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BACTERIAS ÁCIDO LÁCTICAS COMO BIOCONSERVANTE DE CARNE BOVINA

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BACTERIAS ÁCIDO LÁCTICAS COMO BIOCONSERVANTE DE CARNE BOVINA

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CONTENIDO

LISTA DE CUADROS

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

ADN	Ácido desoxirribonucleico.
ARN	Ácido ribonucleico.
CDC	Center for Disease Control and Prevention (centro para el control y prevención de enfermedades).
CFS	Cells-free supernatant (Sobrenadante libre de células).
CFU	Colony-Forming Unit (Unidad formadora de colonia).
CH	Colitis hemorrágica.
EHEC	Enterohemorrhagic *Escherichia coli* (*E. coli* enterohemorrágico).
ETA	Enfermedad transmitida por alimentos.
FDA	U. S. Food and Drug Administration (Administración de alimentos y drogas de EE. UU.).
GRAS	Generally recognized as safe (Generalmente reconocidas como inocuas).
HPP	High hydrostatic pressure (Alta presión hidrostática).
LAB	Lactic acid bacteria (Bacterias ácido lácticas).
MAP	Modified atmosphere packaging (Empacado con atmosfera modificada).
NM	Natural Microbiota (Microbiota natural).
PEF	Pulsed electric fields (Pulsos de campos eléctricos)

DEDICATORIA

Este trabajo se lo dedicado a mi familia.

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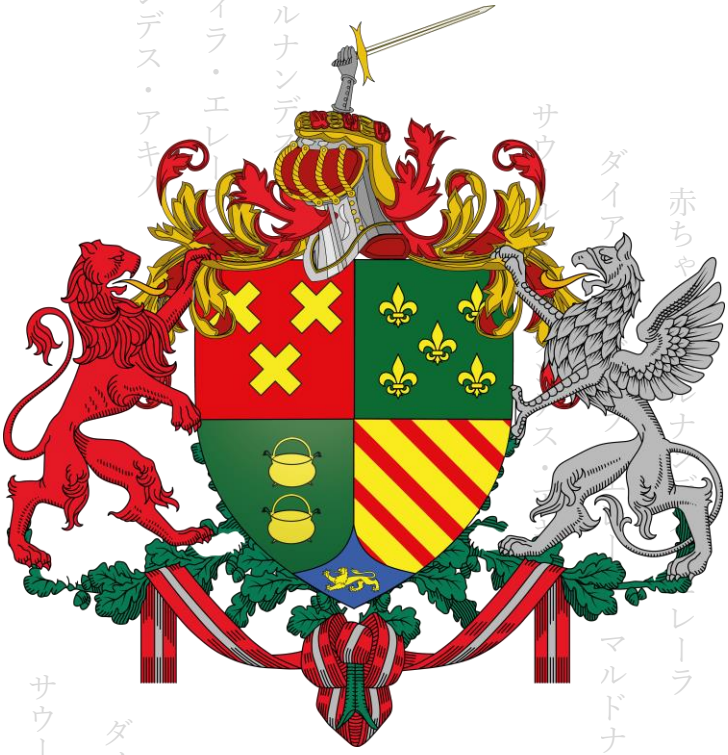

Sha ni hruniná bidó' lá bicaa gui bele,
ne stobi que caya'qui luni.

虎穴に入らずんば虎子を得ず。

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RESUMEN GENERAL

BACTERIAS ÁCIDO LÁCTICAS COMO BIOCONSERVANTES DE CARNE BOVINA

La carne es un alimento que por su rico contenido en nutrientes (grasa, proteína, minerales y agua) es fácilmente colonizada por microorganismos. Un mal procesamiento higiénico durante la cadena productiva favorece la contaminación bacteriana lo que resulta en la reducción de la vida útil de los productos cárnicos. Los métodos alternativos como la bio-conservación buscan aumentar la vida útil y reducir el contenido de bacterias patógenas. Por lo que el objetivo de este estudio fue obtener bacterias acido lácticas (LAB) nativas de carne capaces de inhibir el crecimiento de bacterias patógenas. Para esto se aislaron 115 LAB de carne molida de res de diferentes puntos de venta de la ciudad de Texcoco; de las cuales 44 LAB presentaron antagonismo hacia las bacterias de referencia (Salmonella y E. coli) por el ensayo spot on the lawn. Cinco aislados de LAB, así como su sobrenadante libre de células (CFS) presentaron antagonismo hacia ambas bacterias de referencia, dichas bacterias se identificaron por el sistema API50ch. Posteriormente se usaron los cultivos y los CFS de las LAB con efecto inhibitorio más significativo (Lactobacillus delbrueckii y Lactococcus lactis) para reducir la población de las bacterias de referencia en carne molida de res a temperatura de refrigeración; los cultivos bacterianos presentaron mayor efecto reductivos después del cuarto día, mientras que el CFS redujo la población desde el primer día, aunque el efecto se redujo posteriormente. L. lactis y su CFS presentaron mayor efecto reductivo en comparación con Lb. delbrueckii. Se concluyó que las cepas de Lb. delbrueckii y L. lactis seleccionadas tienen potencial para utilizarse como bio-conservadores que ayuden en la inhibición y reducción de la población de Salmonella y E. coli contaminante en carne molida de res, y su CFS puede ser utilizado como tratamiento de desinfección alternativo o complementario.

Palabras clave: LAB, CFS, *Salmonella*, *E. coli*, inhibición, reducción, carne molida.

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GENERAL ABSTRACT

LACTIC ACID BACTERIA AS BEEF BIOPRESERVATIVE

Meat is food that by its rich content of nutrients (fat, protein, minerals and water) is easily colonized by microorganisms. Poor hygienic processing during the production chain favors bacterial contamination, which reduces the shelf-life of meat products. Alternative methods, such as bio-preservation, seek to increase meat shelf-life and reduce the content of pathogenic bacteria. So, this study aimed to obtain meat native lactic acid bacteria (LAB) capable of inhibiting the growth of pathogenic bacteria. For this purpose, there were isolated 115 LAB from ground beef of different points of sale in the city of Texcoco; of which 44 LAB showed antagonism towards the reference bacteria (Salmonella and E. coli) by the spot on the lawn assay. Five LAB strains, as well as their cell-free supernatant (CFS), showed antagonism towards both reference bacteria; these bacteria were identified by the API50ch system. Subsequently, LAB cultures and CFS of more significant inhibitory effect (Lactobacillus delbrueckii and Lactococcus lactis) were used to population reduction test, in ground beef at refrigeration temperature. Bacterial cultures had a greater reductive effect after the fourth day, while CFS reduced the population since the first day, although the effect was subsequently reduced. L. lactis and its CFS had greater reductive effect than Lb. delbrueckii. It was concluded that the strains of selected Lb. delbrueckii and L. lactis have the potential to be used as bio-preservatives that help in the inhibition and reduction of the population of Salmonella and contaminating E. coli in ground beef, and their CFS can be used as alternative or complementary disinfection treatment.

Keywords: LAB, CFS, *Salmonella*, *E. coli*, inhibition, reduction, ground beef.

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1 INTRODUCCIÓN GENERAL

Uno de los principales problemas de la industria cárnica es la contaminación y deterioro debido a la actividad microbiana, pues la composición química y características biológicas (pH y Aw) de la carne, permiten que sea un excelente medio de cultivo de microorganismos (Devlieghere, Vermeiren, & Debevere, 2004; Rodríguez Agudelo, 2013). A pesar de que los tejidos musculares de animales sanos son estériles, estos se contaminan, adquiriendo su microbiota natural (NM) desde las primeras fases de producción (faenado, manipulación y exposición al medio), y permanece casi inalteradas durante la cadena de proceso (Bruhn et al., 2004; Jackson, 2014; Nychas, Skandamis, Tassou, & Koutsoumanis, 2008)

En este sentido se han reportado diferentes géneros bacterianos contenidos en la carne tales como: *Achromobacter, Streptococcus, Micrococcus, Sarcina, Flavobacterium, Proteus, Bacillus, Chromobacterium*. Además, bacterias que deterioran la carne (*Brochothrix, Enterobacteria y Pseudomonas*), bacterias ácido lácticas (LAB) (*Enterococcus, Lactobacillus, Weissella, Leuconostoc* y *Lactococcus*) (Sobrino-López & Martín-Belloso, 2008; Valcárcel Hervás & Sobrino Gregorio, 2014). La actividad de estas poblaciones bacterianas que comprenden la NM así como sus interacciones producen reducción de la vida útil debido al deterioro prematuro de la carne (Duffy, Cummins, Nally, O' Brien, & Butler, 2006).

