Scientific Research Publishing

Food and Nutrition Sciences, 2025, 16(12), 1848-1865
https://www.scirp.org/journal/fns
ISSN Online: 2157-9458
ISSN Print: 2157-944X

Acid and Heat Mild Stresses Improve the Overall Heat Tolerance of *Leuconostoc mesenteroides*

Ingrid Osorio, Douglas Olson, Kayanush Aryana*

School of Nutrition and Food Sciences, Louisiana State University Agricultural Center, Baton Rouge, USA
Email: *Karyana@agcenter.LSU.edu

How to cite this paper: Osorio, I., Olson, D. and Aryana, K. (2025) Acid and Heat Mild Stresses Improve the Overall Heat Tolerance of *Leuconostoc mesenteroides*. *Food and Nutrition Sciences*, **16**, 1848-1865.
https://doi.org/10.4236/fns.2025.1612108

Received: November 13, 2025
Accepted: December 16, 2025
Published: December 19, 2025

Abstract

The importance of *Leuconostoc mesenteroides* is recognized for its contribution to taste in cultured dairy products and has been cited for its potential role as a probiotic. Objective was to evaluate the effect of prior exposure to various mild stress conditions on the survival of *Leuconostoc mesenteroides* in challenging heat 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 heat conditions. The de Man Rogosa Sharpe Agar was used for plating. The experiments were repeated three times with duplicate readings. Data were analyzed as a Randomized Block Design using the Glimmix procedure and Tukey mean separation. Heat tolerance of *Leuconostoc mesenteroides* at 60˚C was significantly enhanced by subjecting the bacteria to heat mild treatments of 30˚C, 35˚C and 45˚C ($P < 0.05$). The heat tolerance of *Leuconostoc mesenteroides* ssp. *cremoris* was improved by prior exposure to temperatures between 30˚C - 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. This enhanced heat tolerance has potential applications for *Leuconostoc mesenteroides* spp. *cremoris* in food products in which such a challenging condition is encountered.

Keywords

Probiotic, Thermal, Stressor, Food

1. Introduction

Importance of *Leuconostoc mesenteroides* is widely recognized in the dairy foods

industry for their contribution to flavor and aroma in fermented dairy products (Hemme and Foucaud-Scheunemann, 2004), due to its capability of producing CO_2 from carbohydrates, flavor compounds (diacetyl, acetate and ethanol) in many cultured dairy products [1]. *Leuconostoc mesenteroides* has an optimum growth temperature of 25˚C and its optimum growth pH is 5.5 [1]. *Leuconostoc mesenteroides* ssp. *cremoris* has proven to be an extremely potent cytokine producer-10, IFN-γ, IL-12 [2]. The anti-inflammatory effects these cytokines could aid in the treatment of inflammatory conditions such as ulcerative colitis, pouchitis, and irritable bowel syndrome [2]. *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 [3]. After 4 days of feeding *Leuconostoc mesenteroides*, at high cell concentration (10^8 CFU/ml) to lactose-intolerant induced rats, diarrhea disappeared when compared to control in which rats continue to show diarrhea [4]. Ariute *et al.*, [5] reported that *Leuconostoc mesenteroides* F-21 and F-22 exhibited wide genome plasticity, cell adhesion ability, proteolytic activity, proinflammatory and immunomodulation capacity through interaction with TLR-NF-κB and TLR-MAPK pathway components, and no antimicrobial resistance, denoting their potential to be candidate probiotics.

Leuconostoc mesenteroides has shown to have antimicrobial properties against various pathogenic bacteria like Salmonella, Shigella, Vibrio, *E. coli* [6]. Multiple antimicrobial compounds can be produced by *Leuconostoc mesenteroides* such as carbon dioxide, ethanol and acetic acid [7]. Furthermore, the bacteriocins produced by *Leuconostoc mesenteroides* can be used as bio preservatives in food products [8]. Studies have shown a strong activity against *Listeria monocytogenes* and other psychotropic bacteria. Multiple bacteriocins have been isolated and studied such as Leucocin and Mesentericin [4].

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 [9]. Like all living organisms, bacteria have developed different defense mechanisms that enable their survival to these stresses [10]. Stress may be defined as any alteration in the permissive environmental parameters that lead to a response by biological organisms [11]. Lactic Acid Bacteria (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 means to improve the robustness of strains to diverse stress conditions [12]. 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 [7].

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 [13]. In this sense, the adaptive changes caused by one stress may make the organism

fitter 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 [14]. This is industrially used to increase survival and activity of starter cultures during and after manufacture. Cross-protected organisms respond better to novel stressors at different levels [15]. 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*.

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.

Other forms of environmental stress can induce a heat shock-like response in *Leuconostoc mesenteroides* [16]. 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 [16]. The inductions of HSP by various stress indicate the reliance on chaperones as part of the general stress response in *L. mesenteroides* [16].

An understanding of the heat resistance capacity of *Leuconostoc mesenteroides* to survive heat challenging conditions in the external media is important as *Leuconostoc mesenteroides* goes through the heat gradient during food processing. The exposure to different types of mild stress conditions has increased the resistance against the heat challenging conditions (cross-protection) in other lactic acid bacterial species. Increased resistance of *Leuconostoc mesenteroides* ssp. *cremoris* to heat challenging condition would enable the inclusion of this microorganism in more food products without the need of microencapsulating or genetically modifying it, making it more process friendly to the food industry.

