, the acid stress response is characterized by a change in the composition of the fatty acids in the cell membrane making it more rigid to decrease the permeability in the cell. These mechanisms are triggered by the bacteria to promote homeostasis between the pH internal and external environment of the cell (Cotter and Hill, 2003).

2.1.3 Ethanol Tolerance

Several studies have demonstrated the changes in the phospholipid bilayer in the cell membrane when bacteria are in the presence of alcohols (Campos et al., 2009). Mild heat and ethanol exposure induce a similar stress responses in LAB and yeast (Piper, 1995). These responses are characterized by the strong induction of HPS’s by temperature above about 35°C or ethanol levels above of 4–6% (v/v) (Piper, 1995). Heat and ethanol stress cause alike changes to composition of the plasma membrane proteins, decreasing the levels of plasma membrane H⁺-ATPase proteins and inducing the plasma membrane-associated Hsp30 (Díez et al., 2017). The enhancement of proton efflux in the cell characterizes a significant demand in energy, but it still may help to counteract the negative effects for homeostasis of the increased membrane permeability that results from stress (Piper, 1995). Furthermore, the cross-protective effects of ethanol to enhance the
survival in heat stress conditions of *Leuconostoc mesenteroides* ssp. *cremoris* had not yet been reported.

### 2.1.4 Oxidative Tolerance

Oxidative stress refers to the production of reactive oxygen species (ROS) results in negative effects to the cell physiology (Condon, 1987). The four-electron reduction of O$_2$ to H$_2$O enables to the formation of reactive oxygen intermediates such as, superoxide radical anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (HO'). Oxygen and hydrogen peroxide can be responsible for the formation of HO via the Fenton and Haber-Weiss reactions (Condon, 1987). Oxygen can diffuse and reach potential targets and is more reactive with intracellular proteins than H$_2$O$_2$ or HO. Cellular components such proteins, lipids and DNA, are HO targets. In these situations, bacteria can encounter conditions in which the generation of free radicals is higher than the detoxification rate of the cells. These leads to the accumulation of toxic compounds like free radicals and peroxides in the cell (Dowds, 1994). Oxygen is not the stressor but its partial reduction to water results in the formation of reactive oxygen species (ROS) such as hydroxyl and hydrogen peroxide radicals (Corcoran *et al.*, 2008). The production of peroxides and free radicals induce damage in the cell that can affect macromolecules like lipids, proteins, and rupture in the DNA strands (Serrazanetti *et al.*, 2013). Also, the exposure to oxidative damage can cause changes in the DNA bases and subsequent complementarity can lead to mutation. Bacteria have developed different mechanisms to eliminate or prevent these detrimental effects. Under normal conditions the cellular defense mechanism destroy most of the ROS and the cell is constantly repaired, but when extensive oxidative damage takes place ATP depletions makes the cell collapse and die (Effie *et al.*, 2011).
Besides the detrimental effect of oxygen, aeration can have an important effect on the utilization of sugars during the fermentation process of food products (Serrazanetti et al., 2013).

The high levels of manganese in *Leuconostoc mesenteroides* help as a defense against endogenous oxygen and other ROS (Cogan and Jordan, 1994). Moreover, the cross-protective effects of oxidative stress use to enhance the heat stress conditions of *Leuconostoc mesenteroides* ssp. *cremoris* had not yet been reported.

### 2.2 Justification

Heat is an important induced stress which can occur to *Leuconostoc mesenteroides* during the manufacture of dairy products which require heat treatments such as cheeses and cheese dips. The production of flavor and aroma compounds of this microorganism has given its importance in the dairy industry. There has been a recent growing interest in the probiotic characteristics that have been attributed to *Leuconostoc mesenteroides*. The technological suitability of strains is important for their utilization in processed foods. The global market for probiotic products is predicted to reach US$ 44.9 billion by 2018 (Buriti et al. 2016). Therefore, the cellular response caused by heat stress is particularly important in dairy products for this LAB. An understanding of the heat resistance capacity of *Leuconostoc mesenteroides* to survive heat challenging conditions in the external media is thus of great importance. The exposure to different types of mild stress conditions has increased the resistance against the heat challenging conditions (cross-protection) in other LAB species. Increased resistance of *Leuconostoc mesenteroides* ssp. *cremoris* to heat challenging condition would enable the inclusion of this microorganism in more products without the need of microencapsulating or genetically modifying it. Making it a process friendly to the food industry when scaling up the production.
2.3 Hypothesis

- Whether the prior exposure of *Leuconostoc mesenteroides* ssp. *cremoris* to various types of mild stresses (acid, heat, ethanol and oxidative) can enhance its heat tolerance.

2.4 Objectives

- To study the influence of various types mild stress (acid, heat, ethanol and oxidative) at various levels on the enhancement of the heat tolerance of *Leuconostoc mesenteroides* ssp. *cremoris*.

- To define which type and level of mild stress was more helpful to improve the heat tolerance of *Leuconostoc mesenteroides* ssp. *cremoris*.

2.5 Materials and Methods

2.5.1 Experimental Design

Four mild stresses (acid, heat, ethanol and oxidative) were evaluated. Each mild stress had 3 levels of intensity; low, medium and high (pH 5.0, 4.5, 4.0; heat 25, 35, 45°C; ethanol 5, 10, 15% v/v; \( \text{H}_2\text{O}_2 \) 2.5, 5.0, 7.5mM v/v). Each type of mild stress was compared against a negative and a positive control. In the negative control the bacterial culture had no application of a mild stress and was directly exposed to the 60 or 70°C for 3 minutes (hereafter HCC). The positive control accounted for the time the bacterial culture was exposed to the mild stress treatment been evaluated without any level of stress being applied (accounting for the incubation time of the mild stress treatments). After each mild stress treatment, the culture was subjected to the HCC (60 or 70°C for 3 minutes). Counts were enumerated in MRS agar (30°C, 48h) at various time points. Before the application of the mild stress (time zero), after the exposure of the mild stress (AEMS) and immediately after the 60 or 70°C HCC). The experiments were repeated 3 times with duplicate readings. Data were analyzed as a complete block design with repeated measure over time.
2.5.2 Preparation of Media

2.5.2.1 Reconstituted NFDM (10%)

Non-fat dry milk was used as the culture media for all samples. A solution of 10% w/v of milk was prepared by dissolving 100 grams of Great Value® Nonfat dry milk (NFDM) (Walmart, Bentonville, AK) in 1L of distilled water. NFDM solution (700mL) was poured into clean Pyrex bottles and sterilized at 121°C for 20 minutes in an autoclave (AMSCO Scientific, Erie, PA). The bottles were tempered at 30°C for 12h in an aerobic incubator (GCA/ Precision Scientific Chicago, IL). For each treatment, sterile milk was aseptically transferred into sterile 250mL flasks.

2.5.2.2 Agar Preparation

MRS agar was used for the enumeration of all samples. It was prepared according to the manufacturer specifications as follow: 55 grams of MRS broth powder (Fisher Scientific, Fair Lawn, NJ) and 12 g of pure agar powder (Fisher Scientific, Fair Lawn, NJ) were diluted in 1L of distilled water, heating and mixing them in hot plate (Fisher Scientific, Fair Lawn, NJ) with a magnetic stirrer until the solution boiled. It was sterilized at 121°C for 20 minutes. MRS agar was kept in a water bath at 48°C until used.

2.5.2.3 Peptone Water

For all serial dilutions, a solution of 0.1% w/v of peptone water was prepared according to the manufacturer specifications dissolving 1g of peptone powder (BactoTM Peptone, Difco, Dickinson and Co., Sparks, MD) in 1L of distilled water. Peptone solution (9mL) was poured into clean test tubes and sterilized at 121°C for 20 minutes in an autoclave (AMSCO Scientific, Erie, PA).
2.5.3 Treatments and Protocols

2.5.3.1 Heat Challenging Condition (HCC)

The effect of various temperatures and times on the viability of *Leuconostoc mesenteroides* was assessed. The ideal HCC reduced the viability of the bacteria to a level low enough to observe a possible improvement in its resistance. The levels assessed were 60, 70, 80 and 90°C for 2 - 20 minutes. Preliminary studies showed that the treatment that best met the criteria presented above were 60 and 70°C for 3 minutes. Therefore, the HCC consisted of sterile reconstituted NFDM (135mL) that was aseptically transferred to sterilized 250mL Erlenmeyer flasks. 15mL of culture was inoculated into the heated milk and incubated at 60 or 70°C for 3 minutes in a water bath (Fisher Scientific, Fair Lawn, NJ).

2.5.3.2 Negative Control

Negative control culture was not pre-exposed to any mild stress conditions, instead it was directly exposed to the HCC. Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately 10^6 CFU/mL in reconstituted NFDM. A sample of 15mL of the *Leuconostoc mesenteroides* inoculum was immediately transferred to a 250mL Erlenmeyer flask containing 135mL of sterile NFDM (10%). The culture was incubated in a water bath at 60 or 70°C for 3 minutes. Bacterial counts were enumerated in MRS agar (30°C, 48h) at various time points. Counts were determined immediately before and after exposure to HCC.

2.5.3.3 Positive Control

The time that the culture was exposed to the mild stress was taken into consideration before exposing the bacteria to the HCC. Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and
inoculated at approximately $10^7$ CFU/mL in reconstituted NFDM. A sample of 15mL of *Leuconostoc mesenteroides* inoculum was transferred to a 250mL Erlenmeyer flask containing 135mL of sterile NFDM (10%). The culture was incubated for the time specified for each mild stress treatment (10 minutes for mild heat and 2h for acid, ethanol and oxidative mild stresses) at 30°C under aerobic conditions. After the time of exposure to the respective mild stress treatment, 15mL of the control culture was transferred to a 250mL Erlenmeyer flask containing 135mL of sterile NFDM (10%). The culture was incubated at 60 or 70°C for the HCC in a water bath. Counts were enumerated in MRS agar (30°C, 48h) at various time points. Before the application of the mild stress (time zero), after the exposure of the mild stress (AEMS) and immediately after the 60 or 70°C HCC.

**2.5.3.4 Acid Mild Stress Condition**

Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately $10^7$ CFU/mL in reconstituted NFDM. A sample of 15mL of the *Leuconostoc mesenteroides* inoculum was transferred to each of 3 different Erlenmeyer flasks containing 135mL of autoclaved reconstituted NFDM (10%) with modified pH levels of 5.0, 4.5 or 4.0. Culture was incubated for 2h at 30°C in aerobic conditions in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Control was left in unmodified autoclaved NFDM (10%) for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the 2h of acid mild stress treatment, 15mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135mL of NFDM (10%) for the HCC. The culture was incubated at 60 or 70°C for the HCC in a water bath. Counts were enumerated in MRS agar (30°C, 48h) at various
time points. Before the application of the mild stress (time zero), after the exposure of the mild stress (AEMS) and immediately after the 60 or 70°C HCC.

2.5.3.5 Heat Mild Stress Condition

Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately 10⁷ CFU/mL in reconstituted NFDM. A sample of 15mL of the *Leuconostoc mesenteroides* inoculum was transferred to each of 3 different Erlenmeyer flasks containing 135mL of autoclaved reconstituted NFDM (10%) heated in water baths to obtain the final temperature of 25, 35 or 45°C (after inoculation) for 10 minutes. Control was left in autoclaved NFDM (10%) for 10 minutes at 30°C. After the 10 minutes of heat mild stress treatment, 15mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135mL of NFDM (10%) for the HCC. The culture was incubated at 60 or 70°C for the HCC in a water bath. Counts were enumerated in MRS agar (30°C, 48h) at various time points. Before the application of the mild stress (time zero), after the exposure of the mild stress (AEMS) and immediately after the 60 or 70°C HCC.

2.5.3.6 Ethanol Mild Stress Condition

Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately 10⁷ CFU/mL in reconstituted NFDM (10%). 15mL of the *Leuconostoc mesenteroides* inoculum was transferred to each of 3 different Erlenmeyer flasks containing 135mL of autoclaved reconstituted NFDM (10%) modified with ethanol (200° proof) to obtain a 0, 5, 10 or 15% ethanol-modified milk (v/v). Ethanol mild stress treated bacteria were aerobically incubated for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Control was left in unmodified autoclaved
NFDM (10%) for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the ethanol mild stress treatment, 15mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135mL of NFDM (10%) for the HCC. The culture was incubated at 60 or 70°C for the HCC in a water bath. Counts were enumerated in MRS agar (30°C, 48h) at various time points. Before the application of the mild stress (time zero), after the exposure of the mild stress (AEMS) and immediately after the 60 or 70°C HCC.

2.5.3.7 Oxidative Mild Stress Condition

Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately 10^7 CFU/mL in reconstituted NFDM (10%). A sample of 15mL of the *Leuconostoc mesenteroides* inoculum was transferred to four different Erlenmeyer flasks containing 135mL of autoclaved reconstituted NFDM (10%) modified with hydrogen peroxide (9.77mM) to obtain a 0, 2.5, 5.0 or 7.5mM hydrogen peroxide-modified milk (v/v). These oxidative mild stress treatments were aerobically incubated for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Control was left in unmodified autoclaved NFDM (10%) for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the 2h of oxidative mild stress treatments, 15mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135mL of NFDM (10%) for the HCC. The culture was incubated at 60 or 70°C for the HCC in a water bath. Counts were enumerated in MRS agar (30°C, 48h) at various time points. Before the application of the mild stress (time zero), after the exposure of the mild stress (AEMS) and immediately after the 60 or 70°C HCC.
2.5.4 Sample Plating

Counts were enumerated in MRS agar (30°C, 48h) at various time points. Before the application of the mild stress (time zero), after the exposure of the mild stress (AEMS) and immediately after the 60 or 70°C HCC. Samples for bacterial counts were taken from the different time points specified above were serially diluted in sterile peptone water. A sample of 1mL was taken and aseptically poured into sterile petri dishes. MRS agar was poured over the sample. Inoculated plates were incubated aerobically at 30°C for 48 h and counted for data analysis.

2.5.5 Calculations

All the counts described above were transformed to a Survival percentage as previously done by (Flahaut et al., 1998, De Angelis et al., 2001, Wu et al., 2012) with slight modifications. Survival percentage was defined as

\[
\text{Survival (\%)} = \left[\frac{\log \left( \frac{\text{CFU}}{\text{mL}} \right) N_x}{\log \left( \frac{\text{CFU}}{\text{mL}} \right) N_0} \right] \times 100.
\]

Where \( N_x = \log \text{CFU/mL} \) of *Leuconostoc mesenteroides* at given time point of the HCC and \( N_0 \) as the log CFU/mL of the starting cell count (time zero). This ratio was multiplied by 100 to convert it to a percentage. It was use to compare the viability of the bacteria after each given time point in relation to its initial count (time zero).

2.5.6 Statistical Analysis

The type III test of fixed effects of the Glimmix procedure of the Statistical Analysis System (SAS 9.4) was used to detect differences between treatments. Tukey mean separation was used when difference between treatments were found. The level of significance was 0.05.

2.6 Results

Results are presented as 2 separate analyses. In the first analysis, all results were analyzed by each type of mild stress separately. The analysis enabled the examination of which level of mild stress
was better to enhance the heat tolerance within the type of mild stress analyzed. Each level of mild stress was compared against the negative and positive controls. All results presented in this study were transformed to a survival %. That took into consideration the log CFU/mL of bacteria that survived the 60 or 70°C HCC against their respective log CFU/mL of bacteria at the starting point. For the first analysis, only the CFU/mL after exposure to the HCC and the starting CFU/mL were taken into consideration to make the comparisons (calculations section 3.5.3.9).