Adicionalmente al deterioro de la carne causado por bacterias deteriorantes, la contaminación con bacterias patógenas como *Listeria, Salmonella, Escherichia coli, Staphylococcus aureus, Brucella, Clostridium, Bacillus anthracis* y *Pasteurella tuleransis* (Kopper et al., 2014) comprometen la salud del consumidor ya que estos pueden adquirir una enfermedad trasmitida por alimentos (ETAs) (Duffy et al., 2006).

Salmonella es uno de los géneros bacterianos con mayor distribución geográfica a nivel mundial, y la mayoría de sus serovariedades son consideradas

patogénicas (Devlieghere et al., 2004). Por ello en Estados Unidos, el centro de control y prevención de enfermedades (CDC) señala al género *Salmonella* como responsable de aproximadamente 23 mil hospitalizaciones y 450 muertes, de un total de 1.2 millones de casos al año, siendo la mayoría debido a infecciones por alimentos; en México la información que relaciona a la carne con infecciones causadas por *Salmonella,* es escasa y la mayoría esta desactualizada (Nayarit et al., 2016). Por otra parte, *E. coli* es una de las bacterias principalmente aisladas de la carne y es un indicador de contaminación fecal. A pesar de que la mayoría de sus serotipos son inofensivos, algunos son patogénicos y se les atribuye diversos brotes ETAs a nivel mundial (Hiko, Asrat, & Zewde, 2008). Por lo general, este género bacteriano coloniza la carne cuando la canal tiene contacto con materia fecal del ganado durante el proceso de evisceración (Kopper et al., 2014), y dado que la población inicial permanece estable durante la cadena productiva, es necesario inactivar aquellos patógenos que lograron llegar a la carne.

La calidad microbiológica y la vida útil depende de la composición de la NM de la carne. Respecto a lo anterior, el enfoque no es lograr carne con nulo contenido de bacterias, ya que esto sería costoso y poco práctico (Stiles, 1996). Las estrategias contra estos problemas han llevado al desarrollo de diferentes métodos de conservación, la mayoría de ellos se basan en la manipulación de la temperatura y el uso de conservantes químicos (Deak, 2014; Devlieghere et al., 2004). Las principales metodologías utilizadas para reducir la carga bacteriana y evitar su crecimiento son el lavado de la canal con soluciones de ácido láctico y la reducción de temperatura (Buncic et al., 2014). Sin embargo, ante la negativa de muchos consumidores sobre el uso de conservantes químicos en alimentos, se han desarrollado métodos alternativos tales como: pulsos de campos eléctricos (PEF), alta presión hidrostática (HHP), envasado en atmósfera modificada (MAP), envasado activo, compuestos antimicrobianos naturales y la bio-conservación (Devlieghere et al., 2004). Este último, consiste en utilizar microorganismos o su metabolitos para alargar la vida útil y mantener la inocuidad de la carne (Devlieghere et al., 2004). En este sentido, las bacterias ácido lácticas

(LAB) se han utilizado principalmente por su carácter de inocuas (GRAS) (Cleveland, Montville, Nes, & Chikindas, 2001; Favaro & Todorov, 2017). El modo de acción de las LAB incluye la competencia de nutrientes, la adhesión de sustratos y las producción de sustancias antibacterianas (peróxido de hidrógeno, ácidos orgánicos, diacetilo, surfactantes y compuestos antibacterianos proteicos) (Favaro & Todorov, 2017; Hugas, 1998; Stiles, 1996).

Las LAB y sus metabolitos son capaces de inhibir varios microorganismos (*Pseudomonas, Staphylococcus, L. inocua, E. coli*) que deterioran la carne (Ammor, Tauveron, Dufour, & Chevallier, 2006; Buncic et al., 2014; Nychas et al., 2008) y patógenos como *E. coli* O157:H7 (Roldán & Chinen, 2007; Smith, Mann, Harris, Miller, & Brashears, 2005), *Salmonella sp.* (Smith et al., 2005) y *Listeria monocytogenes* (Mahmoud, Vaidya, Corvalan, & Linton, 2008). Actualmente, la nisina y pediocin PA-1, producida por *Lactococcus lactis* y *Pediococcus acidilactici* respectivamente*,* son las únicas bacteriocinas utilizadas de manera comercial como aditivo para extender la vida útil de la carne (Deegan, Cotter, Hill, & Ross, 2006). Sin embargo, se ha considerado que el uso continuo de solo estas sustancias puede inducir la resistencia selectiva y acelerada en las bacterias blanco (Ahmed & Shimamoto, 2015; Cleveland et al., 2001). Para reducir el riesgo de resistencia bacteriana, se ha propuesto buscar nuevas LAB productoras de agentes antimicrobianos (Maragkoudakis et al., 2009). La NM de la carne puede ser un reservorio viable de nuevas cepas, ya que estas LAB crecen bien en la carne, su periodo de adaptación es más corto y algunas podrían producir sustancias antimicrobianas (Zaher & Fujikawa, 2011). Considerando lo antes expuesto, se planteó la presente investigación titulada "***Bacterias ácido lácticas como bioconservante de carne bovina"***. La investigación se realizó en condiciones de laboratorio, y el documento se divide en cinco capítulos. El **Capítulo 1** muestra una introducción en donde se detalla información sobre los antecedentes, importancia, problemática y justificación de la tesis. El **Capítulo 2** proporciona una revisión de literatura donde se analiza la posibilidad de utilizar LAB para inhibir el crecimiento de bacterias que deterioran la carne y sus

implicaciones al ser consideradas en un protocolo de bio conservación. En el **Capítulo 3** se aborda el aislamiento y selección de LAB de carne molida de res que se sometieron a pruebas *in vitro*, para evaluar si inhiben el crecimiento de *Salmonella* y *E. coli,* en co-cultivos así como su CFS. En el **Capítulo 4** se reporta el uso de dos bacterias eficientes para realizar pruebas de inhibición en carne molida a temperatura de refrigeración.

2 ANTIBACTERIAL ACTIVITY OF LACTIC ACID BACTERIA TO IMPROVE SHELF LIFE OF RAW MEAT

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2.1 ABSTRACT

Lactic Acid Bacteria (LAB) are generally recognized as safe. It has been used to increase the shelf-life of fermented products, and its antimicrobial action is based on the metabolites secretions, such as lactic acid, hydrogen peroxide, reuterin, bacteriocins and the like-bacteriocins substances. It has been proven that LAB are able to inhibit deteriorating bacteria of raw meat, but improper handling of live cultures could lead to spoilage. So, the use of their bacteriocins, small antimicrobial peptides, could be an alternative. Besides reducing the number of spoilage bacteria, it seeks to inhibit pathogenic bacteria such as *Salmonella*, enterohemorrhagic *Escherichia coli and Listeria*. The food industry uses few bacteriocins and now bacterial resistance has been reported. For that reason, the search of novel bacteriocins produced by LAB is a priority. Moreover, the natural microbiota of meat could be a reservoir of LAB.

2.2 INTRODUCTION

Spoilage of meat and meat by-products by microbial activity is a common problem in the meat industry (Devlieghere et al., 2004). The food industry applies several processing techniques for inhibition and/or inactivation of microorganisms to guarantee safe products with a long shelf-life (Deak, 2014). Most techniques used are based on temperature manipulation (heat and cold treatment), and chemical

treatment (acids, bases and salts) (Buncic et al., 2014). However, consumers demand tasty, nutritious, natural, and non-chemically treated products. Thus, the food industry has developed new preservation techniques, such as pulsed electric fields (PEF), high hydrostatic pressure (HHP), modified atmosphere packaging (MAP), active packaging, natural antimicrobial compounds and bio preservation (Devlieghere et al., 2004). In bio preservation, shelf-life is extended and meat safety is enhanced by using natural or controlled microbiota (Devlieghere et al., 2004). For these purposes, LAB are the most used microbiota, because these bacteria and their metabolites are generally recognized as safe (GRAS) (Cleveland et al., 2001). LAB and their metabolites capable of inhibiting various spoilage microorganisms (Nychas et al., 2008; Buncic et al., 2014), and even pathogens such as *Escherichia coli* O157:H7 (Roldán et al., 2012) and *Salmonella sp.* (Smith et al., 2005). In recent years, some strains of LAB and their growth-inhibitor factors were isolated, characterized and recommended to extend product shelf-life. However, this situation could contribute to selective and accelerate resistance of spoilage bacteria (Cleveland et al., 2001). To avoid bacterial resistance, some researchers have proposed manipulating the meat-native microbiota for increasing the number of beneficial bacteria. This technique could inhibit a spoilage bacterium with less resistance risk (Zaher and Fujikawa, 2011). This review explores the possibilities of using natural microbiota to isolate bacteriocin LAB producer strains, and its application for extending meat shelf-life.