The hypothesis was 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. The objectives were: 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* and to determine which type and level of mild stress was more helpful to improve the heat tolerance of *Leuconostoc mesenteroides* ssp. *cremoris*.

2. Materials and Methods

2.1. Experimental Design

Four mild stresses (acid, heat, ethanol and oxidative) were separately evaluated. Each mild stress had 3 levels of intensity; low, medium and high (pH 5.0, 4.5, 4.0; heat 25˚C, 35˚C, 45˚C; ethanol 5%, 10%, 15% v/v; H_2O_2 2.5, 5.0, 7.5 mM v/v). Each

type of mild stress was compared against a negative and a positive control. In the negative control the bacterial culture *Leuconostoc mesenteroides* ssp. *cremoris* Vivolac Cremosa CIT/FPC DVS Series (Vivolac Cultures, Greenfield, IN) had no application of a mild stress and was directly exposed to the 60˚C 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˚C or 70˚C for 3 minutes). Counts were enumerated in MRS agar (30˚C, 48 h) at various time points viz. before the application of the mild stress (time zero), after the exposure of the mild stress (AEMS) and immediately after the 60˚C 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.

2.2. Preparation of Media

1) Reconstituted Nonfat Dry Milk (10%)

Non-fat dry milk was used as the culture media for all samples tested. 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 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 12 h in an aerobic incubator (GCA/Precision Scientific Chicago, IL). For each treatment, sterile milk was aseptically transferred into sterile 250 mL flasks.

2) MRS Agar Preparation

De Man-Rogosa-Sharpe (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.

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 1 g of peptone powder (BactoTM Peptone, Difco, Dickinson and Co., Sparks, MD) in 1L of distilled water. Peptone solution (9 mL) was poured into clean test tubes and sterilized at 121˚C for 20 minutes in an autoclave (AMSCO Scientific, Erie, PA).

2.3. Treatments and Protocols

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˚C, 70˚C, 80˚C and 90˚C for 2 - 20 minutes. Preliminary studies showed that the treatment that best met the criteria presented above were 60˚C and 70˚C for 3 minutes.

Therefore, the HCC consisted of sterile reconstituted NFDM (135 mL) that was aseptically transferred to sterilized 250 mL Erlenmeyer flasks. Fifteen mL of culture was inoculated into the heated milk and incubated at 60˚C or 70˚C for 3 minutes in a water bath (Fisher Scientific, Fair Lawn, NJ).

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 15 mL of the *Leuconostoc mesenteroides* inoculum was immediately transferred to a 250 mL Erlenmeyer flask containing 135 mL of sterile NFDM (10%). The culture was incubated in a water bath at 60˚C or 70˚C for 3 minutes. Bacterial counts were enumerated in MRS agar (30˚C, 48 h) at various time points. Counts were determined immediately before and after exposure to HCC.

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 10^6 CFU/mL in reconstituted NFDM. A sample of 15 mL of *Leuconostoc mesenteroides* inoculum was transferred to a 250 mL Erlenmeyer flask containing 135 mL of sterile NFDM (10%). The culture was incubated for the time specified for each mild stress treatment (10 minutes for mild heat and 2 h for acid, ethanol and oxidative mild stresses) at 30˚C under aerobic conditions. After the time of exposure to the respective mild stress treatment, 15 mL of the control culture was transferred to a 250 mL Erlenmeyer flask containing 135 mL of sterile NFDM (10%). The culture was incubated at 60˚C or 70˚C for the HCC in a water bath. Counts were enumerated in MRS agar (30˚C, 48 h) 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˚C or 70˚C HCC.

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 15 mL of the *Leuconostoc mesenteroides* inoculum was transferred to each of 3 different Erlenmeyer flasks containing 135 mL of autoclaved reconstituted NFDM (10%) with modified pH levels of 5.0, 4.5 or 4.0 achieved using 1 M HCl Culture was incubated for 2 h at 30˚C in aerobic conditions in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Control was left in unmodified au-

toclaved NFDM (10%) for 2 h at 30˚C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the 2 h of acid mild stress treatment, 15 mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135 mL of NFDM (10%) for the HCC. The culture was incubated at 60˚C or 70˚C for the HCC in a water bath. Counts were enumerated in MRS agar (30˚C, 48 h) at various time points Namely before the application of the mild stress (time zero), after the exposure of the mild stress (AEMS) and immediately after the 60˚C or 70˚C HCC.

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 15 mL of the *Leuconostoc mesenteroides* inoculum was transferred to each of 3 different Erlenmeyer flasks containing 135 mL of autoclaved reconstituted NFDM (10%) heated in water baths to obtain the final temperature of 25˚C, 35˚C 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, 15 mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135 mL of NFDM (10%) for the HCC. The culture was incubated at 60˚C or 70˚C for the HCC in a water bath. Counts were enumerated in MRS agar (30˚C, 48 h) 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˚C or 70˚C HCC.