In the second analysis, all the types of mild stresses used in the present study were compared against each other. Results enabled the overall comparison of which type of all the mild stresses used (acid, heat, ethanol or oxidative), level of mild stress (control, low, medium or high) and temperature of exposure enabled *Leuconostoc mesenteroides* to withstand better in the HCC. For this analysis, only the positive control. It was used as the base level of stress agent within each type of mild stress (pH 6.8 for acid, 30°C for heat, 0% OH for ethanol and 0mM H$_2$O$_2$ for oxidative). The negative control is no longer taken into consideration since its purpose was fulfilled with the first analysis. Some of the significant interactions among the different types of mild stresses, the levels of mild stress used and the temperature of the HCC are presented.

**2.6.1 Acid Mild Stress**

Figure 2.1 and Figure 2.2 show the results of the exposure to mild acid conditions prior to the exposure to the 60 and 70°C HCC respectively. The prior exposure to mildly acidified media enhanced the survival of *Leuconostoc mesenteroides* to the HCC of 60°C (P < 0.05). Exposing the bacteria to pH 5.0, 4.5 and 4.0 supported the survivability by 62, 62 and 55 % respectively in the 60°C HCC, while the survivability of the negative and the positive controls were 19 and 23 % respectively (Figure 2.1). The use of acid mild treatments did not enhance the survivability of *Leuconostoc mesenteroides* after subsequent exposure to the 70°C HCC (Figure 2.2).
Figure 2.1. Heat tolerance of *Leuconostoc mesenteroides* to 60°C heat challenging condition (HCC) with prior exposure to various levels of mild acid stress for 2h expressed as Survival % = \[\frac{(\log (CFU/mL) N)}{(\log (CFU/mL) N_0)}\] × 100. A-B Means with different letters represent significant differences (P < 0.05).

Figure 2.2. Heat tolerance of *Leuconostoc mesenteroides* to 70°C heat challenging condition (HCC) with prior exposure to various levels of mild acid stress for 2h expressed as Survival % = \[\frac{(\log (CFU/mL) N)}{(\log (CFU/mL) N_0)}\] × 100. A-B Means with different letters represent significant differences (P < 0.05).

### 2.6.2 Heat Mild Stress

Figure 2.3. shows the survivability of *Leuconostoc mesenteroides* to the 60°C HCC when previously exposed to mild heat treatments. Incubating the bacteria at 35 and 45°C enhanced the survivability when compared to the negative control and at 25°C (P < 0.05) Figure 2.3. No
significant differences between the positive control, 35 and 45°C treatments were observed. (P > 0.05). Incubating the bacteria at temperatures at/above its optimal growth temperature (30°C) up to 45°C helped the bacteria to improve its tolerance to HCC (P < 0.05) (Figure 2.3). Incubating the bacteria at 25°C was significantly lower than the effect of the negative control (Figure 2.3). Incubating the bacteria below its optimal condition was more detrimental than exposing the bacteria directly to the challenging condition without any previous mild stress treatment (P < 0.05) (Figure 2.3). However, viability was not improved with the prior exposure to mild heat treatments when bacteria was exposed to the 70°C HCC (Figure 2.4).

Figure 2.3. Heat tolerance of *Leuconostoc mesenteroides* to 60°C heat challenging condition (HCC) with prior exposure to various levels of mild heat stress for 10 minutes expressed as Survival % = [(log (CFU/mL) N)/ (log (CFU/mL) N₀)] × 100.

A-C Means with different letters represent significant differences (P < 0.05).

### 2.6.3 Ethanol Mild Stress

Counts of *Leuconostoc mesenteroides* after HCC of 60 and 70°C with prior exposure to various ethanol levels can be found in Figure 2.5 and Figure 2.6 respectively. Survivability to the 60°C HCC was not improved by the exposure to any level of ethanol when compared to both controls (Figure 2.5). Ethanol was detrimental to the viability of *Leuconostoc mesenteroides*. Significant differences were found with the controls when compared to the ethanol treatments. (P < 0.05). The
exposure to the 70°C treatment was detrimental to the viability of Leuconostoc mesenteroides regardless of the ethanol treatments applied previously (Figure 2.6).

Survival (%) of Leuconostoc mesenteroides to the 70°C HCC after mild heat stresses

<table>
<thead>
<tr>
<th>Survival (%)</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Temperature</td>
<td>25°C</td>
<td>35°C</td>
<td>45°C</td>
<td>55°C</td>
<td>65°C</td>
</tr>
</tbody>
</table>

Figure 2.4. Heat tolerance of Leuconostoc mesenteroides to 70°C heat challenging condition (HCC) with prior exposure to various levels of mild heat stress for 10 minutes expressed as Survival % = \[
\frac{\log \left( \frac{CFU/mL}{N} \right)}{\log \left( \frac{CFU/mL}{N_0} \right)} \times 100
\]. A-B Means with different letters represent significant differences (P < 0.05).

Survival (%) of Leuconostoc mesenteroides to the 60°C HCC after mild ethanol stresses

<table>
<thead>
<tr>
<th>Survival (%)</th>
<th>19%</th>
<th>21%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A</td>
<td></td>
<td>B</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>Concentration</td>
<td>5% Ethanol</td>
<td>10% Ethanol</td>
<td>15% Ethanol</td>
<td>20% Ethanol</td>
<td>25% Ethanol</td>
</tr>
</tbody>
</table>

Figure 2.5. Heat tolerance of Leuconostoc mesenteroides to 60°C heat challenging condition (HCC) with prior exposure to various levels of mild ethanol stress for 2h expressed as Survival % = \[
\frac{\log \left( \frac{CFU/mL}{N} \right)}{\log \left( \frac{CFU/mL}{N_0} \right)} \times 100
\]. A-B Means with different letters represent significant differences (P < 0.05).
Survival (% of *Leuconostoc mesenteroides* to the 70°C HCC after mild ethanol stresses)

<table>
<thead>
<tr>
<th>Survival (%)</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
<th>0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration of ethanol used as mild stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (No mild trt)</td>
</tr>
<tr>
<td>Positive control (0% ethanol incubation)</td>
</tr>
<tr>
<td>5% Ethanol</td>
</tr>
<tr>
<td>10% Ethanol</td>
</tr>
<tr>
<td>15% Ethanol</td>
</tr>
</tbody>
</table>

Figure 2.6. Heat tolerance of *Leuconostoc mesenteroides* to 70°C heat challenging condition (HCC) with prior exposure to various levels of mild ethanol stress for 2h expressed as Survival \[\% = \left(\frac{\log (CFU/mL N)}{\log (CFU/mL N_0)}\right) \times 100\].

A-B Means with different letters represent significant differences (P < 0.05).

### 2.6.4 Oxidative Mild Stress

Counts of *Leuconostoc mesenteroides* after the HCC of 60 and 70°C can be found in Figure 2.7 and 2.8 respectively. Both Figures show that regardless of the treatment used there was no significant improvement in viability of *Leuconostoc mesenteroides* to the HCC 60 or 70°C with the prior use of the various H$_2$O$_2$ concentrations when compared to either of the controls (Figure 2.7).

### 2.6.5 Comparison of Main Effects and Interactions

This second part of the analysis consists of the comparison of all the types and levels of mild stresses to identify which were the best treatments to improve the viability of *Leuconostoc mesenteroides* when exposed to the HCC condition of 60°C or 70°C. For this analysis, only the positive control was used (hereafter control). This change in the analysis allows using the positive control as a level within the types of mild stresses being compared. The negative control is no longer considered. The main effects and their interactions can be found in Table 2.1. The most relevant effects and interactions will be discussed.
### Figure 2.7. Heat tolerance of *Leuconostoc mesenteroides* to 60°C heat challenging condition (HCC) with prior exposure to various levels of mild oxidative stress for 2h expressed as \( \text{Survival } \% = \left[ \frac{\log (CFU/mL) N}{\log (CFU/mL) N_0} \right] \times 100 \).

A-B Means with different letters represent significant differences (P < 0.05).

### Figure 2.8. Heat tolerance of *Leuconostoc mesenteroides* to 70°C heat challenging condition (HCC) with prior exposure to various levels of mild oxidative stress for 2h expressed as \( \text{Survival } \% = \left[ \frac{\log (CFU/mL) N}{\log (CFU/mL) N_0} \right] \times 100 \).

A-B Means with different letters represent significant differences (P < 0.05).

#### 2.6.5.1 Type of Mild Stress

The counts of *Leuconostoc mesenteroides* after the application of the HCC show significant differences depending on which of the type of mild stresses was previously applied (P < 0.05) (Table 2.1). Figure 2.9 compares the survival (%) of *Leuconostoc mesenteroides* to the HCC of both 60 and 70°C depending on the type of mild stresses used previously. Acid and heat were the best treatments to aid in the viability of *Leuconostoc mesenteroides* to the HCC. They showed a
significant improvement when compared to ethanol and oxidative mild stresses (P < 0.05) (Figure 2.9).

Table 2.1. Probability for main effects and their interaction on the heat tolerance of *Leuconostoc mesenteroides* ssp. *cremoris* when exposed to 60 or 70°C with prior exposure to various types and levels of mild stresses.

<table>
<thead>
<tr>
<th>Main Effects</th>
<th>P-Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Mild Stress</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Level of Mild Stress</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HCC Temperature</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Type of Mild Stress × Level of Mild Stress</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Type of Mild Stress × HCC Temperature</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Level of Mild Stress × HCC Temperature</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Type of Mild Stress × Level of Mild Stress × HCC Temperature</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

*P-values less than 0.05 represent significant effect.

![Comparison of the effect of various mild stresses on the survival of *Leuconostoc mesenteroides* to the HCC](image)

Figure 2.9. Heat tolerance of *Leuconostoc mesenteroides* with prior exposure to various types of mild stresses expressed as $\text{Survival} \% = \frac{\log(CFU/mL) N}{\log(CFU/mL) N_0} \times 100$. A-B Means with different letters represent significant differences (P < 0.05).

2.6.5.2 HCC Temperature

The temperature of exposure to the HCC had a significant effect on the survival of *Leuconostoc mesenteroides* (P < 0.05) (Table 2.1). The average survivability to 60°C HCC was of 26% whereas the 70°C was completely lethal to the bacteria, regardless the use of various types of mild stress (Figure 2.10).
Figure 2.10. Average survival of *Leuconostoc mesenteroides* to the 60 and 70°C heat challenging conditions (HCC) expressed as Survival % = \[(\log(CFU/mL) N)/\log (CFU/mL) N_0)\] × 100.

A-B Means with different letters represent significant differences (P < 0.05).

2.6.5.3 Type of Mild stress × Level of Mild Stress × HCC Temperature

Cell counts of *Leuconostoc mesenteroides* after the HCC were affected depending upon the type of mild stress being applied at a specific level of mild stress and the temperature of the HCC (Table 2.1). The 3-way interaction between Type of Mild Stress× Level of Mild Stress × Temperature of HCC had a significant effect on the survivability of *Leuconostoc mesenteroides* ssp. cremoris (P < 0.05) (Table 2.1).

Table 2.2 shows that the viability of *Leuconostoc mesenteroides* was substantially improved when exposed to mild heat of 30, 35, 45°C and pH 4.0, 4.5 and 5.0 prior to the exposure to the 60°C HCC. This holds true when compared to the acid control, heat 25 °C, all ethanol levels and all levels of hydrogen peroxides (P < 0.05). The survivability of *Leuconostoc mesenteroides* exposed to the best treatments range between 63-55% (Table 2.2). However, the same positive results observed with these mild stresses were not observed when the bacteria were subjected to 70°C HCC, instead no mild stress could improve the viability of *Leuconostoc mesenteroides* when exposed to it (Table 2.2).
2.7 Discussion

Tolerance to heat is important because *Leuconostoc* spp. may undergo a processing under heat conditions. In the processing of milk for cheese, *Leuconostoc mesenteroides* which is preferably heat resistant can be used as adjunct cultures to improve flavor development (Hemme and Foucaud-Scheunemann, 2004). Also, the improvement in its heat tolerance could enable its incorporation in new probiotic products such as a processed cheese dips. As with other LAB, *Leuconostoc mesenteroides* responds to stresses by regulating the production of various heat, acid, cold or oxidative shock proteins, including chaperonins and proteases among others (Hemme and Foucaud-Scheunemann, 2004).

Table 2.2. Comparison of the heat tolerance of *Leuconostoc mesenteroides* to the interaction of Type of Mild Stress × Level of Mild Stress × Temperature of Heat Challenging condition expressed as Survival % = [(log(CFU/mL) N)/(log (CFU/mL) N₀)] × 100.

<table>
<thead>
<tr>
<th>Type of mild stress</th>
<th>Level of mild stress</th>
<th>Survival (%) 60°C mean ± std. error</th>
<th>Survival (%) 70°C mean ± std. error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C (control)</td>
<td>62.6 ± 4.4 A</td>
<td>ND*D</td>
</tr>
<tr>
<td>HEAT (10 minutes)</td>
<td>25°C</td>
<td>ND*D</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>35°C</td>
<td>54.5 ± 1.3 A</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>45°C</td>
<td>61.8 ± 3.8 A</td>
<td>ND*D</td>
</tr>
<tr>
<td>pH 6.8 (control)</td>
<td></td>
<td>23.0 ± 7.5 B</td>
<td>ND*D</td>
</tr>
<tr>
<td>ACID (2h HCL)</td>
<td>pH 5.0</td>
<td>61.8 ± 5.0 A</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>pH 4.5</td>
<td>62.2 ± 3.5 A</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>pH 4.0</td>
<td>55.0 ± 2.4 A</td>
<td>ND*D</td>
</tr>
<tr>
<td>ETHANOL (2h ethanol)</td>
<td>0% (control)</td>
<td>20.7 ± 14.6 BC</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>ND*D</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>ND*D</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>15%</td>
<td>ND*D</td>
<td>ND*D</td>
</tr>
<tr>
<td>OXIDATIVE (2h H2O2)</td>
<td>0mM (control)</td>
<td>16.7 ± 12.1 BCD</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>2.5mM</td>
<td>ND*D</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>5mM</td>
<td>ND*D</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>7.5mM</td>
<td>ND*D</td>
<td>ND*D</td>
</tr>
</tbody>
</table>

A-D Means with different letters represent significant differences (P < 0.05). ND* = Non-detectable counts.
The defense mechanisms against heat shock tries to minimize damage, mainly of protein denature (De Angelis et al., 2004). Studies show that heat shock induces a 2- to 100-fold increase in mRNA levels and a 2- to 3-fold increase in protein levels of heat shock induced genes in L. lactis (Kang et al., 2015). In addition, exposure to mild heat temperatures of several LAB cells improves their survival upon a lethal temperature challenge, showing that these cells can trigger a protective heat shock response (Papadimitriou, 2016). The exposure of Leuconostoc mesenteroides to mild heat treatments above its optimal growth temperature (30 °C) up to 45°C improved its survivability to the HCC of 60°C but not to the 70°C (Figure 2.3 and Figure 2.4). Similarly, in an experiment conducted by Kang et al. (2015) L. lactis HE-1 were treated at 37, 42, 47, and 52°C for 15 minutes. The mildly heat-treated cells were exposed to 60°C for 10 minutes to assess the effect of heat on survival. Among the tested temperatures, 42°C was the optimal for heat adaption (Kang et al., 2015).

An increase from 26°C to 37°C in the temperature of the bacterial culture led to an overexpression of HSPs 70 and 60 in Leuconostoc mesenteroides (Salotra et al., 1995). This could help understand why such a difference in survivability (%) was achieved when the bacteria were incubated at temperatures above 30 °C. The conservation of the structure of HSPs 70 and 60 among prokaryotes supports that HSPs perform vital functions for cell survival, particularly under stress (Barnes et al., 1990). HSP60 is induced in the presence of denatured proteins to bind intracellular proteins and protect them from denature (Salotra et al., 1995).