2.3 BACTERIAL SPOILAGE

This can be defined as any change in a food product that makes it unacceptable from the sensory point of consumer view (Dias et al., 2013). Beef meat is one of the most perishable foods**,** because its composition is ideal for growth of a wide variety of microorganisms (Mayr et al., 2003), some of them may cause the spoilage of meat (Ercolini et al., 2006). The physiological status of animals and spread of contamination during slaughter, and, the storage and distribution conditions, like temperature, affect the microbiological quality of meat (Nychas et al., 2008). The signs of microbial spoilage of meat are organoleptic changes like

physical damage, oxidation or appearance of off-flavors and off-odors, resulting from microbial growth and metabolism in the product, principal genera are *Pseudomonas spp., Enterobacteriaceae, Brochothrix thermosphacta* and LAB (Gram et al., 2002; Ercolini et al., 2006). Thus, the natural microbiota and the storage conditions of the meat have an influence on the final organoleptic properties (Ercolini et al., 2006) and shelf-life of meat and meat products (Borch et al., 1996).

Furthermore, preservation parameters of meat affect microbial selection, hence the spoilage microorganism dominant of a product could be predicted (Gram et al., 2002). The fact of modifying some factors, such as temperature, gaseous atmosphere, and meat pH can result in the extension of product shelf-life (Borch et al., 1996). Consequently, the importance of these parameters for bacterial growth should not be underestimated, however interactions among microorganisms of microbiota can determine selection and/or metabolism of spoilage bacteria. Furthermore, different spoilage bacteria species could colonize meat surface through different mechanisms (Ercolini et al., 2009) and interact, mainly could as competition (Bruhn et al., 2004).

Low temperature is the most common applied strategy for preservation of stored meat, mainly from carcasses after slaughter, primal cuts, during transportation to distributors for storage of meat at various wholesale and retail food sites, and finally to the consumer's kitchen (Gram et al., 2002; Ercolini et al., 2006). High growth of bacteria is a prerequisite for meat spoilage, so during all of these phases, chill temperature are used to delay growth of bacteria, and hence meat alteration (Borch et al., 1996). The expected shelf life is related to day range, whilst at the time of rejection, typical odor is putrid and slime is visible on the surface (Bruhn et al., 2004).

2.4 LAB IN MEAT

Generally considered as natural strains of LAB in meat and meat products are: *Carnobacterium piscicola and C. divergens; Lactobacillus sakei, Lb. curvatus and Lb. plantarum, Le. mesenteroides* subsp. *mesenteroides, Le. gelidum and Le. carnosum* (Hugas, 1998).The LAB plays an important role in food fermentations causing flavor and texture changes together with a preservative effect, resulting in an increase in the shelf life of transformed product (Hugas, 1998). The chill-storage under modified atmosphere of red meats has an effect on the meat microbiota, triggering a change from spoilage Gram negative bacilli to fermentative LAB. This change produces an effect on the shelf life extension (Borch et al., 1996). In fresh meat, LAB bring about a mild fermentation process, without producing any changes in the sensorial characteristics, because of the low carbohydrate content and the buffering capacity of meat. In the same way, the growth of LAB in naturally fermented meats after the addition of sugar transform the products through the production of lactic acid by LAB, and then, the subsequent decrease in pH denatures the meat proteins favoring the decrease of water activity (a_W) which ends up in a microbial stabilization of the transformed product (Borch et al., 1996; Hugas, 1998; Favaro and Todorov, 2017).

The metabolic products of LAB and the bacteria itself play an important role in preservation of foods, although the uncontrolled growth of some species of LAB could spoil meats and meat products, *Leuconostoc and Lb. sakei* have been described as slime producing organisms in processed meats, sulfide-producing strains of *Lb. sakei* have been described as spoiling vacuum-packaged meat (Wills et al., 1987). Likewise, the growth of heterofermentative LAB can also cause off-odors and holes after the production of CO_2 (Kandler, 1983).

LAB growth in meat might cause microbial interference to spoilage and pathogenic bacteria, through several mechanisms like nutrient and oxygen competition, also with competition for attachment/adhesion sites and production of a wide range of inhibitory substances, primarily lactic acid or lactic and acetic

acids, acetoin, diacetyl, hydrogen peroxide, reuterin, bacteriocins and bactericins-like (Hugas, 1998).

2.5 LAB INHIBITION POTENTIAL

In food production and commercialization, it crucial that suitable actions are taken to guarantee safety stability during food shelf life. Consumers require foods of high quality, without chemical preservatives, safe, and possessing long shelf-life. The spoilage and short shelf-life is a major part of microbial activity. The deterioration and short shelf life of the meat is mainly due to the deteriorating microorganisms. The natural meat microecosystem is very important, because any bacteria could cause spoilage, even LAB. In fact many types of LAB can be considered deteriorating bacteria because they change the sensory characteristics, such as *Lactobacillus sp., Leuconostoc spp., Lactococcus and Enterococcus* (Ammor et al., 2006; Hamasaki et al., 2003). However, several antagonistic activities may occur in microbiome and control of the microbiota is the key to avoiding these problems. LAB are capable of showing antagonism effects by synthesizing antimicrobial compounds and these productions depends on some factors such as temperature, atmosphere, humidity or interaction with other bacteria (Geremew, et al., 2015; Awojobi et al., 2016, Ammor et al., 2006).

The modification of atmospheric CO_2 between 20-40% benefits to *Lb. sakei* CTC 372 or CTC 711 applied to meat, by reducing the growth of spoilage bacteria almost 80% and also inhibiting spiked *L. monocytogenes* growth, even at refrigeration temperatures (4 °C) (Djenane et al., 2005). Also, temperature is another important factor because at ambient temperatures (20 °C) the LAB fulfills its bioprotective function. On the contrary, at refrigeration temperatures and under vacuum, they are not as efficient and grow at a lower speed (Signorini et al., 2006).

In addition, antifungal capacities have been reported, under laboratory conditions and using culture medium. Several LAB have shown inhibitory capacity against molds of the genus *Penicillum* at 72 h (Kivanc et al., 2014). Also, some species of

genus *Lactobacillus* can inhibit *Aspergillus fumigatus* (Kim, 2005), *A. niger* (Svanström et al., 2013) *A. flavus and P. expansum* (Dalié et al., 2010).

Additionally, multiple bacterial genera are causing diseases outbreaks throughout the world, and the most important bacteria in the meat industry have been *Salmonella* and Enterohemorrhagic *E. coli* (EHEC). The presence of these pathogens is mainly due to poor handling of feces during slaughter. It has been reported that the use of mixed cultures of *L. acidophilus* in TSB medium and ground beef inhibit the growth of *Salmonella* and EHEC in a period of 5 days, maintaining the sensory quality within acceptable ranges (Smith et al., 2005). Also, in medium broth, lactic acid produced by different species of *Lactobacillus* inhibited *S. enterica* ser. Typhimurium (Makras et al., 2006) and if there is oxygen the LAB can produce hydrogen peroxide which increases the effect (Ammor et al., 2006).

Although LABs show a clear inhibitory potential, its use as live cultures is very delicate, which is why the bad selection or bad handling of strains can produce adverse effects such as slime production, off-odors or color changes, even if initial dose are low (10 CFU g^{-1}) (Iulietto et al., 2015; Hamasaki et al., 2003).

Table 1. Bacteria and bacteriocins produces.