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%). 15 mL of the *Leuconostoc mesenteroides* inoculum was transferred to each of 3 different Erlenmeyer flasks containing 135 mL 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 2 h at 30˚C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Control was left in unmodified autoclaved NFDM (10%) for 2 h at 30˚C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the ethanol mild stress treatment, 15 mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135 mL of NFDM (10%) for the HCC. The culture was incubated at 60˚C or 70˚C for the HCC in a water bath. Counts were enumerated in MRS agar (30˚C, 48 h) at various time points viz before the application of the mild stress (time zero), after the exposure of the mild stress (AEMS) and immediately after the 60˚C or 70˚C HCC.

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 15 mL of the *Leuconostoc mesenteroides* inoculum was transferred to four different Erlenmeyer flasks containing 135 mL of autoclaved reconstituted NFDM (10%) modified with hydrogen peroxide (9.77 mM) to obtain a 0, 2.5, 5.0 or 7.5 mM hydrogen peroxide-modified milk (v/v). These oxidative mild stress treatments were aerobically incubated for 2 h at 30˚C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. Control was left in unmodified autoclaved NFDM (10%) for 2 h at 30˚C in an orbital incubator (Multitron Infors, Annapolis Junction, MD) at 120 rpm. After the 2 h of oxidative mild stress treatments, 15 mL from each flask of the mild stress treated bacteria were transferred into its respective Erlenmeyer flasks with 135 mL of NFDM (10%) for the HCC. The culture was incubated at 60˚C or 70˚C for the HCC in a water bath. Counts were enumerated in MRS agar (30˚C, 48 h) 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˚C or 70˚C HCC.

2.4. Sample Plating

Samples for bacterial counts were taken from the different time points specified above were serially diluted in sterile peptone water. A sample of 1 mL 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. Calculations

All the counts described above were transformed to a Survival percentage as previously done [17]-[19], with slight modifications. Survival percentage was defined as

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

where N_x = log 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 used to compare the viability of the bacteria after each given time point in relation to its initial count (time zero).

2.6. Statistical Analysis

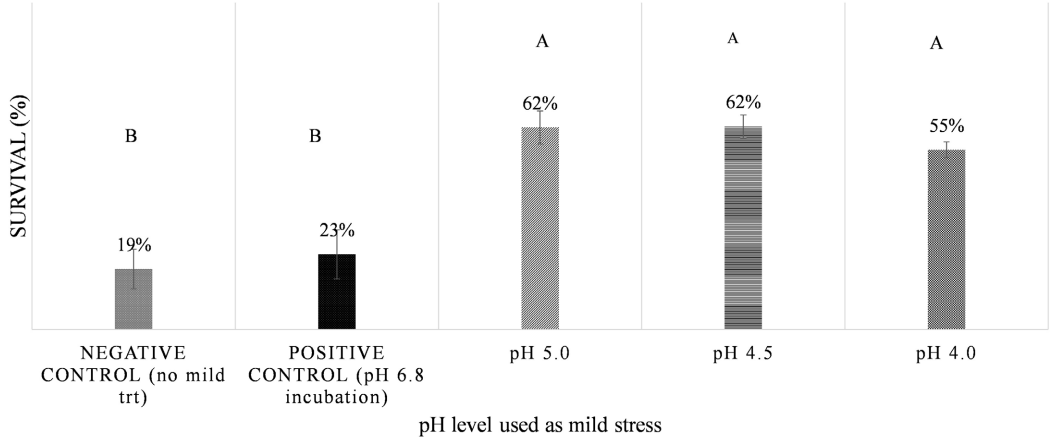
The type III test of fixed effects of the Glimmix procedure of the Statistical Analysis System (SAS 9.4) was used to detect differences between treatments. Tukey mean separation was used when difference between treatments were found. The level of significance was 0.05.

3. Results

Before 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˚C 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 according to formula above).

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_2O_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.

3.1. Acid Mild Stress



[A,B]Means with different letters represent significant differences ($P < 0.05$).

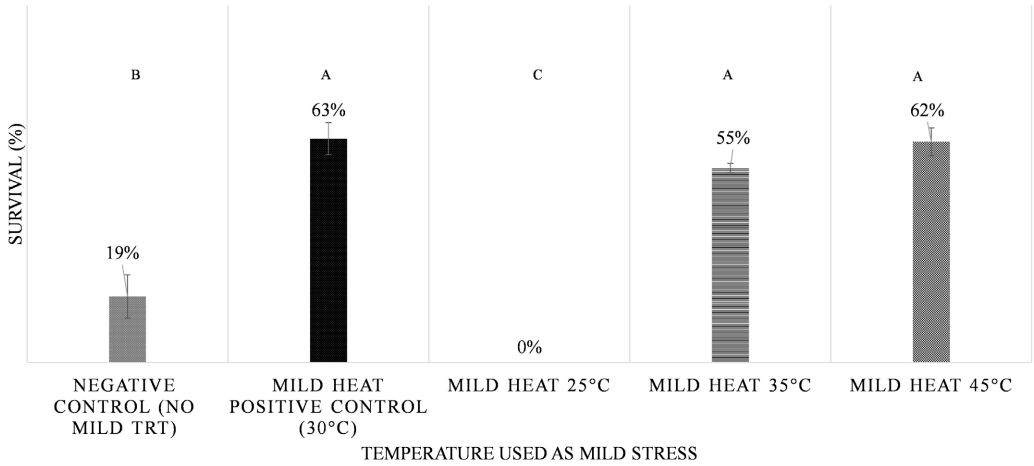
Figure 1. Heat tolerance of *Leuconostoc mesenteroides* to 60˚C heat challenging condition with prior exposure to various levels of acid mild stress for 2 hours.

