UNIVERSIDAD DE LAS AMÉRICAS PUEBLA
ESCUELA DE INGENIERÍA
DEPARTAMENTO DE INGENIERÍA QUÍMICA Y ALIMENTOS

# Evaluación de la actividad antibacteriana, citotóxica y anti-inflamatoria de los sobrenadantes de cepas de bacterias ácido lácticas en sistemas modelo y alimentos

Tesis presentada en cumplimiento parcial de los requisitos para obtener el Grado de Doctor en Ciencia de Alimentos

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UNIVERSIDAD DE LAS AMÉRICAS PUEBLA
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# Evaluation of antibacterial, cytotoxic and anti-inflammatory activity of supernatants of lactic acid bacteria strains in model systems and food

In partial fulfilment of the requirements for the Degree of Doctor of Food Science

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## VALIDATION LETTER

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El Dr. Aurelio López Malo Vigil, Profesor del departamento de Ingeniería Química y Alimentos,

Hace constar:

Que la tesis titulada: "Evaluación de la actividad antibacteriana, citotóxica y anti-inflamatoria de los sobrenadantes de cepas de bacterias ácido lácticas en sistemas modelo y alimentos" presentada por Daniela Arrioja Bretón para obtener el grado de Doctora en Ciencia de Alimentos por la Universidad de las Américas Puebla, ha sido realizada en el Departamento de Ingeniería Química y Alimentos bajo su dirección, reuniendo las condiciones necesarias para ser defendida por su autora.

_____

Dr. Aurelio López Malo Vigil

Director de tesis

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## PROLOGUE

The present work is a doctoral thesis entitled "Evaluation of antibacterial, cytotoxic and anti-inflammatory activity of supernatants of lactic acid bacteria strains in model systems and food". The antimicrobial activity of cell-free supernatants (CFS) as well as their inactivated (paraprobiotic) cells from sixteen strains of lactic acid bacteria (LAB) were studied and evaluated. This antimicrobial activity was studied against six undesirable microorganisms in food: *Escherichia coli, Staphylococcus aureus, Shigella sonnei, Pseudomonas fluorescens, Salmonella* Typhimurium and *Listeria monocytogenes*, in order to select the strains with the highest antimicrobial activity to determine their stability during storage at different temperatures; Subsequently, the cytotoxic and anti-inflammatory activity of the most stable supernatant was fractionated and the nature of the compounds responsible for them was identified. The document is divided into four chapters: 1) Review document: "Paraprobiotics and their application in food", 2) Research document I: "Antimicrobial activity and storage stability of cell-free supernatants of lactic acid bacteria and their applications with fresh beef" , 3) Research document II: "Antimicrobial activity of fractions containing proteins isolated from the culture of *Lactobacillus plantarum* NRRL B-4496" and 4) Research document III: "Antimicrobial activity of *Lactobacillus casei* 21/1 cell-free supernatants on fresh Mexican-style cheese".

The first chapter is a bibliographic review about the definition of parapribiotics, the benefits they have on host health, how they are produced and their possible application in foods, the second chapter discusses the evaluation of the antimicrobial activity of the sixteen LAB (cell-free supernatants), followed by the stability of the supernatants over time and their application in meat. In the third chapter, the antimicrobial activity of the fractionations was evaluated, as well as their cytotoxicity and inflammatory response of selected fractions of CFS produced by *Lb. plantarum* NRRL B-4496; and in the fourth chapter, was studied the effect of *Lb. casei* cell-free supernatants in fresh Mexican-style cheese on the growth of indicator microorganisms (*Escherichia coli*, *Staphylococcus aureus, Salmonella* Typhimurium, and *Listeria monocytogenes*).

**INDEX**

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**ABSTRACT**

Lactic acid bacteria (LAB) are known to produce flavors, aromas, textures and nutritional changes in food, the interest in them has increased in recent years as bio-preservatives, due to the production of secondary metabolites (organic acids, bacteriocins, hydrogen peroxide, reuterin, diacetyl, ethyl alcohol, among others), capable of inhibiting pathogenic bacteria.

This study evaluated the effectiveness of cell-free supernatants (CFS) produced by sixteen strains of LAB as antimicrobials *in vitro* (agar well diffusion test) against *Escherichia coli*, *Staphylococcus aureus*, *Shigella sonnei, Pseudomonas fluorescens, Salmonella* Typhimurium, or *Listeria monocytogenes*. CFS from *Lb. plantarum, Lb. sakei*, and *Lb. rhamnosus* were found to be the most effective, and the antimicrobial properties were eliminated when pH was adjusted to 6.5 for most of tested CFS while only *Lb. plantarum* CFS maintained their antimicrobial activity, which was lost when treated with proteinase K; Antibacterial activity was significantly ($p < 0.05$) reduced between time zero and after 20 weeks of storage at three studied temperatures; the greatest reductions in antibacterial activity were observed at 35 ºC. Using CFS from *Lb. plantarum* to marinate beef corroborates its antimicrobial activity. Meat color differences were important in raw beef; while on grilled meat, changes were scarcely detected. Furthermore, the CFS from *Lb. plantarum* fractions showed no pro-inflammatory activity when exposed to human macrophages.

Finally, CFS of *Lb. casei* were studied against four indicator microorganisms: *Escherichia coli, Salmonella* Typhimurium, *Staphylococcus aureus,* and *Listeria monocytogenes* on inoculated fresh Mexican-style cheese during seven days of storage at 4 °C ± 1.0 °C. *S.* Typhimurium was observed to be the most sensitive microorganism to *Lb. casei* CFS.

**RESUMEN**

Se sabe que las bacterias del ácido láctico (BAL) producen sabores, olores, texturas y cambios nutricionales en los alimentos, el interés en ellas ha aumentado en los últimos años como bio-conservantes, debido a la producción de metabolitos secundarios (ácidos orgánicos, bacteriocinas, peróxido de hidrógeno, reuterina, diacetil, alcohol etílico, entre otros), capaces de inhibir bacterias patógenas.

Este estudio evaluó la efectividad de los sobrenadantes libres de células (SLC) producidos por dieciséis cepas de BAL como antimicrobianos *in vitro* (prueba de difusión en pozos de agar) contra *Escherichia coli, Staphylococcus aureus, Shigella sonnei, Pseudomonas fluorescens, Salmonella* Typhimurium o *Listeria monocytogenes*. Se demostró que los SLC de *Lb. plantarum, Lb. sakei* y *Lb. rhamnosus* fueron efectivos, y las propiedades antimicrobianas se eliminaron cuando el pH se ajustó a 6.5 para la mayoría de los SLC analizados, mientras que únicamente el SLC de *Lb. plantarum* mantuvo su actividad antimicrobiana, que se perdió cuando se trató con proteinasa K; la actividad antibacteriana se redujo significativamente ($p < 0.05$) entre el tiempo cero y después de 20 semanas de almacenamiento a tres temperaturas estudiadas; las mayores reducciones en la actividad antibacteriana se observaron a 35 $^{o}$C. Al utilizar el SLC de *Lb. plantarum* para marinar carne de res, se comprobó su actividad antimicrobiana. Las diferencias de color de la carne fueron importantes en la carne cruda; mientras que en la carne a la parrilla, apenas se detectaron cambios. Además, las fracciones del SLC de de *Lb. plantarum* no mostraron actividad pro-inflamatoria cuando se expusieron a macrófagos humanos.

Finalmente, SLC de *Lb. casei* se estudió contra cuatro microorganismos indicadores: *Escherichia coli, Salmonella* Typhimurium, *Staphylococcus aureus* y *Listeria monocytogenes* en queso fresco estilo mexicano inoculado y almacenad0 durante siete días a 4 ° C ± 1.0 ° C. Se observó que *S.* Typhimurium es el microorganismo más sensible a los SLC de *Lb. casei.*

## GENERAL INTRODUCTION

Microbiological contamination of food is a major problem, both economically and in public health. This phenomenon is generally a mixed process involving bacteria, yeasts, and molds.

Bacteria such as *Escherichia coli, Staphylococcus aureus, Shigella sonnei, Pseudomonas fluorescens, Salmonella* Typhimurium, and *Listeria monocytogenes* are indicators of food contamination, capable of causing spoilage and foodborne illness.

There are several techniques commonly used to preserve food; among which are physical methods such as: drying, freeze-drying, refrigeration and freezing storage, modified atmospheres, or heat treatments, among others. Chemical methods such as organic acids (citric, sorbic and benzoic), sulphates, nitrates and sodium salts (Sharif et al., 2017), are also methods to control microbial growth.

However, the resistance of microorganisms to these chemical methods has been increasing in recent years (Lossada et al., 2017; Mirzaagha et al., 2011). On the other hand, there is a growing interest on the part of consumers to choose foods with cleaner labels, which has promoted the research and development of bio-preservatives, which include enzymes and animal proteins such as: lysozyme and lactoferrin, plants or derivatives of these, such as phytoalexins, some spices and essential oils; and microorganisms such as LAB (Schwenninger et al., 2008, Sauceda, 2011; Mani-López et al., 2018).

LABs are known to produce a wide variety of secondary metabolites (ethanol, hydrogen peroxide, carbon dioxide, reuterin, bacteriocins, lactic acid, acetic acid, benzoic acid, hydroxyphenyl lactic acid, hydroxy fatty acids, and phenyl lactic acid, among others), to which antibacterial properties have been attributed (Jay, 1982; Piard and Desmazeaud, 1992). On the other hand, they have potential use in the food industry, as they are generally recognized as safe (GRAS) by the US Food and Drug Administration, this makes them a good alternative to replace synthetic preservatives in foods while minimizing regulatory hurdles and offering consumers clean label products (Schnürer and Magnusson, 2005; Zacharof and Lovitt, 2012). Numerous studies have shown that direct application of LAB, or

even its cell-free supernatants (CFS), inhibits undesirable bacteria in foods such as chicken breast, beef, bread, and cheese (Topisirovic et al., 2006; Cizeikiene et al., 2013, da Costa et al., 2018; Mani-López et al., 2018).

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## GENERAL JUSTIFICATION

Due the production of secondary metabolites, lactic acid bacteria have been studied as an alternative of bio-preservatives to inhibit the growth of spoilage and/or pathogenic microorganisms related to food products. Most studies have been focused on the isolation and direct application of one or more secondary metabolites. However, very few secondary metabolites are legally authorized for use in food.

Currently the consumer is looking for products with cleaner labels, avoiding synthetic preservatives, which is why in the last few years different alternatives have been studied that are natural, effective and in turn are reasonably acceptable.

## OBJECTIVES

### General objective

Evaluate the antimicrobial and cytotoxic activity, as well as the inflammatory response of cell-free supernatants of lactic acid bacteria strains, against different bacterial groups in model systems and food.

### Specific objectives

- Evaluate the antimicrobial effectiveness of cell-free supernatants from different strains of lactic acid bacteria, against different bacterial groups, in order to select the strains with the highest antimicrobial activity.
- Determine the effect of time and storage temperature on the antimicrobial activity of the cell-free supernatants, product of the growth of the selected strains of lactic acid bacteria.
- Determine the cytotoxicity and the inflammatory activity of selected cell-free supernatants using human-derived macrophages.
- Determine the effect of the incorporation of the lactic acid bacteria cell-free supernatants in food products, on their antimicrobial activity against selected bacterial groups according to the type of food.

**REVIEW PAPER**

**“Paraprobiotics and their application in food”**

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**Abstract**

Paraprobiotics are inactivated cells of probiotic microorganisms, which confer a benefit to the health of the consumer host, within which are: reduction of symptoms in immunological and gastrointestinal disorders, control of allergies, among others, these benefits have been observed in animals and humans. Different probiotic strains have been used for the production of paraprobiotics, using several inactivation methods such as thermal treatments, high pressures, sonication and ultraviolet irradiation. The use of paraprobiotics is a current and relevant topic since their applications can present various advantages as well as challenges.

***Keywords:*** paraprobiotics, inactivation methods, benefits and application.

## Introduction

In recent years the term "paraprobiotics" has been introduced, which refers to non-viable (intact or broken) probiotic microbial cells or crude cell extracts that, when administered or consumed in adequate amounts, produce a health benefit of the host (Sawada et al., 2015). They are microorganisms that completely lost their viability after exposure to factors that alter their cellular structures, by applying an inactivation method such as heat treatments, high pressures, ultraviolet irradiation or sonication (de Almada, Almada, Martinez and Sant'Ana, 2016).

The study of these agents for the science and food industry is of great importance, since different investigations indicate that paraprobiotics could provide various benefits to the health of consumers. In addition, its use represents advantages with respect to probiotics, such as less interaction (or no interaction) with the other components of the food; the possibility of adding paraprobiotics before heat treatment of food; its incorporation in a greater variety of products; that their addition does not imply that the foods in which they are added have to be refrigerated; among others (Ishikawa et al., 2010; Sawada et al., 2015).

The purpose of this work is to compile relevant information about the paraprobiotics, their production, the benefits that their consumption produces for the host, as well as their use and possible applications in food.

**Bibliographic review**

## 1. Paraprobiotics

### 1.1. Definition

The FAO / WHO (2002) definition of probiotics only includes living microorganisms, which when administered in adequate amounts, confer a benefit to the host. However, non-viable cells (intact or broken) have been reported to also provide benefits to the host (Adams, 2010; Ananta and Knorr, 2009). These cells have been named "paraprobiotics" and have been defined as inactivated cells or fractions of microbial cells that, when administered in adequate amounts, confer a benefit to the host (Taverniti and Guglielmetti, 2011). They are also called in the literature as "inactivated probiotics" or "phantom probiotics", which are microorganisms that completely lost their viability after suffering alterations in their cellular structures (fragmentation of DNA strands, membrane rupture or mechanical damage to the cell wall), due to the application of some inactivation methods (de Almada et al., 2016).
Paraprobiotics contain fragments, which are assigned the term "probiotic cell fragments" or PCF; among which are the theicoic acids, nucleotides, components of DNA and / or RNA. Some of these fragments are even structures with physiological functions, such as the peptide-glucan cell wall (Shigwedha et al., 2014).
So far, research has been carried out on paraprobiotics, in which around 60 different strains of probiotics have been used for the production of paraprobiotics, as well as different genera of microorganisms, highlighting the *Lactobacillus* genus as the most studied, followed by the genus *Bifidobacterium* (Table I).

### 1.2. Importance

As shown in Table I, the benefits of paraprobiotics to host health have been observed in various investigations. Most tests have been carried out in vitro, as well as in vivo in rats,

mice and even fish and pigs, but also, although in smaller numbers, in some groups of humans.

In the reviewed studies it has been observed that probiotics, paraprobiotics and PCF are modifiers of various biological responses in the host, contributing to the reduction of symptoms of various immunological disorders, such as in low-grade inflammations; neuro-psychiatric disorders such as Alzheimer's, chronic depression, mild memory loss and general cognitive decline; orthopedic disorders such as general muscle fatigue syndromes and chronic pain; respiratory disorders like bronchitis, sinusitis; metabolic disorders as in diabetes; certain cardiovascular disorders; intestinal diseases such as celiac disease and colon cancer. They are also thought to help modulate the immune system; anti-inflammatory activity against colitis; caries reduction; relief of symptoms of chronic diarrhea; improvement in allergic symptoms; lowering cholesterol; suspension of body weight gain; the relief or elimination of constipation; increased adhesion of intestinal cells (which inhibits pathogens); among others (Shigwedha, Sichel, Jia, Al-Shura, and Zhang 2015).

Likewise, Shigwedha *et al.* (2014) mention that there is clinical evidence on the influence on host health of inactivated *Lactobacillus rhamnosus* V cells, which are effective in the prevention and treatment of infectious diseases, as well as allergies, fatigue and fibromyalgia.

### 1.3. Methods of obtaining paraprobiotics

There are different methods of inactivation of microbial cells, which have been used by researchers to obtain paraprobiotics, among which are heat treatments; Ultraviolet irradiation, high pressures, and sonication have also been used (Ananta and Knorr, 2009; Chiu *et al.,* 2013; de Almada *et al.,* 2016). These methods cause the inactivation of microorganisms by different mechanisms. It is very important to highlight that the inactivation method used to produce paraprobiotics must be capable of retaining the health benefits provided by the probiotic microorganism (Raz and Rachmilewitz, 2005). Table I shows the inactivation methods used on different microorganisms, as well as the conditions under which they were inactivated, and the benefits observed in the host. It can be seen that heat treatment is the most widely used and studied method of production of paraprobiotics, while ultraviolet irradiation, followed by sonication, are the least used.

Once the chosen inactivation method has been applied, different techniques are used to determine the viability of the bacteria. An example of these is contacting bacteria with a fluorescent dye (for example: SYTO 9, SYTOX, or the like) that permeates the cell membrane, allowing live bacteria to be identified by a green fluorescence. Propidium iodide, which does not permeate the cell membrane, can also be used, allowing the committed cells to be identified with a red fluorescence. Another technique used is the counting of viable plated cells (Raz and Rachmilewitz, 2005).

### 1.3.1. Heat treatment

Thermal treatment is the most widely used method to inactivate microorganisms, which consists of applying heat for a certain period of time. This inactivation mechanism affects most of the cell structure, since it involves damage to the cell membrane, loss of ions and nutrients, aggregation of ribosomes, breakdown of DNA filaments, inactivation of enzymes, as well as protein coagulation. In the production of paraprobiotics, this inactivation method has been reported to increase the roughness and roughness of the cell, which influences the immunomodulatory properties of the microbial cell (Ou, Lin, Tsai and Lin, 2011).

Table I shows the diversity of heat treatments that have been applied to inactivate probiotics. Some of them include pasteurization conditions (temperatures below 100 °C) while others use commercial sterilization conditions (temperatures above 100 °C); Treatment times vary, and this surely depends on the matrix or medium in which the probiotics are suspended.

Ou *et al.* (2011) observed in mice that the immunomodulatory properties of probiotic lactic acid bacteria (LAB), grown in MRS broth, subjected to different temperatures for different times (60 ºC, 30 min; 80 ºC, 20 min; 95 ºC, 5 min and 100 ºC, 5 min), depend on the strain evaluated. On the other hand, it was observed that there is a relationship between the increase of the roughness and the roughness of the cell, and the reduction of the beneficial health effects provided by the paraprobiotics. Furthermore, the heat treatment conditions for the inactivation of the probiotic also affect the adhesion capacity of the microorganisms in the intestinal mucosa of the host, which is important in many of the probiotic effects that promote

health; at higher inactivation temperatures of probiotics, the adhesion capacity decreases, but the immunomodulatory properties are not affected (Ou *et al.,* 2011).

Ananta and Knorr (2009) mention that the inactivation of *Lactobacillus rhamnosus* ATCC 53103 is different when it is carried out at different temperatures. In their study they showed that in the thermal treatments applied at 60 ºC, there is no degradation of the cytoplasmic membrane of the cell, although there was inactivation; while at higher temperatures this degradation does exist. So, the temperature directly influences the properties of paraprobiotics; As higher temperatures are applied for cell inactivation, there is an increase in cell degradation and a decrease in the benefits observed in the host.

### 1.3.2. Ultraviolet irradiation

Ultraviolet irradiation is widely used to disinfect water and food, since it results in the inactivation of pathogenic bacteria such as *Listeria monocytogenes, Salmonella, Staphylococcus aureus* and *Mycobacterium avium* subsp. *paratuberculosis*, which can be in these products and affect their organoleptic characteristics of the products and even consumer health (Franz, Specht, Cho, Graef and Stahl, 2009); it has also been used for the production of paraprobiotics (Good *et al*., 2014).

The inactivation of probiotic cells with this method involves the formation of photo-products in the DNA, as well as the generation of the pyrimidine dimer, which is formed between adjacent pyrimidine molecules in the same DNA chain, interrupting the transcription processes and DNA translation, leading to mutagenesis and cell death; it also leads to protein denaturation (Franz *et al.,* 2009; Brimpa, Sfika and Vantarakis, 2013).

The conditions reported in the literature for the production of paraprobiotics (Table I), vary from 5 to 30 minutes of exposure to ultraviolet irradiation; however, the effect of the variation in the time of exposure to ultraviolet radiation is not mentioned, with the benefits of the paraprobiotic on the health of the host to whom it is administered.

As an example, there can be mentioned a study in which *Lactobacillus acidophilus* was inactivated by exposing the cells of this microorganism to ultraviolet radiation for 30 minutes. Subsequently, the cells were supplied to neonates with enterocolitis, observing that the incidence of this disease decreased (Awad *et al.,* 2010).

In another study, Good *et al.* (2014) used ultraviolet irradiation (conditions not specified), to inactivate *Lactobacillus rhamnosus* HN001 cells. These researchers observed that when the inactivated cells were administered to premature piglets and neonatal mice, the severity of necrotizing enterocolitis decreased, improving the macroscopic morphology of the mucosa, as well as the response of cytokines in the latter.

### 1.3.3. High pressures

This inactivation method consists in that the product, in this case probiotics, is sometimes high levels of hydrostatic pressure, (100 to 1000 MPa), continuously for a certain period of time (Herrero and Ávila, 2016). Whatever occurs in the microorganism at high pressures and contributes to its inactivation is damage to the cell membrane, denaturation of proteins, decrease in intracellular pH, loss of solutes, inactivation of enzymes, as well as changes in ribosomes and nucleotides. of the cell (de Almada *et al.,* 2016). No evidence was found in the literature if there is a relationship between the application of different pressures and the benefits of paraprobiotics to the health of the host that consumes them.

In the study by Ananta and Knorr (2009) two inactivation methods, heat treatment and high pressures, on *Lactobacillus rhamnosus* ATCC 53103 for the production of paraprobiotics, are compared by means of flow cytometry analysis. These researchers observed that with pressures of 400 MPa or higher, it is possible to deactivate the cells of these bacteria. Furthermore, it showed that the 400 MPa treatments for this microorganism are not lethal, while the 600 MPa treatments inactivated the microorganism more than seven logarithmic cycles (Ananta and Knorr, 2009).

### 1.3.4. Sonication

Sonication is a physical method, which consists in subjecting the microorganism suspended in a liquid medium to sonic waves; When a sonic wave meets the liquid medium, it creates sonic regions of compression and expansion. Due to this change, a cavity formation begins, and gas bubbles are generated in the medium. A point is reached where the provided ultrasonic energy is not sufficient to retain the vapor phase in the bubble, and condensation

occurs. The condensed molecules collide, creating shock waves. The resulting pressure changes are the main inactivating effect of the microorganisms (Piyasena, Mohareb and McKellar, 2003). This method of inactivation causes cell wall rupture, cell membrane damage, and DNA damage in the microorganism (de Almada *et al.,* 2016).