Bacteriocin	**Producer microorganism**	**Potential application**
Nisin (IA)	*Lactococcus lactis*	Food biopreservative and bovine mastitis prevention
Nukacin 3299 (IA)	*Staphylococcus simulans*	Prevention of streptococcal bovine mastitis
Mutacin, 1140 (IA)	*Streptococcus mutans*	Prevention of dental caries
Hyicin 3682 (IA)	*Staphylococcus hyicus*	Control of phytopathogens
Mersacidin (IB)	*Bacillus spp.*	Prevention and control of methicillin-resistant S. aureus
Lacticin 3147 (IC)	*Lactococcus lactis*	Bovine mastitis prevention
Pediocin PA-1 (IIa)	*Pediococcus acidilactici*	Food biopreservative
Aureocin A53 (IId)	*Staphylococcus aureus*	Prevention and control of bovine mastitis
Lysostaphin (IIIa)	*S. simulans subsp. staphylolyticus*	Prevention and control of human and animal infections caused by *S. aureus*
Enterocin AS-48 (IV)	*Enterococcus faecalis*	Food biopreservative

(Adapted from Cleveland et al., 2001; Devlieghere et al., 2004; Bastos et al., 2015)

An alternative to avoid poor outcomes using live cultures, is use of crude extracts or purified metabolites, like bacteriocins (Yong et al., 2015). The use of crude fractional extracts of LAB has become an inhibitor against different meat spoilage bacteria such as *Leuconostoc spp*.(Yong et al., 2015; Lee and Kim, 2011), pathogens such as *Staphylococcus aureus* (Yong et al., 2015), EHEC (Smith et al., 2005), *Salmonella sp.* (Makras et al., 2006; Smith et al., 2005) and *L. monocytogenes* (Djenane et al., 2005). Meanwhile, it has been reported that the use of bacteriocins is more effective if dosed in the appropriate amount. however, these substances are specific and cannot be used massively (Iulietto et al., 2015). Search of new technologies for food conservation and the use of substances produced by microorganisms deserves more research.

2.6 BACTERIOCINS

Bacteriocins are proteinaceous toxins produced by bacteria in ribosomes for inhibiting growth of closely related bacterial strains (Hugas, 1998). These are polypeptides which are functionally, structurally, and ecologically diverse. Each bacterium synthesizes a different kind of bacteriocin (Table 1). The regulation is by operons, which are responsible for synthesis and autoimmunity (Deegan et al., 2006).

Five classes of bacteriocins have been proposed, and most of them are classified in Classes I and II. (Deegan et al., 2006; López et al., 2008). Class I. These bacteriocins are heat-stable and small (<5 kDa) peptides and generally known as lantibiotics due at presence of non-proteogenic thioether amino acids lanthionine (Lan) or methyllanthionine (MeLan) (McAuliffe et al., 2001; Lee and Kim, 2011). The lantibiotics typically have no antibacterial effect against Gram-negative bacteria, while conversely in closely related Gram-positive are most effective (Lee and Kim, 2011; Wiedemann et al., 2001). The lantibiotics are divided into two subclasses based on chemical structure and functionality. Subclass A: included peptides relatively elongated, flexible and positively charged, these act by disrupting cytoplasmic membrane forming pores of sensitive target species

(McAuliffe et al., 2001). Subclass B: these peptides are more rigid, have a globular structure and they are either negatively charged or have no net charge. These act by interfering with essential enzymatic reactions of sensitive bacteria, like cell wall biosynthesis (McAuliffe et al., 2001; Lee and Kim, 2011; Bierbaum and Sahl, 2009). Recently, the based on action mode of lantibiotics sub-classification has been questioned, by the discovery that mutant variant of nisin can act through both mechanisms (pore formation and inhibition of cell wall biosynthesis), this due which nisin can bind of precursor protein wall, lipid II, and then proceed to pore formation (Wiedemann et al., 2001).

Class II. Non-lantibiotics, these bacteriocins are small (<10 kDa) and heat-stable peptides composed of 30 to 60 amino acids and do not contain Lan or MeLan (Deegan et al., 2006; López et al., 2008). Between these bacteriocins the chemical structure is very heterogeneous, and their sub classification is difficult. However, they can be sub classified in some groups. The subclass IIa: pediocin-like (or *Listeria*-active), is composed of 37 to 48 amino acid with a terminal portion, and contains one or two α-helix (Lee and Kim, 2011).

The subclass IIb: two-component bacteriocins. Among the former group, pediocin AcH/ PA-1. Pediocin-like peptides show a high degree of homology (40–60%), when corresponding amino acid sequences, they are aligned. In particular, the cationic N-terminal domain contains the homologous region pediocin box (YGNGVXCXXXXCXV), with the two cysteine residues forming a disulphide bridge (Deegan et al., 2006; López et al., 2008). Class IIb refers to two-component bacteriocins that require two peptides to work synergistically. These peptides themselves have little or no activity, and there appears to be no sequence similarities between complementary peptides. Both lactacin F and lactococcin G are members of this subgroup (Deegan et al., 2006). The subclass IIc: these bacteriocins have a covalent union between C and N terminal portion, which result in a cyclic structure (Cotter et al., 2012).

2.7 MODE OF ACTION

The bacteriocins act binding on specific receptors located on cell wall of target bacteria, after which several isolated or adjuvant mechanisms act to kill the bacteria. (Souza et al., 2005; Hernández-Dominguez et al., 2008). Bacteriocins are positively charged molecules with hydrophobic patches. Electrostatic interactions, with negatively charged phosphate groups on target cell membranes are thought to contribute to the initial binding with the target membrane (Hugas, 1998; Cleveland et al., 2001). Microbial cell killing due bacteriocin action could occur as the consequence of unbalanced cytoplasmic membrane function (affecting energy use and permeability), inhibition of nucleic acid synthesis, interference on the protein synthesis and changing cell translator mechanism. There are still some bacterial lineages that could suffer cell lyse (Basanta et al., 2009), especially lantibiotics, that inhibit target cells by forming pores in the membrane, depleting the transmembrane potential and/or the pH gradient, resulting in the leakage of cellular materials (Cleveland et al., 2001). Nisin and pediocin AcH are examples of bacteriocin owner of prominent antimicrobial spectrum, which are able to exert inhibitory activity on the growth of bacteria (Ramírez and Ulloa, 2011).

2.8 USE IN MEAT

Nisin is one of the few bacteriocin that is used in the industry as a biopreservative. However, it has not yet been implemented in meat. Nisin can inhibit the growth of Gram-positive bacteria so they help reduce the problem in meat. Also, the greatest interest in the use of bacteriocins are inhibition of meat pathogens (Devlieghere et al., 2004). Some bacteriocins of genus *Lactobacillus* have an antimicrobial effect against varieties of *Salmonella*, such as Enteritidis, Heidelberg, Newport and Typhimurium, and can inhibit their growth for several days (Kim et al., 2015). The bacteriocin of *Pediococcus acidilactici* can inhibit *L. monocytogenes* in meat for a period of 28 days, under refrigeration conditions (Nielsen et al., 1990). Also, *Lb. sakei* 706 and BacFL31 bacteriocins besides inhibiting the growth, its presented a bioprotective effect avoiding a new

colonization of *Listeria and Salmonella* (Schillinger et al., 1991; Chakchouk-Mtibaa et al., 2017). Otherwise, *B. thermosphacta* bacteriocin was used to avoid cross-contamination of meat with the plastic containers, reducing the pathogen population from $\log_{10}$ 7 to 3, after two days (Siragusa et al., 1999). Similarly, the bacteriocin application from spoilage meat bacteria, *Leuconostoc carnosum*, in the meat vacuum packaging is able to reduce the number of viable cells of pathogen at 21 days period (Budde et al., 2003).

Although, some studies have reported efficacy in use of pure bacteriocins, that is not the only use way. Other investigators have reported good results reducing viable cell of *Salmonella, E. coli, L. monocytogenes* and EHEC, using associations of bacteriocins with chelators, such as nitrates, citrates or EDTA (Cutter and Siragusa, 1995; Moon et al., 2002; Belfiore et al., 2007). And, combinations of other methodologies such as active-package, controlled atmosphere and HPP (Zhang and Mustapha, 1999). Though this in practice would increase the cost of meat preservation.

2.9 MICROBIAL RESISTANCE

Bacteriocin-producing strains generally are immune to their own antibacterial products through immunological systems, whose encoding genes are in bacteriocin gene cluster (Deegan et al., 2006). Furthermore, bacteriocins may also have cross-immunity, as some subclass IIa and subclass IIb, and lantibiotics such as Pep5 and epicidin 280. However, the cross-immunity for at least some bacteriocins subclass IIb depends on the receptor in the target cell (Ahmed and Shimamoto, 2015). Mechanisms involved in immunity are not known for most bacteriocins. In most immunological systems, self-protection depends on a single small protein (Bastos et al., 2015). These proteins have been detected either anchored to the membrane surface or embedded in the membrane (Ahmed and Shimamoto, 2015; Bastos et al., 2015). Some are largely exported, remaining trapped at the membrane, and within the cell wall compartment (Van Reenen and Dicks 2011). The roles proposed for these proteins include blocking the insertion

of the bacteriocin into the membrane or protection of a specific target, shielding it from the bacteriocin (Bastos et al., 2015).