Heat shock resistance of bacteria differs based in genetic differences between species, the physiological state of the cells, and chemical and physical factors such as pH, water activity, salt content, and preservatives (Browne and Dowds, 2001). When cells of L. plantarum DPC2739 were subjected to adaptation at 42°C for 1 h, the thermotolerance increased by 3 logs compared to the
thermotolerance of non-adapted cells (control). The resistance to heat of *L. plantarum* DPC2739 depended mainly on induction of protein synthesis (De Angelis *et al.*, 2004). Tolerance to 72°C for 90 s decreased noticeably when a bacteriostatic chloramphenicol (1 mg/L) was added during adaptation. 2DE analyses displayed that there were increases in the levels of expression of 31 and 18 proteins of adapted mid-exponential- and stationary-phase cells of *L. plantarum* DPC2739, respectively, when compared to the controls (De Angelis *et al.*, 2004).

When lactic acid bacteria are subjected to acid stress one of the first mechanisms used by the bacteria to defend against the detrimental effects of acid is to induce the heat shock chaperones (De Angelis *et al.*, 2001). The effects of the acid mild treatments were significantly better to the survival of *Leuconostoc mesenteroides* to the 60°C HCC (Figure 2.1) The best treatments to improve the viability of *Leuconostoc mesenteroides* were the prior exposure to mild heat and acid when exposed to the 60°C HCC (Figure 2.9). Exposing the bacteria to pH 5.0, 4.5 and 4.0 for 2h was as beneficial as exposing the bacteria to temperatures above 30 through 45°C for ten minutes prior to the 60°C HCC (Table 2.2). A strong connection in the response and synergy of these 2 stresses has been reported (van de Guchte *et al.*, 2002, Corcoran *et al.*, 2008). The results of the present study are in similar to the ones in a study conducted by Zotta *et al.* (2008) in which acid and heat adaptation of most *S. thermophilus* strains enhanced the survival of heat stressed cells compared to control (Zotta *et al.*, 2008).

Studies have shown that bacterial growth is greatly affected by solvents. Synthesis of heat shock proteins such as GroES and GRoEL have been reported to be induced by high solvent concentrations (Salotra *et al.*, 1995). The response of cells to heat shock and alcohols show similarities, both stresses alter the fluidity of the cell membrane (Campos *et al.*, 2009).
A synergy between heat and ethanol-induced damages has been reported, and that this synergy results in the adverse influences of ethanol being more severe at higher temperatures and vice versa (Piper, 1995). Ethanol toxicity is generally attributed to the disruption of membrane structure (Campos et al., 2009). However, both heat shock and ethanol exposure will cause, in addition to membrane disordering, increases in protein denaturation. The viability in the HCC of 60 or 70°C was not improved by the used of ethanol concentrations from 5-15% v/v or hydrogen peroxide from 2.5-7.5mM when compared to the results of some acid and heat mild treatments (Figure 2.10 and Table 2.2). HSP synthesis in *Leuconostoc mesenteroides* was found to be stimulated in response to ethanol treatment (Salotra et al., 1995). Addition of ethanol (4% v/v) resulted in a relative overexpression of 70- and 60-kDa proteins with a reduction in total protein synthesis (Salotra et al., 1995). Although the expression of genes was demonstrated in their study cross-protection was not evaluated. Ethanol induces HSPs in diverse organisms as *E. coli*, yeast and mammalian cells (Piper, 1995). The genes induced in various LAB and some yeast strains by ethanol seem to be mostly identical to those induced by heat shock (Piper, 1995). The threshold concentration for ethanol to cause appreciable heat shock protein induction in vegetative yeast cultures growing at 25°C is between 4% and 6% (v/v) (Piper, 1995). However, none of those beneficial effects could be observed by using the concentrations tested in the present study (Table 2.2). Phenolic compounds are known to affect the cell membrane leading to leakage of cell constituents such as proteins, nucleic acids, and inorganic ions such as potassium or phosphate. These compounds may diffuse through the membrane rising its permeability (Denyer and Hugo, 1991).

The levels of oxidative stress were detrimental to the survivability of *Leuconostoc mesenteroides* (Table 2.2) for both 60 and 70°C HCC when compared to some acid and heat mild stresses. The
low survival upon exposure to oxidative stress may be due to the harshness of the stress used. H$_2$O$_2$ is a weak oxidant, but it is exceedingly diffusive and has a long lifetime (Jaroni and Brashears, 2000). The H$_2$O$_2$ contributes to oxidative damage either directly or as a precursor of hydroxyl radicals. It especially potent in causing oxidative damage to DNA (Kang et al., 2015).

The detrimental effects of oxidative stress are comparable to those obtained by D'Angelo et al. (2017) evaluated 29 strains of *Leuconostoc* spp. (*lactis, mesenteroides, pseudomensenteroides and citreum*) for their resistance against a single level of each stress including oxidative, heat, acid, alkaline, osmotic stresses (D'Angelo et al., 2017). The strains studied showed a wide variability in stress resistance especially for temperature, acidic and oxidative stress factors (D'Angelo et al., 2017). In their results, oxidative stress was profoundly detrimental to the survivability of several *Leuconostoc* strains. In their experiments *Leuconostoc* strains were subjected to an oxidative shock of 0.3% (H$_2$O$_2$) for 30 minutes. However D'Angelo et al. (2017), found in their study that there was no cross-protection effect between heat and oxidative stress. Dowds (1994) found that oxidative response appeared to be coupled to the synthesis of 2 major heat shock proteins (DnaK and GRoEL chaperons). He explained that the cross protection between oxidative and heat stress take place in a phase dependent manner but that in their experiment that relationships did not took place in *Bacillus subtilis* when exposed to heat shock of 48°C. (Dowds, 1994)

The cell morphology of cells exposed to mild heat treatments and non-exposed *L. lactis* HE-1 cells were analyzed by SEM before and after heat treatment (Kang et al., 2015). After heat treatment (60 °C), the cells exposed to the optimal heat adaptation temperature (42°C ) displayed stiffer envelopes and fissured surfaces than did the cells that were exposed to non-optimal temperatures (37 and 52°C ) (Kang et al., 2015).
2.8 Conclusions

Our findings highlight the need to take into consideration the technological properties of probiotic strains for their successful incorporation in processing conditions. The heat tolerance of *Leuconostoc mesenteroides* ssp. *cremoris* was improved by prior exposure to temperatures between 30-45°C which led to improved viability (63-55%) upon heat shock at 60°C for 3 minutes. In addition, we observed cross-protection in *Leuconostoc mesenteroides* to heat treatment at 60°C induced by acid pH 5.0, 4.5 and 4.0. The results of this study suggest that the heat tolerance of *Leuconostoc mesenteroides* ssp. *cremoris* involved heat shock and general stress responses which were successfully triggered by heat and acid mild stresses. Nonetheless the viability upon exposure to 70°C was not improved by the prior exposure to any of the mild stress conditions evaluated in the present study. In this respect, if incorporation in the manufacture of processed cheese dips is to be chosen, determining thermostolerance parameters and the implementation of cross-protection techniques should be useful for predicting the behavior of the probiotic strains during subsequent processing.
CHAPTER 3: EVALUATION OF *LEUCONOSTOC MESENTEROIDES*'S TOLERANCE TO ACIDIC CHALLENGING CONDITIONS

3.1 Review of Literature

3.1.1 Acid Tolerance

Acid has been used for its food preservation properties for a long time, as in food fermentations. The fermentation development encompasses the metabolization of carbohydrates to produce various products including organic acids, alcohol, and carbon dioxide (Cotter and Hill, 2003). These products can limit the growth of spoilage and pathogenic microorganisms in the food product. Acids have good antimicrobial activity because in their undissociated form they can move through the cell membrane (Fernandez *et al.*, 2008). Since the pH is commonly higher than that of the growth medium, the weak acids dissociate and release protons leading to acidification of the cytoplasm (De Angelis *et al.*, 2001). For their effectiveness in preventing bacterial growth, acids are commonly added directly to foods for their preservation (De Angelis *et al.*, 2001).

The influence of pH stress has been well characterized in bacteria (Corcoran *et al.*, 2008). Since LAB produce large amounts of lactic acid to reduce the pH response to acid stress has become readily predictable. Damage is eased by a series of metabolic changes to maintain homeostasis between the internal and external pH (Weimer, 2011). Prior exposure to mild levels of acid induces bacterial survival many microbes (Effie *et al.*, 2011). In *lactococci*, prior exposure to acid improves the survival to almost 100% for a subsequent exposure to a higher level of acid for longer time (Corcoran *et al.*, 2008).

In LAB, acid tolerance (hereafter AT) upsurges in at least 2 different physiological states. During logarithmic growth, an adaptive response called L-ATR can be induced by incubation at a mild acidic pH. The other state is after entry into the stationary phase, because there is an induction of
general stress response (Hartke et al., 1995). Most of the LAB species tested possess an L-ATR (Hartke et al., 1995). The induction of the L-ATR can protect the bacteria not only from acid stress but also from heat, osmotic or oxidative shocks (Corcoran et al., 2008, Papadimitriou, 2016). This protective effect of L-ATR can vary among species and does not always protect from the same stress (Quivey et al. 1995, Flahaut et al. 1996). Therefore, efforts have been made to improve the robustness of bacteria to these kinds of stress, especially for probiotic microorganisms.

The sensing of mild acidification can prevent the potentially lethal consequences of acidic conditions in bacteria (Cotter and Hill, 2003). Bacteria that are warned in advance by exposure to mild acidified media can be better prepared through the induction of a variety of protection mechanisms. These include mechanisms that change the cell membrane composition, extrude protons, protect macromolecules, modify metabolic pathways, and generate alkalis (Fernandez et al., 2008). When bacteria are subjected to acid shock without warning, they are forced into an action which involves a heavy reliance on proton pumps, most notably the F1F0-ATPase, in order to maintain the internal pH (hereafter pHi) stable long enough to allow the induction of supplementary mechanisms (Wu et al., 2012).

An understanding of the acid resistance capacity of Leuconostoc mesenteroides to survive challenging acidic conditions in the external media or in low-pH foods is thus of great importance.

3.1.2 Heat Tolerance

When cells are exposed to heat shock they induce the production of HSP’s which help with the correct folding of polypeptides, assembly of protein complexes, degradation and translocation of proteins (Carper et al., 1987).

Some of the most notable chaperones are DnaK, GroEL, GroES. The greatest negative effects of high temperature is protein denaturation, furthermore membranes and nucleic acids have also been
identified as cellular sites of heat injury (Barnes et al., 1990). Heat stress also effects the transmembrane proton gradient, resulting in a decrease in the pH. (Ferrando et al., 2016).

*Leuconostoc* species, commonly considered of significant commercial value in food industry for its production of flavor and aroma in various products, lately have been recognized for its potential as probiotic bacteria. In a study conducted by Salotra et al. (1995) the expression of HSPs in *Leuconostoc mesenteroides* in response to heat shock was evaluated. The study showed that the bacteria overexpressed DnaK and GroEL homolog of *E. coli* in response to heat shock, cold shock and chemical stress (Salotra et al., 1995). Although the heat response of *Leuconostoc mesenteroides* had been previously studied the cross-protection still has not been extensively evaluated. The effect of mild heat stress treatments on the enhancement of the resistance to acid challenging condition had not been evaluated prior to the present study.

### 3.1.3 Ethanol Tolerance

Ethanol is known to be an effective antimicrobial agent and to act at the lipid-water interface, altering the stability and integrity of bacterial cell membranes (Campos et al., 2009). Ethanol toxicity is generally attributed to the negative effect of ethanol on the cell membrane resulting in a loss of membrane integrity (Piper, 1995). The cross-protective effects of ethanol to enhance the acid conditions of *Leuconostoc mesenteroides* ssp. *cremoris* had not yet been studied.

### 3.1.4 Oxidative Tolerance

Microorganisms maintain a defense system in place against reactive oxygen species (ROS) such as superoxide and hydrogen peroxide, products from aerobic metabolism. These compounds have various targets in the cell. The primary damage is to the DNA, which leads to the productions of strand breaks and apurinic and apyrimidinic sites, also ROS can cause the oxidation of membrane lipids and inactivate enzymes (Flahaut et al., 1998). To repair the damage produced by oxidative
damage, aerobic bacteria have developed enzymatic components as scavengers for the reactive oxygen species and have acquired different DNA repair systems (Condon, 1987). It is known that oxidative stressed induced 2 stimulons one is the OxyR which is mainly triggered by hydrogen peroxide the other is the SoxRS which is in turn induced by the presence of superoxides. Studies indicate that LAB are like other organisms that have been investigated in that they respond to mild/sub-lethal concentrations of hydrogen peroxide by inducing a protective system which helps them survive concentrations of hydrogen peroxide which otherwise would be lethal (Condon, 1987). The cross-protection response of the oxidative stress against other stress factors has been investigated in several lactic acid bacteria (Condon, 1987). There is no information regarding the cross-protective effect of the concentrations of hydrogen peroxide being used in the present study regarding the acid tolerance of Leuconostoc mesenteroides ssp. cremoris.

3.2 Justification

Acid is an important environmental stress which can occur in Leuconostoc mesenteroides during fermentation of foods and beverages products. For probiotic Leuconostoc an acid environment is also encountered in the stomach after consumption, and the development of probiotics products renewed the interest in LAB survival in the digestive tract. Therefore acid stress and cellular damage are particularly important in dairy products for LAB. Specifically regarding probiotic functional food, the global market is predicted to reach US$ 44.9 billion by 2018 (Buriti et al. 2016). The technological suitability of strains is important for their utilization in low-pH foods. An understanding of the acid resistance capacity of Leuconostoc mesenteroides to survive acidic challenging conditions in the external media or in low-pH foods is thus of great importance. The exposure to different types of mild stress conditions has increased the resistance against the acid challenging conditions (cross-protection) in other LAB species. The increase resistance of


*Leuconostoc mesenteroides* ssp. *cremoris* to acid challenging condition would enable the inclusion of this microorganism in more products without the need of microencapsulating or genetically modifying it. Making it a process friendly to the food industry when scaling up the production.

### 3.3 Hypothesis

- Whether the prior exposure of *Leuconostoc mesenteroides* ssp. *cremoris* to various types of mild stresses (acid, heat, ethanol, oxidative) can enhance its acid tolerance.

### 3.4 Objectives

- To study the influence of various types mild stress conditions (acid, heat, ethanol, oxidative) at various levels on the enhancement of the acid tolerance of *Leuconostoc mesenteroides* ssp. *cremoris*.
- To define which type and level of mild stress was more helpful to improve the acid tolerance of *Leuconostoc mesenteroides* ssp. *cremoris*.

### 3.5 Materials and Methods

#### 3.5.1 Experimental Design

Four mild stresses (acid, heat, ethanol and oxidative) were evaluated. Each mild stress had 3 levels of intensity; low, medium and high (pH 5.0, 4.5, 4.0; heat 25, 35, 45°C; ethanol 5, 10, 15% v/v; H$_2$O$_2$ 2.5, 5.0, 7.5mM v/v). Each type of mild stress was compared against a negative and a positive control. In the negative control the bacterial culture had no application of a mild stress and was directly exposed to the acid challenging condition. The positive control accounted for the time the bacterial culture was exposed to the mild stress treatment been evaluated without any level of stress being applied (accounting for the time of exposure). After each mild stress treatment, the culture was subjected to the acid challenging condition (hereafter ACC) (pH 3.5 for 2h at 30°C).
Counts were determined before the application of the mild stress treatment (time zero), after the exposure to the mild stress treatment (AEMS), immediately after exposure to ACC (0hACID), 1 h after exposure to ACC (1hACID) and 2h after ACC (2hACID). The experiments were repeated 3 times with duplicate readings. Data were analyzed as a complete block design with repeated measures over time.