Although so far, this inactivation method for probiotic microorganisms has not been widely used, according to the work of Shin *et al.* (2010), carried out in rats, *Bifidobacterium longum* SPM1207 cells isolated from Korean adults, inactivated by sonication (unspecified conditions) for 5 minutes, have the potential to be used to generate paraprobiotics as a serum cholesterol lowering agent. On the other hand, they suggest that this inactivated strain can help to avoid the increase of body weight and to alleviate or eliminate constipation, which should be studied further (Shin *et al.,* 2010).

## 2. Application of paraprobiotics in food

Currently there are food supplements on the market for paraprobiotics like "Del-Immune V®", manufactured by Pure Research Products, LLC, Boulder, Colorado, USA, it is a food supplement for immediate support of the immune system that contains *Lactobacillus rhamnosus* V lysate. Another example is CytoFlora®, which contains the cell walls of *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus bulgaricus, Lactobacillus salivarius, Lactobacillus casei, Lactobacillus reuteri, Lactobacillus sporogenes, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium longum* and *Streptococcus thermophilus* (Shigwedha *et al.,* 2014). No examples were found of foods to which paraprobiotics have been added as such.

### 2.1. Potential use of paraprobiotics in food

de Almada *et al.* (2016) mention that the presence of probiotic microorganisms in different types of food products has some limitations, such as guaranteeing the survival of these microorganisms during the shelf life of foods that can be a stressful substrate for the microorganism. Also, the addition of probiotics to foods should be done after having undergone heat treatments, to ensure the survival of probiotic microorganisms, which

increases the chances of contamination of the food. Therefore, the application of paraprobiotics in food can offer some advantages compared to that of probiotics, such as less interaction (or no interaction) with the components of the food, which can increase its useful life; add the paraprobiotics before subjecting the food to heat treatment; the application in a greater variety of products; as well as facilitating storage and transportation (Ishikawa *et al.,* 2010).

### 2.2. Challenges of applying paraprobiotics in food

According to Almada *et al.* (2016), the main challenges for the application of paraprobiotics in food is the selection of probiotic strains, for the generation of paraprobiotics, according to the expected health benefit of the host. It is also important to mention that, although different methods have been used for the inactivation of probiotics, it is necessary to study in greater depth if all the inactivation methods are appropriate for all species / strains; the influence of inactivation methods and their conditions, on the stability and effectiveness of the benefits that paraprobiotics grant to the host; and the potential use in different types of food. On the other hand, another challenge is to determine the presentation in which paraprobiotics are marketed and added to food. The proposal, review and approval of regulations that regulate the use of paraprobiotics in foods should also be considered.

## Conclusions and final comments

So far, several investigations have been carried out on the paraprobiotics of different strains of probiotic microorganisms and on the inactivation methods to obtain them, as well as on the different benefits that their consumption produces for the host. However, the use and application of paraprobiotics in foods is a great area of opportunity for researchers, since there is no reported information on their incorporation into foods, even though they are already used in the pharmaceutical industry and are marketed as supplements. food.

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**Table I. Research on paraprobiotics, inactivation methods and observed benefits.**

| Microorganism/ Product | Inactivation method | Inactivation conditions | Benefits | References |
|---|---|---|---|---|
| *Bacillus amyloliquefaciens* FPTB16 and *Bacillus subtilis* FPTB13 | Heat treatment | 60 ºC during 2 h | Stimulation of the cell's immune responses (*in vitro*) | Kamilya, Baruah, Sangma, Chowdhury and Pal, (2015) * |
| *Bifidobacterium brevis* Yakult (BbrY) and *Bifidobacterium bifidum* Yakult (BbiY) | Heat treatment | 100 ºC during 30 min | Anti-inflammatory activity against ulcerative colitis (UC) observed in cells obtained from patients diagnosed with UC (*in vitro*) | Imaoka *et al.* (2008) * |
| *Bifidobacterium longum* SPM1207 | Sonication | Sonication during 5 min | Cholesterol reduction, suspension of body weight gain and relief or elimination of constipation in rats | Shin *et al.* (2010) * |
| *Lactobacillus* LB (Lacteol Fort, France) capsules | - | - | Treatment for diarrhea in humans with irritable bowel syndrome | Tarrerias *et al.* (2011) * |
| *Lactobacillus acidophilus* LB campsules | Heat treatment | - | Relief of symptoms of chronic diarrhea (reduced bowel movements, abdominal pain and bloating; improvement of stool consistency and feeling of incomplete evacuation) in humans | Xiao *et al.* (2002) * |
| *Lactobacillus paracasei* 33 capsules | Heat treatment | 70 ºC during 30 min | Improve health well-being and reduce symptoms in humans with allergic rhinitis | Peng and Hsu, (2005) * |
| Dietary supplement with *Bacillus pumilus* SE5 | Heat treatment | 95 ºC during 60 min | Suppression of pathogenic bacteria in the fish gut microbiota | Yang, Xia, Ye, Zou and Sun, (2014) * |

**Table I.** Research on paraprobiotics, inactivation methods, and observed benefits (continued)

| Microorganism/ Product | Inactivation method | Inactivation conditions | Benefits | References |
|---|---|---|---|---|
| Diet supplemented with *Lactobacillus delbrüeckii* subsp. *lactis* CECT 287 and *Bacillus subtilis* CECT 35 | Heat treatment | 60 ºC during 1 h | Local and systemic immunostimulatory effect in fish | Salinas *et al.* (2008) * |
| Diet supplemented with *Lactobacillus paracasei* DSMZ16671 | Heat treatment | Pasteurization with prolonged heating at 80 ºC | Reduction of caries, due to the inhibition of the colonization of *Streptococcus mutans* in rat dental plaques | Tanzer *et al.* (2010) * |
| Diet supplemented with *Psychrobacter sp*. SE6 | Heat treatment | 95 ºC during 60 min | Induction of the activation of immunity in the intestinal mucosa in fish | Sun, Xia, Yang, Wang and Zou, (2014) * |
| *Enterococcus faecalis* FK-23 | Heat treatment | - | Relief of nasal symptoms, reduction of nasal eosinophilia and increase of CD4 + CD25 + cells in model of allergic rhinitis in mice | Zhu *et al.* (2012) * |
| *Enterococcus faecalis* YM-73 and *Lactobacillus salivarius* AP-32 | Heat treatment | 60 ºC during 30 min; 80 ºC during 20 min; 95 ºC during 5 min and 100 ºC during 5 min | Immuno-modulating activity in cells (Caco-2) (*in vitro*) | Ou *et al.* (2011) * |
| *Enterococcus faecium* JWS 833 | Heat treatment | 110 ºC during 15 min | Immuno-modulating property (*in vitro*) | Choi, Shin, Lee and Lee, (2012) * |
| Fermented milk with *Lactobacillus gasseri* CP2305 | Heat treatment | 95 ºC during 30 s | Regulation of intestinal function in humans prone to constipation | Sawada *et al.* (2015) * |

**Table I.** Research on paraprobiotics, inactivation methods, and observed benefits (continued)

| Microorganism/ Product | Inactivation method | Inactivation conditions | Benefits | References |
|---|---|---|---|---|
| *Lactobacillus fermentum* VET9A, *Lactobacillus plantarum* VET14A, and *Lactobacillus rhamnosus* VET16A | Heat treatment | 80 ºC during 30 min | Exclusion of enteropathogens (*Ehrlichia canis*, *Salmonella enterica serovar* Typhimurium and *Clostridium perfringens*) in the intestinal mucus of dogs (*in vitro*) | Grzeskowiak, Collado, Beasley and Salminen, (2014) * |
| *Lactobacillus acidophilus* (LAP5, LAF1 and LAH7) | Heat treatment | 100 ºC during 30 min | Inhibition of *Salmonella* by immuno-modulation of activated macrophages in mice | Lin, Yu, Lin, Hwang and Tsen, (2007) * |
| *Lactobacillus acidophilus* A2, *Lactobacillus gasseri* A5 and *Lactobacillus salivarius* A6 | Heat treatment | 100 ºC during 15 min | Modulation of the immune response (*in vitro*) | Chuang *et al.* (2007) * |
| *Lactobacillus brevis* SBC8803 | Heat treatment | 121 ºC during 20 min | Improvement of ethanol-induced injury and liver fat accumulation in mice | Segawa, Wakita, Hirata, and Watari, (2008) * |
| *Lactobacillus brevis* SBC8803 | Heat treatment | 121 ºC during 20 min | Reduction of intestinal inflammation and improvement of intestinal damage in mice with colitis (*in vitro* e *in vivo*) | Ueno *et al.* (2011) * |
| *Lactobacillus casei* CRL 431 | Heat treatment | 80 ºC during 30 min | Immuno-modulation of the respiratory immune system in mice | Villena, Barbieri, Salva, Herrera and Alvarez, (2009) * |

**Table I.** Research on paraprobiotics, inactivation methods, and observed benefits (continued)

| Microorganism/ Product | Inactivation method | Inactivation conditions | Benefits | References |
|---|---|---|---|---|
| *Lactobacillus casei* Shirota | Heat treatment | 100 ºC during 30 min; 95 ºC during 30 min | Inhibition of allergen-induced IgE production, possible role in prevention of IgE-mediated allergy (*in vitro*). Immuno-modulating effect in mice with an allergy model, possible role in the prevention of respiratory allergies | Shida *et al.* (1998); Lim, Li, Huang, Lee, Lee and Chua, (2009) * |
| *Lactobacillus casei* Zhang (LcZ) | Heat treatment | 70 ºC during 30 min | Effect on the immune system in macrophages (*in vitro*), which suggests its use against viral infections | Wang, Xie, Wang, Li, Sun, Zhang, and Zhang, (2013) * |
| *Lactobacillus gasseri* OLL2809 | Heat treatment | 75 ºC during 60 min | Suppression of peritoneal loss of eosinophils, leading to improvement of allergic symptoms (allergic rhinitis) in mice | Sashihara *et al.* (2008) * |
| *Lactobacillus gasseri* TMC0356 | Heat treatment | 90 ºC during 5 min | Enhancement of cell immunity in mice, leading to enhancement of natural defenses against respiratory infections | Kawase, He, Miyazawa, Kubota, Yoda and Hiramatsu (2012b) * |
| *Lactobacillus gasseri* TMC0356 | Heat treatment | 70 ºC during 30 min or 90 ºC during 5 min | Immuno-modulating effect (*in vitro*) | Miyazawa, He, Kawase, Kubota, Yoda and Hiramatsu, (2011) * |
| *Lactobacillus gasseri* TMC0356-70 and TMC0356-90 | Heat treatment | 70 ºC during 30 min and 90 ºC during 5 min | Protection against H1N1 influenza virus infection by stimulating respiratory and intestinal immune responses in mice | Kawase, He, Kubota, Yoda, Miyazawa and Hiramatsu, (2012a) * |

**Table I.** Research on paraprobiotics, inactivation methods, and observed benefits (continued)

| Microorganism/ Product | Inactivation method | Inactivation conditions | Benefits | References |
|---|---|---|---|---|
| *Lactobacillus paracasei* IMPC2.1 and *Lactobacillus rhamnosus* GG | Heat treatment | 95 ºC during 1h | Pro-apoptotic and anti-proliferative effect in colon and gastric cancer cells (*in vitro*) | Orlando, Refolo, Messa, Amati, Lavermicocca, Guerra, and Russo, (2012) * |
| *Lactobacillus pentosus* b240 | Heat treatment | 121 ºC during 15 min | Suppression of S. *pneumoniae*-induced pneumonia, improvement of inflammatory tissue responses and reduction of respiratory tissue damage in mice | Tanaka *et al.* (2011) * |
| *Lactobacillus plantarum* 06CC2 | Heat treatment | Boil during 1 h | Relief of influenza symptoms in mice due to the immunomodulatory effect | Takeda *et al.* (2011) * |
| *Lactobacillus plantarum* b240 | Heat treatment | 121 ºC during 15 min | Prevention of *S.* Typhimurium infection in mice | Ishikawa *et al.* (2010) * |
| *Lactobacillus plantarum* KTCT3104 and *Lactobacillus curvatus* KTCT3767 | Heat treatment | 100 ºC during 30 min | Attenuation of airway inflammation by modulating intestinal immunity in mice | Hong, Kim, Cho and Kim, (2010) * |
| *Lactobacillus plantarum* L-137 | Heat treatment | 70 ºC during 10 min | Protection against influenza virus infection in mice due to improved innate immunity of the respiratory tract | Maeda *et al.* (2009) * |
| *Lactobacillus plantarum* L-137 | Heat treatment | 80 ºC during 20 min | Improvement of immunomodulatory activity *in vitro* and *in vivo* (in mice) | Fujiki, Hirose, Yamamoto, and Murosaki, (2012) * |
| *Lactobacillus plantarum* MYL26 | Heat treatment | 65 ºC during 30 min | Attenuation of inflammation in cells Caco-2 (*in vitro*) | Chiu *et al*. (2013) * |

**Table I.** Research on paraprobiotics, inactivation methods, and observed benefits (continued)

| Microorganism/ Product | Inactivation method | Inactivation conditions | Benefits | References |
|---|---|---|---|---|
| *Lactobacillus reuteri* | Heat treatment and gamma irradiation | 80 ºC during 20 min; cobalt source 60 during 20 h at 8.05 Gy/min | Inhibitory effect on visceral pain in rats | Kamiya *et al.* (2006) * |
| *Lactobacillus rhamnosus* GG | Heat treatment | 80 ºC during 20 min | Immunomodulatory action (*in vitro*). Reduced level of pro-inflammatory mediators and increased lipopolysaccharide-induced anti-inflammatory markers of *E. coli* in rats | Bloise, Torricelli, Novembri, Borges, Carrarelli, Reis and Petraglia, (2010); Li, Russell, Douglas-Escobar, Hauser, Lopez and Neu, (2009) * |
| *Lactobacillus rhamnosus* GG | UV | UV during 20 min; UV during 5 min | Improvement of the immune response *in vitro*; Modulation of Caco-2 cell inflammation by reduced interleukin-8 production (*in vitro*) | Van Hoffen *et al.* (2010); López, Li, Kataria, Russell and Neu, (2008) * |
| *Lactobacillus rhamnosus* HN001 | UV | - | Attenuation of the severity of necrotizing enterocolitis in premature piglets and newborn mice | Good *et al.* (2014) * |
| *Lactobacillus sakei* probio 65 | Heat treatment | 121 ºC during 15 min | Inhibitory effect on inflammation and skin lesions, atopic dermatitis in mice and in vitro activation of mast cells | Kim, Park, Park, Park, Kim and Pyo, (2013) * |
| *Lactobacillus salivarius* E4191 | Heat treatment | 95 ºC during 1h | Protective effect on intestinal epithelial cells (anti-inflammatory activity) by inhibition of interleukin-8 secretion (*in vitro*) | Oh, Jeun, Lee, Chun and Kim (2012) * |
| *Lactobacillus lactis* G50 | Heat treatment | 100 ºC during 30 min | Improving intestinal immunity and suppressing the growth of enteric bacteria-producing H2S in mice | Kimoto-Nira, Mizumachi, Okamoto, Sasaki and Kurisaki, (2009) * |

**Table I.** Research on paraprobiotics, inactivation methods, and observed benefits (continued)

| Microorganism/ Product | Inactivation method | Inactivation conditions | Benefits | References |
|---|---|---|---|---|
| *Lactobacillus lactis* subsp. *cremoris* H61 | Heat treatment | 100 ºC during 30 min | Potential to suspend aging-related effects (anti-aging effect) in mice | Kimoto-Nira, Suzuki, Kobayashi, Sasaki, Kurisaki and Mizumachi, (2007) * |
| *Leuconostoc mesenteroides* 1RM3 | Heat treatment | 100 ºC during 10 min | Prevention of *L. monocytogenes* invasion and entero-gastric infection in Caco-2 cells in mice and *in vitro* | Nakamura, Kuda, An, Kanno, Takahashi and Kimura, (2012) * |
| *Saccharomyces boulardii* | Heat treatment | 121 ºC during 15 min | Maintenance of intestinal integrity and modulation of the immune system of mice | Generoso *et al.* (2011) * |
| "Sachet" containing inactivated probiotic microorganism (Dialac) | - | - | Improved absorption of lactose in children | Rampengan, Manoppo and Warouw, (2010) * |
| Skim milk supplemented with *Lactobacillus rhamnosus* HN001 | Heat treatment | 100 ºC during 15 min | Improvement in the immune system in mice | Gill and Rutherfurd (2001) * |
| Supplementation with *Lactobacillus acidophilus* LB (sachet) | Heat treatment | - | Beneficial in treating children diagnosed with rotavirus-induced diarrhea | Lie'vin-Le Moal, Sarrazin-Davila, and Servin, (2007) * |
| Supplementation with *Lactobacillus casei* GG | Heat treatment | 85-100 ºC during 10 min | Treatment of rotavirus diarrhea in children | Kaila, Isolauri, Saxelin, Arvilommi and Vesikari (1995) * |
| *Lactobacillus acidophilus* GG suplement | UV | UV during 30 min | Prevention of necrotizing enterocolitis and sepsis in newborns | Awad *et al.* (2010) * |
| *Lactobacillus paracasei* K71 suplement | Heat treatment | - | Beneficial effects on symptoms of atopic dermatitis in humans | Moroi *et al.* (2011) * |
| *Lactobacillus paracasei* K71 suplement | Heat treatment | - | Beneficial effects on symptoms of atopic dermatitis in humans | Moroi *et al.* (2011) * |

**Table I.** Research on paraprobiotics, inactivation methods, and observed benefits (continued)

| Microorganism/ Product | Inactivation method | Inactivation conditions | Benefits | Referencias |
|---|---|---|---|---|
| *Lactobacillus paracasei* K71 suplement | Heat treatment | - | Beneficial effects on symptoms of atopic dermatitis in humans | Moroi *et al.* (2011) * |
| *Lactobacillus gasseri* OLL2809 tablets | Heat treatment | 75 ºC during 60 min | Reduction of symptoms in humans with high predisposition to allergies due to systemic modulation of the immune system | Gotoh *et al.* (2009) * |
| *Lactobacillus pentosus* b240 tablets | Heat treatment | 121 ºC during 15 min | Reduced incidence of cold due to immuno-protective effect, effect observed in humans | Shinkai *et al.* (2013) * |
| Yogurt with lactic acid bacteria (Mix-BAL) (*Lactobacillus bulgaricus, Streptococcus thermophilus* and *Lactobacillus acidophilus*) | Heat treatment | 65 ºC during 60 min | Protective role in intestinal epithelial barrier dysfunction induced by pro-inflammatory cytokines *in vitro* | Zeng, Jiang, Zhu and Chu, (2015) * |
| Delpro® food supplement and immunoregulatory formulation | - | - | Decreased gastrointestinal symptoms in children with autism | West, Roberts, Sichel and Sichel, (2013) |
| *Lactobacillus rhamnosus* V | - | - | Prevention and treatment of infectious diseases, common allergies (food allergy, bronchitis, hay fever and asthma), hepatitis C, fatigue crónica y fibromialgia en humanos | Pidgorskyy, Shynkarenko-Sichel, Timoshok and Spivak (2008) |

* Cited by de Almada *et al.* (2016).

* Adapted from: de Almada *et al.* (2016)

**RESEARCH PAPER I**

**"Antimicrobial Activity and Storage Stability of Cell-free Supernatants from Lactic Acid Bacteria and their Applications with Fresh Beef"**

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**Antimicrobial Activity and Storage Stability of Cell-free Supernatants from Lactic Acid Bacteria and their Applications with Fresh Beef**

D. Arrioja, E. Mani-López, E. Palou, and A. López-Malo

**Abstract**

This study aimed to evaluate the effectiveness of cell-free supernatants (CFS) produced by selected strains of lactic acid bacteria (LAB) as antimicrobials *in vitro* against *Escherichia coli*, *Staphylococcus aureus*, *Shigella sonnei, Pseudomonas fluorescens, Salmonella* Typhimurium, or *Listeria monocytogenes*. The agar-well diffusion method was performed using CFS from sixteen LAB. Neutralization of CFS pH as well as treatment with proteinase K were utilized to determine the nature of CFS' antimicrobial compounds. Then stability of the three most effective CFS during storage at various temperatures (15, 25, or 35 $^{o}$C) was determined through agar-well diffusion assays each week for 20 weeks. In addition, antimicrobial activity of CFS from *Lb. plantarum* was tested in inoculated (*E. coli*, *Staph. aureus*, *S.* Typhimurium, or *L. monocytogenes*) beef pieces; using this CFS to marinate beef pieces. Furthermore, the effect on beef color (raw or grilled beef, marinated or not with *Lb. plantarum* CFS) was determined. CFS from *Lb. plantarum, Lb. sakei*, and *Lb. rhamnosus* were found to be the most effective, with inhibition halos greater than 20.2 ± 2.0, 20.8 ± 2.9, and 17.1 ± 3.6 mm, respectively. Antimicrobial properties were eliminated when pH was adjusted to 6.5 for most of tested CFS while only *Lb. plantarum* CFS maintained their antimicrobial activity, which was lost when treated with proteinase K; according to these results, the antimicrobial activity of tested CFS can therefore be mainly attributed to organic acids. Antibacterial activity was significantly ($p < 0.05$) reduced between time zero and after 20 weeks of storage at three studied temperatures; the greatest reductions in antibacterial activity were observed at 35 $^{o}$C. Antimicrobial activity against *L. monocytogenes* was less affected by time and temperature for the three most effective CFS. *Lb. plantarum* CFS was effective in reducing the microbial load of inoculated bacteria in beef, mainly *S.* Typhimurium and *L. monocytogenes*. Meat color differences were important in raw beef; while on grilled meat, changes were scarcely detected.

**Keywords:** antimicrobial activity, cell-free supernatant, storage stability, lactic acid bacteria, bio-preservative.

## Introduction

Currently there is a trend in consumer preference to choose fresh, preservative-free, less processed foods. In order to meet the consumer demands while still ensuring food safety, research into the use of natural antimicrobials to replace synthetic ones is important. Many bio-preservatives have been studied, including (among others) essential oils, enzymes, and microorganisms (Mani-López, Palou & López-Malo, 2018; Muhialdin, Hassan, Bakar & Saari, 2016).