Figure 1 shows the results of the exposure to mild acid conditions prior to the exposure to the 60˚ HCC. The prior exposure to mildly acidified media enhanced the survival of *Leuconostoc mesenteroides* to the HCC of 60˚C ($P < 0.05$). Expos-

ing 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 1). The use of acid mild treatments did not enhance the survivability of *Leuconostoc mesenteroides* after subsequent exposure to the 70˚C HCC. This indicated that prior subjection of *Leuconostoc mesenteroides* to mild acid stress of pH's 4.0, 4.5 and 5.0 improved its survival at 60˚C.

3.2. Heat Mild Stress

Figure 2 shows the survivability of *Leuconostoc mesenteroides* to the 60˚C HCC when previously exposed to mild heat stresses at 25˚C, 35˚C and 45˚C. Incubating the bacteria at 35˚C and 45˚C enhanced the survivability when compared to the negative control and at 25˚C ($P < 0.05$) (Figure 2). No significant differences between the positive control, 35˚C 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). Incubating the bacteria at 25˚C was significantly lower than the effect of the negative control (Figure 2).



[A,B,C]Means with different letters represent significant differences ($P < 0.05$).

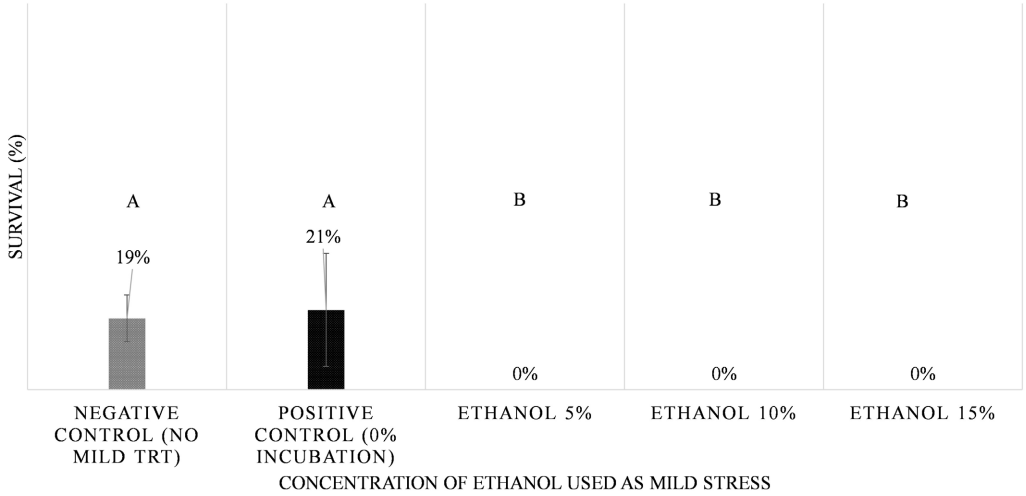
Figure 2. Heat tolerance of *Leuconostoc mesenteroides* to 60˚C heat challenging condition with prior exposure to various levels of heat mild stress for 10 minutes.

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). However, viability was not improved with the prior exposure to mild heat treatments when bacteria was exposed to the 70˚C HCC. This indicated that prior subjection of *Leuconostoc mesenteroides* to mild heat stress of 35˚C and 45˚C improved its survival at 60˚C.

3.3. Ethanol Mild Stress

Counts of *Leuconostoc mesenteroides* after HCC of 60˚ with prior exposure to

various ethanol levels can be found in Figure 3. Survivability to the 60˚C HCC was not improved by the exposure to any level of ethanol when compared to both controls (Figure 3). 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.

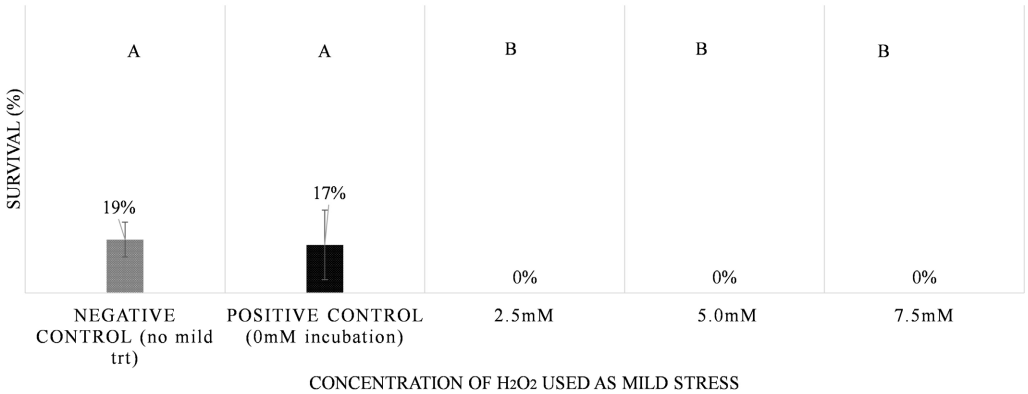


[A,B]Means with different letters represent significant differences ($P < 0.05$).

Figure 3. Heat tolerance of *Leuconostoc mesenteroides* to 60˚C Heat challenging condition with prior exposure to various levels of ethanol mild stress for 2 hours.