3.5.2 Preparation of Media

3.5.2.1 Reconstituted NFDM (10%)

Non-fat dry milk was used as the culture media for all samples. A solution of 10% w/v of milk was prepared by dissolving 100 grams of Great Value ® Nonfat dry milk (NFDM) (Walmart, Bentonville, AK) in 1L of distilled water. NFDM solution (700mL) was poured into clean Pyrex bottles and sterilized at 121°C for 20 minutes in an autoclave (AMSCO Scientific, Erie, PA). The bottles were tempered at 30°C for 12h in an aerobic incubator (GCA/ Precision Scientific Chicago, IL). For each treatment, sterile milk was aseptically transferred into sterile 250mL flasks

3.5.2.2 Agar Preparation

MRS agar (Fisher Scientific, Fair Lawn, NJ) was used for the enumeration of all samples. It was prepared according to the manufacturer specifications as follow: 55 grams of MRS broth powder (Fisher Scientific, Fair Lawn, NJ) and 12 g of pure agar powder (Fisher Scientific, Fair Lawn, NJ) were diluted in 1L of distilled water, heating and mixing them in hot plate (Fisher Scientific, Fair Lawn, NJ) with a magnetic stirrer until the solution boiled. It was sterilized at 121°C for 20 minutes. MRS agar (Fisher Scientific, Fair Lawn, NJ) was kept in a water bath at 48°C until used.

3.5.2.3 Peptone Water

For all serial dilutions, a solution of 0.1% w/v of peptone water was prepared according to the manufacturer specifications dissolving 1g of peptone powder (BactoTM Peptone, Difco,
Dickinson and Co., Sparks, MD) in 1L of distilled water. Peptone solution (9mL) was poured into clean test tubes and sterilized at 121°C for 20 minutes in an autoclave (AMSCO Scientific, Erie, PA).

3.5.3 Treatments and Protocols

3.5.3.1 Acid Challenging Stress Condition (ACC)

The effect of various acidity levels on the viability of *Leuconostoc mesenteroides* was assessed. The ideal ACC reduced the viability of the bacteria to a level low enough to observe a possible improvement in its resistance. The levels assessed were pH 2.5, 3.0, 3.5 and 4.5 from 30-120 minutes. Preliminary studies showed that the treatment that best met the criteria presented above was pH 3.5 (after bacterial inoculation) for 120 minutes.

Sterile NFDM (700mL) was acidified using 6N hydrochloric acid. Acidified milk (135mL) was aseptically transferred to sterilized 250mL Erlenmeyer flasks. 15mL of culture was inoculated into the acidified milk and incubated at 30°C for 2h in aerobic conditions.

3.5.3.2 Negative Control

Bacterial culture was not pre-exposed to any mild stress conditions, instead it was directly exposed to the ACC. Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately 10^6 CFU/mL in reconstituted NFDM. A sample of 15mL of the *Leuconostoc mesenteroides* inoculum was immediately transferred to a 250mL Erlenmeyer flask containing 135mL of sterile acidified NFDM (10%) to obtain a final pH of 3.5. The culture was incubated at pH 3.5 for 2h at 30°C under aerobic conditions in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Bacterial counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ)
(30°C, 48h) at various time points. Counts were determined immediately before exposure to ACC (time zero) and 2h after ACC (2hACID).

3.5.3.3 Positive Control

Accounted for the time that the culture was exposed to each type of mild before exposing the bacteria to the ACC. Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately 10^7 CFU/mL in reconstituted NFDM. A sample of 15mL of *Leuconostoc mesenteroides* inoculum was transferred to a 250mL Erlenmeyer flask containing 135mL of sterile NFDM (10%) at approximately pH 6.8. The culture was incubated for the time specified for each type of mild stress treatment (10 minutes for mild heat and 2h for acid, ethanol and oxidative mild stresses) at 30°C under aerobic conditions in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the time of exposure to the respective mild stress treatment, 15mL of the control culture was transferred to a 250mL Erlenmeyer flask containing 135mL of sterile acidified NFDM (10%) to obtain a final pH of 3.5. The culture was incubated at pH 3.5 for 2h at 30°C under aerobic conditions in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ) (30°C, 48h) at various time points. Before the application of the mild stress (time zero), after the exposure of the mild stress (AEMS), immediately after the inoculation in the challenging stress condition (0hACID), after 1 h of being exposed to challenging stress condition (1hACID) and 2h of exposure to the challenging stress (2hACID).

3.5.3.4 Acid Mild Stress Condition

Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately 10^7 CFU/mL in
reconstituted NFDM. A sample of 15mL of the *Leuconostoc mesenteroides* inoculum was transferred to each of 3 different Erlenmeyer flasks containing 135mL of autoclaved reconstituted NFDM (10%) with modified pH levels of 5.0, 4.5 or 4.0. Culture was incubated for 2h at 30°C in aerobic conditions in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Control was left in unmodified autoclaved NFDM (10%) for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the 2h of acid mild stress treatment, 15mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135mL of acidified NFDM (10%) for the ACC (pH of 3.5 for 2h). Counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ) (30°C, 48h) at various time points. Counts were determined before the application of the mild stress treatment (time zero), after the exposure to the mild stress treatment (AEMS), immediately after exposure to the ACC (0hACID), 1 h after the ACC (1hACID) and 2h after the ACC (2hACID).

### 3.5.3.5 Heat Mild Stress Condition

Pure culture of *Leuconostoc mesenteroides* ssp. cremoris Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately $10^7$ CFU/mL in reconstituted NFDM., a sample of 15mL of the *Leuconostoc mesenteroides* inoculum was transferred to each of 3 different Erlenmeyer flasks containing 135mL of autoclaved reconstituted NFDM (10%) heated in water baths to obtain the final temperature of 25, 35 or 45°C (after inoculation) for 10 minutes. Control was left in autoclaved NFDM (10%) for 10 minutes at 30°C. After the 10 minutes of heat mild stress treatment, 15mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135mL of acidified NFDM (10%) for the ACC (pH of 3.5 for 2h). Counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ) (30°C, 48h) at various time points. Counts were determined before the application
of the mild stress treatment (time zero), after the exposure to the mild stress treatment (AEMS), immediately after exposure to ACC (0hACID), 1 h after ACC (1hACID) and 2h after ACC (2hACID).

3.5.3.6 Ethanol Mild Stress Condition

Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately $10^7$ CFU/mL in reconstituted NFDM (10%). 15mL of the *Leuconostoc mesenteroides* inoculum was transferred to each of 3 different Erlenmeyer flasks containing 135mL of autoclaved reconstituted NFDM (10%) modified with ethanol (200° proof) to obtain a 0, 5, 10 or 15% ethanol-modified milk (v/v). Ethanol mild stress treated bacteria were aerobically incubated for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Control was left in unmodified autoclaved NFDM (10%) for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the ethanol mild stress treatment, 15mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135mL of acidified NFDM (10%) for the ACC (pH of 3.5 for 2h). Counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ) (30°C, 48h) at various time points. Counts were determined before the application of the mild stress treatment (time zero), after the exposure to the mild stress treatment (AEMS), immediately after exposure to ACC (0hACID), 1 h after ACC (1hACID) and 2h after ACC (2hACID).

3.5.3.7 Oxidative Mild Stress Condition

Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately $10^7$ CFU/mL in reconstituted NFDM (10%). A sample of 15mL of the *Leuconostoc mesenteroides* inoculum was
transferred to four different Erlenmeyer flasks containing 135mL of autoclaved reconstituted NFDM (10%) modified with hydrogen peroxide (9.77mM) to obtain a 0, 2.5, 5.0 or 7.5mM hydrogen peroxide-modified milk (v/v). These oxidative mild stress treatments were aerobically incubated for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Control was left in unmodified autoclaved NFDM (10%) for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the 2h of oxidative mild stress treatments, 15mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135mL of acidified NFDM (10%) for the ACC (pH of 3.5 for 2h). Counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ) (30°C, 48h) at various time points. Counts were determined before the application of the mild stress treatment (time zero), after the exposure to the mild stress treatment (AEMS), immediately after exposure to ACC (0hACID), 1 h after ACC (1hACID) and 2h after ACC (2hACID).

3.5.3.8 Sample Plating

Counts were determined before the application of the mild stress treatment (time zero), after the exposure to the mild stress treatment (AEMS), immediately after exposure to ACC (0hACID), 1 h after ACC (1hACID) and 2h after ACC (2hACID). Samples for bacterial counts were taken from the reconstituted NFDM for the different time points specified above and serially diluted in sterile peptone. A sample of 1mL was taken and aseptically poured into sterile petri dishes. MRS agar (Fisher Scientific, Fair Lawn, NJ) was poured over the sample. Inoculated plates were incubated aerobically at 30°C for 48 h and counted for data analysis.
3.5.3.9 Calculations

All the counts described above were transformed to a Survival percentage as previously done by (Wu 2012, De Angelis 2004, Flahaut 1998) with slight modifications. Survival percentage was defined as

\[
\text{Survival} \% = \frac{(\log (\text{CFU/mL}) N_x)/(\log (\text{CFU/mL}) N_0)}{\text{CFU/mL}} \times 100.
\]

Where \( N_x = \log \text{CFU/mL} \) of \( \text{Leuconostoc mesenteroides} \) at given time point (AEMS, 0hACID, 1hACID or 2hACID) and \( N_0 \) as the log CFU/mL of the starting cell count (time zero). This ratio was multiplied by 100 to convert it to a percentage. It was use to compare the viability of the bacteria after each given time point in relation to its initial count (time zero).

3.5.4 Statistical Analysis

The type III test of fixed effects of the Glimmix procedure of the Statistical Analysis System (SAS 9.4) was used to detect differences between treatments. Tukey media separation was used when difference between treatments were found. The level of significance was 0.05.

3.6 Results

Results are presented as 2 separate analysis. In the first analysis, all results were analyzed separately by type of mild stress. This helped to identify if the prior exposure to a given type of mild stress helped the bacteria to perform better upon the exposure to the ACC. Furthermore, the analysis enabled the examination of which level of mild stress was better to enhance the acid tolerance within the type of mild stress analyzed. Each level of mild stress was compared against the negative and positive controls. All results presented in this study were transformed to a survival % . That took into consideration the log CFU/mL of bacteria that survived the acid challenge condition against their respective log CFU/mL of bacteria at the starting point. For the first
analysis, only the CFU/mL after the 2h exposure of the bacteria to the ACC and the starting CFU/mL were taken into consideration to make the comparisons (calculations section 3.5.3.9).

In the second analysis, all the types of mild stresses used in the present study were compared against each other. Results enabled the overall comparison of which type of all the mild stresses used (acid, heat, ethanol or oxidative), level of mild stress (control, low, medium or high) and temperature of exposure enabled *Leuconostoc mesenteroides* to withstand better in the ACC. For this analysis, only the positive control. It was used as the base level of stress agent within each type of mild stress (pH 6.8 for acid, 30°C for heat, 0% OH for ethanol and 0mM H₂O₂ for oxidative). The negative control was no longer taken into consideration since its purpose was fulfilled with the first analysis. Some of the significant interactions among the different types of mild stresses, the levels of mild stress used and the time of exposure are presented. Besides the type mild stresses and the levels mild stress, the different times of exposure to the ACC were also taken into consideration to determine the survival (%).

3.6.1 Acid Mild Stress Condition

Survival (%) to the ACC with prior exposure to various levels of mildly acidified media is presented in Figure 3.1. Based on the survival (%), the prior exposure to pH 5.0 had a positive effect on the survival of *Leuconostoc mesenteroides* to the ACC when compared to the negative control (Figure 3.1). Moreover, the effect of pH 5.0 on the survival (%) of *Leuconostoc mesenteroides* was no different than the positive control (pH 6.8), pH 4.5 or pH 4.0 (Figure 3.1). *Leuconostoc mesenteroides’s* survivability was enhanced (P < 0.05) by the exposure to pH 5.0 for 2h prior to the ACC when compared to the negative control (absence of time of exposure to the mild stress treatment) (Figure 3.1).
Figure 3.1. Tolerance of *Leuconostoc mesenteroides* to acid challenging condition (ACC) with prior exposure to various levels of mild acid treatments for 2h expressed as *Survival % = [(log(CFU/mL) N)/(log (CFU/mL) N0)] × 100.*

A-B Means with different letters represent significant differences (P < 0.05).

### 3.6.2 Heat Mild Stress Condition

The survival (%) to the ACC with prior exposure to various levels of heat mild stress are presented on Figure 3.2. The counts of *Leuconostoc mesenteroides* after the ACC indicate that there was no effect in the application of the various levels of mild heat stress, the positive control or the negative control (P > 0.05) (Figure 3.2). None of the different levels of mild stresses used in the present experiment improved the survival when compared to either of the controls (Figure 3.2).

### 3.6.3 Ethanol Mild Stress Condition

The counts of *Leuconostoc mesenteroides* after the exposure to ACC with or without prior exposure to various levels of ethanol (v/v) are presented in Figure 3.3. When compared to the negative control, there were no significant differences (P > 0.05) in the survival (%) of *Leuconostoc mesenteroides* when exposed to 0, 5 and 10% ethanol (v/v) prior to the ACC. Hence, the exposure to 0, 5 and 10% ethanol (v/v) for 2h did not improve the tolerance of *Leuconostoc mesenteroides* to the ACC. However, a significantly harmful effect (P < 0.05) was found when the
bacteria was exposed to 15% ethanol for 2h prior to the acid challenge when compared to both types of controls, 5 and 10% ethanol (Figure 3.3). Ethanol (15% v/v) showed detrimental effects on the survival of the culture used. Overall the ethanol mild stress treatment did not help in the enhancement of the acid tolerance of *Leuconostoc mesenteroides*.

**Survival of Leuconostoc mesenteroides to ACC after heat mild stresses**

![Graph showing survival of Leuconostoc mesenteroides after various conditions](image)

Figure 3.2. Tolerance of *Leuconostoc mesenteroides* to acid challenging condition (ACC) with prior exposure to various levels of mild heat treatments for 10 minutes expressed as Survival % = \([\log(\text{CFU/mL}) N] / (\log (\text{CFU/mL}) N_0)\] × 100.

A-B Means with different letters represent significant differences (\(P < 0.05\)).

### 3.6.4 Oxidative Mild Stress Condition

The counts of *Leuconostoc mesenteroides* after the exposure to the ACC were affected using the different levels of the oxidative mild stress as shown on Figure 3.4. The application of the oxidative mild stress treatments was detrimental to the acid tolerance of *Leuconostoc mesenteroides* when compared to the controls (Figure 3.4). Both the controls (positive and negative) outperformed the mild stress treatments (\(P < 0.05\)). When looking at the effect of the positive control, Figure 3.4 shows that there were significant differences in the survival (%) when compared to the negative control and the exposure to oxidative mild stress (\(P < 0.05\)). The 2h of incubation at optimal
conditions without the application of any level of hydrogen peroxide helped *Leuconostoc mesenteroides* to perform better than the negative control and rendered better results than modifying the media with hydrogen peroxide to achieve levels of 2.5, 5.0 and 7.5mM. In a similar way, the negative control also showed a significant difference in the survival (%) when compared to the all the levels of hydrogen peroxide in the media. For the bacteria, it was better to be immediately exposed to the ACC than first going through the incubation period in the hydrogen peroxide-modified media but worse than the positive control as stated above (Figure 3.4). There were no significant differences (P > 0.05) among the use of different levels of hydrogen peroxide to improve the survival of the bacteria to the ACC (Figure 3.4). The use of 2.5, 5.0 and 7.5mM of hydrogen peroxide were all detrimental for *Leuconostoc mesenteroides*.