Lactic acid bacteria (LAB) are Gram-positive cocci or rods that are widely utilized in the commercial production of fermented foods; which, in addition to producing flavors, odors, textural and nutritional changes in foods, are also known for their antagonistic effect against pathogenic bacteria. Lactic acid is the major metabolite produced by these bacteria (Souza, Azevedo, Domínguez, Converti & Oliveira, 2017), though these species also have the ability to produce other antimicrobial metabolites such as other organic acids (acetic, formic, and propionic, among others), bacteriocins (nisin, reutericyclin, pediocin, lacticin, and sakacin), reuterin, diacetyl, and/or ethyl alcohol (Jay, 1982; Piard & Desmazeaud, 1992). Besides, selected LAB have been designated as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration, making LAB a good alternative to replace synthetic preservatives in foods while both minimizing regulatory hurdles and offering consumers products with clean labels (Schnürer & Magnusson, 2005; Zacharof & Lovitt, 2012). These characteristics have attracted much interest toward the use of LAB as an alternative bio-preservative. Numerous studies have shown that direct application of LAB, or even just their cell-free supernatant (CFS), inhibits undesirable bacteria in chicken breast, beef, bread, and cheese (Cizeikiene, Juodeikiene, Paskevicius & Bartkiene, 2013; Da Costa et al., 2018; Mani-López et al., 2018; Topisirovic et al., 2006).

Kareem, Hooi Ling, Teck Chwen, May Foong & Anjas Asmara (2014) investigated the *in vitro* inhibitory properties of the CFS of *Lb. plantarum* RG11, RG14, RI11, UL4, TL1, and RS5 in combination with inulin, against *S. enterica* S-1000, *L. monocytogenes* L-MS, *E. coli* E-30, and vancomycin-resistant *Enterococci*. They found that the CFS was able to inhibit

both Gram-positive and Gram-negative microorganisms. Beristain-Bauza, Mani-López, Palou & López-Malo, (2016) studied the antimicrobial activity of protein films in combination with the CFS of *Lb. rhamnosus* against *L. monocytogenes* Scott A, *Staph. aureus* ATCC 29413, *E. coli* ATCC 25922, and *S. enterica* serovar Typhimurium ATCC 14028 in fresh beef cubes. Other researchers have studied possible applications for preserving other food products such as cheese and ground chicken breast, using CFS from *Lb. plantarum* 49 and *Lb. paracasei* 108, looking at the relationship between the antimicrobial activity of the CFS against *L. monocytogenes* and *S.* Enteritidis and the LAB ability to produce organic acids such as lactic and acetic acid (Da Costa et al., 2018). Despite the extensive research in this field, very few studies have evaluated the stability of CFS at different storage temperatures (Fernández, Chanci, Wilches & Cardona, 2014), which is an important factor to consider when examining possible applications of LAB CFS in food.

In the food industry, marinades for meat are generally based on mixtures of water, salt, and phosphates; these ingredients increase the tenderness, juiciness, and yield of the meat. The mode of marinade application can vary, with methods such as injection technology, immersion, and vacuum tumbling being employed in different scenarios. Various reports have studied the effects of marinating chicken (Alvarado & McKee, 2007), pork, beef (Sharedeh, Gatellier, Astruc & Daudin, 2015), and even horse meat (Stanisławczyk, Rudy & Gil, 2019), while also examining the different methods of application in terms of the control of pathogenic microorganisms and the change of sensory attributes. Thus, the aims of this study were to evaluate the effectiveness of CFS produced by selected strains of LAB as antimicrobials against *E. coli* ATCC 25922, *S.* Typhimurium ATCC 14028, *Staph. aureus* ATCC 29213, *Staph. aureus* ATCC 25923, *Sh. sonnei* ATCC 25931, *Ps. fluorescens* ATCC 13525, and *L. monocytogenes* Scott A, as well as to evaluate the stability of the CFS in terms of their antimicrobial activity during storage at different temperatures. Another aim was to identify the nature of the compounds responsible for the CFS antimicrobial properties and evaluate the ability of CFS to control indicator bacteria in fresh beef. In addition, an evaluation of marinated beef (raw or grill cooked) was performed to determine if differences in color could be detected when the marinade contained *Lb. plantarum* CFS or not.

## Materials and Methods

### *Bacterial strains, culture, and growth conditions*

The cultures of LAB utilized in this study (*Lactococcus lactis subsp. lactis* NRRL B-633, *Lc. lactis* subsp. *cremoris* NRRL B-634, *Pediococcus acidilactici* NRRL B-1116, *P. pentosaceus* NRRL B-14009, *Leuconostoc mesenteroides* subsp. *mesenteroides* NRRL B-1118, *Lb. sakei* NRRL B-1917, *Lb. fermentum* NRRL B-1932, *Lb. reuteri* NRRL B-14171, *Lb. plantarum* NRRL B-4496, *Lb. acidophilus* NRRL B-4495, *Lb. casei* NRRL B-1922, *Lb. brevis* ATCC 367*, Lb. casei* 21/1, *Lb. amylovorus* ATCC 33621*, Lb. sanfranciscensis* ATCC 27651*,* and *Lb. rhamnosus* NRRL B-442) as well as the indicator strains (*E. coli* ATCC 25922, *S.* Typhimurium ATCC 14028, *Staph. aureus* ATCC 29213, *Staph. aureus* ATCC 25923, *Sh. sonnei* ATCC 25931*, Ps. fluorescens* ATCC 13525*,* and *L. monocytogenes* Scott A) were obtained from the Food Microbiology Laboratory of the Universidad de las Americas Puebla (Puebla, Mexico). LAB were reactivated in de Man, Rogosa and Sharpe (MRS) broth (Difco$^{TM}$ BD, Sparks, Maryland). Indicator strains were reactivated in trypticase soy broth (TSB, Bioxon BD, Mexico). Both groups of bacteria were incubated at 35 ± 1.0 °C, for 48 h for LAB and 24 h for indicator strains.

### *Preparation of cell-free supernatant*

For the sixteen LAB cultures ($10^6$ CFU/mL), 30 mL of CFS was collected by centrifuging the culture at 8,000 *x g* for 10 min (Marathon 21K/R, Fisher Scientific, Germany), filter sterilizing through a 0.45 µm Millipore membrane filter, and the CFS volume was concentrated 10-fold by vacuum evaporation on a Buchi R-210/215 rotary evaporator (Buchi, Flawil, Switzerland) at 70 °C ± 1.0 °C and 25 cm Hg. Concentrated supernatants were evaluated on the same day. Uninoculated MRS broth was also concentrated following the same protocol to generate a negative control.

### *Antimicrobial screening*

#### *Agar-well diffusion method*

Antimicrobial activity of LAB supernatants was evaluated using the agar-well diffusion method (Tagg & McGiven, 1971). Briefly, 0.1 mL of the indicator bacteria culture ($10^6$

CFU/plate) was spread on TSA plates. Four wells (8 mm diameter) were punched in the plate; three were filled with 100 µL of concentrated CFS of the chosen LAB, while the fourth was filled with 100 µL concentrated MRS broth as a negative control. Plates were incubated at 37 °C ± 1.0 °C for 24 h. Each experiment was run in duplicate. Bacterial growth was monitored, and the inhibition zone diameter (mm) around the wells (including the well) was measured with a digital caliper (Mitutoyo Corp., Kawasaki, Japan; three times in different directions).

*Nature of antimicrobial compounds present in CFS*

*pH sensitivity*

To identify the nature of the antimicrobial metabolites in the CFS, their pH was neutralized to determine if their activity was due to organic acids. The pH of 4 mL of each CFS was determined by electrode immersion with a pH meter (PC45, Conductronic, Mexico) according to the method 981.12 of AOAC (2000). In addition, 4 mL of each CFS was taken and adjusted to pH 6.5 with a 40% NaOH (w/v) solution. Neutral and normal CFS were prepared as previously described, and antimicrobial activity was assessed with the agar-well diffusion method.

*Enzyme sensitivity (Proteinase K test)*

After organic acid antimicrobial activity was inhibited (by neutralizing pH), only one CFS (from *Lb. plantarum* NRRL B-4496) maintained antimicrobial activity. To determine if the compound responsible was bacteriocin-like, 4 mL of the CFS from *Lb. plantarum* NRRL B-4496 were taken, the pH was adjusted to 6.5 with a 40% NaOH (w/v) solution, the CFS was prepared as previously described, and 20 µg/mL proteinase K (Sigma-Aldrich, St. Louis, MO, USA) was added before incubation at 37 °C ± 1.0 °C for 2 h. Following this, the treated CFS was heated further (120 °C ± 1.0 °C) for 5 min. Afterwards, the antimicrobial activity was determined with the agar-well diffusion method. As all antimicrobial activity was lost, proteinase K was determined to have hydrolyzed the peptides in the active antimicrobial compound and as such, treatments with other enzymes such as peroxidase were not tested.

*CFS characterization*

The analysis of the organic acids present in the CFS was performed using a gas chromatograph (Agilent Technologies 6850N, USA) coupled to a selective mass detector (Agilent 5975C, USA). A 30 m apolar HP-ms capillary column, 5% phenyl methyl polysiloxane, 250 µm in diameter and 0.25 µm thick, and helium (1.1 mL / min) as the carrier gas were used. The oven was maintained at 300 °C, the temperature ramp in the column started at 60 °C for 2 min and then increased to 250 °C at a rate of 10 ° C / min. One µL of each esterified CFS was injected using a 10:1 split injection with an injector temperature of 205 °C. The mass spectrometer was operated at 70 eV and the mass range was 30 to 425 uma. The components were identified by comparing their mass spectrum fragmentation patterns with the database of the National Institute of Standards and Technology Spectral Database (NIST, 2010)

*CFS storage stability*

To determine storage stability of the selected CFS, three different temperatures were used (15, 25, or 35 ± 1.0 °C). The pH and antimicrobial activity were determined as previously described every seven days for five months.

*Antimicrobial activity of selected CFS in raw beef*

*Meat and marinade preparation*

Fresh raw top round beef was purchased in a local market in Puebla (Puebla, Mexico) and used the same day of purchase. To reduce the native microbiota, a layer of approximately 1 cm of meat was removed from the external surface, and meat was cut into 5 g average weight pieces for testing.

A standard marinade was formulated with 0.35% sodium chloride, 0.45% Hamine® (commercial mixture of sodium phosphates, McCormick-PESA, Cd. de Mexico, Mexico), and potable water at room (23 ± 2 °C) temperature (Pokharel, Brooks, Martin & Brashears, 2016). The marinade was prepared in a single batch. In addition, a *Lb. plantarum* NRRL B-

4496 CFS marinade was formulated with 0.35% sodium chloride, 0.45% Hamine®, and 10-fold concentrated *Lb. plantarum* NRRL B-4496 CFS.

*Inoculation and initial microbial counts on meat*

Three pieces of ~5 g of raw beef (for each microorganism) were inoculated (~$10^5$ CFU/piece) by immersion into *E. coli*, *Staph. aureus* ATCC 29213, *S.* Typhimurium, or *L. monocytogenes* suspensions in peptone water (45 mL) for one minute. After the immersion step, the pieces were drained for 10 min. For initial counts, each inoculated meat piece was put in a sterile plastic bag (Whirl-Pak1, Nasco, Fort Atkinson, WI, USA), and homogenized for 2 min in a stomacher 80 lab blender (Seward Ltd., West Sussex, England) with 45 mL of sterile peptone water. Microbial counts were performed for each indicator bacteria. Adequate decimal dilutions were prepared into peptone water and 0.1 mL was spread on Baird-Parker agar for *Staph. aureus,* XLD agar for *Salmonella,* Oxford agar for *L. monocytogenes,* and MacConkey agar for *E. coli*. Plates were incubated for 18–24 h at 37 °C. In addition, to verify the commercial and sanitary quality of meat, counts of total mesophilic aerobic bacteria were completed using standard methods agar (Bioxon, BD, Edo. de Mexico, Mexico); inoculated plates were incubated for 24 h at 37 °C. For total coliforms counts, violet red bile agar (Difco BD, Sparks, MD) was utilized and plates were incubated at 37 °C for 24 h. To determine the initial counts of indicator strains on meat, the same technique and culture medium were used as previously described.

*Meat marination*

Meat pieces inoculated with each indicator bacteria were put in plastic bags and the marinade mixture (standard or CFS) was added in a 5:1 ratio (w/v). Bags were stored at 4 °C for 14 h. After that, microbial counts were performed as previously described. For meat color determination, non-inoculated meat pieces were marinated in the same way. Afterwards, marinated beef pieces were grilled for 1 min each side in a 100 °C pre-heated pan (previous tests demonstrated that this was sufficient for the meat to reach 70 °C in the center). Color evaluations were carried out fifteen minutes after the meat was grilled.

*Meat color determination*

A previously calibrated colorimeter (CR 400, Konica Minolta, Japan) was used in reflectance mode to perform color measurements of beef pieces which were either CFS or standard marinated, and grilled or raw, to obtain the CIELab parameters (L*, a*, and b*). The measurements were made in triplicate. The net color difference (ΔE) was determined by Eq. 1 between the beef pieces (CFS marinated and standard marinated, grilled, or raw).

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \qquad \text{Eq. 1}$$

where $L^*_0$, $a^*_0$, $b^*_0$ are color parameters of the control (standard marinated beef pieces) and L*, a*, b* are color parameters of CFS marinated pieces.

*Statistical analysis*

Statistical software Minitab (v.17, LEAD Technologies Inc., USA) was used to perform an analysis of variance and Fisher's test with a 95% level of confidence. ANOVA and Fisher's tests were performed with the results of the marinated meat-color parameters, the stability of the CFS during storage by comparing inhibition halos at time zero and after 20-weeks, as well as among the inhibition halos of each CFS against the indicator microorganisms tested. Further, for each evaluated LAB, the results of the inhibition halos were combined in three different ways, firstly all the results regardless of the type of indicator microorganism tested, then for all those Gram-positive microorganisms, and finally for the Gram-negative ones. In each case, Student's t -tests (two-sample) were performed between LAB pairs ($H_0$: $\mu_1 - \mu_2 = 0$; $H_1$: $\mu_1 - \mu_2 > 0$), and the p-value associated with each test was recorded. From all the comparisons made, the geometric mean of the obtained p-values was calculated and multiplied by 100. With these values, rankings (overall scores) about the performance of the studied CFS from LABs against the pathogens were established.

## Results and Discussion

### *LAB antimicrobial activity*

The effect of the CFS against the tested indicator bacteria is shown in Table 1. The LAB tested showed antagonistic activity against the indicator microorganisms, in agreement with previous studies (Da Costa et al., 2018; Sahraoui, Fayolle, Leriche, Le Flèche-Matéos & Sadoun, 2015). The CFS produced by *Lb. plantarum* NRRL B-4496, *Lb. sakei* NRRL B-1917, and *Lb. rhamnosus* NRRL B-442 was found to have the greatest inhibition zones against the seven tested bacteria; the supernatant of *Lb. plantarum* NRRL B-4496 displayed the highest inhibition against *S.* Typhimurium (24.89 mm), *Pseudomonas* (19.5 mm), *Staph, aureus* ATCC 25923 (18.93 mm), and *Sh. sonnei* (15.63 mm); while *Staph. aureus* ATCC 29213, *E. coli*, and *L. monocytogenes* were more inhibited by the CFS produced by *Lb. sakei* NRRL B-1917 (with inhibition halos of 22.77, 20.82, and 22.90 mm, respectively) (Table 1).

By assessing both the results of the pH neutralization as well as the CFS composition, the antimicrobial activity of the studied CFS can be attributed to organic acids (mainly acetic and lactic acids). Performed analysis establish that acetic acid (~0.11 %) and lactic acid (~2.06%) were present. It has previously been reported that the primary antimicrobial effect produced by LAB is through the production of different organic acids (Omemu & Faniran, 2011), which may interfere with the maintenance of cell membrane potential, inhibit active transport, reduce intracellular pH, and hinder a variety of metabolic functions (Mani-López, García & López-Malo, 2012). Of the seven tested indicator bacteria, *L. monocytogenes* was the most sensitive ($p < 0.05$) to eleven supernatants of the studied LAB (Table 1).

**Table 1.** Diameter (mm) of inhibition zones generated by supernatants of studied lactic acid bacteria (LAB) against selected indicator microorganisms.

| LAB | *Escherichia coli* | *Salmonella* Typhimurium | *Shigella sonnei* | *Pseudomonas fluorescens* | *Listeria monocytogenes* | *Staphylococcus aureus* ATCC 29213 | *Staphylococcus aureus* ATCC 25923 |
|---|---|---|---|---|---|---|---|
| *Lactococcus lactis subsp. cremoris* | 12.96 ± 1.33 $^{e}$ | 13.77 ± 1.67 $^{d, e}$ | 14.36 ± 0.63 $^{c, d}$ | 13.91 ± 0.86 $^{d}$ | 15.55 ± 1.52 $^{a, b}$ | 15.96 ± 2.27 $^{a}$ | 14.98 ± 0.79 $^{b, c}$ |
| *Lc. lactis subsp. lactis* | 13.78 ± 1.96 $^{b}$ | 12.24 ± 3.28 $^{c}$ | 11.11 ± 1.15 $^{d}$ | 13.01 ± 1.40 $^{b, c}$ | 14.91 ± 0.90 $^{a}$ | 13.30 ± 1.43 $^{b}$ | 13.13 ± 0.90 $^{b, c}$ |
| *Lactobacillus acidophilus* | 14.06 ±1.24 $^{c}$ | 13.54 ± 2.51 $^{c}$ | 11.53 ± 1.35 $^{d}$ | 10.89 ± 0.38 $^{d}$ | 15.74 ± 2.31 $^{b}$ | 17.56 ± 0.89 $^{a}$ | 13.91 ± 1.63 $^{c}$ |
| *Lb. casei* NRRL B-1922 | 16.52 ± 1.84 $^{b}$ | 17.17 ± 1.07 $^{b}$ | 11.06 ± 1.37 $^{d}$ | 11.61 ± 0.62 $^{d}$ | 20.03 ± 4.00 $^{a}$ | 16.43 ± 0.95 $^{b}$ | 14.20 ± 1.13 $^{c}$ |
| *Lb. fermentum* | 13.71 ± 1.63 $^{c, d}$ | 15.34 ± 3.11 $^{a, b}$ | 13.24 ± 1.24 $^{d}$ | 13.91 ± 1.07 $^{c, d}$ | 16.62 ± 1.23 $^{a}$ | 16.74 ± 3.93 $^{a}$ | 15.05 ± 0.86 $^{b, c}$ |
| *Lb. plantarum* | 20.23 ± 2.03 $^{b, c}$ | 24.89 ± 2.82 $^{a}$ | 15.63 ± 1.03 $^{d}$ | 19.5 ± 1.09 $^{b, c}$ | 20.39 ± 3.57 $^{b, c}$ | 20.53 ± 2.32 $^{b}$ | 18.93 ± 1.22 $^{c}$ |
| *Lb. reuteri* | 16.42 ± 3.02 $^{b}$ | 16.03 ± 3.24 $^{b,c}$ | 14.66 ± 1.07 $^{c}$ | 14.58 ± 1.14 $^{c}$ | 18.77 ± 1.41 $^{a}$ | 15.49 ± 3.08 $^{b,c}$ | 15.96 ± 0.73 $^{b,c}$ |
| *Lb. rhamnosus* | 17.15 ± 3.58 $^{c}$ | 18.89 ± 1.37 $^{b}$ | 14.54 ± 0.68 $^{d}$ | 15.59 ± 0.83 $^{d}$ | 19.51 ± 1.47 $^{b}$ | 21.83 ± 4.11$^{a}$ | 18.87 ± 1.58 $^{b}$ |
| *Lb. sakei* | 20.82 ± 2.95 $^{b}$ | 20.91 ± 2.68 $^{b}$ | 14.14 ± 1.32 $^{d}$ | 16.13 ± 1.18 $^{c}$ | 22.90 ± 2.93 $^{a}$ | 22.77± 5.58 $^{a}$ | 16.18 ± 1.61 $^{c}$ |
| *Leuconostoc mesenteroides subsp. mesenteroides* | ---- | ---- | ---- | 8.37 ± 0.38 $^{b}$ | 12.49 ± 3.05 $^{a}$ | ---- | ---- |
| *Pediococcus acidilactici* | ---- | 7.90 ± 0.10 $^{b}$ | ---- | 8.36 ± 0.55 $^{a}$ | 7.80 ± 0.26 $^{b}$ | ---- | ---- |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *P. pentosaceus* | 12.08 ± 1.18 [c, d] | 13.04 ± 1.96 [b, c] | 11.11 ± 0.76 [d, e] | 10.30 ± 2.46 [e] | 17.13 ± 4.47 [a] | 16.66 ± 1.67 [a] | 13.65 ± 1.53 [b] |
| *Lb. brevis* | ---- | ---- | 9.01 ± 0.53 [d] | 11.05 ± 1.12 [b] | 14.81 ± 1.15 [a] | 11.25 ± 0.94 [b] | 11.39 ± 0.89 [b] |
| *Lb. casei* 21/1 | 16.14 ± 1.6 [e] | 18.76 ± 1.13 [b] | 14.1 ± 1.11 [f] | 17.76 ± 0.83 [c, d] | 22.02 ± 1.02 [a] | 17.21 ± 1.39 [d] | 18.19 ± 0.91 [b, c] |
| *Lb. amylovorus* | 14.58 ± 0.55 [d] | 14.52 ± 0.96 [d] | 12.34 ± 1.42 [e] | 17.53 ± 2.59 [b] | 22.15 ± 1.39 [a] | 16.23 ± 1.31 [c] | 15.79 ± 1.07 [c] |
| *Lb. sanfranciscensis* | ---- | ---- | 8.81 ± 0.44 [c] | 9.81 ± 0.88 [b] | 10.48 ± 0.94 [a] | ---- | ---- |

---- No inhibition zone was observed.

Means in the same row followed with the same letter are not significant different ($p > 0.05$) according to Fisher's test.

Similar results have been reported in other studies, Anas, Eddine & Mebrouk, (2008) observed that the supernatants of *Lb. plantarum, Lb. paracasei* subsp. *paracasei*, or *Lb. rhamnosus* were capable of inhibiting the growth of *Staph. aureus*, and that the supernatants of *Lb. plantarum* were more effective in reducing pathogenic populations. Kareem et al. (2014) reported that the supernatants of *Lb. plantarum* RG11, RG14, RI11, UL4, TL1, and RS5 in combination with inulin had an inhibitory effect on the population of Gram-positive and Gram-negative pathogenic microorganisms. Beristain-Bauza et al. (2016) observed that increasing the concentration of supernatants resulted in higher reductions in the amount of indicator microorganisms (*E. coli* ATCC 25922, *Staph. aureus* ATCC 29413, *S.* Typhimurium ATCC 14028, or *L. monocytogenes* Scott A).