3.4. Oxidative Mild Stress

Counts of *Leuconostoc mesenteroides* after the HCC of 60˚C can be found in Figure 4. Regardless of the amount of H_2O_2 used as mild oxidative stress there was no significant improvement in viability of *Leuconostoc mesenteroides* to the HCC 60˚C or 70˚C when compared to either of the controls.



[A,B]Means with different letters represent significant differences ($P < 0.05$).

Figure 4. Heat tolerance of *Leuconostoc mesenteroides* to 60˚C Heat challenging condition with prior exposure to various levels of hydrogen peroxide as mild stresses for 2 hours.

3.5. 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 1. The most relevant effects and interactions are discussed.

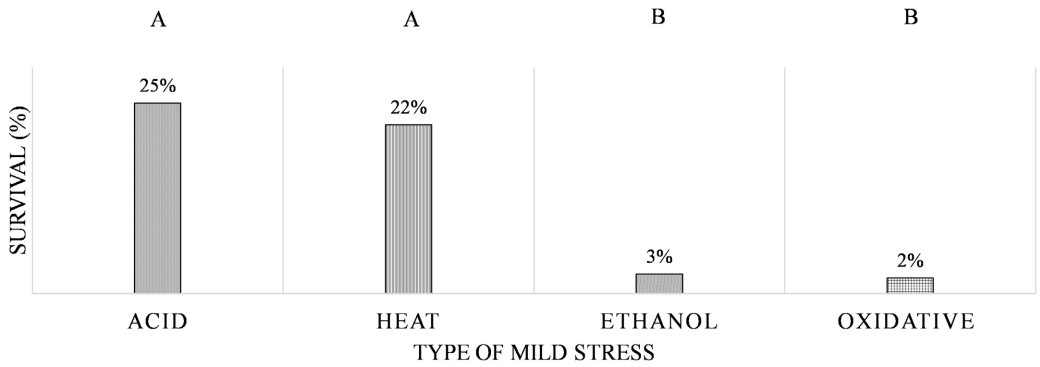
3.6. Type of Mild Stress

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 1). Figure 5 compares the survival (%) of *Leuconostoc mesenteroides* to the HCC of both 60˚C 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 5).

Table 1. Probability for main effects and their interaction on the heat tolerance of *Leuconostoc mesenteroides* spp. *cremoris* when exposed to 60˚C or 70˚C HCC with prior exposure to various types and levels of mild stresses.

Main Effects	P-Value
Type of Mild Stress	<0.0001
Level of Mild Stress	<0.0001
HCC Temperature	<0.0001
Type of Mild Stress × Level of Mild Stress	<0.0001
Type of Mild Stress × HCC Temperature	<0.0001
Level of Mild Stress × HCC Temperature	<0.0001
Type of Mild Stress × Level of Mild Stress × HCC Temperature	<0.0001

P-value less than 0.05 represents significant effect.

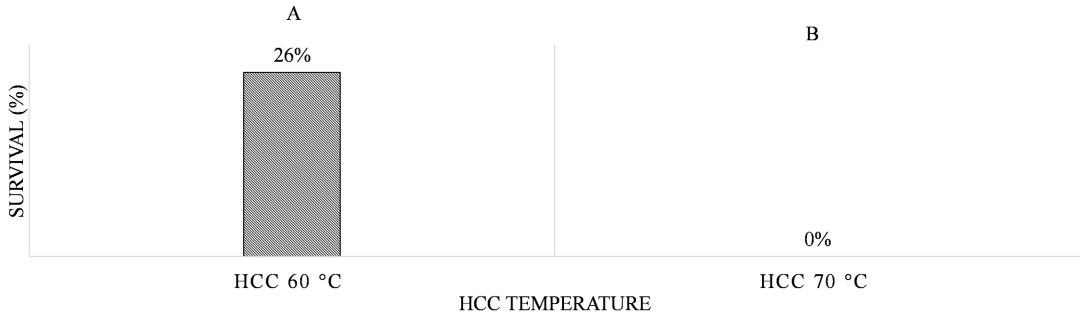


[A,B]Means with different letters represent significant differences ($P < 0.05$).

Figure 5. Heat tolerance of *Leuconostoc mesenteroides* with prior exposure to various types of mild stresses.

3.7. HCC Temperature

The temperature of exposure to the HCC had a significant effect on the survival of *Leuconostoc mesenteroides* ($P < 0.05$). The average survivability to 60˚C HCC was of 26% whereas the 70˚C was completely lethal to the bacteria, regardless of the use of various types of mild stress (Figure 6).



[A,B]Means with different letters represent significant differences ($P < 0.05$).

Figure 6. Average survival of *Leuconostoc mesenteroides* to the 60˚C and 70˚C heat challenging conditions.

3.8. Type of Mild Stress × Level of Mild Stress × HCC Temperature Interaction Effect

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 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 1).

The viability of *Leuconostoc mesenteroides* was substantially improved when exposed to mild heat of 35˚C, 45˚C and pH 4.0, 4.5, 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%. 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 70˚C HCC.

4. 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 [7]. Also, the improvement in its heat tolerance could enable its incorporation in new probiotic products such as a processed cheese dip. 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 [7].