Figure 3.3. Tolerance of *Leuconostoc mesenteroides* to acid challenging condition (ACC) with prior exposure to various levels of mild ethanol treatments for 2h expressed as Survival % = \[
\frac{\log \left( \frac{CFU/mL}{N} \right)}{\log \left( \frac{CFU/mL}{N_0} \right)} \times 100
\]

Means with different letters represent significant differences (P < 0.05).
Survival of *Leuconostoc mesenteroides* to ACC after oxidative mild stresses

Figure 3.4. Tolerance of *Leuconostoc mesenteroides* to acid challenging condition (ACC) with prior exposure to various levels of mild ethanol treatments for 2h expressed as Survival % = \[
\left(\frac{\log(\text{CFU/mL} \, N)}{\log(\text{CFU/mL} \, N_0)}\right) \times 100.
\]
A-B Means with different letters represent significant differences (P < 0.05).

3.6.5 Comparison of Main Effects and Interactions

This second part of the analysis consists of the comparison of all the types and levels of mild stresses to identify which were the best treatments to improve the viability of *Leuconostoc mesenteroides* when exposed to the ACC. For this analysis, only the positive control was used (hereafter control). This change in the analysis allows using the positive control as a level within the types of mild stresses being compared. The negative control is no longer considered. The main effects and their interactions can be found in Table 3.1. The most relevant effects and interactions will be discussed.

Table 3.1. Probability values for main effects and their interaction on the acid tolerance of *Leuconostoc mesenteroides* ssp. cremoris when exposed to various types of mild stresses, levels of mild stress for 2h.

<table>
<thead>
<tr>
<th>Effect</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Mild stress</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Level of Mild Stress</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Time of exposure to the ACC</td>
<td>0.0206</td>
</tr>
<tr>
<td>Type Mild stress × Level of Mild Stress</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Type of Mild stress × Time of exposure to the ACC</td>
<td>0.0083</td>
</tr>
</tbody>
</table>
Table 3.1 continued

<table>
<thead>
<tr>
<th>Effect</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level of Mild stress × Time of exposure to the ACC</td>
<td>0.0887</td>
</tr>
<tr>
<td>Type of Mild stress × Level of Mild stress × Time of exposure to the ACC</td>
<td><strong>0.0009</strong></td>
</tr>
</tbody>
</table>

*P-values less than 0.05 represent significant effect.

3.6.5.1 Type of Mild stress

The counts of *Leuconostoc mesenteroides* after the application of the ACC show significant differences depending on which of the type of mild stresses was previously applied (P < 0.05) (Table 3.1). Figure 3.5 compares the survival (%) of *Leuconostoc mesenteroides* to the ACC depending on the type of mild stresses used previously. Acid significantly enhanced the survivability of *Leuconostoc mesenteroides* in the ACC when compared to heat, ethanol and oxidative mild treatment (Figure 3.5). Heat was different from oxidative but not different from ethanol. As seen in the Figure 3.5 oxidative showed the least viability of *Leuconostoc mesenteroides*. Oxidative was significantly less effective than any of the other mild stress treatments (P < 0.05).

**General comparison of the effect of various types of mild stresses on the survival of *Leuconostoc mesenteroides* to the ACC**

![Figure 3.5](image-url)

Figure 3.5. Acid tolerance of *Leuconostoc mesenteroides* with prior exposure to various types of mild stresses expressed as Survival % = [(log(CFU/mL) N)/(log (CFU/mL) N₀)] ×100.

A-C Means with different letters represent significant differences (P < 0.05).
3.6.5.3 Type of Mild stress × Level of Mild Stress × Time of Exposure to the ACC

Cell counts of *Leuconostoc mesenteroides* after the ACC were affected depending upon the type of mild stress being applied at a specific level of mild stress over the 2h of exposure (Table 3.1). The 3-way interaction between Type of Mild Stress × Level of Mild Stress × Time of Exposure to the ACC had a significant effect on the survivability of *Leuconostoc mesenteroides ssp. cremoris* (P < 0.05) (Table 3.1).

Table 3.2 shows that the acid tolerance of *Leuconostoc mesenteroides* was the same regardless of the levels of acid or heat used prior to the ACCs. The effect when *Leuconostoc* was exposed to mild acid stresses of pH 5.0, 4.5 or 4.0 and the control (pH 6.8) from 0 - 2h was the same as exposing it to mild heat levels of 25, 35 or 45°C or the control (30°C) for 10 minutes. However, changes in the survival (%) of *Leuconostoc mesenteroides ssp. cremoris* can be seen with the use of ethanol and hydrogen peroxide (Table 3.2). The effect of prior exposure to 15% ethanol (v/v) on the acid tolerance of *Leuconostoc mesenteroides* was detrimental at 2h of exposure when compared to the effects of all levels of acid and heat mild treatments on its acid tolerance (Table 3.2) *Leuconostoc mesenteroides ssp. cremoris* was able to withstand the ACC for 2h when previously exposed to 0, 5 and 10% ethanol v/v, but at 15% ethanol (v/v) the bacteria showed a decrease in survivability at the 2h of exposure to the acid challenge (pH 3.5 at 30°C) (Table 3.2).

In general, all levels of hydrogen peroxide used in this study (2.5, 5.0, 7.5mM) were detrimental to the viability of *Leuconostoc mesenteroides* when used prior to the ACC (Table 3.2). Except for the control (0mM H₂O₂ v/v), by the first h of the ACC all bacteria had died. The effect of oxidative stress was so detrimental to the survivability of *Leuconostoc mesenteroides* (P < 0.05) that at 7mM all cells had died by the time they were transferred to the ACC (Table 3.2). The 5mM hydrogen peroxide level was also detrimental to the survivability of *Leuconostoc mesenteroides* to a lesser
extent than that of the 7mM H₂O₂. When compared to all other types of mild stresses used and all the different levels evaluated, oxidative stress rendered the most detrimental effects on the survivability of *Leuconostoc mesenteroides* ssp. *cremoris* to the ACC (pH 3.5 for 2h at 30°C).

Table 3.2. Survivability of *Leuconostoc mesenteroides* expressed as Survival % =\[\left(\frac{\log(CFU/mL) N}{\log(CFU/mL) N_0}\right)\times 100\] upon the interactions between type of mild stresses × level of mild stress× time exposure to the acid challenging condition (ACC) after prior exposure to various types of mild stress treatments.

<table>
<thead>
<tr>
<th>Mild stress</th>
<th>Mild stress level</th>
<th>Time zero acid survival (%) mean ± std. error</th>
<th>1 h in acid survival (%) mean ± std. error</th>
<th>2h in acid survival (%) mean ± std. error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid (2h)</td>
<td>pH 6.8 (control)</td>
<td>86.1 ± 3.7A</td>
<td>86.6 ± 3.5A</td>
<td>93.0 ± 4.6A</td>
</tr>
<tr>
<td></td>
<td>pH 5.0</td>
<td>90.1 ± 13.5A</td>
<td>89.0 ± 14.9A</td>
<td>94.2 ± 9.7A</td>
</tr>
<tr>
<td></td>
<td>pH 4.5</td>
<td>88.5 ± 2.6A</td>
<td>95.2 ± 4.2A</td>
<td>92.2 ± 5.2A</td>
</tr>
<tr>
<td></td>
<td>pH 4.0</td>
<td>83.6 ± 4.7A</td>
<td>88.8 ± 5.3A</td>
<td>85.8 ± 4.9A</td>
</tr>
<tr>
<td></td>
<td>30°C (control)</td>
<td>78.8 ± 1.1A</td>
<td>77.8 ± 1.3A</td>
<td>80.8 ± 5.9A</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>79.0 ± 2.2A</td>
<td>77.1 ± 2.1A</td>
<td>74.0 ± 1.6A</td>
</tr>
<tr>
<td></td>
<td>35°C</td>
<td>80.0 ± 2.2A</td>
<td>77.5 ± 3.2A</td>
<td>76.5 ± 6.1A</td>
</tr>
<tr>
<td></td>
<td>45°C</td>
<td>81.5 ± 5.1A</td>
<td>76.6 ± 1.9A</td>
<td>76.8 ± 3.2A</td>
</tr>
<tr>
<td></td>
<td>OH 0% (control)</td>
<td>80.8 ± 13.8A</td>
<td>91.3 ± 1.3A</td>
<td>86.7 ± 7.6A</td>
</tr>
<tr>
<td></td>
<td>OH 5%</td>
<td>57.5 ± 41.2A</td>
<td>81.5 ± 3.6A</td>
<td>80.8 ± 2.1A</td>
</tr>
<tr>
<td></td>
<td>OH 10%</td>
<td>84.3 ± 4.9A</td>
<td>76.3 ± 10.6A</td>
<td>53.0 ± 39.6AB</td>
</tr>
<tr>
<td></td>
<td>OH 15%</td>
<td>67.8 ± 6.0A</td>
<td>49.2 ± 23.1AB</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>H₂O₂mM (control)</td>
<td>87.0 ± 6.8A</td>
<td>91.8 ± 2.0A</td>
<td>98.0 ± 6.8A</td>
</tr>
<tr>
<td></td>
<td>H₂O₂ 2.5mM</td>
<td>55.1 ± 39.0AB</td>
<td>ND*D</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>H₂O₂ 5mM</td>
<td>22.8 ± 32.5BC</td>
<td>ND*D</td>
<td>ND*D</td>
</tr>
<tr>
<td></td>
<td>H₂O₂ 7.5mM</td>
<td>ND*D</td>
<td>ND*D</td>
<td>ND*D</td>
</tr>
</tbody>
</table>

A–D Means with different letters represent significant differences (P < 0.05).
ND* = Non-detectable counts.
3.7 Discussion

When compared to the negative control the use of pH 5.0 improved the viability of *Leuconostoc mesenteroides* (Figure 3.1). The results obtained in the present study are similar to those reported by Fernandez *et al.* (2008). In which the prior exposure of *Lb. bulgaricus* to mild acid conditions of pH 4.3, 4.9 and 5.5 for 40 minutes enhanced it survival about 100- to 1000- fold in media with pH 3.8 for 40 minutes. Fernandez *et al.* (2008) reported that the best mild stress was pH 4.9. As in the present study pH 5.0 rendered the highest survival (%) when compared to the negative control (Figure 3.1). Moreover, in their results based on proteomic analysis of the acid tolerance response (ATR) of *Lb. bulagricus*, exposed previously to a pH 4.9 for 40 minutes an average of 21 distinct protein transcription spots were identified when compared to non-adapted cells (control) (Fernandez *et al.*, 2008).

Proteins that were in higher amounts in the acid mild treated culture samples were chaperone proteins such as GroES, GroEL, DnaK, GrpE, and ClpL (Fernandez *et al.*, 2008).

Adapted cells (30 minutes at pH 4.75) of *Lb. delbrueckii ssp. bulgaricus* were approximately 250- fold more tolerant to the challenging acid stress (30 minutes at pH 3.5) than control (De Angelis and Gobbetti, 2004). Cells adapted at pH 5.0 for 90 had a higher resistance to the acid challenge (1600-fold) compared to non-adapted cells (De Angelis *et al.*, 2001).

Growth is a self-limiting factor in bacteria and it is more noticeable in natural environment than when they are grown under controlled conditions. Natural stresses like acidity and starvation can caused by cell growth itself, while other stresses (e.g. temperature, osmotic shock or oxygen) are environmental stresses (Corcoran *et al.* 2008). Homeostasis of pH is essential for growth and survival of all biological cells, including *Leuconostoc* and it is achieved through the bacterial acidification of the external medium and maintenance of a neutral pH. *Leuconostoc* species have
demonstrated to be less efficient in maintaining a neutral pHi than other lactic acid bacteria. As the external pH decreased, the pHi of *Leuconostoc mesenteroides* decreases, in contrast to that of *Lactococcus lactis* (Hemme and Foucaud-Scheunemann, 2004). Growth stopped when pHi values of 5.4 and 5.7 were reached independently from the composition of the media (Hemme and Foucaud-Scheunemann, 2004). Moreover, studies have shown that good homeostasis capacity is species related (Hache *et al.*, 1999). *Leuconostoc mesenteroides* has a poor ability to maintain a neutral pHi in pH levels ≤ 4.0. At pH 4.0 the pHi of *Leuconostoc mesenteroides* dropped to 5.2 whereas *Leuconostoc lactis* could maintain a pHi of 6 (Hache *et al.*, 1999). This could explain why *Leuconostoc mesenteroides* was able to have a better performance at pH 5.0 than at 4.5 or 4.0 in the present study (Figure 3.1).

Cell membrane is an important element in the acid tolerance mechanism in lactic acid bacteria. The induction of genes involved in the production of fatty acid (fabH, fabI) were identified when various LAB were exposed to acidic conditions (Cotter and Hill, 2003). It has been reported that ATR changes the natural ratio of fatty acids in the cell membrane composition. These changes are characterized by an increased production of saturated fatty acids and a decrease in the production of unsaturated fatty acids. These types of modifications enhance the rigidity and impermeability of the cell membrane (Hemme and Foucaud-Scheunemann, 2004). Acids can passively diffuse through the cell membrane and then dissociate into protons to which the cell membrane is impermeable (Presser *et al.* 1997). The intracellular accumulation of protons may lower the pHi and thus affecting the proton motive force (pmf), that is used as an energy source in several transmembrane transport processes. The internal acidification also decreases the activity of enzymes and can damage proteins and DNA.
There were no significant differences in the survival of *Leuconostoc mesenteroides* by exposing the bacteria to the heat mild treatments when compared to both controls (Figure 3.2). *Lactobacillus helveticus* cells exposed to heat shocks between (37-48°C) resulted in a significant increase in expression of HSP’s but decreased rapidly between 10 and 20 minutes following the heat shock and remained at very low levels thereafter (van de Guchte et al. 2002). The exposure of *L. platarum* to heat (42°C for 1 h) exhibited higher growth at pH 5.0 when compared to control (non-adapted cells) (De Angelis et al., 2001). They performed a 2-dimensional gel electrophoresis for proteins induced by the control and the heat adapted cells. Results reveled changes in the level of expression of 31 proteins in mid-exponential phase and 18 in stationary phase (De Angelis et al., 2001). Twelve of these proteins were commonly induced by heat stress adaptation, some of the proteins identified were DnaK, GroEL and Csp (De Angelis et al., 2001). However, these positive results were not obtained in the present study (Figure 3.2). They also carried all their experiment in milk and obtained better results in milk when compared to MRS broth (De Angelis et al., 2001). D'Angelo et al. (2017), performed a study in which they tested the effect of 1 level of heat mild stress on single levels of challenging stresses of acid, heat, oxidative and osmotic stresses. D'Angelo et al. (2017), found that when *Leuconostoc* cells were pre-adapted to mild heat (40°C for 30 minutes) they were more resistant to acid stress treatment (pH 4 for 30 minutes).

The effect of ethanol on membrane permeability has been extensively studied in several microorganisms and results show that the main damages include the leakage of bacterial cell constituents such as proteins, nucleic acids, and inorganic ions (Denyer and Hugo,1991). *Leuconostoc*’s acid tolerance was no improved by subjecting the bacteria to 5, 10 or 15% ethanol when compared to both control (Figure 3.3). The use of 15% ethanol was significantly detrimental to the survivability of *Leuconostoc mesenteroides* (Figure 3.3). *Lactococcus lactis* NZ9700 was
unable to grow in the presence of 8% and 10% ethanol. However, it was able to grow in the presence of 2% ethanol with a growth rate of about half of that of control samples without ethanol in the culture broth (Díez et al., 2017). According to Díez et al. (2017), the strain still produced nisin, but only 25% when compared to control conditions in the absence of ethanol. Culture broth pH values were lower (pH 4.88) for cultures in the absence of ethanol after 24 h incubation, than for cultures containing 2%–6% ethanol (pH 5.08–5.15), which correlated with the higher cell density of ethanol-free cultures (Díez et al., 2017).