Another immunity mechanism reported for some class II bacteriocins depends on the activity of multi-drug transporter proteins, which participate in bacteriocin immunity by removing bacteriocin that enters the cytoplasmic membrane from the outside (Bastos et al., 2015). The frequency at which susceptible organisms develop resistance to a given bacteriocin is therefore a very important issue to consider when bacteriocin-based biocontrol strategies are proposed. Bacteriocins have not been extensively used in the clinical setting (Deegan et al., 2006; Bastos et al., 2015). Nisin has long been used as a food biopreservative, but nisin resistance has not been reported among food-spoilage microorganisms in the food industry (Deegan et al., 2006).

Therefore, understanding the potential for bacteriocin resistance development has been possible primarily from experiments performed under laboratory conditions (Deegan et al., 2006). Once a new preservative is found safe and effective, it is critical to ensure the longevity of its use by preventing the proliferation of resistant cells. Nowadays, cells exhibit resistance to several antibiotics and the transfer of resistance between microorganisms has been documented. Nontheless, bacteriocins are not antibiotics, there is concern that exposure to bacteriocins will render cells more resistant to antibiotics (Hugas, 1998; Cleveland et al., 2001). There is substantial variation in the frequency of spontaneous mutations resulting in bacteriocin resistance, depending on the microorganism, the bacteriocin and the strain tested, and the assay used, including the bacteria/bacteriocin ratio as well as environmental conditions. The mechanisms involved in acquired resistance are also quite diverse (Bastos et al., 2015).

The frequency of nisin (100 IU mL^{-1}) resistance development in *L. monocytogenes* strain ScottA was found to drop (at least, 100-fold) when the strain was grown at 10 ºC and in the presence of decreasing pH (5.5 and 6.0) and NaCl concentration

(2.0 and 0.5%). Growth on NaCl (6.5%) at 30 ºC also reduced the frequency of nisin (500 IU mL^{-1}) resistance development. Nisin resistance seems to have little impact on bacterial fitness, and the resistance seems to be stable in most mutants in the absence of selection. That indicates how some bacteria develop resistance to bacteriocins (Bastos et al., 2015).

2.10 TOXICITY

Bacteriocins have been consumed for a long time, principally through fermented food. The approval of some bacteriocins as nisin was based on data regarding its safety, not on the history of common use. Acute, subchronic, and chronic toxicity studies, as well as reproduction, sensitization, *in vitro*, and cross-resistance studies showed that bacteriocins are safe for human consumption at an acceptable daily intake of 2.9 mg $person^{-1}$ day^{-1} (Cleveland et al., 2001).

These substances to be used as food additives must fulfill such requirements some characteristics as: all proposed additives must be toxicologically tested and evaluated in all pertinent aspects, including accumulative, synergistic and potential effects. Only those additives considered safe at the intended level of insertion must be released to use; all food additives must be again evaluated when arisen new information about their use and safety. Food additives must be kept in conformity with specifications approved by Codex Alimentarius Commission. Justification of additive use must be based strongly in the requisites of food safety of different characters of consumers, and it also must present an economically and technically feasible alternative; the temporary or permanent approval of use of a food additive must consider the limitation for specific foods, the purpose, use conditions, decrease of necessary level to reach the desirable effects and the acceptable daily intake for human, and still must consider the probable intake of special consumers (Souza et al., 2005; Deegan et al., 2006).

Consequently, while bacteriocins have application in many food systems, foods should not be preserved by bacteriocins alone, but rather as part of a system with multiple hurdles. Since LAB are commonly found in meat, bacteriocins produced

by these bacteria have been explored and isolated. Nevertheless, most bacteriocins have been isolated from food-associated LAB and they are not necessarily effective in all food systems. However, several bacteriocins certainly do have potentials in food applications, when used under proper conditions (Souza et al., 2005). Nisin presents is characterized as nontoxic, thermic and has storage stability; digestive enzymes degradation, spoilage factor and larger spectrum sensitive of Gram-positive bacteria (Souza et al., 2005). On the contrary, it is less active to inhibit most of Gram-negative bacteria, yeast and molds (Nychas et al., 2008).

Even though bacteriocins antimicrobial roles have been recognized, it has still a rare application in food conservation. This fact is probably due to the absence of detailed studies of their particularities (Zhou et al., 2010). Among major studied bacteriocins are nisin, acidocin, bavaracin, curavaticin, sakacin, although they are not still well characterized. Nisin is the single bacteriocin commercially used, as a biopreservative, and considered safe by World Health Organization, and has received the denomination of GRAS by Food and Drug Administration (Souza et al., 2005). Nisin or its combination with lower levels of nitrate can prevent the growth of Clostridium (Ávila et al., 2014). However, some researchers concluded that nisin is not effective in meat applications, due to high pH (Hugas, 1998; Cleveland et al., 2001; Zhou et al., 2010), and inability to be uniform distributed, as well as interference by meat components such as phospholipids; whilst other studies find contradictory results (Zhou et al., 2010).

2.11 CONCLUSIONS

LAB have inhibitory potential and represent an alternative to avoid spoilage and increase the shelf-life of raw meat. Native microbiome can be a good LAB reservoir to isolate bacteria with inhibition capacity. However, bad handling, bad use or meat microbiome variation can limit the use of live cultures, and these factors can be different among geographic regions and what could favor inappropriate results, such as lime, off-odors, off-flavor and color changes.

Therefore, the best way to use as a biopreservative on meat is in the form of extracts or pure antimicrobial substances.

The bacteriocins can also be used as a biopreservative became it does not produce organoleptic changes in the meat and reduce the growth of deteriorating bacteria, and some can inhibit pathogens. But it is necessary to characterize and evaluated novel bacteriocins and develop efficient conservation methodologies to avoid resistance.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be regarded as a potential conflict of interest.

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3 EFFECT OF MEAT NATIVE LACTIC ACID BACTERIA ON THE GROWTH KINETIC OF *Salmonella sp.* AND *Escherichia coli*

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3.1 ABSTRACT

In this study, lactic acid bacteria (LAB) strains were isolated from natural microbiota (NM) of ground meat and screened their ability to inhibit two reference bacteria (*Escherichia coli* and *Salmonella* sp.) under in vitro conditions. The 44 strains were shown to inhibit at least one of the reference bacteria by spot on the lawn assay. Five bacteria were selected that were able to inhibit both reference bacteria. The selected bacteria were identified by API50ch and their inhibitory capacity were determined by growth kinetics in co-culture, likewise their cell-free supernatant (CFS) were screened by agar wells diffusion assay. In co-culture, selected LAB altered the growth rate and reduce the maximum population of reference bacteria. On the other hand, LAB CFS also showed antimicrobial activity, the crude extract, heat-treated and neutralized pH was tested. Only the crude extract with neutralized pH showed less activity. These LAB and their metabolites can help improve the microbiological quality of the meat.

Key words: Natural microbiota, kinetics, CFS, population, *in vitro.*

3.2 INTRODUCTION

Natural microbiota (NM) of meat is compound by several bacterial genera, *Achromobacter, Streptococcus, Micrococcus, Sarcina, Flavobacterium, Proteus, Bacillus, Chromobacterium* are mainly reported, also spoilage bacteria

(*Brochothrix, Enterobacteria and Pseudomonas*), pathogenic bacteria (*Listeria, Salmonella, E. coli, Staphylococcus aureus, Brucella, Clostridium, Bacillus anthracis and Pasteurella tuleransis*) and LAB (*Enterococcus, Lactobacillus, Weissella, Leuconostoc and Lactococcus*) (Valcárcel Hervás and Sobrino Gregorio, 2014).

Inside the pathogenic bacteria, *Salmonella* and *E. coli* are the most important genera in meat (Devlieghere, Vermeiren, and Debevere, 2004). *Salmonella* its related with 93.8 million of worldwide human infection cases, and 155,000 annually deaths (Majowicz et al., 2010). In the *Salmonella* infections outbreaks, beef is the most commonly implicated food (Jackson, 2014). In the same way, *E. coli* is the most frequent bacteria isolated from meat and its used as indicator of fecal contamination, their pathogenic serotypes (enterohemorrhagic, enteropathogenic, diarrheagenic and verotoxigenic) are responsible for multiple outbreaks of food-borne diseases throughout the world (Hiko, Asrat, and Zewde, 2008), only in USA is attributed about 40,000 annually deaths, and ground beef is commonly food vector (Russo and Johnson 2003).

The continuous fight against meat spoilage bacteria and shelf life extension has led to development of different preservation methods, most of them are based on temperature manipulation, cold chain, and use of chemical preservatives (Devlieghere et al., 2004). Due to the refusal of many consumers about use of chemical preservatives to extend meat shelf life, alternative methods have been developed, among one of them, the bio-preservation method, that consist in use of LAB or its metabolites to control the growth of pathogens and spoilage microorganisms. (Favaro and Todorov, 2017). Action mode includes nutrient competition, substrate adhesion and antibacterial production substances (hydrogen peroxide, organic acids, diacetyl, surfactants and proteinic antibacterial compounds) (Favaro and Todorov, 2017; Hugas, 1998; Stiles, 1996).