The defense mechanisms against heat shock tries to minimize damage, mainly of protein denature [20]. 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* [21]. 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 [22]. 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 (**Figure 2**) but not to the 70˚C. Similarly, in an experiment conducted by Kang *et al.*, [21]. *L. lactis* HE-1 were treated at 37˚C, 42˚C, 47˚C, 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 [21]. Beniamino *et al.*, [23] isolated ropy slime producing *Leuconostoc mesenteroides* from cooked meat products and reported no growth of these *Leuconostocs* at 44˚C.

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* [16]. 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 [24]. HSP60 is induced in the presence of denatured proteins to bind intracellular proteins and protect them from denaturation [16]. Living cells respond to an 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 [25]. The major HSPs, include chaperones DnaK, GroEL, and GroES, as well as the Clp family of proteins, are a main factor in protein quality control in both stressed and unstressed bacteria [16]. Heat resistance to the induction general stress responses. HSP has various roles in cell physiology such as ribosome stability, temperature sensing, and control of ribosomal function [20].

Heat shock resistance of bacteria differs based on genetic differences between species, the physiological state of the cells, and chemical and physical factors such as pH, water activity, salt content, and preservatives [26]. 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 [20]. Tolerance to 72˚C for 90 s decreased noticeably when a bacteriostatic chloramphenicol (1 mg/L) was added during adaptation. Two-Dimensional Gel Electrophoresis 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 [20]. Bussarin and Sundip-Kumar [27] reported that heat tolerance of thermostable *Lactobacillus plantarum* ssp1 (ID1L) could potentially

be enhanced by 0.3 M NaCl for 30 min and could be sprayed at high temperatures. Li *et al.*, [28] reported that *Leuconostoc mesenteroides* SN-8 produced exo-polysaccharide and this exo-polysaccharide was heat resistance to temperature of 80˚C.

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 [18]. The effects of the acid mild treatments were significantly better to the survival of *Leuconostoc mesenteroides* to the 60˚C HCC (**Figure 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 5**). Exposing the bacteria to pH 5.0, 4.5 and 4.0 for 2 h was as beneficial as exposing the bacteria to temperatures above 30˚C through 45˚C for ten minutes prior to the 60˚C HCC. A strong connection in the response and synergy of these 2 stresses has been reported [10] [14]. The results of the present study are in similar to the ones in a study conducted by Zotta *et al.*, [29] in which acid and heat adaptation of most *S. thermophilus* strains enhanced the survival of heat stressed cells compared to control [29].

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 [16]. The response of cells to heat shock and alcohols show similarities, both stresses alter the fluidity of the cell membrane [30].

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 [31]. Ethanol toxicity is generally attributed to the disruption of membrane structure [30]. 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˚C or 70˚C was not improved by the used of ethanol concentrations from 5% - 15% v/v or hydrogen peroxide from 2.5 - 7.5 mM when compared to the results of some acid and heat mild treatments (**Figure 6**). HSP synthesis in *Leuconostoc mesenteroides* was found to be stimulated in response to ethanol treatment [16]. Addition of ethanol (4% v/v) resulted in a relative overexpression of 70- and 60-kDa proteins with a reduction in total protein synthesis [16]. 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 [31]. The genes induced in various LAB and some yeast strains by ethanol seem to be mostly identical to those induced by heat shock [31]. 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) [31]. However, none of those beneficial effects could be observed by using the concentrations tested in the present study. 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 [32]. Ethanol is amphipathic, so it can insert into

lipid bilayers causing increased membrane fluidity, loss of membrane integrity, leakage of ions and metabolites, collapse of proton gradient needed for ATP generation [32] hence killing *Leuconostoc mesenteriodes* cells.

The levels of oxidative stress were detrimental to the survivability of *Leuconostoc mesenteroides* for both 60˚C 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_2O_2 is a weak oxidant, but it is exceedingly diffusive and has a long lifetime [33]. The H_2O_2 contributes to oxidative damage either directly or as a precursor of hydroxyl radicals. It is especially potent in causing oxidative damage to DNA [21]. Reactive oxygen species (ROS) disrupts cellular components, cellular membranes, oxidizes amino acids and inactivates vital enzymes [21].

Dowds [34] found that oxidative response appeared to be coupled to the synthesis of 2 major heat shock proteins (DnaK and GRoEL chaperons) and explained that the cross protection between oxidative and heat stress takes place in a phase dependent manner. This study is based on a single strain which can be a limitation. Future research should test other strains to be able to generalize these stress responses.

5. 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˚C - 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 thermotolerance parameters and the implementation of cross-protection techniques should be useful for predicting the behavior of the probiotic strains during subsequent processing. Normally bacterial cultures are added after pasteurization/heat treatment. While the product is hot (60˚C) if this culture is added it is expected to show the above mentioned benefits.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Vedamuthu, E.R. (1994) The Dairy *Leuconostoc*: Use in Dairy Products. *Journal of*

Dairy Science, **77**, 2725-2737. https://doi.org/10.3168/jds.s0022-0302(94)77215-5

[2] Kekkonen, R.A., Kajasto, E., Miettinen, M., Veckman, V., Korpela, R. and Julkunen, I. (2008) Probiotic *Leuconostoc mesenteroides* ssp. Cremoris and *Streptococcus thermophilus* Induce IL-12 and IFN-γ Production. *World Journal of Gastroenterology*, **14**, 1192-1203. https://doi.org/10.3748/wjg.14.1192