The toxicity of oxygen is caused by the formation of reactive oxygen species like $O_2^-$ (superoxide), and OH (hydroxyl radical), that harm proteins, lipids and nucleic acids, becoming one of the major reasons of cell ageing and death. (Flahaut et al., 1998). Living organisms have develop mechanisms to prevent the formation of these ROS, eliminate them (by enzymatic degradation or scavenging), or repairing the damage caused by them (De Angelis et al., 2001).

The application of the oxidative mild stress treatments was detrimental to the acid tolerance of *Leuconostoc mesenteroides* when compared to the controls (Figure 3.4). Both the controls (positive and negative) outperformed the mild stress treatments (P < 0.05). When looking at the effect of the positive control, Figure 3.4 shows that there were significant differences in the survival (%) when compared to the negative control and the exposure to oxidative mild stress (P < 0.05). Some studies show that the cross-protection response of lactic acid bacteria varies among species depending on the different types and levels antioxidative mechanism (De Angelis and Gobbetti, 2004). Different LAB have developed different mechanisms to counteract the negative effects of oxidative damages (van de Guchte et al., 2002). For example, the high manganese content in *Leuconostoc mesenteroides* and some other LAB serves as a competent oxygen scavenger and therefore compensating for the absence if superoxide dismutase which is an enzyme
in charge of the elimination of ROS (Archibal et al. 1981). Some studies have shown that the transcription of the *L. lactis* sodA gene encoding for superoxide dismutase was found to be induced also through aeration and this same gene has been identified through its inductions at low pH (van de Guchte et al., 2002).

Table 3.2 shows that the acid tolerance of *Leuconostoc mesenteroides* was the same regardless of the levels of acid or heat used prior to the ACCs. The effect when *Leuconostoc* was exposed to mild acid stresses of pH 5.0, 4.5 or 4.0 and the control (pH 6.8) from 0-2h was the same as exposing it to mild heat levels of 25, 35 or 45°C or the control (30°C) for 10 minutes. When lactic acid bacteria are subjected to acid stress one of the first mechanisms used by the bacteria to defend against the detrimental effects of acid is to induce the heat shock chaperones (De Angelis et al., 2004).

The robustness of *Leuconostoc spp.* has shown to be strongly dependent on the species D'Angelo et al. (2017) evaluated 29 strains of *Leuconostoc spp.* (*lactis, mesenteroides, pseudomesenteroides and citreum*) for their resistance against a single level of each stress including oxidative, heat, acid, alkaline, osmotic stresses (D'Angelo et al., 2017). The strains studied showed an extensive variability in stress tolerance particularly for temperature, acidic and oxidative stress factors (D'Angelo et al., 2017). The most robust strains belong to the species *lactis* followed by *mesenteroides* and *pseudomesenteroides* being the most sensitive of them all (D'Angelo et al., 2017). Results suggested that *Leuconostoc mesenteroides* was more susceptible to acid and heat shock treatments than *Leuconostoc lactis* (D'Angelo et al., 2017). *Leuconostoc mesenteroides* strains showed a high susceptibility to acid when compared to the other strains evaluated (D'Angelo et al., 2017).
However, changes in the survival (%) of *Leuconostoc mesenteroides* ssp. *cremoris* can be seen with the use of ethanol and hydrogen peroxide (Table 3.2). The effect of prior exposure to 15% ethanol (v/v) on the acid tolerance of *Leuconostoc mesenteroides* was detrimental at 2h of exposure when compared to the effects of all levels of acid and heat mild treatments on its acid tolerance (Table 3.2). *Leuconostoc mesenteroides* ssp. *cremoris* was able to withstand the ACC for 2h when previously exposed to 0, 5 and 10% ethanol v/v, but at 15% ethanol (v/v) the bacteria showed a decrease in survivability at the 2h of exposure to the acid challenge (pH 3.5 at 30°C) (Table 3.2). High environmental alcohol concentrations have detrimental effects on bacterial growth, viability, and metabolism due to the leakage of components within the cell membrane (Ingram, 1989).

According to Ingram (1989) the main target of toxic levels of extracellular alcohol is the cell membrane (Ingram, 1989). The composition of the fatty acid in the cell membrane of *E. coli* K-12 changed drastically when the cell was grown in the presence of alcohols and that the proportion of 18:1 fatty acids increased at the expense of saturated fatty acids (Ingram, 1989). Ethanol tolerant cells of *Oenococcus oeni* contain a higher share of unsaturated fatty acids and a decreased proportion of total cell lipid content, decreasing membrane fluidity (Da Silveira *et al.*, 2003). In general, all levels of hydrogen peroxide used in this study (2.5, 5.0, 7.5 mM) were detrimental to the viability of *Leuconostoc mesenteroides* when used prior to the ACC (Table 3.2). Except for the control (0mM H₂O₂ v/v), by the first h of the ACC all bacteria had died. The effect of oxidative stress was so detrimental to the survivability of *Leuconostoc mesenteroides* (P < 0.05) that at 7mM all cells had died by the time they were transferred to the ACC (Table 3.2). The 5mM hydrogen peroxide level was also detrimental to the survivability of *Leuconostoc mesenteroides* to a lesser extent than that of the 7mM H₂O₂. When compared to all other types of mild stresses used and all the different levels evaluated, oxidative stress rendered the most detrimental effects on the
survivability of *Leuconostoc mesenteroides* ssp. *cremoris* to the ACC (pH 3.5 for 2h at 30°C). Comparable to the results found in the present study, D’ Angelo *et al.* (D’Angelo *et al.*) found that oxidative stress had the most detrimental effects on the survival of *Leuconostoc* spp. followed by heat and acid respectively. In their study all the strains in stationary phase displayed a higher tolerance to the stress factors than those in exponential phase (D'Angelo *et al.*, 2017).

Studies suggest that acid stress response mechanism results in the removal of protons (H⁺), alkanization of the external environment, changes in the composition of the fatty acid in cell membrane, production of general shock proteins and induction of chaperones (Cotter and Hill, 2003). These mechanisms fight the detrimental effects of a reduction in pH, which is related to the loss of activity of glycolytic enzymes (which severely affects the ability to produce ATP) and structural damage to the cell membrane and macromolecules (van de Guchte *et al.*, 2002). The importance of the role of the cell membrane is demonstrated by the changes in the composition of the fatty acid profiles in the cell membrane in response to a reduction in pH (Fernandez *et al.*, 2008). The surge in the production of the straight-chain fatty acids C14:0 and C16:0 as well as the reduced C18:0 levels accompanying acid adaptation which may be responsible for the enhanced cross-protective effects to acid stress (Fernandez *et al.*, 2008).

**3.8 Conclusions**

The counts of *Leuconostoc mesenteroides* after the ACC where greatly influenced by the type of mild stress being applied. The results obtained from the individual analysis of each type of mild stress showed that the previous exposure to pH 5.0 enhances the survival of *Leuconostoc mesenteroides* to the ACC (pH 3.5 for 2h at 30°C). The use of heat, ethanol and hydrogen peroxide at all levels did not enhance the survival of *Leuconostoc mesenteroides*. When analyzing the behavior of *Leuconostoc mesenteroides* on the 3-way interaction (type × level × time) the results
showed that the survival to the ACC of the bacteria was the same when exposed to acid heat and ethanol up to 10% for 2h. Even though the resistance of *Leuconostoc mesenteroides* was not enhanced by these treatments, important information was generated. The results suggest that these bacteria are very robust and can cope with stressful conditions successfully. These results give light to the possible inclusion of *Leuconostoc mesenteroides* ssp. *cremoris* to more products in which such conditions are encountered. Based on the low performance with all the levels hydrogen peroxide it is not recommended to use the oxidative stress at the levels used as a mean to improve the tolerance to pH 3.5 of these bacteria.
4.1 Review of Literature

Osmotic changes are one of the stresses live probiotics may encounter in food formulations and during processing (Sunny-Roberts and Knorr, 2008). The production and storage conditions may represent environmental stresses such as osmotic stress takes place when some osmotically active agents, like sucrose, are in the food matrix. Sucrose is a disaccharide composed of glucose and fructose. It is a food sweetener and used in food products because its and its functional properties (Randazzo et al., 2013). Response to osmotic stress, induced by sucrose, of a potential probiotic strain such as *Leuconostoc mesenteriodes* is of interest given the potential application for dairy dessert.

Bacterial cells gather solutes in their cytoplasm at amounts higher than those essential for the metabolism of the cell to assure that the direction of the flow of water during growth is into the cell (Le Marrec, 2011). Sucrose is a compatible solute, which can be gathered at high levels in the cytoplasm of osmotically stressed cells and when accumulated, are not significantly detrimental to the functioning of cytoplasmic enzymes (Sunny-Roberts and Knorr, 2008). Growing cells exhibit a high turgor pressure directed outward that places the cell membrane near the expanding peptidoglycan wall. Maintaining a constant positive turgor is considered necessary for cell expansion, growth, and division (Glaasker et al., 1998). Changes in extracellular water activity have direct consequences on the cytoplasm and immediately trigger fluxes of water along the osmotic gradient. Bacterial cell membrane possesses a high permeability to water but not for many solutes (Sunny-Roberts and Knorr, 2008). By diffusion through the cell membrane, water can enter
and go out of the cell until an equilibrium is reached between internal and external osmotic concentrations (Sunny-Roberts and Knorr, 2008).

Stress-sensing system and defense mechanism of bacteria are used to prepare them for challenging stress conditions or to tolerate sudden changes in the environment (Effie et al., 2011). Instability in the environment increases the production of stress metabolites, which can help the bacteria to survive the detrimental conditions (Glaasker et al., 1998). Water flow in hypotonic environments can result in swelling and bursting of the cell or under hypertonic conditions in loss of turgor, plasmolysis, and dehydration (Le Marrec, 2011). An accelerated water movement can be obtained by diffusion through water-selective channels embedded in the membrane called aquaporins (Le Marrec, 2011). They facilitate water fluxes in both directions in response to a decrease or increase in osmotic pressure. Aquaporins belong to a family of transporters called major intrinsic protein (MIP). This family also includes glycerol facilitators and aquaglyceroporins, which aid in the passage of several small molecules, such as glycerol and other polyols, dihydroxyacetone, CO₂, urea, and ammonium (Glaasker et al., 1998). The synthesis of aquaporins is induced after bacteria are subjected to hyperosmotic environment showing their role in osmotic stress response.

*Leuconostoc mesenteroides* has shown to possess promising probiotic characteristics such as good acid and bile tolerance; and the ability to adhere to the intestinal epithelium cell line Caco-2 cells (de Paula et al., 2014). Technologically, it provides with flavor and aroma in dairy products (Hemme and Foucaud-Scheunemann, 2004). The tolerance to sucrose concentrations in basic syrups found in e.g. can fruit cocktail between 14-18° Brix could represent the incorporation of this potential probiotic bacteria in products which are contained in syrups.
4.2 Justification

There are no studies that evaluate the survivability of *Leuconostoc mesenteroides* in sucrose solutions neither has the cross-protection ability been evaluated in osmotic conditions caused by high concentrations of sugar. An understanding of the resistance capacity of *Leuconostoc mesenteroides* to survive osmotic challenging conditions in the external media or in sweetened foods is thus of great importance. The exposure to different types of mild stress conditions has not been evaluated in order increased the resistance against the osmotic challenging condition (cross-protection) in *Leuconostoc mesenteroides*. The increase resistance of *Leuconostoc mesenteroides* ssp. *cremoris* to osmotic challenging condition would enable the inclusion of this microorganism in more products without the need of microencapsulating or genetically modifying it. Making it a process friendly to the food industry when scaling up the production.

4.3 Hypothesis

- Whether the prior exposure of *Leuconostoc mesenteroides* ssp. *cremoris* to various types of mild stresses (acid, heat, ethanol, oxidative) can enhance its osmotic tolerance (sucrose).

4.4 Objectives

- To study the influence of various types mild stress conditions (acid, heat, ethanol, oxidative) at various levels on the enhancement of the osmotic tolerance of *Leuconostoc mesenteroides* ssp. *cremoris*.
- To define which type and level of mild stress was more helpful to improve the osmotic tolerance of *Leuconostoc mesenteroides* ssp. *cremoris*. 
4.5 Materials and Methods

4.5.1 Experimental Design

Four mild stresses (acid, heat, ethanol and oxidative) were evaluated. Each mild stress had 3 levels of intensity; low, medium and high (pH 5.0, 4.5, 4.0; heat 25, 35, 45°C; ethanol 5, 10, 15% v/v; \( \text{H}_2\text{O}_2 \) 2.5, 5.0, 7.5mM v/v). Each type of mild stress was compared against a negative and a positive control. In the negative control the bacterial culture had no application of a mild stress and was directly exposed to the OCC. The positive control accounted for the time the bacterial culture was exposed to the mild stress treatment been evaluated without any level of stress being applied (accounting for the time of exposure). After each mild stress treatment, the culture was subjected to the OCC (20% sucrose for 2h at 30°C).

Counts were determined before the application of the mild stress treatment (time zero), after the exposure to the mild stress treatment (AEMS), immediately after exposure to OCC (0hSUCROSE), 1 h after exposure to OCC (1hSUCROSE) and 2h after OCC (2hSUCROSE). The experiments were repeated 3 times with duplicate readings. Data were analyzed as a complete block design with repeated measure over time.

4.5.2 Preparation of Media

4.5.2.1 Reconstituted NFDM (10%)

Non-fat dry milk was used as the culture media for all mild stress treatment samples. A solution of 10% w/v of milk was prepared by dissolving 100 grams of Great Value ® Nonfat dry milk (NFDM) (Walmart, Bentonville, AK) in 1L of distilled water. NFDM solution (700mL) was poured into clean Pyrex bottles and sterilized at 121°C for 20 minutes in an autoclave (AMSCO Scientific, Erie, PA). The bottles were tempered at 30°C for 12h in an aerobic incubator (GCA/
Precision Scientific Chicago, IL). For each treatment, sterile milk was aseptically transferred into sterile 250mL flasks.

4.5.2.2 Sucrose Solution (20%)

A solution of 20% w/v of sucrose was prepared by dissolving 200 grams of Great Value® Sugar (Walmart, Bentonville, AK) in 1L of distilled water. Sucrose solution (700mL) was poured into clean Pyrex bottles and sterilized at 121°C for 20 minutes in an autoclave (AMSCO Scientific, Erie, PA). The bottles were tempered at 30°C for 12h in an aerobic incubator (GCA/ Precision Scientific Chicago, IL). For each treatment, sterile sucrose solution was aseptically transferred into sterile 250mL flasks.

4.5.2.3 Agar Preparation

MRS agar (Fisher Scientific, Fair Lawn, NJ) was used for the enumeration of all samples. It was prepared according to the manufacturer specifications as follow: 55 grams of MRS broth powder (Fisher Scientific, Fair Lawn, NJ) and 12 g of pure agar powder (Fisher Scientific, Fair Lawn, NJ) were diluted in 1L of distilled water, heating and mixing them in hot plate (Fisher Scientific, Fair Lawn, NJ) with a magnetic stirrer until the solution boiled. It was sterilized at 121°C for 20 minutes. MRS agar (Fisher Scientific, Fair Lawn, NJ) was kept in a water bath at 48°C until used.