Table 2 presents the results of the calculation of the geometric mean of the p-values after performing Student's t-tests. It can be seen that in general, the best CFS in terms of inhibition of the indicator bacteria tested were *Lb. plantarum*, *Lb. sakei*, and *Lb. rhamnosus* (lower geometric means). These LABs act quite well against Gram-positive and Gram-negative bacteria.

On the other hand, some of the CFS tested did not have the desired activity to inhibit the tested indicator bacteria (higher values of the geometric means); some CFS have poor performance and inhibit poorly (null or small inhibition halos) and/or only act against one or few of the tested indicator microorganisms (Tables 1 and 2)

**Table 2. Geometric mean (multiplied by 100) of p-values obtained after Student's two-sample t-tests.**

| LAB strain | Geometric mean* | | | | | |
|---|---|---|---|---|---|---|
| | All tested | Rank | Gram + | Rank | Gram - | Rank |
| *Lactobacilus casei* NRRL B-1922 | 9.08 | 11 | 3.02 | 6 | 12.53 | 11 |
| *Lactobacillus fermentum* | 1.07 | 7 | 6.26 | 8 | 3.49 | 7 |
| *Lactobacillus plantarum* | 0.02 | 1 | 0.33 | 3 | 0.16 | 1 |
| *Lactobacillus rhamnosus* | 0.10 | 3 | 0.27 | 2 | 0.61 | 4 |
| *Lactobacillus reuteri* | 0.47 | 6 | 3.47 | 7 | 1.35 | 5 |
| *Lactobacillus sakei* | 0.03 | 2 | 0.19 | 1 | 0.27 | 2 |
| *Lactobacillus acidophillus* | 3.44 | 9 | 8.66 | 10 | 9.50 | 9 |
| *Lactococcus lactis* subsp. *cremoris* | 1.76 | 8 | 9.61 | 11 | 3.89 | 8 |
| *Lactococcus lactis* subsp. *lactis* | 8.46 | 10 | 15.85 | 12 | 10.31 | 10 |
| *Leuconostoc mesenteroides* subsp. *mesenteroides* | 99.42 | 16 | 39.81 | 14 | 99.74 | 16 |
| *Pediococcus acidilactici* | 83.85 | 15 | 99.90 | 16 | 61.50 | 15 |
| *Pediococcus pentosaceus* | 15.81 | 12 | 8.44 | 9 | 15.70 | 12 |
| *Lactobacillus brevis* | 15.81 | 12 | 25.12 | 13 | 33.94 | 13 |
| *Lactobacillus casei* 21/1 | 0.21 | 4 | 0.52 | 4 | 0.57 | 3 |
| *Lactobacillus sanfranciscensis* | 43.70 | 14 | 63.10 | 15 | 56.53 | 14 |
| *Lactobacillus amylovorus* | 0.36 | 5 | 0.63 | 5 | 2.02 | 6 |

* multiplied by 100, lower values indicate better performance

As observed in Tables 1 and 2 for most studied supernatants, tested Gram-negative microorganisms exhibited more resistance than the Gram-positive ones. This difference is very likely due to the differences in cell-wall structure and composition between Gram-negative and Gram-positive organisms (Beristain-Bauza et al., 2016). The three most effective CFS (*Lb. sakei* NRRL B-1917, *Lb. plantarum* NRRL B-4496, and *Lb. rhamnosus* NRRL B-442) were selected for further evaluation.

After CFS from *Lb. sakei* NRRL B-1917 and *Lb. rhamnosus* NRRL B-442 were neutralized with sodium hydroxide solution (adjusted to pH 6.5), antibacterial activity against all of the tested indicator microorganisms was eliminated. In contrast, neutralized *Lb. plantarum* NRRL B-4496 CFS maintained antimicrobial activity against only *L. monocytogenes*, though 11% less activity (18.10 ± 1.81 mm) was observed in comparison to that seen with CFS at the native pH (3.89). After neutralization, Da Costa et al. (2018) observed antagonistic activity was reduced, and therefore that organic acids must play a primary role in the inhibitory effects against pathogenic microorganisms. Similar findings were reported by Teixeira de Carvalho, Aparecida de Paula, Mantovani, & Alencar de Moraes, (2006), who tested several LAB supernatants isolated from naturally fermented Italian salami against *L. monocytogenes*; only one of their studied CFS maintained inhibitory activity after being neutralized (pH 6.5). Bian, Molan, Maddox & Shu (2011) and Kalalou, Zerdani & Faid (2010) reported antimicrobial activity of CFS with a neutral pH from *Lb. reuteri* DP616 and *Lb. plantarum* against Gram-positive (*L. monocytogenes* Scott A or *Staph. aureus* ATCC 2592) and Gram-negative bacteria (*E. coli* O157:H7 or *S.* Typhimurium ATCC 14028), respectively. Organic acids are able to diffuse through the plasma membrane of pathogenic microorganisms and inhibit glycolysis, as the key enzymes in this pathway are sensitive to low pH (Stoyanova, Ustyugova & Netrusov, 2012).

Peptide or protein antimicrobial activity of the CFS can be inferred by looking at changes in antimicrobial activity after treatment with proteinase K – if activity is eliminated, the mechanism of action is protein-based. In this study, only the CFS from *Lb. plantarum* NRRL B-4496 was found to contain a peptide-based compound with antibacterial activity against *L. monocytogenes*. To our knowledge, no previous bacteriocin or bacteriocin-like compound has been reported for the strain NRRL B-4496 of *Lb. plantarum*; further research is being

conducted in our laboratory to identify this compound. It is interesting to note; however, that plantaricins had been previously reported for other strains of *Lb. plantarum* (Siezen & van Hylckama Vlieg, 2011).

*CFS stability during storage*

CFS of *Lb. plantarum* NRRL B-4496 and *Lb. sakei* NRRL B-1917 CFS were tested against four indicator microorganisms (*E. coli, S.* Typhimurium, *L. monocytogenes,* and *Staph. aureus* ATCC 29213), while CFS from *Lb. rhamnosus* NRRL B-442 was tested only against *L. monocytogenes* and *Staph. aureus* ATCC 29213. In every case, it was observed that the antibacterial activity was significantly ($p < 0.05$) reduced between fresh (time zero) CFS when compared to that seen after twenty weeks of storage. In general, stability (as determined by antimicrobial activity) during storage was inversely proportional to temperature, as higher reductions in antimicrobial activity were observed for supernatants stored at 35 °C (average losses of 20 to 25%) when compared to those stored at 25 ºC (average losses of 19 to 21.5%) or at 15 ºC (average losses of 16 to 18.4%) (Figures 1–3). In spite of the observed drop in antibacterial activity after storage, close to 75% of the original antimicrobial activity was preserved even at the highest tested storage temperature (35 ºC). This high stability was expected due to the stability of acetic/lactic acid (De Villiers, Wurster & Narsai, 1998), which are the major antimicrobial compounds in the CFS. De Villiers et al. (1998) studied the stability of lactic acid (<5%) in aqueous solutions with various excipients at different pH values, and estimated shelf lives greater than 79 years at 25 ºC which could be even longer if pH remains low.

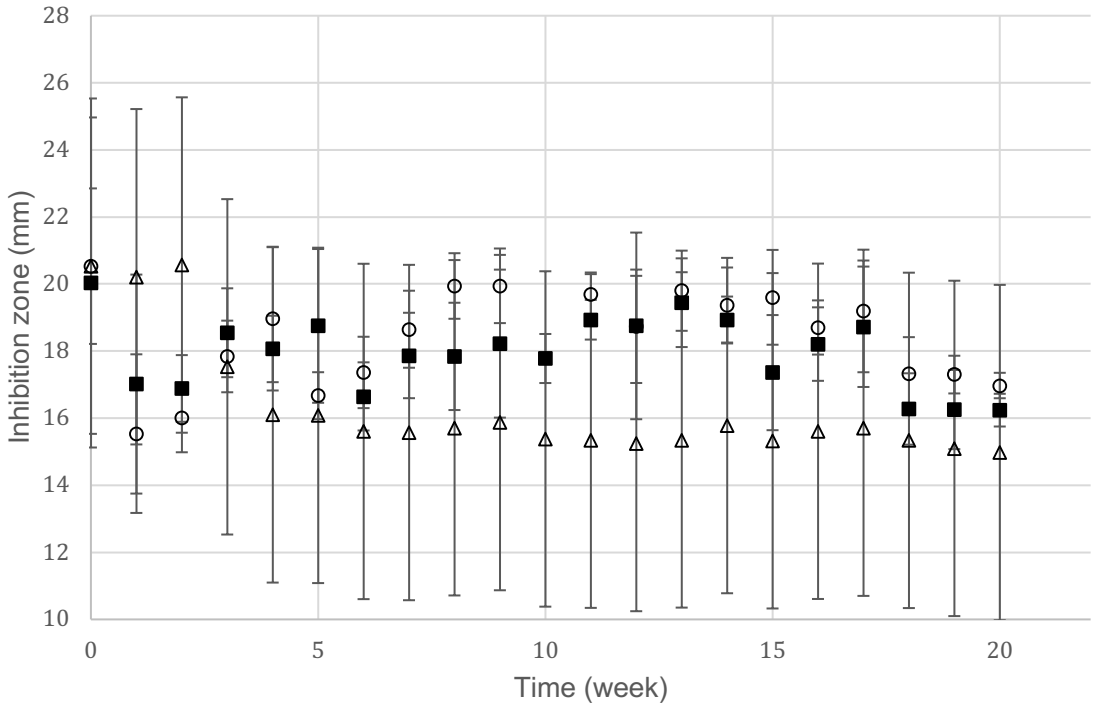
Significant ($p < 0.05$) reduction in antimicrobial activity against *Staph. aureus* ATCC 29213 was seen in CFS from *Lb. sakei* when stored at 15, 25, or 35 °C, as inhibition zones decreased (comparing inhibition halos at time 0 against at 20-weeks) by 5.43, 6.93, or 7.47 mm, respectively. *Lb. sakei* mainly produces lactic acid, but acetic acid is also formed, as this LAB has the ability to synthesize these acids from glucose and ribose, respectively (McLeod, Zagorec, Champomier-Vergès, Naterstad & Axelsson, 2010). Although the pH of CFS from *Lb. sakei* only decreased $0.15 \pm 0.02$ units during storage, it is possible that acetic acid was gradually lost at 25 °C and 35 ºC, and as a result, *Staph. aureus* ATCC 29213 was less inhibited toward the end of storage. Further, *Staph. aureus* is more sensitive to acetic acid

than lactic acid at the same pH value (Medved'ová & Valík, 2012). Similar behavior was observed for CFS from *Lb. rhamnosus*; inhibition zones decreased by 5.05, 5.45, or 5.90 mm (between time 0 and after 20-weeks) when stored at 15, 25, or 35 °C, though these decrements were less pronounced. The same reason explains this loss in activity, since *Lb. rhamnosus* is also a facultative heterofermentative *Lactobacillus* that produces lactic and acetic acid (Ceapa et al., 2016). For this CFS, the pH decreased 0.13 ± 0.03 units after 5 months of storage.

In contrast, CFS from *Lb. plantarum* NRRL B-4496 had larger reductions in inhibition zones against *S.* Typhimurium (4.69, 4.47, or 6.82 mm at 15, 25, or 35°C, respectively). *Lb. plantarum* is also a facultative heterofermentative LAB (Melgar-Lalanne, Rivera-Espinoza, & Hernández-Sánchez, 2012) that also produces a bacteriocin-like compound. In spite of *Salmonella* not being sensitive to this bacteriocin-like compound, it is possible that a synergistic effect exists between the organic acids and peptides in the CFS, and that this effect was lost during storage, resulting in the reduction of antibacterial activity. On the other hand, *E. coli* and *L. monocytogenes* presented lower reductions in their inhibition zones, being 2.49, 3.19, or 4.22 mm and 3.66, 4.87, or 4.03 mm, when studied CFS were stored at 15, 25, or 35 °C ± 1, respectively. These bacteria are more sensitive to acid pH values; at the end of the storage experiment, the pH of the CFS from *Lb. plantarum* NRRL B-4496 was 3.74 ± 0.03 (ΔpH = 0.15 ± 0.03). Therefore, it would be expected that this CFS would be more effective against these species than the CFS from another studied LAB.

No previous studies about stability of CFS from LAB in MRS broth appear to have been published. However, Gerez, Fornaguera, Obregozo, Font de Valdez & Torino (2015) evaluated the stability of a semi-liquid bio-preservative (wheat flour broth) that resulted from fermentation with *Lb. plantarum* CRL 778, intended for packaged bread preservation. The characteristics of this bio-preservative maintained stability over 14 days of storage at 4 °C ± 1, while the concentration of organic acids significantly decreased after 7 days of storage. The results from this study along with those from Gerez et al. (2015) differ due to the studied LAB and test strains. Commercial products resulting from the growth of LAB in a liquid blend of water and wheat flour had a shelf life of nine months at ≤ 25 ºC (Puratos Group, 2018). The CFS in this study had similar stabilities to this mixture; results indicate that they

may be able to reach 9 months of shelf life, though this needs to be tested further to be confirmed.


Inhibition zone (mm)
28
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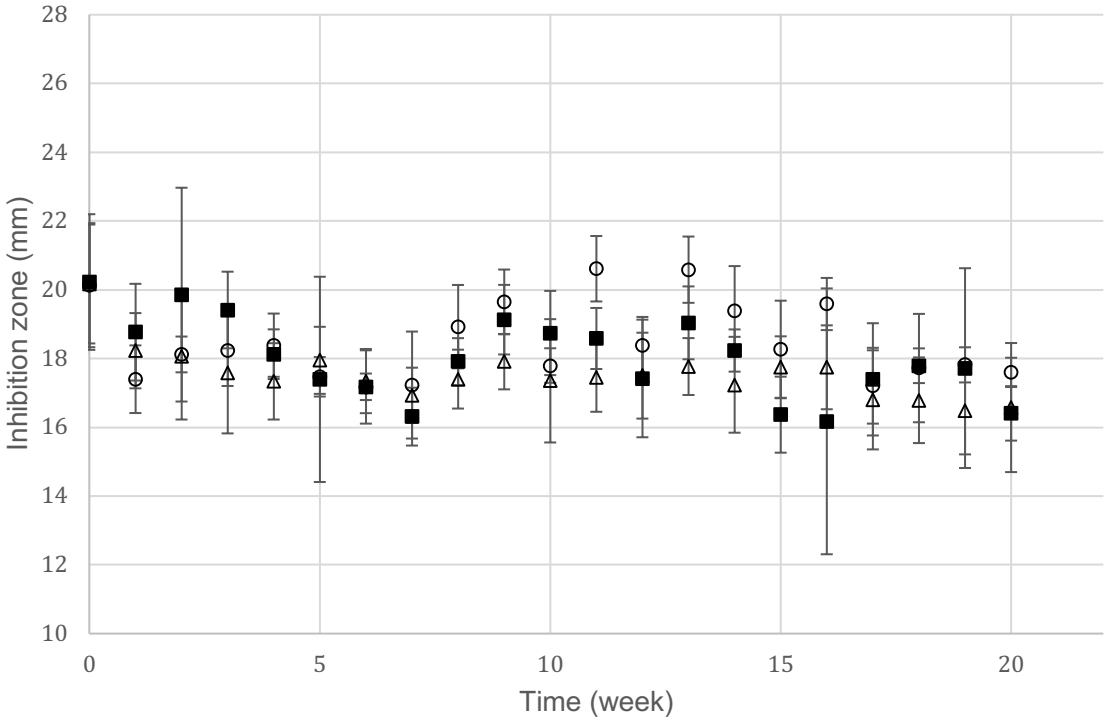
Time (week)

a)


Inhibition zone (mm)
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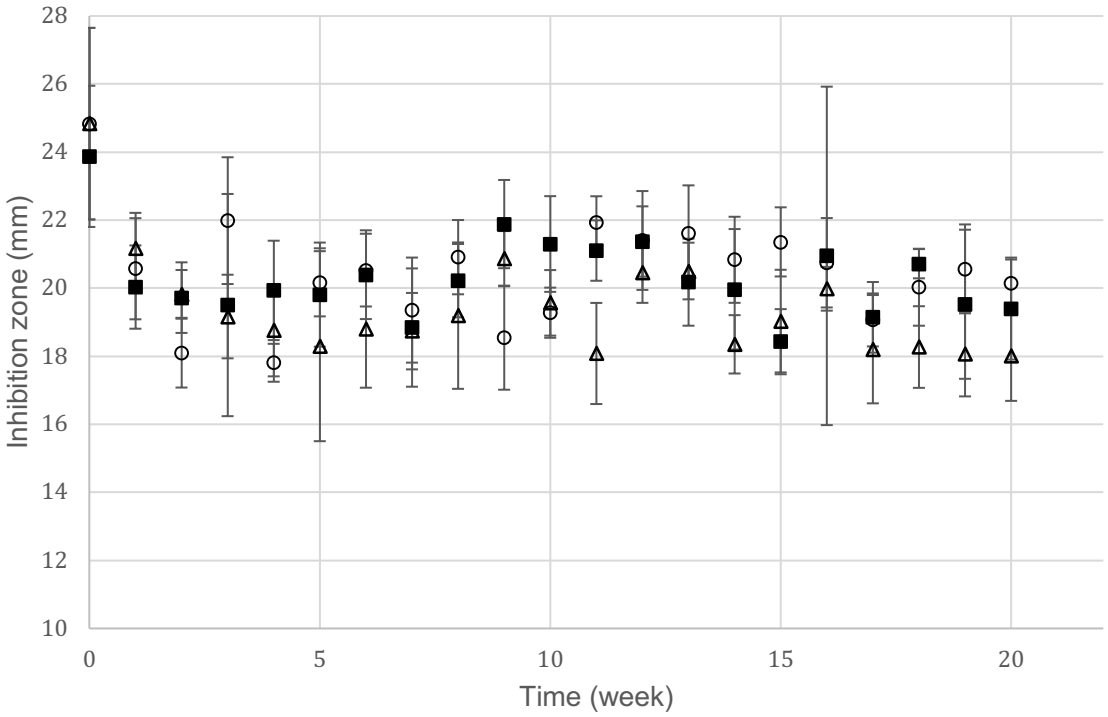
Time (week)

b)

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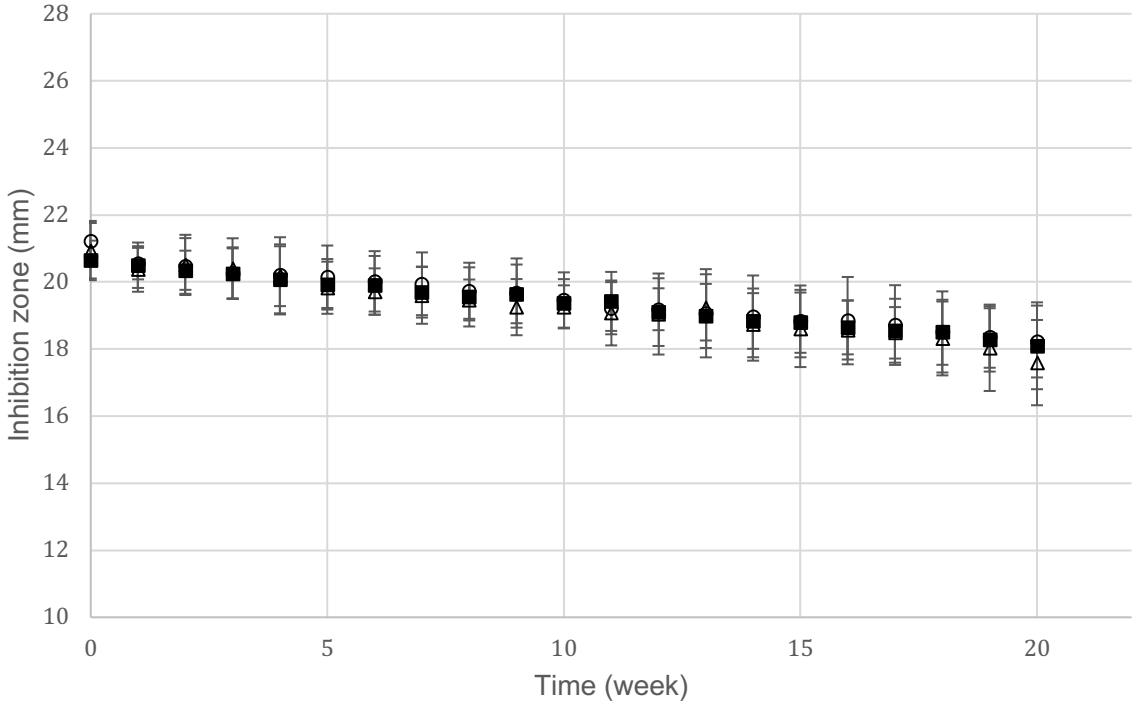
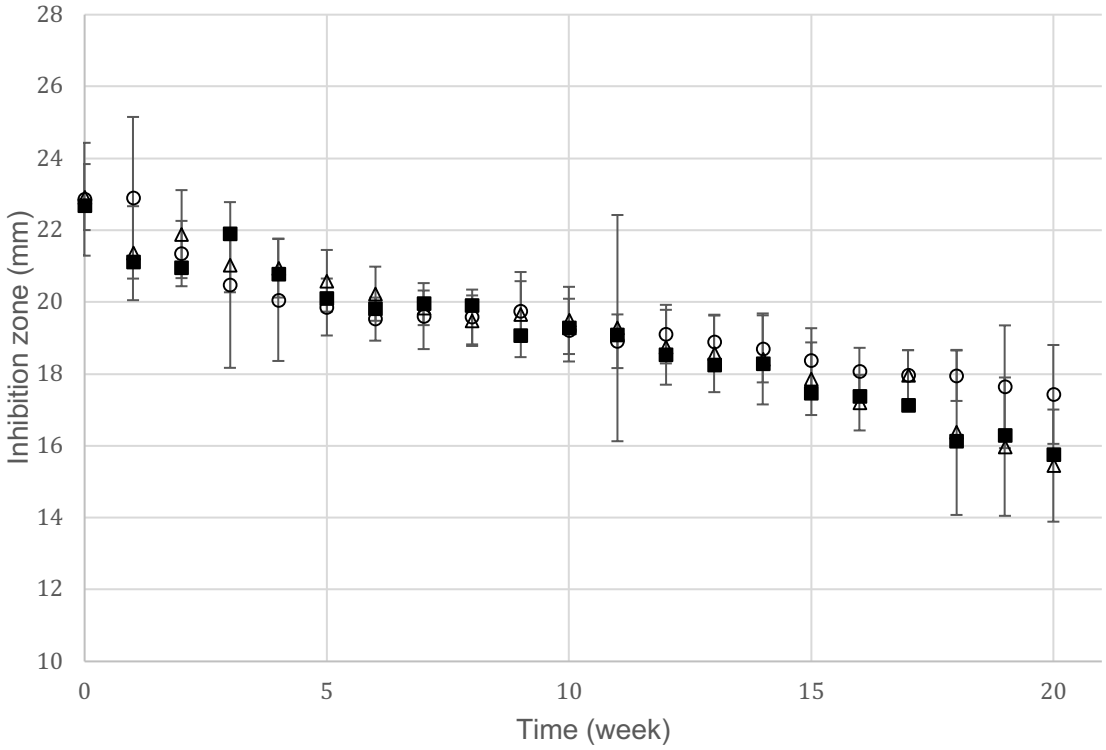



d)

**Fig. 1.** *Lb. plantarum* NRRL B-4496 cell-free supernatant stability as inhibition zones against *Staph. aureus* ATCC 29413 (a), *E. coli* ATCC 25922 (b), *S.* Typhimurium ATCC 14028 (c), or *L. monocytogenes* SCOTT A (d) during storage at 15 (○), 25 (■), or 35 °C (△).