The study of the effectiveness of treatments with LAB in meat and meat products, as well as its potential effect to limit the growth of spoilage and pathogens bacteria,

has been reported by some authors (Hugas, 1998; Pérez, Guerrero, and Ponce, 2008; Signorini, Ponce-Alquicira, and Guerrero-Legarreta, 2006) without altering sensory characteristics. The most abundant metabolite of LAB is lactic acid. When used like sanitizing can reduce of spoilage bacteria concentration and inhibit growth rate of pathogens such as *Escherichia coli*, and *Salmonella sp*. (Favaro and Todorov, 2017; Gutierrez, 2012; Jones, Hussein, Zagorec, Brightwell, and Tagg, 2008). Despite the effectiveness of lactic acid, if it is not dosed correctly, it can alter the organoleptic characteristics of meat, so its use is limited. On the other hand, LAB antimicrobial peptides, such as bacteriocins, to reduce spoilage and pathogenic bacteria are an alternative that does not alter the meat sensory characteristics. Bacteriocins are substances generally recognized as safe (GRAS) (Favaro & Todorov, 2017; Gutierrez, 2012).

Natural microbiota of meat is a complex ecosystem, several kinds of microorganisms are implicated, and some LAB population have inhibition capacities. Some authors (Alegría, Delgado, Roces, López, and Mayo, 2010; Simova, Beshkova, Angelov, and Dimitrov, 2008; Yan and Lee, 1997) indicate that some LAB species show antagonistic activity against pathogens such as *L. monocytogenes*, *E. coli* (Riaz, Nawaz, and Hasnain, 2010) and *Salmonella* (Favaro and Todorov, 2017). The use of LAB from NM of meat to preserve it, is not new, however, majority research works that use LAB or its CFS are of bacteria isolated from fermented products or ATCC strains. The objective of this work was evaluated the effect of meat native LAB in growth parameters of *Salmonella* and *E. coli* in co-cultures and estimate inhibition effect of LAB cultures and its CFS, using *in vitro* techniques.

3.3 MATERIALS AND METHODS

Sampling and bacteria isolation. Fifty samples of raw ground beef were aseptically obtained (NOM-109-SSA1-1994) from butchers and meat retailers in Texcoco de Mora, Mexico, and which were transported and storage (-20 °C) in the laboratory of Livestock Microbiology, Chapingo Autonomous University.

Aliquots (25 g) of each sample were placed on sterile bottles and homogenized with peptone solution (225 mL, Bioxon) in a blender for 1 min. Serial dilutions were made with saline solution (0.85%) and pouring in de Man, Rogosa and Sharpe (MRS) agar (Dibico), after incubated 48 h at 37 °C. LAB characteristic colonies were taken and isolated by streaking in MRS agar. All bacteria strains were stored in appropriate media with glycerol (30%) at -20 °C.

Inhibition test. Inhibition assay of bacteria were performed by spot on the lawn assay (Harris, et al., 1999), conserved strains were activated by streak on agar medium and incubating for 24 h at 37 °C and no contamination were verified. LAB were incubated in MRS broth over night at 37 °C, the culture was diluted with saline solution (0.85%) until it had a turbidity of 4 on the McFarland scale. Indicator bacteria (*Salmonella* and *E. coli*) were spread over Muller-Hinton (Dibico) agar Petri dish, and LAB were spotted (10 µL) onto surface. The dish was incubated until indicator bacteria lawn was visible. The LAB those that had an inhibition zone around the spot, which indicates antimicrobial activity, were selected and stored (Shokryazdan et al. 2014).

Supernatant test. Five strains which can show antimicrobial activity for both reference bacteria were selected and identified by API 50CH (Biomerieux) and *apiweb* tool (Biomerieux). These LAB's were activated and streaked in MRS agar plate and incubated 24h at 37 °C. Good growth colonies were taken and cultured in MRS broth for 72 h at 37 °C in low oxygen conditions. Cultures were bathed in a boiling water for 5 min, the pH was neutralized with NaOH (0.1 M) and centrifuged at 10,000 rpm for 10 minutes at 4 °C, the resulting supernatants were stored at -20 °C. The antimicrobial activity of cells-free supernatants (CFS), heat treated (CFSH) and heat treated and neutralized pH (7.0, ± 0.5) (CFSHN) were tested by agar wells diffusion assay (NCCLS, 1993). The inhibition diameter zone (IDZ) were expressed in arbitrary unit (AU) and were measured using the following formula.

$$\text{AU mL}^{-1} = \frac{\text{IDZ x } 1000}{\text{Volume in the well } (\mu\text{L})}$$

Where:
AU mL^{-1}= arbitrary units per milliliter.
IDZ= inhibition diameter zone.

Co-culture kinetics. Growth kinetics were performed, for pure reference bacteria and co-culture with the selected LAB, they were aerobically cultured in BHI at 37 °C for 48 h in duplicate. The adjustment of growth curves and parameters calculation were made using the new logistic model (NLM) (Fujikawa, 2010).

$$\frac{dN}{dt} = rN\{1 - (\frac{N}{Nmax})^m\}\{1 - (\frac{Nmin}{N})^n\}$$

Where:

t=time
N=is the population at time t
r=is rate constant of growth
Nmax=is the maximum population
Nmin=is the minimal population
m=is a deceleration curvature parameter
n=is *lag* phase parameter

The variables of growth kinetics and arbitrary units were analyzed using a totally random design, based on general linear model in the SAS statistical software (9.4, 2014). Each treatment had two repetitions. The averages of treatment were analyzed using Tukey's multiple comparisons test, and results were considered significand when $P<0.05$.

3.4 RESULTS

Isolation and Identification of Lactic Acid Bacteria from ground meat.

Total of isolated LAB strains (115) were screened for antagonism activity, 44 had an inhibitory effect against at least one reference bacterium, of which, five strains inhibited the growth of both bacteria. Its identification were performed by API50ch kit and *apiweb* tool (Biomerieux) Table 2.

Table 2. Identification of meat native LAB, isolated from ground meat of Texcoco, Mexico.

Isolate	Genus and species
B9	*Lactobacillus delbrueckii*
B27	*Lactococcus lactis*
B29	*Lactobacillus fermentum*
B40	*Lacoccus lactis*
B44	*Leuconostoc mesenteroides*

Kinetics growth parameters of LAB

The maximum growth (N_{max}) of the bacteria, rate of constant growth (r) and lag phase (*Lag*) differed ($P<0.05$) among treatments (Table 3). The highest N_{max} was found in the treatment B9 (7.82 *log* CFU mL^{-1}) followed by B27 and B29 (7.59 and 7.01 *log* CFU mL^{-1}). The N_{max} was similar among the treatments B40 y B44 (6.11 y 6.46 *log* CFU mL^{-1}). Similar to N_{max}, the highest rate of constant growth (r) ($P<0.05$) were recorded in B9, B27 and B29 (0.97, 0.98 and 1.05 h^{-1}) followed by B40 (0.81h^{-1}). Meanwhile, treatments B44 had the lowest r (0.024 h^{-1}) ($P<0.05$). Finally, B9 required a longer period of time (*Lag*) to initiate the growth (4.6 h) ($P<0.05$) with respect to B44, B40, B27 and B9 treatments (Table 3).

Table 3. Kinetics growth parameter of the LAB strains, at 48h.

	LAB strains				
Parameter	**B9**	**B27**	**B29**	**B40**	**B44**
r	0.97^{a}	0.98^{a}	1.05^{a}	0.81^{b}	0.45^{c}
Lag	0.28^{b}	0.44^{b}	4.26^{a}	0.52^{b}	0.58^{b}
N_{max}	7.82^{a}	7.59^{b}	7.01^{c}	6.11^{d}	6.46^{d}
pH	4.14^{c}	4.57^{a}	4.18^{c}	4.15^{c}	4.34^{b}

Different letters in the lines indicate a statistically significant difference (Tukey test, $P<0.05$).

r= Rate of constant growth.

Lag= Lag period.

N_{max}= Maximum cell population (*log* CFU mL^{-1}).

Co-culture kinetics.