4.5.2.4 Peptone Water

For all serial dilutions, a solution of 0.1% w/v of peptone water was prepared according to the manufacturer specifications dissolving 1g of peptone powder (BactoTM Peptone, Difco, Dickinson and Co., Sparks, MD) in 1L of distilled water. Peptone solution (9mL) was poured into clean test tubes and sterilized at 121°C for 20 minutes in an autoclave (AMSCO Scientific, Erie, PA).
4.5.3 Treatments and Protocols

4.5.3.1 Osmotic Challenging Condition (OCC)

For all OCC treatments a solution of 20% w/v of sucrose was prepared by dissolving 200 grams of Great Value® Sugar (Walmart, Bentonville, AK) in 1L of distilled water. Sucrose solution (700mL) was poured into clean Pyrex bottles and sterilized at 121°C for 20 minutes in an autoclave (AMSCO Scientific, Erie, PA). The bottles were tempered at 30°C for 12h in an aerobic incubator (GCA/ Precision Scientific Chicago, IL). For each OCC treatment, 135mL sterile sucrose solution was aseptically transferred into sterile 250mL flasks. 15mL of mildly-treated bacteria were transferred into the flask. The culture was incubated for 2h at 30°C under aerobic conditions in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ) (30°C, 48h) at various time points. Immediately after the inoculation in the challenging stress condition (0hSUCROSE), after 1 h of being exposed to challenging stress condition (1hSUCROSE) and 2h of exposure to the challenging stress (2hSUCROSE).

4.5.3.2 Negative Control

Culture was not pre-exposed to any mild stress conditions, instead it was directly exposed to the OCC. Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately $10^6$ CFU/mL in reconstituted NFDM. A sample of 15mL of the *Leuconostoc mesenteroides* inoculum was immediately transferred to a 250mL Erlenmeyer flask containing 135mL of sterile distilled water modified with 20% sucrose. The culture was incubated for 2h at 30°C under aerobic conditions in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Bacterial counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ) (30°C, 48h) at various
time points. Counts were determined immediately before exposure to OCC (0hSucrose) and 2h after OCC (2hSUCROSE).

4.5.3.3 Positive Control

The time the bacterial culture was exposed to the mild stress conditions was taken into consideration before exposing the bacteria to the OCC. Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately $10^7$ CFU/mL in reconstituted NFDM. A sample of 15mL of *Leuconostoc mesenteroides* inoculum was transferred to a 250mL Erlenmeyer flask containing 135mL of sterile NFDM (10%) at approximately pH 6.8. The culture was incubated for the time specified for each mild stress treatment (10 minutes for mild heat and 2h for acid, ethanol and oxidative mild stresses) at 30°C under aerobic conditions in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the time of exposure to the respective mild stress treatment, 15mL of the control culture was transferred to a 250mL Erlenmeyer flask containing 135mL of sterile distilled water modified with sucrose 20%. The culture was incubated for 2h at 30°C under aerobic conditions in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ) (30°C, 48h) at various time points. Before the application of the mild stress (time zero), after the exposure of the mild stress (AEMS), immediately after the inoculation in the challenging stress condition (0hSUCROSE), after 1 h of being exposed to challenging stress condition (1hSUCROSE) and 2h of exposure to the challenging stress (2hSUCROSE).

4.5.3.4 Acid Mild Stress Condition

Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately $10^7$ CFU/mL in
reconstituted NFDM. A sample of 15mL of the *Leuconostoc mesenteroides* inoculum was transferred to different Erlenmeyer flasks containing 135mL of autoclaved reconstituted NFDM (10%) with modified pH levels of 5.0, 4.5 or 4.0. Culture was incubated for 2h at 30°C in aerobic conditions in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Control was left in unmodified autoclaved NFDM (10%) for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the 2h of acid mild stress treatment, 15mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135mL of sterile distilled water modified with sucrose 20% for 2h. Counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ) (30°C, 48h) at various time points. Counts were determined before the application of the mild stress treatment (time zero), after the exposure to the mild stress treatment (AEMS), immediately after exposure to the OCC (0hSUCROSE), 1 h after the OCC (1hSUCROSE) and 2h after the OCC (2hSUCROSE).

### 4.5.3.5 Heat Mild Stress Condition

Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately 10^7 CFU/mL in reconstituted NFDM, a sample of 15mL of the *Leuconostoc mesenteroides* inoculum was transferred to each of 3 different Erlenmeyer flasks containing 135mL of autoclaved reconstituted NFDM (10%) heated in water baths to obtain the final temperature of 25, 35 or 45°C (after inoculation) for 10 minutes. Control was left in autoclaved NFDM (10%) for 10 minutes at 30°C. After the 10 minutes of heat mild stress treatment, 15mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135mL of sterile distilled water modified with sucrose 20% for 2h. Counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ) (30°C, 48h) at various time points. Counts were determined before the application of
the mild stress treatment (time zero), after the exposure to the mild stress treatment (AEMS), immediately after exposure to OCC (0hSUCROSE), 1 h after OCC (1hSUCROSE) and 2h after OCC (2hSUCROSE).

4.5.3.6 Ethanol Mild Stress Condition

Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately $10^7$ CFU/mL in reconstituted NFDM (10%). 15mL of the *Leuconostoc mesenteroides* inoculum was transferred to each of four different Erlenmeyer flasks containing 135mL of autoclaved reconstituted NFDM (10%) modified with ethanol (200° proof) to obtain a 0, 5, 10 or 15% ethanol-modified milk (v/v). Ethanol mild stress treated bacteria were aerobically incubated for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the ethanol mild stress treatment, 15mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135mL of sterile distilled water modified with sucrose 20% for 2h. Counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ) (30°C, 48h) at various time points. Counts were determined before the application of the mild stress treatment (time zero), after the exposure to the mild stress treatment (AEMS), immediately after exposure to OCC (0hSUCROSE), 1 h after OCC (1hSUCROSE) and 2h after OCC (2hSUCROSE).

4.5.3.7 Oxidative Mild Stress Condition

Pure culture of *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) was thawed and inoculated at approximately $10^7$ CFU/mL in reconstituted NFDM (10%). A sample of 15mL of the *Leuconostoc mesenteroides* inoculum was transferred to four different Erlenmeyer flasks containing 135mL of autoclaved reconstituted NFDM (10%) modified with hydrogen peroxide (9.77mM) to obtain a 0, 2.5, 5.0 or 7.5mM
hydrogen peroxide-modified milk (v/v). These oxidative mild stress treatments were aerobically incubated for 2h at 30°C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the 2h of oxidative mild stress treatments, 15mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135mL of sterile distilled water modified with sucrose 20% for 2h. Counts were enumerated in MRS agar (Fisher Scientific, Fair Lawn, NJ) (30°C, 48h) at various time points. Counts were determined before the application of the mild stress treatment (time zero), after the exposure to the mild stress treatment (AEMS), immediately after exposure to OCC (0hSUCROSE), 1 h after OCC (1hSUCROSE) and 2h after OCC (2hSUCROSE).

**4.5.3.8 Sample Plating**

Counts were determined before the application of the mild stress treatment (time zero), after the exposure to the mild stress treatment (AEMS), immediately after exposure to OCC (0hSUCROSE), 1 h after OCC (1hSUCROSE) and 2h after OCC (2hSUCROSE). Samples for bacterial counts were taken from the reconstituted NFDM for the different time points specified above and serially diluted in sterile peptone. A sample of 1mL was taken and aseptically poured into sterile petri dishes. MRS agar (Fisher Scientific, Fair Lawn, NJ) was poured over the sample. Inoculated plates were incubated aerobically at 30°C for 48 h and counted for data analysis.

**4.5.3.9 Calculations**

All the counts described above were transformed to a Survival percentage as previously done by Wu 2012, De Angelis 2004, Flahaut 1998 Flahaut *et al.* (1998) with slight modifications. Survival percentage was defined as

\[
\text{Survival (\%)} = \left[\frac{\log(N_x)}{\log(N_0)}\right] \times 100,
\]

Where \(N_x\) = log CFU/mL of *Leuconostoc mesenteroides* at given time point (AEMS, 0hSUCROSE, 1hSUCROSE or 2hSUCROSE) and \(N_0\) as the log CFU/mL of the starting cell count.
(time zero). This ratio was multiplied by 100 to convert it to a percentage. It was use to compare the viability of the bacteria after each given time point in relation to its initial count (time zero).

4.5.4 Statistical analysis

The type III test of fixed effects of the Glimmix procedure of the Statistical Analysis System (SAS 9.4) was used to detect differences between treatments. Tukey media separation was used when difference between treatments were found. The level of significance was 0.05.

4.6 Results

Results are presented as 2 separate analysis. In the first analysis, all results were analyzed by each type of mild stress separately. This helped to identify if the prior exposure to a given type of mild stress helped the bacteria to perform better upon the exposure to the OCC. Furthermore, the analysis enabled the examination of which level of mild stress was better to enhance the osmotic tolerance within the type of mild stress analyzed. Each level of mild stress was compared to the negative and positive controls. All results presented in this study were transformed to a survival%. That compares the log CFU/mL that survived the osmotic challenge condition over their respective log CFU/mL at the starting point; being 100% the starting point. Hence, survivability greater than 100% means that the bacteria grew during the application of the treatments. For the first analysis, only the CFU/mL after the 2-h exposure of the bacteria to the OCC and the starting CFU/mL were taken into consideration to make the comparisons (calculations section 3.5.3.9).

In the second analysis, all the types of mild stresses used in the present study were compared to each other. Results enabled the overall comparison of which type of all the mild stresses used (acid, heat, ethanol or oxidative), level of mild stress (control, low, medium, or high) and time of exposure enabled Leuconostoc mesenteroides to withstand better in the OCC. For this analysis, only the positive control was used as the basis for comparison. The most relevant interactions
among the different types of mild stresses, the levels of mild stress used and the time of exposure are presented. Besides the type mild stresses and the levels mild stress, the different times of exposure to the OCCs were also taken into consideration to determine the survival (%).

4.6.1 Acid Mild Stress

The effect of subjecting *Leuconostoc mesenteroides* to acid conditions prior to the OCC can be found in Figure 4.1. Results demonstrate that there were no significant differences among the negative control, positive control, pH 5.0 and pH 4.5 (P > 0.05), while the use of pH 4.0 resulted in significantly lower survivability (P < 0.05) (Figure 4.1). The exposure to an acid pH 4.0 prior to the OCC was detrimental to the survival of the culture. These results suggest that the application of the mild stresses did not improve the viability of *Leuconostoc mesenteroides* to OCCs (sucrose 20% w/v). However, the good survivability results of the controls show that the *Leuconostoc mesenteroides* has a good tolerance to the OCC and could be incorporated in products which contain a pH as low as 4.5 without affecting its viability.

![Survival of Leuconostoc mesenteroides to ACC after acid mild stresses](image)

Figure 4.1. Tolerance of Leuconostoc mesenteroides to acid challenging condition (ACC) with prior exposure to various levels of mild ethanol treatments for 2h expressed as Survival % = \([\log(CFU/mL) N]/(\log (CFU/mL) N_0)\) × 100.

A-B Means with different letters represent significant differences (P < 0.05).
4.6.2 Heat Mild Stress

The effect of subjecting *Leuconostoc mesenteroides* to heat conditions prior to the OCC can be found in Figure 4.2. The survivability (%) of *Leuconostoc mesenteroides* cells was not affected by the application of the heat mild stress treatment (P > 0.05). As shown in Figure 4.2 there were no differences in viability of the bacteria between the negative control, the positive control and any of the other levels of mild heat treatment (25, 35 or 45°C). These results suggest that the application of the heat mild stresses did not improve the viability of *Leuconostoc mesenteroides* to OCC. However, the good survivability (%) shows that the *Leuconostoc mesenteroides* has a good tolerance to the OCC and could be incorporated in products which receive a heat treatment as high as 45°C during 10 minutes without affecting its viability.

**Figure 4.2.** Tolerance of *Leuconostoc mesenteroides* to acid challenging condition (ACC) with prior exposure to various levels of mild heat treatments for 10 minutes expressed as Survival % = [(log(CFU/mL) N)/(log (CFU/mL) N₀)] × 100. A-B Means with different letters represent significant differences (P < 0.05).

4.6.3 Ethanol Mild Stress

The effect of using various levels of ethanol as a mild stress treatment are shown in Figure 4.3. No significant differences were found with the application of the negative and positive controls and the ethanol mild stress treatment at 0, 5, or 10% (P > 0.05). Survival (%) was lower with exposure
to OCC after 15% ethanol treatment (P < 0.05) (Figure 4.3). These results suggest that the application of the ethanol mild stresses did not improve the viability of *Leuconostoc mesenteroides* to OCC. However, the good survivability results of the controls show that the *Leuconostoc mesenteroides* has a good tolerance to the OCC and could be incorporated in products which contain up to 10% of alcohol without affecting its viability. Furthermore, bacterial growth was observed with the positive control, 5 and 10% ethanol (6.3 and 4% respectively) when compared to the initial counts of the treatments.

![Survival of *Leuconostoc mesenteroides* to ACC after ethanol mild stresses](image)

**Figure 4.3.** Tolerance of *Leuconostoc mesenteroides* to acid challenging condition (ACC) with prior exposure to various levels of mild ethanol treatments for 2h expressed as Survival % = [(log(CFU/mL) N)/log (CFU/mL) N0)] × 100.

A-B Means with different letters represent significant differences (P < 0.05).

### 4.6.4 Oxidative Mild Stress

The effect of the oxidative mild stress on the viability of *Leuconostoc mesenteroides* in the OCC is found in Figure 4.4. The positive control had beneficial effects on the viability of *Leuconostoc mesenteroides* (P < 0.05) when compared to the negative control, 2.5 and 5.0mM. There were no significant differences between the negative control, 2.5 and 5.0mM, therefore the OCC was not detrimental to the viability of the bacteria (Figure 4.4). However, when exposed to 7.5mM 80% of the bacteria survived which is statistically lower than the rest of treatments (P < 0.05). These results
suggest that the application of the oxidative mild stresses did not improve the viability of *Leuconostoc mesenteroides* to OCC. However, the good survivability results of the controls show that the *Leuconostoc mesenteroides* has a good tolerance to the OCC and could be incorporated in products which may contain up to 5.0 mM without affecting its viability. The survivability above 100% indicates that there was growth during the time of exposure to the treatments.

Survival of *Leuconostoc mesenteroides* to ACC after oxidative mild stresses

![Survival Graph](image)

**Figure 4.4.** Tolerance of *Leuconostoc mesenteroides* to acid challenging condition (ACC) with prior exposure to various levels of mild ethanol treatments for 2h expressed as Survival % = \[\frac{\log (CFU/mL) N}{\log (CFU/mL) N_0}\] \times 100.

A-C Means with different letters represent significant differences (P < 0.05).

### 4.6.5 Comparison of Main Effects and Interactions

This second part of the analysis consists of the comparison of all the types and levels of mild stresses over the 2h of exposure to the OCC to identify which were the best treatments. For this analysis, only the positive control was used (hereafter control). This change in the analysis allows using the positive control as a level within the types of mild stresses being compared. Hence, the negative control is no longer considered.
Table 1.1. Probability values for main effects and their interaction on the osmotic tolerance of *Leuconostoc mesenteroides* ssp. *cremoris* when exposed to various types of mild stresses and levels of mild stress for 2h.