Inhibition zone (mm)
28
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16
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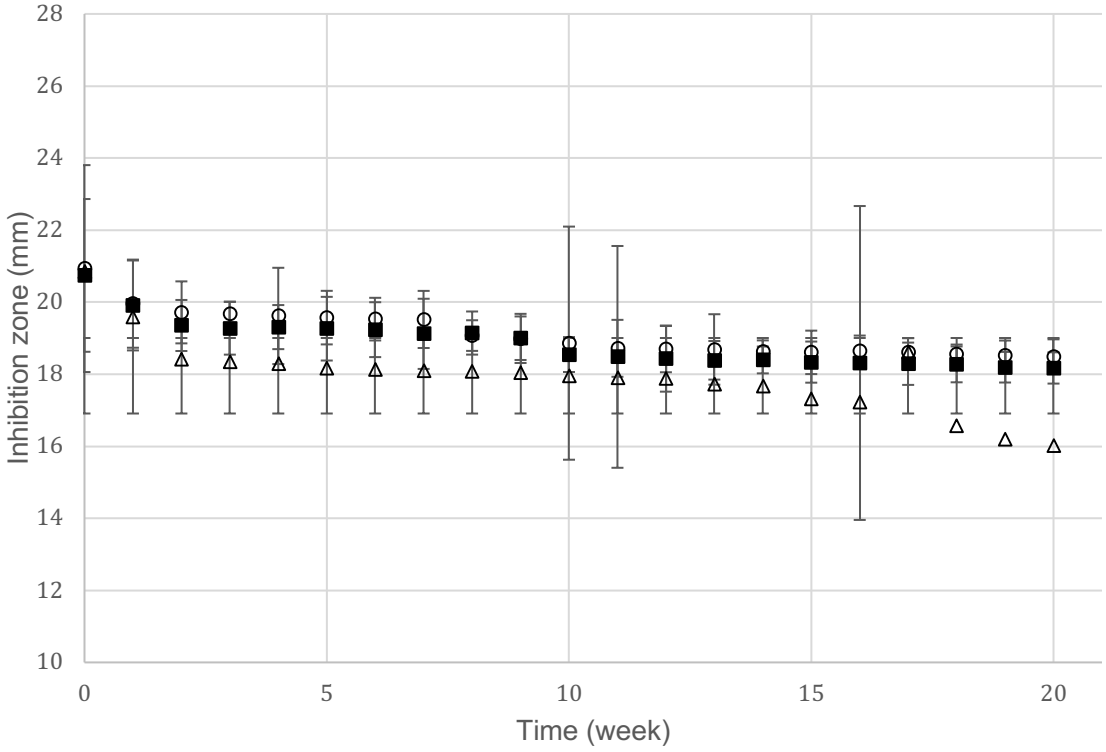
5
10
15
20
Time (week)

a)


Inhibition zone (mm)
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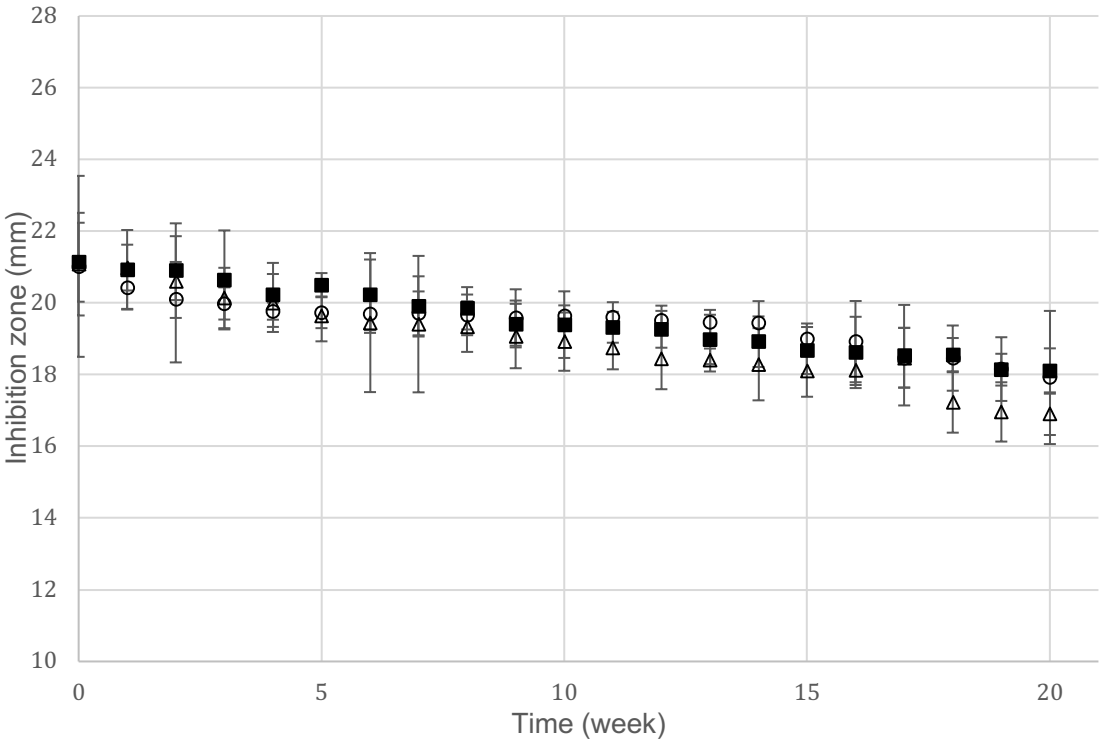
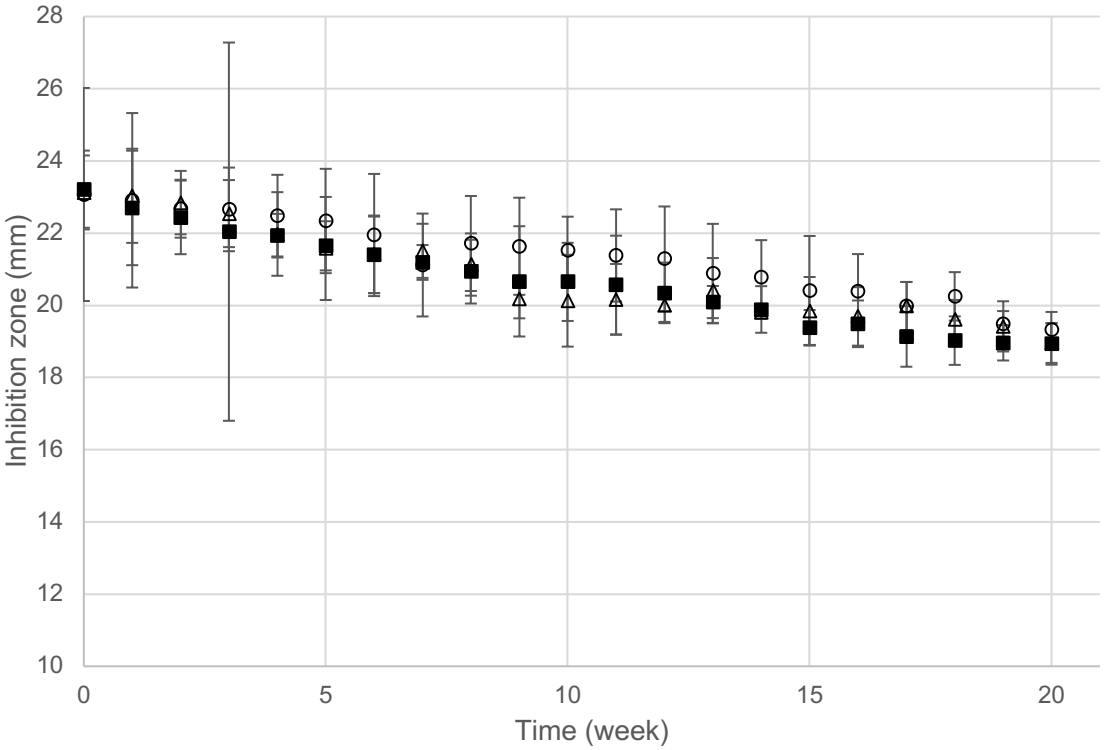
Time (week)

b)

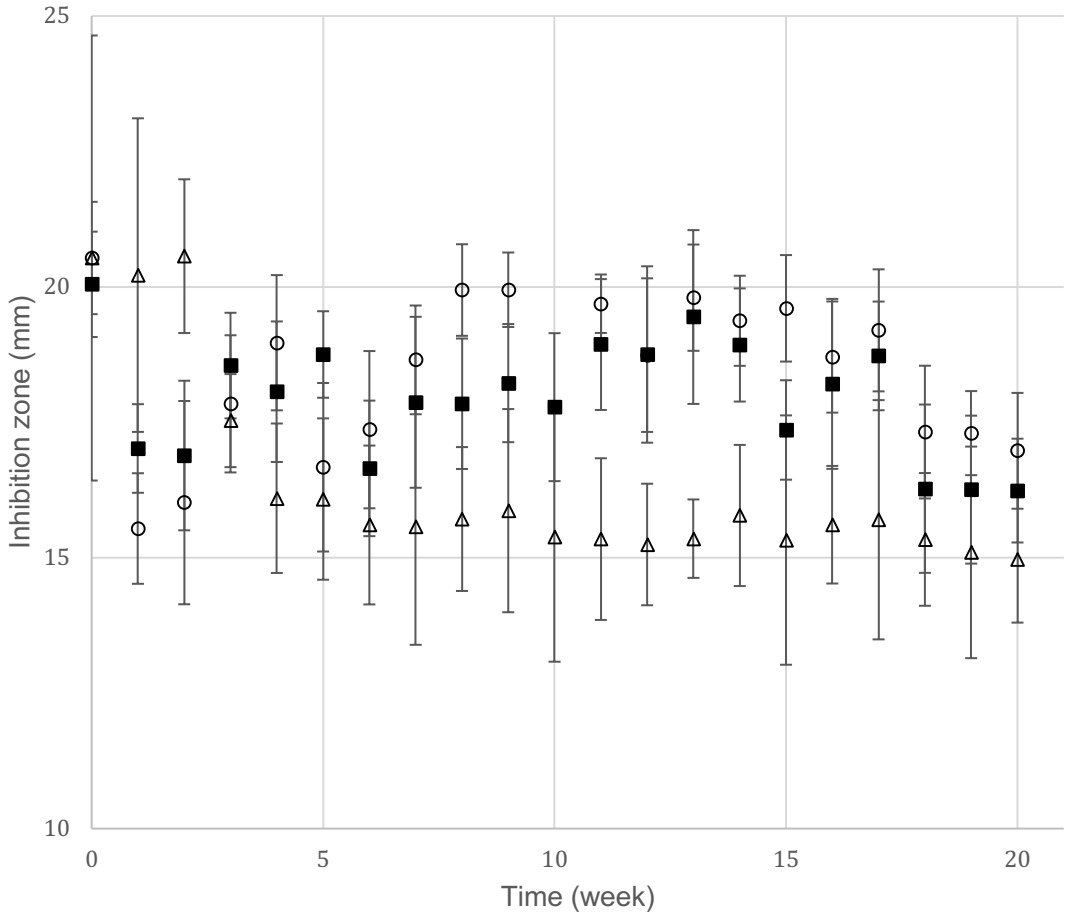



c)

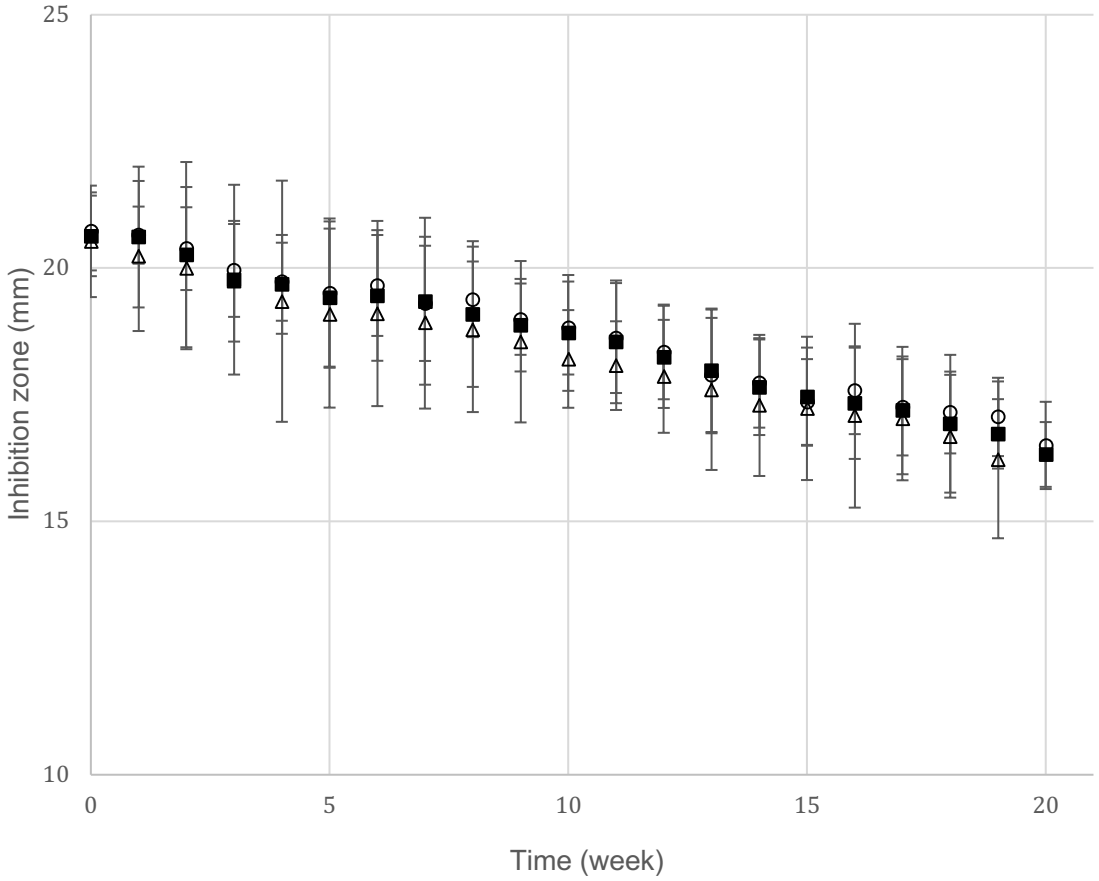



d)

**Fig. 2.** *Lb. sakei* NRRL B-1917 cell-free supernatant stability as inhibition zones against *Staph. aureus* ATCC 29413 (a), *E. coli* ATCC 25922 (b), *S.* Typhimurium ATCC 14028 (c), or *L. monocytogenes* Scott A (d) during storage at 15 (○), 25 (■), or 35 °C (△).




a)




b)

**Fig. 3.** *Lb. rhamnosus* NRRL B-442 cell-free supernatant stability as inhibition zones against *Staph. aureus* ATCC 29413 (a) or *L. monocytogenes* Scott A (b) during storage at 15 (○), 25 (■), or 35 °C (△).

*CFS antimicrobial activity in meat*

Initial counts of total mesophilic aerobic bacteria were 2.57 ± 0.96 $\log_{10}$ CFU/g, and total coliforms were 2.6 ± 0.82 $\log_{10}$ CFU/g, while counts of *E. coli*, *Salmonella*, *Staph. aureus* ATCC 29213, and *Listeria* were under detection level (<100 CFU/g), indicating that the bacterial counts of the fresh beef non-inoculated were satisfactory for the purpose of the study.

The pH of the beef pieces marinated with *Lb. plantarum* CFS was 3.80. Generally, the antagonist effects of CFS from *Lactobacillus* species on bacterial growth improve when the pH decreases near to pKa values, as this produces optimal antimicrobial conditions (lactic acid pKa 3.83; acetic acid pKa 4.76). *S.* Typhimurium was the most sensitive organism studied, followed by *L. monocytogenes*, while *Staph. aureus* ATCC 29213 and *E. coli* were the most resistant to CFS activity (Figure 4). Application of CFS to meat confirmed the sensitivity of *S.* Typhimurium observed in *in vitro* tests; at the beginning, counts of *Salmonella* were 5.87 $\log_{10}$ CFU/g for inoculated beef pieces, and decreased 3.74 $\log_{10}$ CFU/g cycles after 14 h of refrigerated marination (Figure 4). It is well known that organic acids such as lactic or acetic acid can be used on meat to control *Salmonella* proliferation, as these acids are capable of crossing the cell membrane where they dissociate in the cytoplasm, causing cytoplasmic acidification, changes of metabolic activities such as protein synthesis, and inhibition of ATP synthesis (Mani-López et al., 2012).

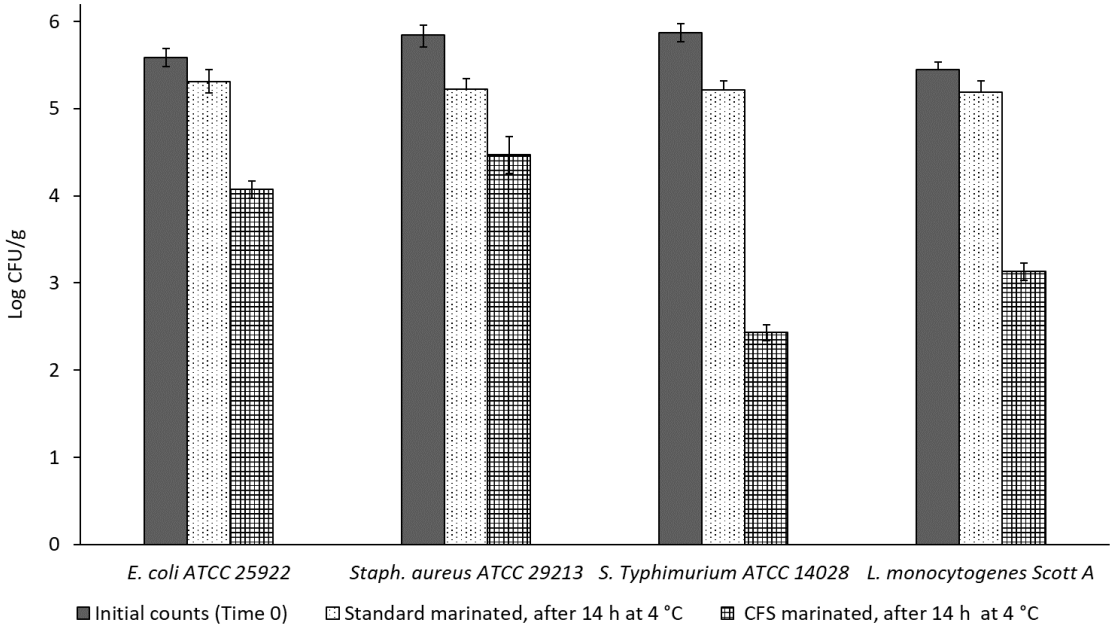



**Fig. 4.** Antimicrobial effect of standard or cell-free supernatants (CFS) marinade solutions in fresh beef pieces.

The initial count of *L. monocytogenes* was 5.44 $\log_{10}$ CFU/g; marination with CFS caused cell counts to decline 2.3 $\log_{10}$ CFU/g cycles after 14 h in refrigerated conditions. Rhoades, Kargiotou, Katsanidis & Koutsoumanis (2013) studied the effect of five different marinades (soy sauce base marinade with or without lactic acid, red wine base marinade without or with 0.5% v/v oregano essential oil, and sterile saline solution) to control *S. enterica* and *L. monocytogenes* in raw beef; they observed greater inhibition of *L. monocytogenes* than *S. enterica* when using the marinade with lactic acid. Alvarado & McKee (2007) studied the effect of two different marinating solutions on chicken, and the one with acetic and lactic acid proved to be more effective against *L. monocytogenes*, while the one with citric acid had less effect. *E. coli* initial counts (5.58 $\log_{10}$ CFU/g) were reduced by 1.93 $\log_{10}$ CFU/g cycles after 14 h of marinade in CFS. Previous studies have observed that after spraying lactic acid on the surface of beef, surface pH values decrease to 3.1 and provide at least 1-log reduction of *E. coli* O157:H7 and *Salmonella* (Kalchayanand et al., 2018).

Initial counts of *Staph. aureus* ATCC 29213 were 5.84 $\log_{10}$ CFU/g and decreased 1.33 $\log_{10}$ CFU/g cycles after 14 h of refrigerated CFS marinade. Akbar & Anal (2014) showed that *Lc. lactis* subsp. *lactis* is capable of inhibiting pathogenic bacteria such as *Staph. aureus* on

poultry meat products; they observed one $\log_{10}$ CFU/g cycle reduction during the first 7 days, along with a pH decrease (6.65 to 5.75). The differences between these studies could be due to different components present in the studied CFS, the effectiveness of antimicrobials, as well as the different application mode of the CFS.

The standard marinade mixture reduced microbial counts between 0.2 to 0.5 $\log_{10}$ CFU/g cycles after 14 h at 4 °C ± 1. These reductions were very low and may be attributed to sodium chloride activity on the microbial cells.