[3] Agarwal, K. and Bhasin, S. (2002) Feasibility Studies to Control Acute Diarrhoea in Children by Feeding Fermented Milk Preparations Actimel and Indian Dahi. *European Journal of Clinical Nutrition*, **56**, S56-S59. https://doi.org/10.1038/sj.ejcn.1601664

[4] de Paula, A.T., Jeronymo-Ceneviva, A.B., Todorov, S.D. and Penna, A.L.B. (2014) The Two Faces of *Leuconostoc mesenteroides* in Food Systems. *Food Reviews International*, **31**, 147-171. https://doi.org/10.1080/87559129.2014.981825

[5] Ariute, J.C., Coelho-Rocha, N.D., Dantas, C.W.D., de Vasconcelos, L.A.T., Profeta, R., de Jesus Sousa, T., et al. (2023) Probiogenomics of *Leuconostoc mesenteroides* Strains F-21 and F-22 Isolated from Human Breast Milk Reveal Beneficial Properties. *Probiotics and Antimicrobial Proteins*, **17**, 500-515. https://doi.org/10.1007/s12602-023-10170-7

[6] Rani, P.S. and Agrawal, R. (2008) Effect on Cellular Membrane Fatty Acids in the Stressed Cells of *Leuconostoc mesenteroides*: A Native Probiotic Lactic Acid Bacteria. *Food Biotechnology*, **22**, 47-63. https://doi.org/10.1080/08905430701863977

[7] Hemme, D. and Foucaud-Scheunemann, C. (2004) *Leuconostoc*, Characteristics, Use in Dairy Technology and Prospects in Functional Foods. *International Dairy Journal*, **14**, 467-494. https://doi.org/10.1016/j.idairyj.2003.10.005

[8] de Paula, A.T., Jeronymo-Ceneviva, A.B., Silva, L.F., Todorov, S.D., Franco, B.D.G.M. and Penna, A.L.B. (2014) *Leuconostoc mesenteroides* SJRP55: A Potential Probiotic Strain Isolated from Brazilian Water Buffalo Mozzarella Cheese. *Annals of Microbiology*, **65**, 899-910. https://doi.org/10.1007/s13213-014-0933-9

[9] Serrazanetti, D.I., Gianotti, A., Montanari, C. and Gottardi, D. (2013) Dynamic Stresses of Lactic Acid Bacteria Associated to Fermentation Processes. In: Kongo, M., Ed., *Lactic Acid Bacteria—R&D for Food, Health and Livestock Purposes*, IntechOpen. https://www.intechopen.com/chapters/42318

[10] Corcoran, B., Stanton, C., Fitzgerald, G. and Ross, R. (2008) Life under Stress: The Probiotic Stress Response and How It May Be Manipulated. *Current Pharmaceutical Design*, **14**, 1382-1399. https://doi.org/10.2174/138161208784480225

[11] Panoff, J., Thammavongs, B., Guéguen, M. and Boutibonnes, P. (1998) Cold Stress Responses in Mesophilic Bacteria. *Cryobiology*, **36**, 75-83. https://doi.org/10.1006/cryo.1997.2069

[12] D'Angelo, L., Cicotello, J., Zago, M., Guglielmotti, D., Quiberoni, A. and Suárez, V. (2017) *Leuconostoc* Strains Isolated from Dairy Products: Response against Food Stress Conditions. *Food Microbiology*, **66**, 28-39. https://doi.org/10.1016/j.fm.2017.04.001

[13] Rangel, D.E.N. (2010) Stress Induced Cross-Protection against Environmental Challenges on Prokaryotic and Eukaryotic Microbes. *World Journal of Microbiology and Biotechnology*, **27**, 1281-1296. https://doi.org/10.1007/s11274-010-0584-3

[14] van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S.D. and Maguin, E. (2002) Stress Responses in Lactic Acid Bacteria. *Antonie van Leeuwenhoek*, **82**, 187-216. https://doi.org/10.1023/a:1020631532202

[15] Hartke, A., Bouche, S., Laplace, J., Benachour, A., Boutibonnes, P. and Auffray, Y.

(1995) UV-Inducible Proteins and UV-Induced Cross-Protection against Acid, Ethanol, H_2O_2 or Heat Treatments in Lactococcus Lactis Subsp. Lactis. *Archives of Microbiology*, **163**, 329-336. https://doi.org/10.1007/bf00404205

[16] Salotra, P., Singh, D.K., Seal, K.P., Krishna, N., Jaffe, H. and Bhatnagar, R. (1995) Expression of DnaK and GroEL Homologs in *Leuconostoc esenteroides* in Response to Heat Shock, Cold Shock or Chemical Stress. *FEMS Microbiology Letters*, **131**, 57-62. https://doi.org/10.1111/j.1574-6968.1995.tb07754.x

[17] Flahaut, S., Laplace, J.M., Frère, J. and Auffray, Y. (1998) The Oxidative Stress Response in *Enterococcus faecalis*: Relationship between H_2O_2 Tolerance and H_2O_2 Stress Proteins. *Letters in Applied Microbiology*, **26**, 259-264. https://doi.org/10.1046/j.1472-765x.1998.00325.x