<table>
<thead>
<tr>
<th>Effect</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Mild stress</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Level of Mild Stress</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Time of exposure to the OCC</td>
<td>0.2401</td>
</tr>
<tr>
<td>Type Mild stress * Level of Mild Stress</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Type of Mild stress x Time of exposure to the OCC</td>
<td>0.1224</td>
</tr>
<tr>
<td>Level of Mild stress x Time of exposure to the OCC</td>
<td>0.0033</td>
</tr>
<tr>
<td>Type of Mild stress x Level of Mild stress x Time of exposure to the OCC</td>
<td>0.2087</td>
</tr>
</tbody>
</table>

*P-values less than 0.05 represent significant effect.

### 4.6.5.1 Type of Mild Stress

The survival (%) of *Leuconostoc mesenteroides* after the application of the OCC show significant differences depending on which of the type of mild stresses was applied previously (P < 0.05) (Table 4.1). Figure 4.5 compares the survival (%) of *Leuconostoc mesenteroides* to the OCC depending on the type of mild stresses used previously. The heat was the best treatment to aid in the viability of *Leuconostoc mesenteroides* to the OCC. It shows a significant improvement when compared to acid, ethanol, and oxidative mild stresses (P < 0.05) (Figure 4.5).

![General comparison of the effect of various types of mild stresses on the survival at *Leuconostoc mesenteroides* to the OCC](image)

Figure 4.5. Comparison of the osmotic tolerance of *Leuconostoc mesenteroides* with prior exposure to various types of mild treatments expressed as *Survival %* =\([\log(CFU/mL) N]/(\log (CFU/mL) N_0)\) × 100.

A-C Means with different letters represent significant differences (P < 0.05).
4.6.5.2 Level of Mild Stress

The survival (%) of *Leuconostoc mesenteroides* after the application of the OCC show significant differences depending on which of the level of mild stresses was previously applied (P < 0.05) (Table 4.1). Figure 4.6 compares the survival (%) of *Leuconostoc mesenteroides* to the OCC depending on the level of mild stresses the bacteria were exposed previously. There were no significant differences in the survival of the bacteria to the OCC when exposed to the control, low or medium levels of the mild treatments (P > 0.05) (pH 6.8-4.5, 25-35°C, 0-10% OH and 0-5.0mM H₂O₂). However, the highest levels were significantly detrimental to the viability of *Leuconostoc mesenteroides* to the OCC (P < 0.05) (Figure 4.6) (pH 4.0, 45°C, 15% OH, 7.5mM H₂O₂).

General comparison of the effect of various levels of mild stresses on the survival of *Leuconostoc mesenteroides* to the OCC

![Graph showing survival (%) of *Leuconostoc mesenteroides* with prior exposure to various levels of mild stresses](image)

Figure 4.6. Comparison of the osmotic tolerance of *Leuconostoc mesenteroides* with prior exposure to various levels of mild treatments expressed as Survival % = [(log(CFU/mL) N)/(log (CFU/mL) N₀)] × 100.

Means with different letters represent significant differences (P < 0.05).

4.6.5.3 Time of Exposure to the OCC

The time of exposure to the OCC had no significant effect on the survival of *Leuconostoc mesenteroides* (P > 0.05) (Table 4.1). In general, no positive or detrimental effect was observed the longer the time the bacteria spent in the OCC (Figure 4.7).
Effect of the acid challenging condition on the survivability of *Leuconostoc mesenteroides*

![Graph showing survival percentage over time](image)

Figure 4.7. Evaluations of the osmotic tolerance of *Leuconostoc mesenteroides* over 2h expressed as $\text{Survival} \% = [(\log \left( \text{CFU/mL} \right) N) / \log \left( \text{CFU/mL} \right) N_0] \times 100$.

$\text{A-C}$ Means with different letters represent significant differences ($P < 0.05$).

4.6.5.4 Type of Mild stress × Level of Mild Stress

Survival (%) of *Leuconostoc mesenteroides* cells after the OCC was affected depending upon the type of mild stress being applied at a specific level of mild stress (Table 4.1). The 2-way interaction between Type of Mild Stress × Level of Mild Stress had a significant effect on the survivability of *Leuconostoc mesenteroides* ssp. cremoris ($P < 0.05$) (Table 4.2). The survivability of *Leuconostoc mesenteroides* was statistically the same across all types of mild stress and all levels except by the pH 4.0, ethanol 15% and oxidative stress with 7.5mM hydrogen peroxide which were significantly detrimental to the survivability of the bacteria ($P < 0.05$) (Table 4.2)

Table 4.2. Comparison of the osmotic tolerance of *Leuconostoc mesenteroides* produced by the interaction of various types and various levels of mild stresses expressed as $\text{Survival} \% = [(\log \left( \text{CFU/mL} \right) N) / \log \left( \text{CFU/mL} \right) N_0] \times 100$.

<table>
<thead>
<tr>
<th>LEVEL OF MILD STRESS</th>
<th>ACID</th>
<th>HEAT</th>
<th>OH</th>
<th>H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>102.7 ± 3.8$^A$</td>
<td>100.2 ± 1.3$^A$</td>
<td>104.2 ± 1.5$^A$</td>
<td>103.9 ± 109$^A$</td>
</tr>
<tr>
<td>LOW</td>
<td>102.1 ± 4.0$^A$</td>
<td>104.0 ± 1.4$^A$</td>
<td>102.9 ± 3.1$^A$</td>
<td>101.3 ± 3.6$^A$</td>
</tr>
<tr>
<td>MEDIUM</td>
<td>98.8 ± 5.2$^A$</td>
<td>103.2 ± 2.73$^A$</td>
<td>102.7 ± 2.3$^A$</td>
<td>96.4 ± 4.9$^A$</td>
</tr>
<tr>
<td>HIGH</td>
<td>72.7 ± 12.2$^C$</td>
<td>98.5 ± 2.5$^A$</td>
<td>72.9 ± 21.0$^C$</td>
<td>84.1 ± 5.9$^B$</td>
</tr>
</tbody>
</table>

$\text{A-C}$ Means with different letters represent significant differences ($P < 0.05$).
4.6.5.5 Level of Mild stress × Time of exposure to the OCC

Cell survival (%) of *Leuconostoc mesenteroides* after the OCC was affected by the interaction between the level of mild stress being applied over the 2h of exposure (Table 4.1). The 2-way interaction between Level of Mild Stress × Time of Exposure to the OCC had a significant effect on the survivability of *Leuconostoc mesenteroides* ssp. cremoris (P < 0.05) (Table 4.3). The exposure to the high levels of stress-agent was already significantly detrimental since the immediate exposure to the OCC (Table 4.3).

Table 4.3. Comparison of the osmotic tolerance of *Leuconostoc mesenteroides* produced by the interaction between various levels of mild stresses over the 2h of exposure to the OCC expressed as Survival % = [(log(CFU/mL) N) / (log (CFU/mL) N0)] × 100.  

<table>
<thead>
<tr>
<th>Time of Exposure</th>
<th>Control</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-h</td>
<td>102 ± 1.5A</td>
<td>101.9 ± 3.1A</td>
<td>99.54 ± 5.3A</td>
<td>86.6 ± 20.0B</td>
</tr>
<tr>
<td>1-h</td>
<td>102.5 ± 1.8A</td>
<td>103.2 ± 3.6A</td>
<td>100.3 ± 3.5A</td>
<td>83.2 ± 13.4B</td>
</tr>
<tr>
<td>2-h</td>
<td>103.7 ± 4.1A</td>
<td>102.5 ± 3.1A</td>
<td>100.6 ± 5.4A</td>
<td>76.2 ± 21.0C</td>
</tr>
</tbody>
</table>

A-C Means with different letters represent significant differences (P < 0.05).

4.7 Discussion

The analysis of the results by single type of mild stress (first analysis) as well as the analysis of the interactions (second analysis) indicates that the exposure to the OCC did not have a negative effect on the viability of *Leuconostoc mesenteroides*. In all the analysis performed by single type of mild stress (first analysis) the same pattern was found; there were no significant differences between the negative and positive controls, low and medium levels of stress-agent within a given type of mild stress except for the highest levels of stress-agents which were significantly detrimental. In acid mild stress the survivability of *Leuconostoc mesenteroides* ranged from 103-63% the highest being the positive control and the lowest being exposure to pH 4.0 (Figure 4.1). Figure 4.2 shows that with the heat mild treatment no significant differences were found among both controls and all levels of mild heat stress (P > 0.05). The survivability ranged from 105-100%
(Figure 4.2). With ethanol, the survivability of *Leuconostoc mesenteroides* in the OCC was only different when exposed to 15% ethanol (v/v) (P < 0.05). The highest viability was obtained with the positive control which was 106% and the lowest with the 15% ethanol (v/v) with was 62%. (Figure 4.3). With the prior exposure to the oxidative stress, the viability in the OCC raged from 106-80% the highest being the positive control and the lowest being the 7.5mM concentration of hydrogen peroxide which rendered the lowest viability (P < 0.05) (Figure 4.4).

These results suggest that the changes in viability obtained were not influenced by the exposure to the OCC but essentially by the exposure to the different levels of stress-agents within a type of mild stress (Table 4.1). That table shows that the time of exposure to the OCC was not significant (P > 0.05). However, the type of mild stress and levels of mild stress were significant. Therefore, the changes in viability observed caused entirely by those 2 main effects and not by the time of exposure OCC (Table 4.1).

The most beneficial type of mild stress to the viability of *Leuconostoc mesenteroides* was heat (Figure 4.5) (P < 0.05). The incubation in the OCC after the mild heat treatment promoted a viability of 102% which was significantly higher (P < 0.05) than that obtained with the acid (94%), ethanol (96%) and oxidative (96%) (Figure 4.5).

As expected the most detrimental level of mild stress was the highest level of mild stress-agent (Figure 4.6) which was significantly lower than the rest of level of mild stresses (P < 0.05). There were no significant differences in the control, low and medium levels (P > 0.05) (Figure 4.6).

Table 3.2 shows that the interactions between the Types of Mild Stress × Levels of Mild Stress combination were statistically the same (P > 0.05) except for the oxidative 7.5mM, followed by acid pH 4.0 and ethanol 15% stresses (P < 0.05).
In the food industry, LAB are often exposed to osmotic stress with sugars (Sunny-Roberts and Knorr). The sugar content estimate in some sugar-containing foods include cookies (30–50%), honey (40–80%), candies (23–90%) and jams (30–70%) (Sunny-Roberts and Knorr, 2008). Also it can be found in syrups used for the preservations of canned products such as fruits between 14-22 °Brix (FAO/WHO, 2000). Glaasker et al. (1998) compared the effects of osmotic stress on the growth of *L. plantarum* by increasing the medium’s osmolarity with high concentrations of salts and iso-osmotic concentrations of lactose and sucrose. Hyperosmotic conditions obtained by sugar stress were less negative and more transient because cells could readily equilibrate the extracellular and intracellular concentrations of lactose and sucrose (Glaasker et al., 1998). In a study with *Lactobacillus rhamnosus* and *L. lactis* (Prasad et al. 2003); On the minute time scale, sugars caused osmotic stress. However, on a longer time scale, the external and internal sugars equilibrated. Growth of the bacteria was not limited at sugar concentrations at which equimolar salt concentrations were already detrimental (Prasad et al., 2003).

These results are consistent with various studies in which the effect of sucrose and other sweeteners was evaluated with respect to the viability of probiotic bacteria. In a study conducted by Popa and Ustunol (2011) different sources of sweeteners were evaluated on *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacteria*. In general, the sweeteners were not a detrimental agent to the viability of these bacteria (Popa and Ustunol, 2011). Similar results were obtained by Chick *et al.* (2001) in which the effect of 5% clover honey in supporting the growth of *S. salivarius* ssp. *thermophilus* (St-133) was investigated and compared with sucrose and fructose, it was concluded that all sweeteners promoted the growth of this bacteria (Chick *et al.* 2001).
In a study conducted by Riazi and Ziar (2008) a higher increase (P < 0.05) in growth and metabolic activity of *S. thermophilus* and *L. bulgaricus* was observed in the presence of honey. Cell viability was improved by 5% for *S. thermophilus* and 10% for *L. bulgaricus* in pure honey-sweetened yogurt over 28 days of refrigerated storage (Riazi and Ziar, 2008).

Production of organic acids, principally lactic acid, and other volatiles during fermentation is important in dairy products since acid production helps determines several of the chemical characteristics of the product as well as their sensory properties and can be used as an indicator of cell activity (Randazzo *et al*., 2013). Although in the present study this metabolic aspect was not evaluated several studies suggest that the presence of sweeteners do not inhibit the metabolic activity of various lactic acid bacteria but some sweeteners promote it more than others. However, this has not been evaluated in *Leuconostoc mesenteroides*. Studies support that metabolism of LAB is not compromised by the exposure to sugar. *Lactobacillus casei, Lactobacillus brevis* and *Lactobacillus plantarum* were evaluated for lactic acid production and bacterial growth in the presence of sucrose, glucose and stevia leaf extract (Davoodi *et al*., 2016). The highest bacterial growth and lactic acid production in these 3 strains were obtained with high concentration of sucrose (20%). *Lactobacillus casei* and *Lactobacillus brevis* could produce more lactic acid compared to *Lactobacillus plantarum*. All bacteria studied was able to produce lactic acid in the presence of sucrose, glucose and stevia leaf extract in an amount-dependent manner (Davoodi *et al*., 2016).

An important characteristic of *Leuconostoc mesenteroides* is the production of lactic, diacetyl and acetic acid as end products of sugar fermentation (Hemme and Foucaud-Scheunemann, 2004). Although these results have not been evaluated or obtained in *Leuconostoc mesenteroides* some
interesting results have been obtained from the evaluation of *Bifidobacteria* (Popa and Ustunol, 2011).

Studies show that usually the cell morphology is not compromised by exposure to high sucrose concentrations (Sunny-Roberts and Knorr, 2008). Cells grown under the control conditions (MRS broth) presented the characteristic rod-shaped morphology, characteristic of lactic acid bacteria. Osmotic stress treatment with sucrose, 0.6 and 1.5 M, did not cause a significant change in cell morphology (Sunny-Roberts and Knorr, 2008). Furthermore, in comparison with the control cells, all sucrose-treated cells had integral membranes (Sunny-Roberts and Knorr, 2008).

**4.9 Conclusions**

Under the investigated conditions, *Leuconostoc mesenteroides* survived with no significant loss of cultivability/viability. This was a proof that these cells responded to sudden changes in their environmental osmotic conditions. The good performance of *Leuconostoc mesenteroides* in a high concentration of sucrose (20%) suggest the promising possibility of incorporating these bacteria in dairy desserts that could contain up to 20% sucrose. Furthermore, these products could have a pH as low as 4.5, contain up 10 % ethanol and have a concentration of oxidative agents as high as 5.0mM without compromising the viability of *Leuconostoc mesenteroides*. However, further studies are needed to confirm if the stated attributes (survival and functionality) can still be maintained by sucrose-treated cells in the gastrointestinal tract.
OVERALL CONCLUSION

The enhancement of its heat tolerance and the robustness displayed to the acid and osmotic challenging conditions suggest the possible applications of *Leuconostoc mesenteroides sub spp. cremoris* in food products in which such challenging conditions are encountered.
REFERENCES


VITA

Ingrid Osorio was born in Tegucigalpa, Honduras in 1991. In 2011, she enrolled as an undergraduate student at the Escuela Agricola Panamericana, Zamorano to pursue the Food Science and Technology major. During her senior year she participated in an internship at Louisiana, State University in Louisiana, USA. After graduation, in 2015 she was granted the opportunity to pursue a Master’s degree in Dairy Foods under the supervision of Dr. Kayanush Aryana. The author plans to graduate with a Master’s degree in Animal, Dairy and Poultry Sciences at Louisiana State University.