*Meat color*

One of the most important attributes to consider is food color. Consumers evaluate this factor when selecting meat, and therefore it is associated with food quality (Carpenter, Cornforth, & Whittier, 2001). The results of this study showed that redness (a*) and yellowness (b*) values changed significantly, and lightness (L*) decreased ($p < 0.05$) for raw beef pieces with CFS marinade, these pieces of meat were observed to have a dark and brownish color compared to the standard marinade, while for grilled beef redness pieces with CFS marinade (a*) values changed significantly and lightness (L*) decreased ($p < 0.05$) which means that the CFS marinade increased the redness of the grilled beef (Table 3); similar results have been observed for pork meat marinated with *P. acidilactici, P. pentosaceus*, or *Lb. sakei* (Mozuriene et al., 2016). On the other hand, the pH of the beef with the CFS marinade was 3.8 ± 0.01, while it was 4.8 ± 0.02 with the standard marinade. It has been shown that when pH decreases, myoglobin is easily oxidized to metamyoglobin, giving the color of the meat a lower color intensity; lower values of pH are also related to beef seeming more red and yellow (Page, Wulf & Schwotzer, 2001), agreeing with the color change of raw beef. According to Marcus (1998), perceptibility of color differences is detected if $\Delta E > 1.57$; in the case of this experiment, color differences were more pronounced in raw beef, while for grilled beef the difference was scarcely detected.

**Table 3. Color parameters and net color differences (ΔE) of beef CFS marinated or standard marinated, grilled or raw.**

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | Grilled beef | | | |
| CFS marinade | | | Standard marinade | | | |
| **L*** | **a*** | **b*** | **L*** | **a*** | **b*** | **ΔE** |
| 43.71 ± 2.2[a] | 8.70 ± 2.8[a] | 14.95 ± 5.2[a] | 54.04 ± 2.6[b] | 5.85 ± 0.7[b] | 12.64 ± 1.5[a] | 1.76 ± 1.5 |
| | | | Raw beef | | | |
| 42.1 ± 1.2[a] | 12.69 ± 2.3[a] | 8.19 ± 0.8[a] | 47.94 ± 1.6[b] | 6.10 ± 0.8[b] | 10.82 ± 0.8[b] | 9.2 ± 2.0 |

Means in the same row followed with the same letter are not significant different ($p < 0.05$) according to Fisher´s test.

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2}$$

## Conclusions

The observed antimicrobial activity of tested CFS in this study is mainly due to the presence of organic acids, and in the case of studied *Lb. plantarum* NRRL B-4496, a possible bacteriocin-like compound. An important finding is that the studied CFS can be produced and stored at room temperatures (23 ± 2 °C) for relatively long periods, with a low decrease in their antimicrobial activity over time. The marinade with *Lb. plantarum* NRRL B-4496 CFS was efficient in reducing microbial load for meat inoculated with the four tested bacterial strains. As shown in this study, CFS marinade has potential use as a bio-preservative on beef, which can enhance the safety and quality of beef products.

## Acknowledgements

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## Conflict of Interest

No conflict of interest declared associated with this research.

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**RESEARCH PAPER II**

**"Antimicrobial activity of protein-containing fractions isolated from *Lactobacillus plantarum* NRRL B-4496 culture"**

Brazilian Journal of Microbiology

Year 2020

**Antimicrobial activity of protein-containing fractions isolated from *Lactobacillus plantarum* NRRL B-4496 culture**

D. Arrioja-Bretón, E. Mani-López, H. Bach and A. López-Malo

## Abstract

The interest in lactic acid bacteria, including *Lactobacillus plantarum* NRRL B-4496, has increased in recent years as bio- preservatives, due to the production of secondary metabolites capable of inhibiting pathogenic bacteria. The objectives of this study were to evaluate the antimicrobial activity, cytotoxicity and the anti-inflammatory response of *Lb. plantarum* NRRL B-4496 cell-free supernatant (CFS). Furthermore, the CFS was fractionated by size exclusion chromatography using Sephadex G-25, and a minimal inhibitory volume test was determined against a panel of pathogenic bacteria. The cytotoxicity and the inflammatory activities of the fractions were evaluated using the human-derived THP-1 cell line. Results of this study indicates that CFS of *Lb. plantarum* NRRL B-4496 possesses antimicrobial protein compounds against the pathogen *Listeria monocytogenes* and showed no toxicity nor a pro-inflammatory response to human macrophages. The obtained results contribute to the development of novel bio-preservatives, *Lb. plantarum* cell-free supernatant or its fractions, with a potential use in the food industry.

**Keywords:** *Lactobacillus plantarum*, Protein fractions, Antimicrobial activity, Cytotoxicity, Inflammatory activity

## Introduction

In recent years, a precipitated increase in drug-resistant infections to antibiotics has presented a serious challenge for researchers from different areas of study such as medicine, molecular biology, food science, and antimicrobials therapies. The ability of bacteria to develop different mechanism of resistance and the loss of efficacy of antibiotics/antimicrobials to inhibit pathogenic microorganisms bring out the urgent need to develop alternatives to evolve substances/ compounds as control agents [1, 2].

Over the last decades, peptides have been studied for their antimicrobial activity and new development of natural antimicrobials and/or peptides (AMPs) with a vast spectrum of antiviral, antibacterial, antifungal, and anti-parasitic targets have been published [3–5].

In another perspective, there is a trend in the preference of consumers to choose fresh, preservative free, and less processed foods, which makes the research on natural antimicrobials relevant in order to replace current antimicrobials, while ensuring food safety. For this purpose, bio-preservatives such as essential oils, enzymes and microorganisms among others have been studied [6–8].

A good example is lactic acid bacteria (LAB) which have the ability to produce secondary metabolites that have been shown to possess antimicrobial activities such as organic acids, bacteriocins, hydrogen peroxide, reuterin (or 3- hydroxypropionaldehyde), diacetyl and ethyl alcohol among others [9–11]. Moreover, selected LAB has been recognized as safe for its traditional use in food fermentation process and was designed QPS (Qualified Presumption of Safety) with a direct application in food and pharmaceutical industries.

Previous studies have reported the antimicrobial activities of peptides isolated from lactobacilli. For example, peptides isolated from *Lb. helveticus* PR4 showed a broad spectrum of inhibition against *Enterococcus faecium, Bacillus megaterium, Escherichia coli, Listeria innocua, Salmonella* spp., *Yersinia enterocolitica*, and *Staphylococcus aureus* [12]. In another study, the peptide plantaricin K25 isolated from *Lb. plantarum* K25 showed antibacterial activity against Gram-positive and -negative bacteria [13].

To the best of our knowledge, no previous reports recognize bacteriocins or antimicrobial peptides/proteins from *Lb. plantarum* NRRL B-4496; moreover, previous work in our laboratory shows a strong antimicrobial activity of this *Lactobacillus* strain (data not shown). Therefore, following our program to identify novel peptides for use in food preservation, we aimed to study the antimicrobial activities of the cell-free supernatant (CFS) of *Lb. plantarum* NRRL B-4496. Moreover, we assessed the cytotoxicity and the inflammatory response of the CFS using a human macrophage model.

## Materials and methods

### Strains and media culture

*Lb. plantarum* NRRL B-4496 used in this study was provided by the Food Microbiology Laboratory strain collection of the Universidad de las Americas Puebla (Puebla, Mexico). Other bacterial strains used in this study were methicillin-resistant *Staphylococcus aureus* (ATCC 700698), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Acinetobacter baumannii* (ATCC BAA-747), *Pseudomonas aeruginosa* (ATCC 14210), and *Listeria monocytogenes* (Scott A). *Salmonella* Typhimurium (ATCC 13311) was only used to test the antimicrobial activity of CFS fractions. The fungal strains *Candida albicans* (ATCC 10231), *Cryptococcus neoformans* var. *grubii* (provided by Dr. Karen Bartlett, University of British Columbia, BC, Canada), *Aspergillus fumigatus* (ATCC 1022), and *Trichophyton rubrum* (ATCC 18758), representing human pathogenic fungi were used in this study. Bacterial strains were maintained in Mueller-Hinton broth (Becton- Dickinson, Sparks, MD) supplemented with 1.5% agar (Becton-Dickinson, Sparks, MD) at 4 °C. Fungal strains were maintained in Sabouraud broth (Becton-Dickinson, Sparks, MD) supplemented with 1.5% agar and incubated at 28 °C. Fungal spores' suspensions were prepared according to published protocols [14].

### Cell-free supernatant preparation

*Lb. plantarum* NRRL B-4496 was cultivated ($10^6$ CFU $mL^{-1}$, plate count) in 30 mL of MRS broth (Becton-Dickinson, Sparks, MD) and incubated at 35 °C, 48 h. The CFS culture was obtained by centrifugation at 8000×g during 10 min (Marathon 21 K/R, Fisher Scientific, Germany), filter sterilized through 0.45 µm Millipore membrane filter, and CFS concentrated 10-fold by vacuum evaporation on a Buchi R-210/215 rotary evaporator (Buchi, Flawil, Switzerland) at 70 °C ± 1.0 °C and 25 cm Hg. Concentrated supernatants were lyophilized on a freeze-dryer (Labconco Corp., Kansas, MO).

Gel filtration chromatography of fractions

To obtain the fractions, CFSs were reconstituted in 1.5 mL PBS buffer and fractionated through a glass column (60 × 1.5 cm) filled with Sephadex G-25 (Pharmacia, Uppsala) in PBS with a final volume of ($10^6$ $cm^3$). Gel filtration chromatography by size-exclusion was performed as described Hamilton [15]. The column was connected to a fraction collector FRAC-100 (Pharmacia Biotech, Piscataway, NJ) and fourteen fractions of 1 mL each were collected with a flow rate of 200 μL $min^{-1}$, in a total elution time of 1.2 h.

Protein quantification and molecular weight calculations

The protein amount in each fraction was determinate by the Bradford method. The binding of the dye with the proteins was evaluated by spectrophotometry at an absorbance of 595 nm [16]. A calibration curve using bovine serum albumin was used to calculate the protein concentrations.

Fractions 2, 3, 5 and 6 were subjected to SDS- polyacrylamide gel electrophoresis (PAGE, 12%) [17] in a Hoffer apparatus and 12 μL of the samples were loaded. Protein bands were silver stained, and molecular mass standards were obtained from Bio-Rad (Hercules, CA).

Minimal inhibitory volume

The minimum inhibitory volume (MIV) was defined as the minimum volume of CFS or its fractions at which no microbial growth was observed (no turbidity observed in the well). MIVs were determined by a microdilution assay. The CFS *Lb. plantarum* NRRL B-4496 and its fractions volumes of 2, 5 and 50 μg $mL^{-1}$ were assayed in a final volume of 200 μL per well. Bacterial strains were grown at 37 °C for 18 h at 1×g and their densities were adjusted to an optical density of 0.05 at 600 nm ($1 \times 10^7$ CFU $mL^{-1}$). In the case of the filamentous fungi, 5 μL of a spore suspension ($1 \times 10^6$ spores $mL^{-1}$) were used as inoculum and incubated at 28 °C for 48 h. Amikacin (100 μg $mL^{-1}$) or gentamicin (50 μg $mL^{-1}$) (for bacteria), and amphotericin (100 μg $mL^{-1}$) or terbinafine (125 μg $mL^{-1}$) (for fungi) were used as positive controls. Experiments were performed in triplicate.

pH sensibility

CFS pH was measured and adjusted to pH 6.5 with a 40% NaOH (w/v) solution to test the antimicrobial activity of neutralized or not CFS.

Proteinase K test

The pH of the reconstituted CFS was adjusted to 6.5 with a 40% NaOH (w/v) solution. The proteinase digestion was per- formed by adding 3 µL of proteinase K (Fermentas, Hanover, MD) in a 200 µL well, the plate was incubated at 37 °C during 2 h. Afterwards, the antimicrobial activity of the neutralized- hydrolyzed CFS was determined by means of a minimum inhibitory volume test.

Cytotoxic assay

The cytotoxicity of the CFS fractions was evaluated using the human-derived monocytes THP-1 cell line (ATCC TIB- 202). We have implemented this model in our lab and the following publications are provided as an example: PDIM: 25417600, 27,794,508, 28,122,038, 31,111,047, and 31,205,934. The cytotoxic evaluation was performed following published protocols [18]. Briefly, $5 \times 10^4$ cells were dispensed per well in a 96-well plate with a final volume of 200 µL. CFS fractions were tested at a final volume of 100 µL. THP-1 cells exposed to hydrogen peroxide (10 µL of a 5% solution) were used as a positive control, whereas untreated cells were used as negative control. The analysis of the toxicity was performed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma). Experiments were performed in triplicate. Final concentrations of DMSO per well were always ≤1%.

Inflammatory assay

Inflammatory and anti-inflammatory assays were performed using activated THP-1 cells at a final density of $7.5 \times 10^4$ cells per well following published protocols [18]. Cells treated with 1% DMSO were considered as negative control (previous study showed DMSO suppresses the expression of many pro-inflammatory responses [19]), whereas 100 ng $mL^{-1}$ of LPS from *E. coli* (Sigma-Aldrich, St. Louis, MO) was used as a positive control, since

LPS stimulates immune responses by interacting with the membrane receptor CD14 to induce the generation of cytokines [20]. Experiments were carried out in triplicate and the final concentrations of DMSO per well were always ≤ 1%. Fractions were tested at a final volume of 100 μL, which was selected based on the survival of the cell in the cytotoxic experiments.

Statistical analysis

Statistical software Prism 8.2.1 (GraphPad Software, Inc.) was utilized to perform analysis of variance (ANOVA) and the mean differences were calculated using Tukey's multiple comparison test ($\alpha = 0.05$).

**Results and discussion**

Antimicrobial activities of CFS *Lb. plantarum* NRRL B-4496

The CFSs of *Lb. plantarum* NRRL B-4496 with pH neutralized or not, was tested against selected pathogenic bacteria and fungi (Table 1). Results showed that the non-neutralized CFS were more effective against methicillin-resistant *S. aureus, S. aureus*, and *L. monocytogenes,* followed by *E. coli* (Table 1) probably due to lactic acid antimicrobial activity. The molds and yeasts tested in this study (clinical pathogens) showed to be resistant to the CFSs. Interestingly, other studies have found a direct correlation between the antifungal activity and the lactic acid concentration. However, the microorganisms responded in a different way to antimicrobial compounds such as CFS due to their nature (wild type or collection strain), antimicrobial resistance, matrix, environmental conditions, among others. For example, the activity of CFSs from eighty-eight different *Lb. plantarum* strains isolated from different food matrices showed that lactic acid concentrations of 5 g $L^{-1}$ to 25 g $L^{-1}$, (which this variability depends on the incubation time of *Lb. plantarum*) were able to control the growth of several strains from the Spanish Type Culture Collection (CECT, Paterna, Spain): *Aspergillus niger* (CECT 2805), *A. flavus* (CECT 20802), *Fusarium culmorum* (CECT 2148), *Penicillium roqueforti, P. expansum* (CECT 2278), and *P. chrysogenum* (CECT 2669), and *Cladosporium* spp. (UFG 163, isolated from an oat based matrix). The researchers observed that higher production of lactic acid in the CFS (after 24 h of incubation) improved antifungal activity [21].

In our study, the neutralized CFS showing anti-*Listeria* activity appear to be related to the presence of peptides or proteins since the activity is abolished after treatment with proteinase K (Table 1). The antimicrobial activity detected for *Listeria* in CFS (neutralized or not) is lost when treated with the enzyme that hydrolyzed antimicrobial peptides or proteins.

**Table 1. Antimicrobial and antifungal activities of the CFS of *Lb. plantarum* NRRL B-4496 expressed as the minimum volume for inhibition (μg $mL^{-1}$).**

| Microorganism | | Treatment | | |
|---|---|---|---|---|
| | | Native pH | Neutralized pH | Proteinase K |
| Bacteria ($1x10^7$ CFU $mL^{-1}$) | Methicillin-resistant *Staphylococcus aureus* ATCC 700698 | 2 | R | R |
| | *Staphylococcus aureus* ATCC 25923 | 2 | R | R |
| | *Escherichia coli* ATCC 25922 | 5 | R | R |
| | *Listeria monocytogenes* Scott A | 2 | 50 | R |
| | *Acinetobacter baumannii* ATCC BAA-747 | R | R | R |
| | *Pseudomonas aeruginosa* ATCC 14210 | R | R | R |
| Fungi ($1x10^6$ spores $mL^{-1}$) | *Candida albicans* ATCC 10231 | R | R | R |
| | *Cryptococcus neoformans* var. *grubii* | R | R | R |
| | *Trichophyton rubrum* ATCC 18758 | R | R | R |
| | *Aspergillus fumigatus* ATCC 1022 | R | R | R |

R= maximal concentration tested 200 μl

Experiments were performed in triplicate.

In order to isolate the fractions responsible for the antibacterial activity, the CFS of the culture was subjected to fractionation through size exclusion chromatography. A total of fourteen fractions of 1 mL each were collected.

After testing all the fourteen fractions (upon pH neutralization), only the CFS fractions 2, 3, 5, and 6 showed antimicrobial activity against *L. monocytogenes* (Table 2) with a minimum volume of 50 μL. The rest of the CFS fractions showed no antibacterial activity against the tested bacteria.

**Table 2. Antimicrobial activity ($1x10^7$ CFU $mL^{-1}$) of the CFS fractions of *Lb. plantarum* NRRL B-4496 expressed as the minimum volume for inhibition (µg $ml^{-1}$)**

| Fraction | *Listeria monocytogenes* | *Escherichia coli* | *Salmonella* Typhimurium | Methicillin-resistant *Staphylococcus aureus* | *Staphylococcus aureus* |
|---|---|---|---|---|---|
| 1 | + | + | + | + | + |
| 2 | - | + | + | + | + |
| 3 | - | + | + | + | + |
| 4 | + | + | + | + | + |
| 5 | - | + | + | + | + |
| 6 | - | + | + | + | + |
| 7 | + | + | + | + | + |
| 8 | + | + | + | + | + |
| 9 | + | + | + | + | + |
| 10 | + | + | + | + | + |
| 11 | + | + | + | + | + |
| 12 | + | + | + | + | + |
| 13 | + | + | + | + | + |
| 14 | + | + | + | + | + |

Experiments were performed in triplicate.

- No microbial growth observed; + Microbial growth observed

In the literature, studies have reported that AMPs produced by LABs inhibited the growth of *L. monocytogenes.* For example, the bacteriocin LiN333 produced by *Lb. casei* isolated from Jianshui Cai (Chinese fermented food), inhibited the growth of *L. monocytogenes* at pH <10 [22], whereas plantaricin ZJ316 produced by *Lb. plantarum* ZJ316 showed antibacterial activities not only for *Listeria* spp., but also to a panel of Gram-negative and –positive bacteria [23]. Also, it is been reported that most of the bacteriocins studied are stable in acidic conditions but decreasing their activity when the medium is either in neutral or alkaline conditions [24]. In our study, we report that the activity of the protein-containing fractions was active upon neutralization of the pH.

The protein concentrations of the fractions were 157, 915, 328, and 196 μg $mL^{-1}$ in fraction 2 (>100 kDa), 3 (~45 kDa), 5 (30–45 kDa), and 6 (15 kDa), respectively (Fig. 1). The rest of the collected fractions showed no presence of proteins.

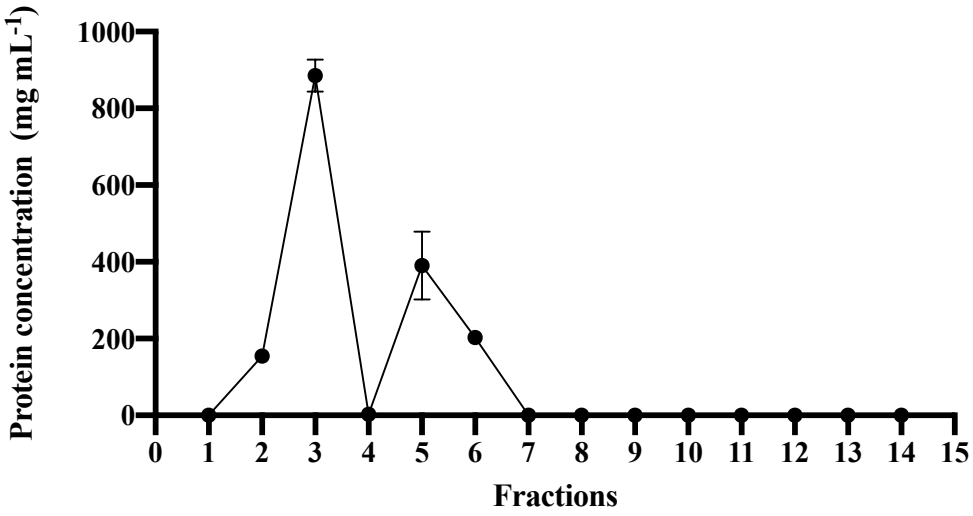


**Fig. 1**. Protein concentration in CFS fractions of *Lb. plantarum* NRRL B-4496. The protein concentration of CFS fractions obtained by exclusion chromatography were measured using the Bradford reagent. Protein concentrations were calculated according to a calibration curve of BSA.

Other studies have reported that the antimicrobial peptide plantaricin ZJ316 had a molecular mass of 2.3 kDa, whereas pediocin LB-B1 (molecular masses of 2.5 and 6.5 kDa) produced by *Lb. plantarum* LB-B1 showed an effective bactericidal activity against *L. monocytogenes*

54,002 [25]. Tsai et al. [26] studied the antimicrobial peptide m2163 and m2386 isolated from *Lb. casei* ATCC 334, which showed the antiproliferative activity on the human colorectal cancer cell line SW480.

Other works have reported that certain AMPs showed antibacterial activity against antimicrobial resistant bacteria. For example, although the methicillin resistant *S. aureus* Oxford was reported to be resistant to vancomycin, it was sensitive to nisin [27], whereas lacticin 3147 showed activity against twenty strains of vancomycin-resistant enterococci isolated from patients and obtained from the Antibiotic Resistance Monitoring and Reference Laboratory, Health Protection Agency (HPA), Colindale, UK), with minimal inhibitory concentration values between 1.9 and 7.7 mg $L^{-1}$ [28].

Cytotoxicity and inflammatory activities of CFS fractions

The cytotoxic activity was assayed on the human macrophage cell line THP-1. Results showed that all of the fractions that showed anti-*Listeria* activity were nontoxic to THP-1 cells (Fig. 2), suggesting a potential use in food preservation. Human-derived macrophages are a known model to assess cytotoxicity and inflammatory response upon exposure of the cells to different types of compounds. Macrophages are among the first cells to arrive when an injury or exposure to compounds occurs [18]. Another study reported that an exopolysaccharide isolated from the probiotic *Lb. plantarum* RJF4 showed no toxicity to the rat myoblast L6 cell line and have been proposed to use this polysaccharide as a food additive or for therapeutical applications [29]. Sentürk, Ercan and Yalcin [30] reported an $IC_{50}$ value of the secondary metabolite produced by *Lb. plantarum*, isolated from animal sources, in the MCF-7 cell line of 0.0011 mg $mL^{-1}$.