[18] De Angelis, M., Bini, L., Pallini, V., Cocconcelli, P.S. and Gobbetti, M. (2001) The Acid-Stress Response in *Lactobacillus sanfranciscensis* CB1. *Microbiology*, **147**, 1863-1873. https://doi.org/10.1099/00221287-147-7-1863

[19] Wu, C., Zhang, J., Wang, M., Du, G. and Chen, J. (2012) *Lactobacillus casei* Combats Acid Stress by Maintaining Cell Membrane Functionality. *Journal of Industrial Microbiology and Biotechnology*, **39**, 1031-1039. https://doi.org/10.1007/s10295-012-1104-2

[20] De Angelis, M. and Gobbetti, M. (2004) Environmental Stress Responses in *Lactobacillus*: A Review. *Proteomics*, **4**, 106-122. https://doi.org/10.1002/pmic.200300497

[21] Kang, C., Jeon, H., Shin, Y., Kwon, Y. and So, J. (2015) Heat Adaptation Improves Viability of *Lactococcus lactis* Subsp. *lactis* HE-1 after Heat Stress. *Food Science and Biotechnology*, **24**, 1823-1827. https://doi.org/10.1007/s10068-015-0238-1

[22] Papadimitriou, K., Alegría, Á., Bron, P.A., de Angelis, M., Gobbetti, M., Kleerebezem, M., et al. (2016) Stress Physiology of Lactic Acid Bacteria. *Microbiology and Molecular Biology Reviews*, **80**, 837-890. https://doi.org/10.1128/mmbr.00076-15

[23] Cenci-Goga, B.T., Sechi, P., Iulietto, M.F., Amirjalali, S., Barbera, S., Karama, M., *et al.* (2020) Characterization and Growth under Different Storage Temperatures of Ropy Slime-Producing *Leuconostoc mesenteroides* Isolated from Cooked Meat Products. *Journal of Food Protection*, **83**, 1043-1049. https://doi.org/10.4315/jfp-19-521

[24] Barnes, C.A., Johnston, G.C. and Singer, R.A. (1990) Thermotolerance Is Independent of Induction of the Full Spectrum of Heat Shock Proteins and of Cell Cycle Blockage in the Yeast *Saccharomyces cerevisiae*. *Journal of Bacteriology*, **172**, 4352-4358. https://doi.org/10.1128/jb.172.8.4352-4358.1990

[25] Carper, S.W., Duffy, J.J. and Gerner, E.W. (1987) Heat Shock Proteins in Thermotolerance and Other Cellular Processes. *Cancer Research*, **47**, 5249-5255.

[26] Browne, N. and Dowds, B.C.A. (2001) Heat and Salt Stress in the Food Pathogen Bacillus Cereus. *Journal of Applied Microbiology*, **91**, 1085-1094. https://doi.org/10.1046/j.1365-2672.2001.01478.x

[27] Kosin, B. and Rakshit, S.K. (2010) Induction of Heat Tolerance in Autochthonous and Allochthonous Thermotolerant Probiotics for Application to White Shrimp Feed. *Aquaculture*, **306**, 302-309. https://doi.org/10.1016/j.aquaculture.2010.04.017

[28] Li, Y., Liu, Y., Cao, C., Zhu, X., Wang, C., Wu, R., et al. (2020) Extraction and Biological Activity of Exopolysaccharide Produced by *Leuconostoc mesenteroides* SN-8. *International Journal of Biological Macromolecules*, **157**, 36-44. https://doi.org/10.1016/j.ijbiomac.2020.04.150

[29] Zotta, T., Ricciardi, A., Ciocia, F., Rossano, R. and Parente, E. (2008) Diversity of Stress Responses in Dairy Thermophilic Streptococci. *International Journal of Food Microbiology*, **124**, 34-42. https://doi.org/10.1016/j.ijfoodmicro.2008.02.024

[30] Campos, F.M., Couto, J.A., Figueiredo, A.R., Tóth, I.V., Rangel, A.O.S.S. and Hogg, T.A. (2009) Cell Membrane Damage Induced by Phenolic Acids on Wine Lactic Acid Bacteria. *International Journal of Food Microbiology*, **135**, 144-151. https://doi.org/10.1016/j.ijfoodmicro.2009.07.031

[31] Piper, P. (1995) The Heat Shock and Ethanol Stress Responses of Yeast Exhibit Extensive Similarity and Functional Overlap. *FEMS Microbiology Letters*, **134**, 121-127. https://doi.org/10.1016/0378-1097(95)00431-9

[32] Denyer, S.P. and Hugo, W.B. (1991) Mechanisms of Action of Chemical Biocides: Their Study and Exploitation. Blackwell Scientific Publications, 45-64.

[33] Jaroni, D. and Brashears, M.M. (2000) Production of Hydrogen Peroxide by *Lactobacillus delbrueckii* Subsp. *lactis* as Influenced by Media Used for Propagation of Cells. *Journal of Food Science*, **65**, 1033-1036. https://doi.org/10.1111/j.1365-2621.2000.tb09412.x

[34] Dowds, B.C.A. (1994) The Oxidative Stress Response in *Bacillus subtilis*. *FEMS Microbiology Letters*, **124**, 255-263. https://doi.org/10.1111/j.1574-6968.1994.tb07294.x