Growth kinetic parameters of *Salmonella* was different (P<0.05) compared when its growing in monoculture and its growing in co-culture with LAB (Table 4). Growth rate (r) was not different (P>0.05) between *Salmonella* monoculture and B9 (0.96 and 0.95), but r was lower in B27, B29 and B40 (0.63, 0.70, 069) followed by B44 (0.93). Meanwhile lowest lag phase (*Lag*) were recorded in B27 (0.35) followed by B29, B40, B44 and B9. Moreover, the maximum population (N_{max}) in all LAB co-cultures was lower (P<0.05) than *Salmonella.* Graphically were observed N_{max} reduction after 10 h (Figure 1).

Table 4. Effect of LAB co-culture on *Salmonella* growth kinetics parameters, at 48h.

	Salmonella	B9	B27	B29	B40	B44
r	0.96^{a}	0.95^{a}	0.63^{c}	0.70^{c}	0.69^{c}	0.83^{b}
Lag	1.76^{a}	1.02^{b}	0.35^{c}	0.66^{bc}	1.02^{b}	1.18^{b}
N_{max}	8.38^{a}	7.35^{b}	6.93^{b}	6.50^{b}	6.51^{b}	6.59^{b}

r= growth rate.
Lag= lag phase.
N_{max}= Maximum cell population (*log* CFU mL^{-1}).

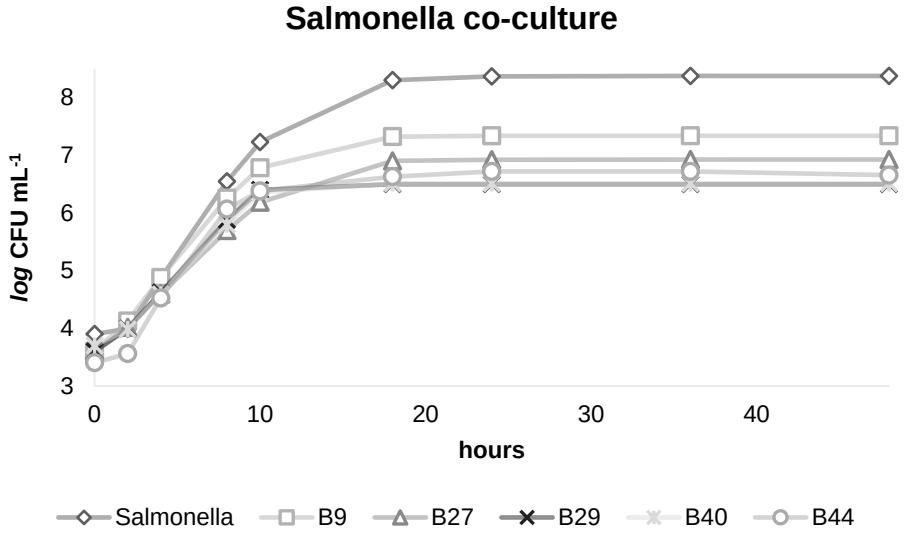

Figure 1. *Salmonella* culture and LAB co-culture growth kinetics, at 48h.

Growth rate (r) of *E. coli* was similar (P>0.05) to B27 and B44 (0.77 and 0.81), but different in B9 and B29 followed by B40 (Table 5). Meanwhile, shortest lag phase (*Lag*) were recorder in B9 and B40 and longest *Lag* in B27, B29, B44 and *E. coli.* However, N_{max} no have differences (P>0.05) among LAB but were less than *E. coli* in monoculture. Graphically were showed a reduction of N_{max} after 10 h (Figure 2).

Table 5. Effect of LAB co-culture on *E. coli* growth kinetics parameters at 48h.

	E. coli	B9	B27	B29	B40	B44
r	0.80[a]	0.60[c]	0.77[a]	0.63[c]	0.69[b]	0.81[a]
Lag	0.76[a]	0.62[b]	0.71[a]	0.74[a]	0.62[b]	0.79[a]
N_{max}	7.90[a]	5.56[b]	6.86[b]	6.01[b]	5.79[b]	6.50[b]

r= growth rate.
Lag= lag phase.
N_{max}= Maximum cell population (*log* CFU mL^{-1}).

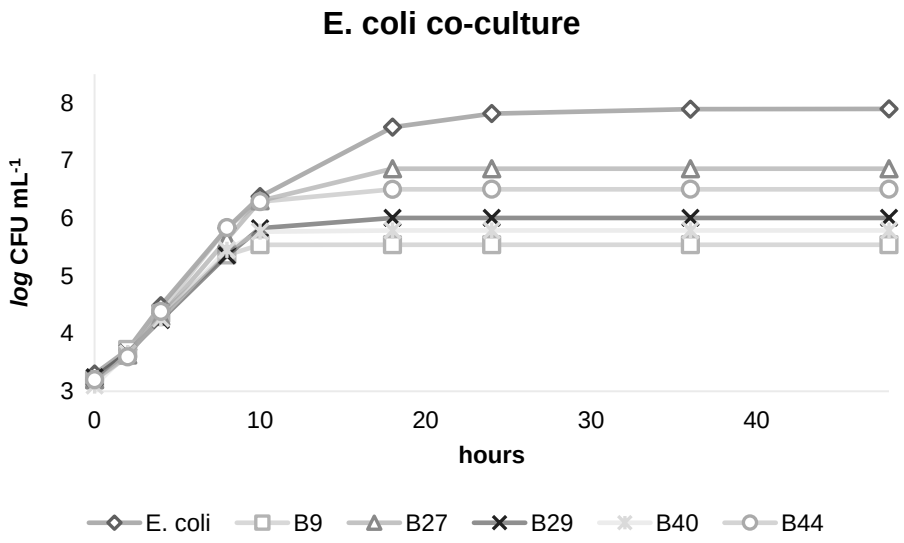

Figure 2. *E. coli* culture and LAB co-culture growth kinetics, at 48h.

Inhibition test

The mean values of inhibition zone radius are shown in Table 6. It is observed biggest *Salmonella* inhibition diameter zone (IDZ) in B40 (6.34 mm) followed by B29, B9, B27 and lowest IDZ were B44 (Table 6).

Table 6. LAB inhibition diameters zone (IDZ) on *Salmonella* and *E. coli*, spot in the lawn test (mm).

	B9	B27	B29	B40	B44
Salmonella	4.16^{ab}	3.64^{ab}	4.26^{ab}	6.34^{a}	3.22^{b}
E. coli	4.3^{a}	3.2^{b}	3.4^{ab}	3.3^{b}	3.3^{b}

Different letters in the lines indicate a statistically significant difference (Tukey test, P<0.05).

Inhibition diameter zone (IDZ) of LAB CFS are shown in for *Salmonella* were in B40 followed by B9 and B29 and lowest in B27 and B44 (Table 7). However, for *E. coli,* B9 was highest IDZ and lowest was B27, no differences (P>0.05) among other LAB strains.

Table 7. Inhibition diameter zone of LAB CFS on *Salmonella* and *E. coli,* agar-wells diffusion assay (AU mL^{-1}).

	B9	B27	B29	B40	B44
Salmonella					
CFS	203.84^{b}	178.36^{c}	208.94^{b}	310.86^{a}	157.98^{c}
CFSH	204.25^{b}	178.72^{c}	209.35^{b}	311.48^{a}	158.29^{c}
CFSHN	202.21^{b}	176.93^{c}	207.26^{b}	308.36^{a}	156.71^{c}
E. coli					
CFS	214.03^{a}	157.98^{c}	168.17^{b}	163.07^{b}	163.07^{b}
CFSH	214.46^{a}	158.29^{c}	168.50^{b}	163.40^{b}	163.40^{b}
CFSHN	212.32^{a}	156.71^{c}	166.82^{b}	161.76^{b}	161.76^{b}

Different letters in the lines indicate a statistically significant difference (Tukey test, P<0.05).
CFS= Cells-free supernatant.
CFSH= Heat treatment cells-free supernatant.
CFSHN= Heat treatment and pH neutralized cells-free supernatant.

3.5 DISCUSSION

According with Zaher & Fujikawa (2011) growth kinetics parameters, rate of growth (r), period of lag phase (*Lag*) and maximum population (N_{max}) are important parameter to describe growth kinetics. LAB with high r, short *Lag* and high N_{max} are parameter desirables for a good medium colonization. Co-culture with LAB were showed reduction of growth parameter of reference bacteria, mainly N_{max}, that reduction can be for a nutrient competition effect (Fujikawa et al.,2014). Also, pH medium reduction is a common mechanism for growth

inhibition, but not is the only way, also LAB can produces antimicrobial substances such as reuterin, surfactants, bacteriocins and bacteriocins-like (Costa, 2013), these are produced during the lag phase and, besides affecting pathogens they also affect spoilage bacteria (Jones et al., 2008).