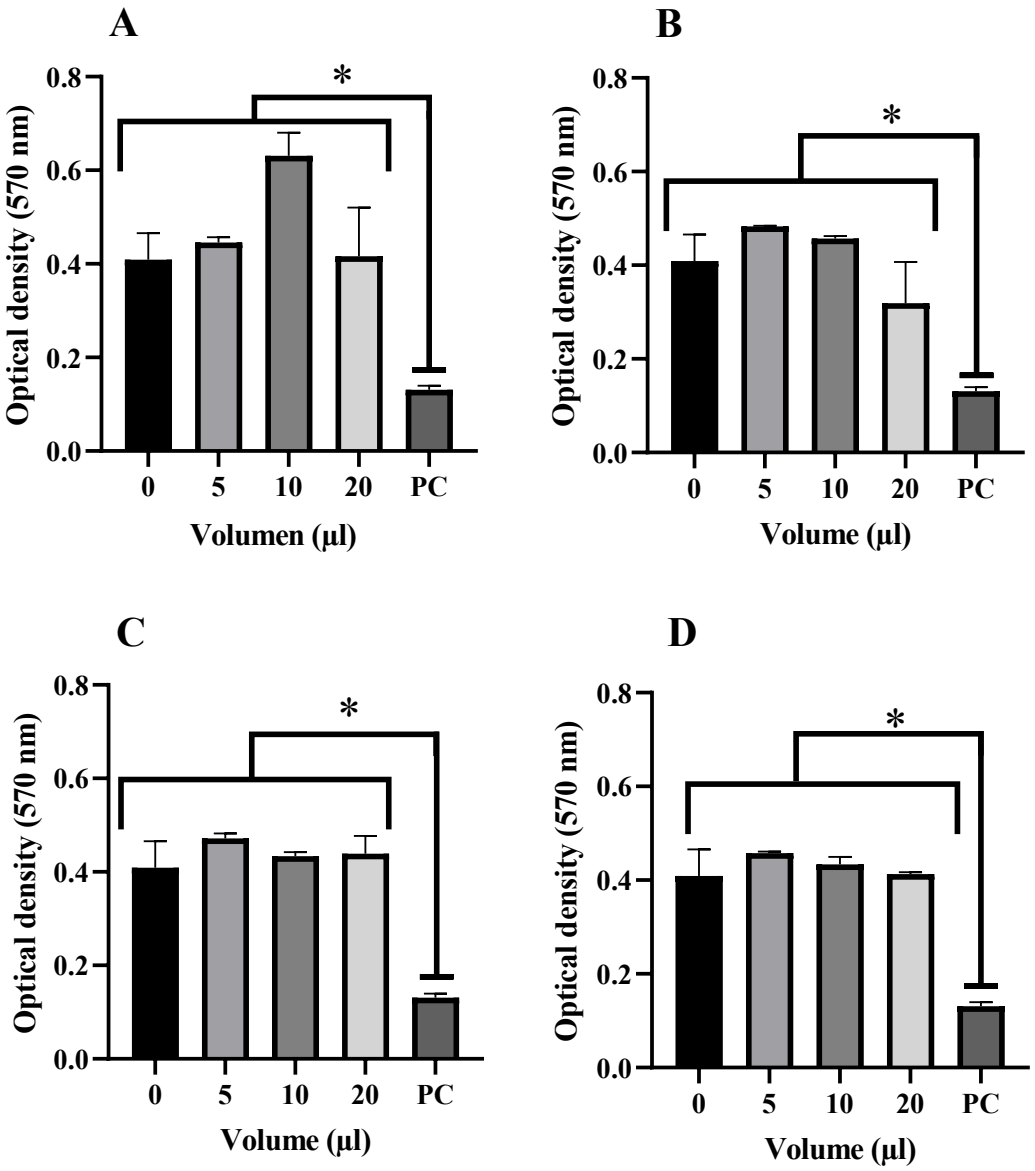


**Fig. 2.** Cytotoxicity of CFS *Lb. plantarum* NRRL B-4496 fractions. The cytotoxicity of the (A) Fraction 2, (B) Fraction 3, (C) Fraction 5, and (D) Fraction 6; were assessed on human-derived macrophage THP-1 cell line using the MTT assay. PC = positive control. The shown mean ± S.D. is from three independent experiments. Same letters are not significant different ($p<0.05$) according to Tukey's test.

THP-1 cells are responsible for eliciting an inflammatory or anti-inflammatory response mediated by the secretion of cytokines [18]. In the case of the inflammatory activity, fractions

were not able to elicit a pro-inflammatory response, as the levels of the pro-inflammatory cytokines IL-6 and TNF-α were not significantly different from the untreated control (negative control) (Fig. 3a and c). No anti-inflammatory activity was measured as the levels of IL-10 remained comparable to the untreated cells (Fig. 3b).

Other studies have reported anti-inflammatory activity of antimicrobial peptides. For example, the effect of the cationic antimicrobial peptide CEMA (cecropin-melittin hybrid) in macrophages treated with lipopolysaccharide (LPS, inflammation inducer) was assessed. Results showed a selectively blockage of the genes induced by LPS, suggesting that the peptide has an anti-inflammatory activity [31]. Moreover, studies on the effect of nisin Z (a lantibiotic peptide) showed a modulation of the host inflammatory responses against *S. aureus* (ATCC 25293), *Salmonella enterica* sv. Typhimurium (SL1344), and *E. coli* (Xen-14, Caliper Life Sciences, Hopkinton, MA, USA) using murine challenge models [32]. Lastly, peptides isolated from fermented milk inoculated with *Lb. plantarum* strains showed anti- inflammatory activity using the albumin inhibition denaturation test in vitro [33].

In another perspective, different strains of *Lb. plantarum* as a potential probiotic have been studied [34]. The authors observed that *Lb. plantarum* Ln4 from kimchi survive at pH 2.5 in the presence of 0.3% pepsin and 0.3% oxgall, showed intestinal cell adhesion to HT-29 cells and, did not produce harmful enzymes, as β-glucuronidase. As well, it has been reported that the daily intake of a capsule with *Lb. plantarum* (CECT 7527, CECT 7528, and CECT 7529, $1.2 \times 10^9$ CFU) after 12 weeks, lowered plasma total cholesterol, LDL-cholesterol (LDL-C), and oxidized LDL-C (17.4, 17.6 and 15.6%, respectively; compared with the placebo group) in participants with hypercholesterolemia [35].

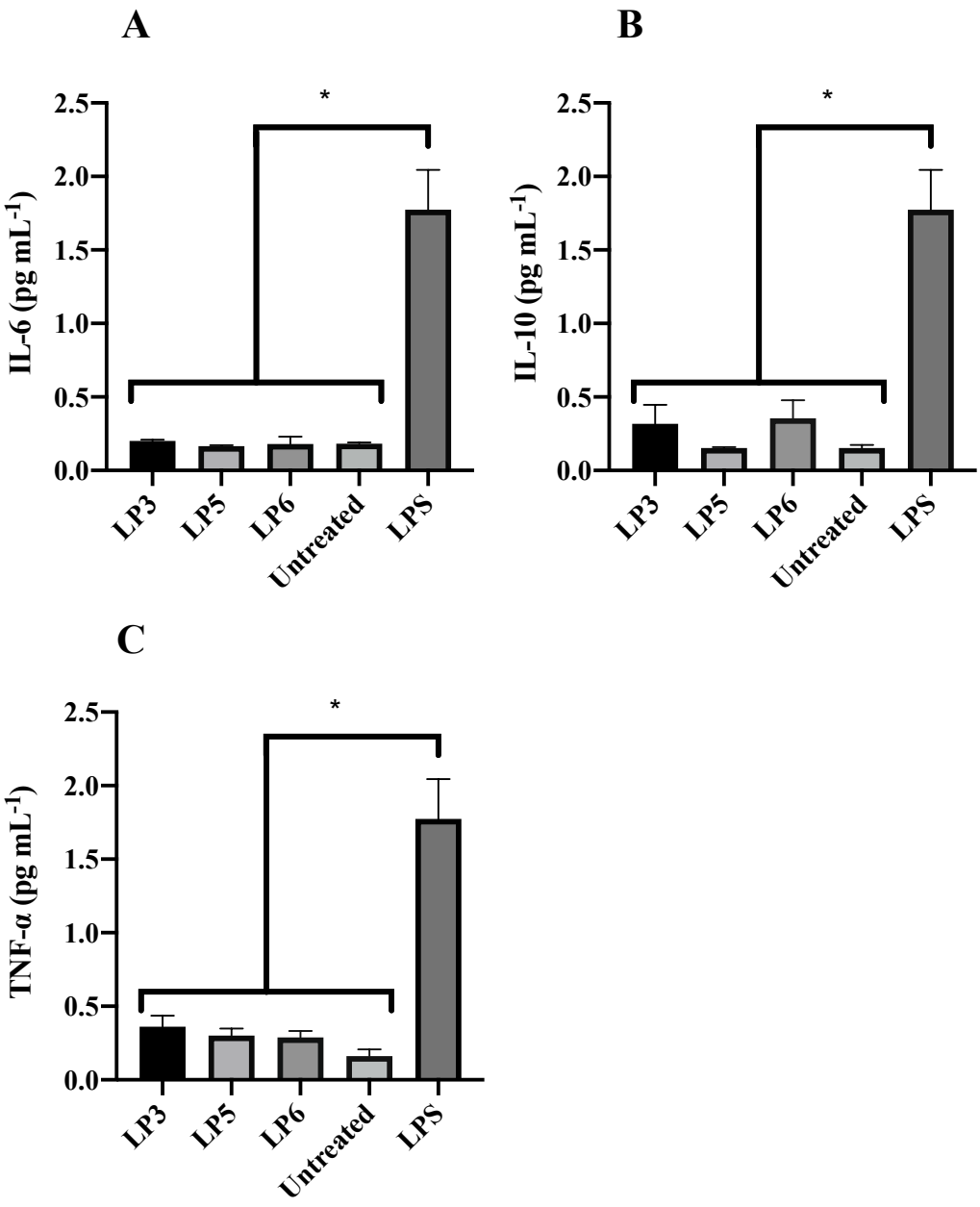


**Fig. 3.** Immunological response of CFS *Lb. plantarum* NRRL B-4496 fractions. The immunological response of the CFS fractions 3, 5 and 6 were assessed on human-derived macrophage THP-1 cell line using ELISA for (A) IL-6, (B) IL-10, and (C) TNF-α. LPS = lipopolysaccharide (positive control). The shown mean ± S.D. is from three independent experiments. Same letters are not significant different ($p<0.05$) according to Tukey's test.

In conclusion, fractions containing proteins or peptides obtained upon fractionation of the crude extract of *Lb. plantarum* NRRL B-4496 inhibited the growth of *L. monocytogenes*.

However, the fractions showing no protein levels did not show any antibacterial activity to any of the tested pathogenic microorganisms. The obtained results contribute to the development of a novel bio-preservative from *Lb. plantarum* CFS and its fractions, which constitute an important area of applications in the food industry. Some of the factors that should be considered in future studies are specificity, manufacturer cost, legislation governing their use, and a guideline for rational design.

**Acknowledgments**

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**Author contribution**

D. Arrioja-Bretón performed research, analyzed data and wrote the paper, E. Mani-López contributed to analyze data and write the paper, H. Bach conceived the study and analyzed data, and A. López-Malo analyzed data and contributed to write the paper. All authors read and approved the final manuscript.

**Compliance with ethical standards**

Conflict of interest No conflict of interest declared.

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**RESEARCH PAPER III**

**"Antimicrobial activity of *Lactobacillus casei* 21/1 cell-free supernatants on fresh Mexican-style cheese"**

Submitted to the Journal of Food Science and Technology

**Antimicrobial activity of *Lactobacillus casei* 21/1 cell-free supernatants on fresh Mexican-style cheese**

D. Arrioja-Bretón, E. Mani-López, E. Palou, and A. López-Malo

**Abstract**

Ensuring the safety of foods such as Mexican-style fresh cheese represents a challenge for the food industry, so various alternatives are being investigated. In the present work, the effect of cell free-supernatants (CFS) of *Lb. casei* were studied against four indicator microorganisms: *Escherichia coli, Salmonella* Typhimurium, *Staphylococcus aureus,* and *Listeria monocytogenes* on inoculated fresh Mexican-style cheese during seven days of storage at 4 °C ± 1.0 °C. Microbial counts for each inoculated microorganism were determined at day zero, four, and seven. *S.* Typhimurium was observed to be the most sensitive microorganism decreasing 1.29±0.05 $\log_{10}$ CFU/g cycles after four days while 1.60±0.04 $\log_{10}$ CFU/g cycles were reduced after seven days of storage; on the other hand, *E. coli* was the most resistant reducing 0.24±0.01 and 0.63±0.04 $\log_{10}$ CFU/g cycles after four and seven days of storage, respectively. Studied CFS are a good alternative as bio-preservative in foods such as fresh cheeses.

**Key words:** bio-preservation, antibacterial activity, cell-free supernatant, cheese

## Introduction

Most of traditional Mexican fresh cheeses are of artisanal production from whole or low-fat cow's milk through casein coagulation with rennet, frequently without a thermal treatment (Moreno-Enriquez et al. 2007); their processing is not commonly standardized, producers handle low production volumes and often utilize unpasteurized milk (de Gante et al. 2016), which represent a health risk (Verraes et al. 2015*).* In the production of fresh Mexican-style cheese, no bacteria are inoculated because is not a fermented product; due its short self-life is usually consumed shortly after it is made (de Gante et al. 2016).

The presence of pathogenic microorganisms in this type of cheese may be due to the use of raw milk or to the manipulation of the cheese during the elaboration process (Kousta et al. 2010); it has been observed the relationship of its consumption with several outbreaks, due to the presence of pathogenic microorganisms such as: *Salmonella* (Villar et al. 1999; Pastore 2008), *Listeria monocytogenes* (MacDonald et al. 2005; Cabedo et al. 2008; Jackson et al. 2018), *Escherichia coli* (Honish et al. 2005; Espié et al. 2006), and *Staphylococcus aureus* (Pereira et al. 1996; Araújo et al. 2002; Akineden et al. 2008).

Lactic acid bacteria (LAB) are generally regarded as safe (GRAS) and qualified presumption of safety (QPS) (Alvarez-Sieiro et al. 2016), many of them have been studied as a bio-preservatives due the secretion of secondary metabolites during their growth on different food products: such as beef (Rhoades et al. 2013), chicken (Alvarado and McKee, 2007), shrimp (Li et al. 2019), and vegetables (Lee et al. 2016), among many others.

Ensuring the safety of cheeses while maintaining their organoleptic characteristics is a challenge for the food industry. LAB cell-free supernatants (CFS) can be an alternative since their secondary metabolites are known to possess antimicrobial activity (Moradi et al. 2019). Thus, the aim of this study was to evaluate the antimicrobial activity of *Lactobacillus casei* 21/1 CFS against four indicator microorganisms: *Escherichia coli*, *Salmonella* Typhimurium, *Staphylococcus aureus*, and *Listeria monocytogenes* on inoculated Mexican-style fresh cheese.

## Materials and methods

### *Bacterial strains, culture, and growth conditions*

*Lactobacillus casei* 21/1 as well as tested indicator strains (*E. coli* ATCC 25922, *S.* Typhimurium ATCC 14028, *Staph. aureus* ATCC 29213, and *L. monocytogenes* Scott A) were obtained from the Food Microbiology Laboratory of the Universidad de las Americas Puebla (Puebla, Mexico). *Lb. casei* 21/1 was re-activated in de Man, Rogosa and Sharpe (MRS) broth (DifcoTM BD, Sparks, Maryland). Indicator strains were reactivated in trypticase soy broth (TSB, Bioxon BD, Mexico). Both groups of bacteria were incubated at 35±1.0 °C, during 48 h for *Lb. casei* 21/1 or 24 h for the indicator strains.

*Preparation of cell-free supernatant (CFS)*

40 mL of CFS *Lb. casei* 21/1 grown in MRS broth and incubated at 35±1.0 °C during 48 h were collected by centrifuging at 15,000 rpm during 15 min (Thermo Scientific Heraeus Megafuge™ 8, DJB Labcare Limited, UK), filter sterilized through a 0.45 μm Millipore membrane filter, and then the CFS volume was concentrated 10- fold by vacuum evaporation on a Buchi R-210/215 rotary evaporator (Buchi, Flawil, Switzerland) at 70±1.0 °C and 25 cm Hg, as described by Arrioja-Bretón et al. (2020). Concentrated supernatants were evaluated on the same day. Uninoculated MRS was used as a negative control.

*Cheese production*

Mexican-style fresh cheese was scaled manufactured with commercial pasteurized (Lala™) whole milk (fat: 3.3%, protein: 3.1%, carbohydrates: 4.7%, 5 g of vitamin D/L, and 666 mg retinol equivalents/L). The milk was heated until reaching a temperature of 39±1.0 °C, then 0.02% of 50% calcium chloride (Reactivos Química Meyer, México) and 0.03% of commercial chymosin (Cuamex®, Chr. Hansen de México SA de CV, Mexico) per liter of milk were added and stirred. Then, milk was left to rest during 20 min until it curdled, the curd was cut into cubes of approximately 2 $cm^3$ and left to rest for other 20 min. Cubes were subsequently placed on a mesh and squeezed to remove the serum. To the resulting paste, 1% salt was added to the total cheese mass. Finally, cheese mass was molded in a circular mold and pressed at 4 °C±1.0 °C for 24 h.

*Physicochemical Analyses*

For characterization of manufactured Mexican-style fresh cheese, AOAC (1995) methods were taken as a reference to determine moisture (method 33.7.03), fat (method 933.05), ash (method 33.7.07), and protein (method 33.7.12 by the Kjeldahl method using a conversion factor of 6.38).

*Cheese inoculation and microbial counts on cheese*

Each side of a piece of the elaborated Mexican-style fresh cheese (~115 g) was inoculated with 500 µL (~$10^5$ CFU/side) of each indicator microorganism (*E. coli*, *Staph. aureus*, *S.* Typhimurium, or *L. monocytogenes*) and drained for 20 min.

*CFS Cheese immersion*

One piece of Mexican-style fresh cheese was inoculated with each indicator microorganism, subsequently cheese pieces were immersed in individual containers with 8 mL of CFS from *Lb. casei* and on the upper side 3 mL of the same CFS were added. Cheese pieces were kept submerged for a period of 20 min. Subsequently, each cheese piece was put in a plastic bag (Whirl-Pak1, Nasco, Fort Atkinson, WI, USA) and stored at 4.0±1.0 °C during the storage period (7 days). After that, microbial counts were performed. Non-inoculated cheese pieces were immersed and stored in the same way.

*Microbial counts on fresh Mexican-style cheese*

Microbial counts were performed for each indicator microorganism on day 0, 4, and 7; one slice (~10 g) of each inoculated piece was put in a sterile plastic bag (Whirl-Pak1, Nasco, Fort Atkinson, WI, USA), and homogenized for 2 min in a Stomacher 80 lab blender (Seward Ltd., West Sussex, England) with 90 mL of sterile peptone water. Adequate decimal dilutions were prepared into peptone water and 100 µL were spread on Baird-Parker agar for *Staph. aureus,* XLD agar for *Salmonella,* Oxford agar for *L. monocytogenes,* or MacConkey agar for *E. coli*. Plates were incubated during 18-24 h at 37±1.0 °C. In addition, to verify the sanitary quality of studied cheese, counts of total mesophilic aerobic bacteria were completed using standard methods agar (Bioxon, BD, Edo. de Mexico, Mexico); inoculated plates were incubated for 24 h at 37±1.0 °C. For total coliforms counts, violet red bile agar (Difco BD, Sparks, MD) was utilized and plates were incubated at 37±1.0 °C during 24 h. As control of the Mexican-style fresh cheese production, previously mentioned microbiological contents were also reported for an uninoculated cheese.

**Results and discussion**

Counts (Table 1) for the indicator microorganisms *E. coli, S.* Typhimurium*, Staph. aureus*, and *L. monocytogenes* on the uninoculated Mexican-style fresh cheese were under detection level (< 100 CFU/g), while initial counts of total mesophilic aerobic bacteria were 2.54±0.09 $\log_{10}$ CFU/g and total coliforms were 2.59±0.16 $\log_{10}$ CFU/g indicating that these bacterial counts were acceptable for the aim of the study. Composition of the manufactured Mexican-style fresh cheese was: moisture 63.42±1.77%, protein 11.48±1.49%, fat 5.40±0.13%, ash 1.85±0.03%, and 17.85% carbohydrates (by difference), while cheese yield was approximately 10%.

Furthermore, microbiological analyses of commercially available Mexican-style fresh cheese samples obtained from local markets (Table 1) indicate that these cheeses, although they were for sale, are not suitable since their consumption represents a risk to consumer health due to the presence of high bacterial counts as well as of pathogenic microorganisms, including *L. monocytogenes.* The Mexican official standard NOM-243-SSA1-2010 (Norma Oficial Mexicana 2010) establishes a maximum allowed level for total coliform bacteria in milk derivatives of 2 $\log_{10}$ CFU/g or mL, aerobic mesophilic 5 $\log_{10}$ CFU/g or mL, and in the case of *Staph. aureus* 2 $\log_{10}$ CFU/g or mL; microbial counts of these commercially available Mexican-style fresh cheeses were above the established maximum limits and can be associated with low hygiene in their food production and/or handling processes.

**Table 1. Microbial counts ($log_{10}$ CFU/g) of selected fresh Mexican-style fresh cheeses.**

| Microorganism | Cheese | | |
|---|---|---|---|
| | A | B | C |
| *E. coli* | 7.05 ± 0.16 | 6.13 ± 0.10 | - |
| *L. monocytogenes* | 5.11 ± 0.10 | 5.27 ± 0.10 | - |
| *Staph. aureus* | 6.64 ± 0.03 | 7.80 ± 0.01 | - |
| Aerobic mesophilic bacteria | 7.85 ± 0.05 | 3.70 ± 0.01 | 2.54 ± 0.09 |
| Coliforms | 7.23 ± 0.46 | 6.18 ± 0.04 | 2.59 ± 0.16 |

-: Under the level of detection.

A: Cheese sample 1 from a local market.

B: Cheese sample 2 from a local market.

C: Uninoculated studied Mexican-style fresh cheese manufactured in our laboratory.

In a previous report, Arrioja-Bretón et al. (2020) reported that during an *in vitro* assay, the antimicrobial activity of studied *Lb. casei* CFS against the four tested indicator microorganisms was significantly different ($p<0.05$); being *L. monocytogenes* the most sensitive microorganism with diameter inhibition zones higher than 22 mm, followed by *S.* Typhimurium while the most resistant tested microorganisms were *Staph. aureus* and *E. coli* with lower inhibition halos. Results of *in vivo* antimicrobial activity (Fig. 1) are in contrast to those previously reported for *in vitro* assays since in our case *S.* Typhimurium was the most sensitive microorganism, followed by *L. monocytogenes* while *E. coli* followed by *Staph. aureus* were the most resistant to *Lb. casei* CFS activity on the studied Mexican-style fresh cheese. Rolim et al. (2015) observed in semi-hard goat cheese major inhibition rates from *L. rhamnosus* against *Staph. aureus* (21.66%) and *L. monocytogenes* (10.23%) after 21 days of storage at 4 °C while an inhibition rate of 5.52% for *S.* Enteritidis. Settanni et al. (2011) controlled the growth of *L. monocytogenes, Salmonella* spp., and coliforms using *Lactobacillus paracasei* NdP78 or *Streptococcus macedonicus* NdP1; they managed to reduce 1.79 and 2.22 $log_{10}$ CFU/g cycles coliforms and *Staph. aureus,* respectively while *Salmonella* and *L. monocytogenes* were not detected.

As you can observe in Fig. 1, *S.* Typhimurium initial counts were 5.83±0.01 $\log_{10}$ CFU/g for inoculated Mexican-style fresh cheese and decreased 1.29±0.05 $\log_{10}$ CFU/g cycles after four days and 1.60±0.04 $\log_{10}$ CFU/g cycles after seven days of storage at 4 °C. In the case of *L. monocytogenes*, the initial counts in the studied cheese were 5.63±0.01 $\log_{10}$ CFU/g, and a reduction of 0.78±0.01 $\log_{10}$ CFU/g cycles was observed after four days while 1.43±0.04 $\log_{10}$ CFU/g cycles were reduced after seven days stored at 4 °C. Dal Bello et al. (2012) successfully controlled the growth of *Listeria monocytogenes* using bacteriocins produced by *Lactococcus lactis* during manufacture of cottage cheese. Kousta, Mataragas, Skandamis, & Drosinos (2010) studied the effect of adding ferulic acid or nisin (4 mg/g) on fresh cheese to inhibit *L. monocytogenes* growth, they observed the reduction of 2.0 or 1.5 logarithmic cycles after 21 days of storage, respectively. In our case, initial counts of *Staph. aureus* were 5.75±0.02 $\log_{10}$ CFU/g and decreased 0.96±0.18 and 1.28±0.03 $\log_{10}$ CFU/g cycles after four and seven days stored at 4 °C, respectively (Fig. 1) while *E. coli* was the most resistant of tested microorganisms to *Lb. casei* CFS, initial counts were 5.57±0.02 $\log_{10}$ CFU/g only decreasing 0.24±0.01 and 0.63±0.04 $\log_{10}$ CFU/g cycles after four and seven days stored at 4 °C, respectively.

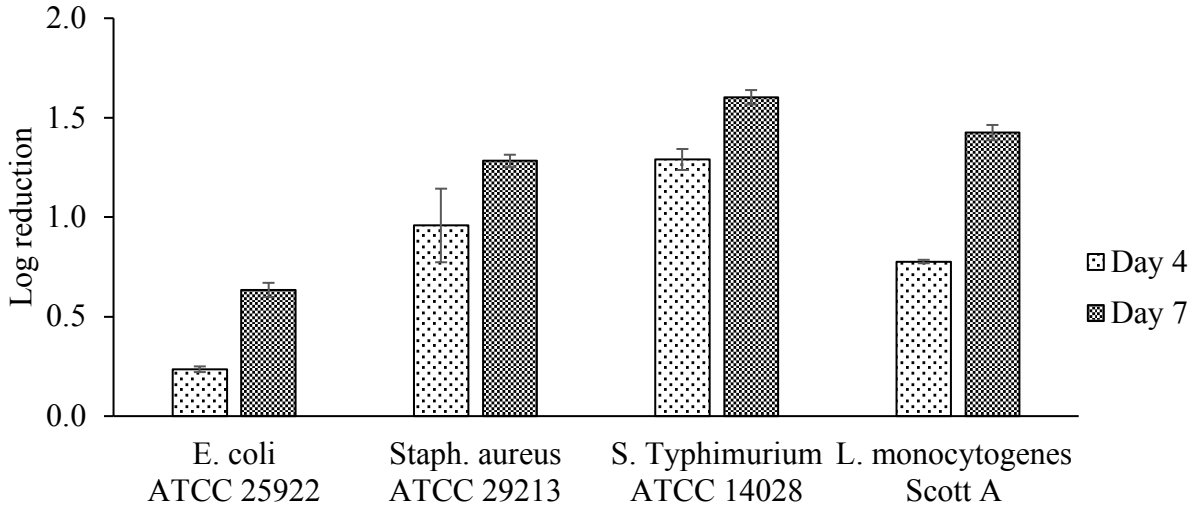


**Fig. 1.** Logarithmic reductions of tested indicator microorganisms (*E. coli* ATCC 25922, *Staph. aureus* ATCC 29213, *S.* Typhimurium ATCC 14028, and *L. monocytogenes* Scott A) in studied Mexican-style fresh cheese at day four or seven stored at 4 °C

## Conclusions

The studied cell-free supernatants from *Lb. casei* are effective against microorganisms causing foodborne illness such *as E. coli, Staph. aureus, S. Typhimurium,* and *L. monocytogenes* thus can be a good alternative as a bio-preservative in foods with risk of contamination such as Mexican-style fresh cheeses. Several other aspects that are necessary to further study include the way in which these supernatants will be incorporated into the cheeses and their contact time, as well as possible changes in the organoleptic characteristics of treated cheeses.

## Acknowledgements

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## GENERAL CONCLUSIONS

It can be concluded that the supernatants of the lactic acid bacteria evaluated so far show inhibition against the indicator bacteria, with the supernatants with the highest inhibition being those of the *Lactobacillus plantarum, Lactobacillus sakei*, *Lactobacillus rhamnosus*, and *Lactobacillus casei* strains.

The observed antimicrobial activity of CFS in this study is mainly due to the presence of organic acids, and in the case of *Lb. plantarum* NRRL B-4496, a possible bacteriocin-like compound. An important finding is that the studied CFS can be produced and stored at room temperature (23 ± 2 °C) for relatively long periods, with a low decrease in its antimicrobial activity over time. The marinade with *Lb. plantarum* NRRL B-4496 CFS was efficient in reducing the microbial load for meat inoculated with the four bacterial strains tested. In addition, the sensory evaluation for CFS marinated meat obtained acceptable scores (more than 6) for all attributes evaluated. As shown in this study, CFS marinade has potential use as a bio-preservative in beef, which can improve the safety and quality of meat products.

Fractions containing proteins or peptides obtained by fractionating the crude extract of *Lb. plantarum* NRRL B-4496 inhibited the growth of *L. monocytogenes*. However, fractions that do not show protein levels did not show any antibacterial activity to any of the pathogenic microorganisms tested. In the direct application of the CFS of *Lb. casei* to fresh Mexican-style cheese a reduction of the microbial load was observed during storage in refrigeration. The most sensitive microorganism tested was *S.* Typhimurium. The use and application of *Lb. casei* and *Lb. plantarum* CFS and its fractions, as bio-preservatives, constitute an important area of applications in the food industry. Some of the factors that should be considered in future studies are the specificity, the cost of the manufacturer, the legislation that governs its use and a guide for rational design.

## GENERAL RECOMMENDATIONS

- Test different methods of paraprobotic production and assess whether the presence of antimicrobial activity depends on this.
- Inoculate lactic acid bacteria in different liquid media and evaluate/compare production of secondary metabolites.
- Characterize the secondary metabolites present in CFS produced by LAB showing antimicrobial activity.
- Evaluate the effectiveness of the cell-free supernatants marination as bio-preservatives in meet products against other microbial strains.
- Optimize cell-free supernatant marinade formulation for better consumer acceptability.
- Design the packaging of the marinated meat product with cell-free supernatants.
- Apply cell-free supernatants in different food products as possible antimicrobials.
- Evaluate the effectiveness of peptides with antimicrobial activity produced by LAB, in different food products.

# ANNEXES

## ANNEX I. Beef sensory evaluation

### Methodology

Sensory evaluation was carried out with non-inoculated marinated meat (described on page 47). The hedonic preference test was performed by 26 untrained panelists, in which a score of 1 represents the attributes most unacceptable and a score of 9 represents the attributes most acceptable. Scores of 6 were considered acceptable. Panelists were asked to evaluate grilled beef for taste, odor, color, texture, and overall acceptability. Panelists also evaluated raw beef pieces for odor, color, and overall acceptability, with the same hedonic scale. Marinated beef pieces were grilled for 1 min each side in a 100 °C pre-heated pan (previous tests demonstrated that this was sufficient for the meat to reach 70 °C in the center). The evaluations were carried out fifteen minutes after the meat was grilled.

Statistical software Minitab (v.17, LEAD Technologies Inc., USA) was used to perform an analysis of variance Tukey's and Fisher test with a 95% level of confidence.

### Results and Discussion

The panel of judges liked the meats in both types of marinade, either grilled or raw; the scores for every evaluated attribute were over 6 (Table 1). The grilled beef with the two types of marinade had the same acceptability ($p > 0.05$) for taste, odor, texture, and overall acceptability. Color of grilled meat was significantly different ($p < 0.05$) between meat marinated with the standard marinade and the CFS marinade; the CFS marinated meat had a higher average score for color ($8.1 \pm 0.8$). For the marinated raw beef, color, texture, and overall acceptability were significantly different ($p < 0.05$) between meat marinated with the two types of marinades; the standard marinade received higher scores, and only odor was not significant different ($p > 0.05$). Panelists commented that beef with the CFS marinade had an

acidic taste. This could be attributed to the presence of acetic/lactic acid in the *Lb. plantarum* CFS (pH 3.8). Mozuriene et al. (2016) observed that natural marinades based on selected LAB increased the overall acceptability scores of pork, and that the overall acceptability scores depend on the LAB used in the marinade. Nevertheless, the different sensory evaluations can be attributed to chemical features of the specific strains and how they combine with food matrices.

**Table 1. Sensory scores of beef CFS marinated or standard marinated, grilled or raw.**

| Attribute | CFS marinade | Standard marinade |
|---|---|---|
| **Grilled beef:** | | |
| Flavor | 6.9 ± 1.9 [a] | 6.8 ± 1.2 [a] |
| Odor | 7.4 ± 1.3 [a] | 7.2 ± 1.4 [a] |
| Color | 8.1 ± 0.8 [a] | 6.9 ± 1.1[b] |
| Texture | 7.5 ± 1.4 [a] | 7.5 ± 1.1[a] |
| Overall acceptability | 7.2 ± 1.6 [a] | 7.3 ± 1.1[a] |
| **Raw beef:** | | |
| Flavor | ---- | ---- |
| Odor | 6.4 ± 1.6 [a] | 7.0 ± 1.4 [a] |
| Color | 6.0 ± 1.9 [a] | 7.9 ± 1.2 [b] |
| Texture | 6.6 ± 1.9 [a] | 7.7 ± 1.1 [b] |
| Overall acceptability | 6.4 ± 1.8 [a] | 7.7 ± 1.1 [b] |

Responses values represent means ± standard deviations (26 subjects).

Means in the same row followed with the same letter are not significant different ($p < 0.05$) according to Fisher's test.

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**ANNEX II. Evidence publications**

Entorno UDLAP

No. 5

February 2018

Actividad antibacteriana

DE BACTERIAS ÁCIDO

LÁCTICAS

Por: Daniela Arrioja-Bretón · Emma Mani-López · Enrique Palou · Aurelio López-Malo

Actualmente existe una tendencia en la preferencia de los consumidores por elegir alimentos frescos y menos procesados, por lo que ha surgido el interés en buscar conservadores diferentes a los sintéticos y asegurar la inocuidad de los alimentos. Con este propósito se han estudiado algunos bioconservadores, entre los que destacan los aceites esenciales, enzimas y microorganismos (Beristain-Bauza *et al.*, 2012, 2017; Mani-López *et al.*, 2017). Entre estos últimos se encuentran las bacterias ácido-lácticas (BAL), las cuales, además de producir sabores, olores, texturas y cambios nutricionales en los alimentos, también son conocidas por su capacidad de producir metabolitos secundarios tales como ácidos orgánicos, bacteriocinas, peróxido de hidrógeno, reuterina, diacetilo y alcohol etílico, entre otros. Varios estudios han demostrado que la aplicación directa de las BAL o de sus metabolitos inhiben bacterias indeseables en alimentos (Mani-López *et al.*, 2018).

El objetivo de este estudio fue evaluar la efectividad como antimicrobianos de diferentes cepas de BAL (sobrenadantes libres de células) contra *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium y *Listeria monocytogenes*. Se evaluó la actividad antimicrobiana mediante una prueba de difusión en pozos de los sobrenadantes de doce cepas de BAL.

## METODOLOGÍA

Las cepas de las BAL (*Lactococcus lactis* subsp. *lactis* NRRL B-633, *Lactococcus lactis* subsp. *cremoris* NRRL B-634, *Pediococcus acidilactici* NRRL B-1116, *Pediococcus pentosaceus* NRRL B-14009, *Leuconostoc mesenteroides* subsp. *mesenteroides* NRRL B-1118, *Lactobacillus sakei* NRRL B-1917, *Lactobacillus fermentum* NRRL B-1932, *Lactobacillus reuteri* NRRL B-14171, *Lactobacillus plantarum* NRRL B-4496, *Lactobacillus acidophilus* NRRL B-4495, *Lactobacilus casei* NRRL B-1922 y *Lactobacillus rhamnosus* NRRL B-442), así como las cepas de las bacterias indicadoras (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29413, *Salmonella* Typhimurium ATCC 14028 y *Listeria monocytogenes* Scott A) fueron obtenidas de la colección de cepas del laboratorio de Microbiología de Alimentos de la Universidad de las Américas Puebla, y se mantuvieron a 5°C en agar MRS y en agar soya tripticaseína, respectivamente.

Los cultivos fueron preparados inoculando las cepas de las BAL en 30 mL de caldo MRS y las bacterias indicadoras en 10 mL de caldo soya tripticaseína, se incubaron a 35°C durante 24 h. Para la obtención del sobrenadante de las BAL, los cultivos fueron centrifugados durante diez minutos a 10,000 rpm. Posteriormente se filtraron al vacío a través de una membrana HVLP de 0.45 µm, para después concentrar hasta reducir el volumen a la décima parte del original, en un rotavapor a una temperatura de 70°C.

La actividad antimicrobiana de los sobrenadantes de las BAL se evaluó a través de una prueba de difusión en pozos sobre placas de agar, la cual consiste en realizar una siembra por extensión de los microrganismos indicadores ($10^5$ UFC/g) en cajas Petri con agar soya trip-

Chapter

# Stability of the Antimicrobial Activity of *Lactobacillus plantarum* NRRL B-4496 Supernatants during Storage against *Staphylococcus aureus* ATCC 29413

*Daniela Arrioja Bretón, Emma Mani-López and Aurelio López-Malo*

## Abstract

*Staphylococcus aureus* is a pathogenic microorganism that causes gastrointestinal diseases due to the production of enterotoxins. The study of lactic acid bacteria such as *Lactobacillus plantarum* has generated interest due to its ability to generate secondary metabolites against pathogenic microorganisms. In this work, the stability of the antimicrobial activity of the *L. plantarum* supernatants was evaluated by means of a well diffusion test. The supernatants were stored at 25 ± 1.0°C for a period of 20 weeks. A significant difference ($P < 0.05$) was observed in the antimicrobial activity of *L. plantarum* supernatants between time 0 and after 20 weeks of storage, although the ability to inhibit *Staphylococcus aureus* was still observed during the storage time.

**Keywords:** lactic acid bacteria, storage, antimicrobial activity, stability

## 1. Introduction

*Staphylococcus aureus* is a spherical, nonsporulating, nonmotile bacterium, facultative aero-anaerobic, Gram-positive, and catalase positive. It belongs to the normal microbiota and is found on the skin and mucous of mammals and birds. This bacterium can be disseminated in the environment of its hosts and survives for long periods in these areas [1].

The determination of *S. aureus* in food products is done in order to establish its potential to cause food poisoning and demonstrate contamination after being processed. This microorganism produces enterotoxins formed in foods under broad conditions of pH, water activity, and redox potential [2].

On the other hand, the use of some of the genera of lactic acid bacteria (LAB), such as *Lactobacillus, Leuconostoc, Lactococcus*, as alternatives in the biopreservation of foods is due to their ability to produce secondary metabolites such as

**ANNEX III. Evidence of participation in congresses**

SEGURIDAD ALIMENTARIA 2017

CONGRESO INTERNACIONAL DE INOCUIDAD ALIMENTARIA
CONGRESO NACIONAL SOBRE SOSTENIBILIDAD ANTE EL DESPERDICIO DE ALIMENTOS

Otorgan la presente

# CONSTANCIA

a: *Arrioja-Bretón, D., López-Malo, A., Mani López, E.*

Por su PARTICIPACIÓN con el trabajo titulado "**Actividad antibacteriana de diversas cepas de bacterias ácido lácticas inactivadas y sus sobrenadantes**" en el Congreso de Seguridad Alimentaria 2017, llevado a cabo del 15 al 17 de Noviembre del 2017, Chihuahua, Chih.

Dr. Pedro Javier Martínez Ramos
**Director de la Facultad de Ciencias Químicas**

Dra. Guadalupe Virginia Nevárez Moorillón
**Presidente AMEPAL, A.C**

Dra. Juliana Morales Castro
**Coordinadora de la Red REDSA PDA**

**SEGURIDAD ALIMENTARIA 2017**

**CONGRESO INTERNACIONAL DE INOCUIDAD ALIMENTARIA**
**CONGRESO NACIONAL SOBRE SOSTENIBILIDAD ANTE EL DESPERDICIO DE ALIMENTOS**

Otorgan la presente

# C O N S T A N C I A

a: Daniela Arrioja Bretón

Por su participación como **ASISTENTE** en el
Congreso de Seguridad Alimentaria 2017, llevado a cabo
del 15 al 17 de Noviembre del 2017, Chihuahua, Chih.

Valor curricular 25 horas

Dr. Pedro Javier Martínez Ramos
**Director de la Facultad de Ciencias Químicas**

Dra. Guadalupe Virginia Nevárez Moorillón
**Presidente AMEPAL, A.C**

Dra. Juliana Morales Castro.
**Coordinadora de la Red REDSA PDA**

AMEPAL A.C.

Latin Food 2018

8th Food Science, Biotechnology & Safety Congress

MEXICAN ASSOCIATION OF FOOD SCIENCE

Food for Health 2018

AWARDS THE PRESENT CERTIFICATE TO:

Arrioja-Bretón, D., Mani-López, E., López-Malo, A.

IN RECOGNITION FOR THEIR PARTICIPATION AS AUTHORS OF THE WORK:

Antimicrobial activity storage stability of cell free supernatants from Lactobacillus plantarum (POSTER)

DR. GUSTAVO ADEL GUTIÉRREZ LÓPEZ
PRESIDENT OF THE AMECA DIRECTING COUNCIL 2017-2019

DR. NICOLÁS OSCAR SOTO CRUZ
VICE PRESIDENT OF THE AMECA DIRECTING COUNCIL PRESIDENT OF THE ORGANIZING DIRECTING COMMITTEE

DRA. LILIA ARELY PRADO BARRAGÁN
SECRETARY OF THE DIRECTING COUNCIL PRESIDENT OF THE SCIENTIFIC COMMITTEE

DR. SANTOS GARCÍA
PRESIDENT OF FOOD FOR HEALTH

Puerto Vallarta, Jalisco, México, 14-16 November 2018


AMECA
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Food for Health 2018
AWARDS THE PRESENT CERTIFICATE TO:
ARRIOJA BRETÓN DANIELA
IN RECOGNITION FOR HIS/HER PARTICIPATION AS DELEGATE TO THIS CONGRESS
DR. GUSTAVO ADEL GUTIÉRREZ LÓPEZ
PRESIDENT OF THE AMECA DIRECTING COUNCIL 2017-2019
DR. NICOLÁS OSCAR SOTO CRUZ
VICE PRESIDENT OF THE AMECA DIRECTING COUNCIL PRESIDENT OF THE ORGANIZING DIRECTING COMMITTEE
DRA. LILIA ARELY PRADO BARRAGÁN
SECRETARY OF THE DIRECTING COUNCIL PRESIDENT OF THE SCIENTIFIC COMMITTEE
DR. SANTOS GARCÍA
PRESIDENT OF FOOD FOR HEALTH
Puerto Vallarta, Jalisco, México, 14-16 November 2018

# Certificate of Participation

**This certifies that**

Daniela Arrioja Breton

**POSTER ATTENDED**

Antimicrobial activity stability during storage of cell free supernatants from Lactobacillus rhamnosus and Lactobacillus plantarum

**Attended IFT19: Feed Your Future® June 2-5, 2019 held in New Orleans, LA USA**

*The IFT19 scientific and applied sessions qualify for Certified Food Scientist (CFS) recertification contact hours (CH). CFS Certificants may claim a maximum of 22 CH for their participation in scientific and technical symposia and poster sessions related to the CFS Content Domains.*

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Daniel Gullicksen

Daniel Gullicksen
IFT Meeting Planner

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*Arrioja-Bretón, E. Mani-López, A. López-Malo, H. Bach*

el presente

# *RECONOCIMIENTO*

por su participación con el trabajo titulado:

Actividad antibacteriana y antifúngica de los sobrenadantes libres de células de *Lactobacillus rhamnosus* NRRL B-442. D

en el:

**CONGRESO INTERNACIONAL DE INOCUIDAD ALIMENTARIA 2019**

"Retos de la Industria Alimentaria Alrededor de la Inocuidad"

llevado a cabo del 11 al 13 de septiembre de 2019,
en la Biblioteca Universitaria "Raúl Rangel Frías" en Monterrey, N.L.

M.C. Nydia Orué Arreola
Presidenta de la AMEPAL A.C.

Dra. María Teresa Jiménez Munguía
Secretaria de la AMEPAL A.C.

Inocuidad Alimentaria 2019 Congreso Internacional