The inhibition assays, both LAB co-culture growth and the CFS agar well diffusion assay, were obtain moderated positive results. On the other hand, the results of CFS as well as the thermally treated and with neutral pH, were weak, this differs from other works (Alegría et al., 2010; Yan and Lee, 1997), mainly because in other works a semi-purification process was carried out that increased the concentration of the inhibitory substance. Despite this, antagonistic CFS activity was observed and in co-culture it was also reduced by at least 1.5 log units.

Lactococcus lactis (B27) was the LAB isolated from meat that had better behavior, its growth was moderately fast, and its maximum population was the lowest, however as regards the activity of its CFS was higher in *Salmonella* than against *E. coli*, the same was observed in coculture, the reports also indicate the ability to produce lacticin (Mirkovic et al., 2016). Among the most prominent genres to be used in bio preservation protocols, those that have the capacity to produce bacteriocins stand out, in addition they are required not to produce spoilage (Porto, Kuniyoshi, Azevedo, Vitolo, and Oliveira, 2017). In the industry already has the use of bacteriocins to sanitize the surface of meat products such as sausages, in view of this *L. lactis* B40 isolated from the meat could be used for this purpose (Ünlü, Nielsen, and Ionita, 2016).

The consumer reluctance to purchase products with chemical preservatives results in use of alternatives (Djadouni and Kihal, 2012). Use of bacteriocins raises the same ethical question, as it would be use of substances foreign to food. However, it must be borne in mind that various types and quantities of bacteriocins produced by LAB are already consumed in foods such as dairy and fermented meat. To date there have been no reported toxicity problems but some cases of

hypersensitivity or allergy (Abdel-Mohsein, Yamamoto, Otawa, Tada, and Nakai, 2010).

3.6 CONCLUSION

Isolated native of meat LAB strains were modified growth kinetics parameters of reference bacteria (*Salmonella* and *E. coli*), mainly maximum population reduction.

Lactococcus lactis and *Lactobacillus delbrueckii* showed the greatest inhibitory effect on the reference bacteria.

Lactococcus lactis and its CFS showed greater inhibition towards *Salmonella*; while *Lactobacillus delbrueckii* and its CFS inhibited the growth of *E. coli*.

3.7 CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be regarded as a potential conflict of interest.

3.8 ACKNOWLEDGEMENTS

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4 INHIBITION OF *SALMONELLA* and *E. coli* ON GROUND BEEF BY LACTIC ACID BACTERIA

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4.1 Abstract

The objective of this work was to evaluate the inhibitory effect of two Lactic Acid Bacteria (*Lb. delbrueckii* and *L. lactis*) cultures and their cell-free supernatants (CFS) over the growth of reference bacteria (*Salmonella* and *E. coli*) in ground meat at fridge temperature. Ground beef was artificially contaminated with the reference bacteria, samples were taken, and two tests were carried out. In the first, LAB cultures were applied and in the second, LAB CFS was applied to contaminated meat. Subsequently samples were stored at 4 °C. The population of reference bacteria was determined at 0, 1, 4, 8 and 14 days. An analysis of repeated measures over time was performed. The reduction range was from 0.3 to 2 log units, while the LAB CFS showed reduction from first day; however, the reductive effect was lower at each sampling date, the reduction range was from 0.6 to 2 log units. *Lb. delbrueckii* and *L. lactis* showed a reduction effect in the population of reference bacteria, the CFS showed a greater effect in the first days, while the cultures showed a moderate effect in the first days and their greater effect was until day 8. Thus, *L. lactis* had a greater effect compared to *Lb. delbrueckii*, CFS effect is more rapid and *Salmonella* is more sensible than *E. coli*.

Key words: storage, fridge, beef, reduction, LAB, CFS.

4.2 Introduction

Meat is a product of easy colonization by bacteria, its microbiological quality and shelf life depend on the initial bacteria load acquired during the process of slaughter, cutting and handling manipulation (Bruhn et al., 2004; Jackson, 2014). If the meat did not have an adequate hygienic process, this would represent economic losses and health risk. Although, foodborne diseases can use different foods as a vehicle, meat and meat products are important sources of foodborne pathogens that cause human illness (Gómez, Ramiro, Quecan, & de Melo Franco, 2016). The most related pathogens with meat are *Salmonella*, *E. coli* pathogenic serovars, *Campylobacter jejuni, Yersinia enterocolitica* and *Listeria monocytogenes* (Nørrung, 2009).

Salmonella is a large genus, more than 2500 serotypes, and almost all are recognized as pathogenic bacteria, although most of human infections (60-90%) are caused by *S. enterica* ser. Typhimurium and *S. enterica* ser. Enteritidis (Jackson, 2014). Salmonellosis is characterized by fever, diarrhea, nausea and abdominal pain. Its importance lies mainly because its infective dose is low ($<10^3$ for *S. enterica* ser. Thyphimurium), tolerate a low range of water activity (20%), can survive in dehydrated meat, and are even able to survive in atmospheres modified with 20% CO_2. However, it does not tolerate low pH (<4) and its growth is almost at low temperatures (Desmarchelier, 2014; Nørrung, 2009).

On the other hand, *E. coli* is a bacterium that is present in animals' intestinal tract, including human. *Per se* is not pathogenic, although it can produce colibacillosis; this disease is characterized by producing diarrhea, its clinical symptoms are like choleric syndrome and usually be recognized as infantile diarrhea in children and traveler's diarrhea in adults (Estrada-Garcia, Hodges, Hecht, & Tarr, 2013). Also, some *E. coli* serovars are recognized as pathogenic and are classified as enteropathogenic, enteroinfective and enterotoxigenic (Gómez-Aldapa et al., 2013). Its infective dose depends on the serovar. It is commonly found in meat

and can survive and grow at refrigeration temperatures and low pH (Desmarchelier, 2014; Nørrung, 2009).

In these context, risks management of meat and its meat preservation is a challenge (Desmarchelier, 2014). Preservation is based in two types of treatments: bactericide, applying organic acids (lactic, acetic, benzoic, citric, formic, propionic acids) (Theron & Lues, 2007), and bacteriostatic, reducing temperature of meat (cold chain). These treatments are more used because they are cheap and easy to apply in contrast with recent bactericide (high hydrostatic pressure, radiation) and bacteriostatic (modified atmosphere) alternative methods are usually more expensive due to equipment and infrastructure that are required (Xiao & Yang, 2017).

In the present, consumers demand quality foods that are healthy, natural, safety, chemical-free and environmentally friendly, which is a challenge because quality deterioration is a complex course for perishable food (Desmarchelier, 2014; Nørrung, 2009; Xiao and Yang, 2017). Meat is an easy bacteria colonization product, its shelf life depends on first bacterial load, during slaughter and cutting, and technics of hygienic manipulation. If meat do not have an adequate process, it can result in bad quality and shelf life reduction, what would represent economic losses and health risk. For all the above, the objective of this work was to evaluate the inhibitory effect of LAB cultures as well as their CFS to reduce the growth of *Salmonella* and *E. coli* in ground beef under fridge conditions.

4.3 Materials and Methods

4.3.1 Study area and ground beef

Ground beef was purchased at a retail store in Texcoco de Mora, Mexico. Meat was examined for contamination with *Salmonella* sp. and *E. coli* by conventional method (NOM-114-SSA1-1994). After thorough mixing, meat was placed in sterile plastic bottles and storage at -20 °C. For use, bottles with meat were thawed at fridge temperature (4 °C) overnight.

4.3.2 Bacterial cell preparations

The strains of *Salmonella sp*., *Escherichia coli*, and two LAB strains *Lactobacillus delbrueckii* (B9) and *Lactococcus lactis* (B40) were used, isolated of a previous study. Bacteria were conserved in plastic tubes with glycerol (25%) at -20 °C. For activation, bacteria strains were streaked in agar plates with appropriate medium, De Man, Rogosa and Sharpe (MRS) for LAB and nutritive agar for reference bacteria (*E. coli* and *Salmonella*), and incubated 24 h at 37 °C. After, well-grown colonies were taken and incubated in appropriate broth, MRS broth for LAB and brain heart infusion (BHI) for reference bacteria and incubated at 37 °C until turbidity was as 4 McFarland scale. 1 mL of culture was washed trice with sodium chloride solution 0.85% w/v (saline) by centrifugation at 10,000 rpm at 4 °C for 15 min, harvested bacteria were resuspended in 1 mL of saline solution for immediate use.

4.3.3 Cells-free supernatants

LAB B1 and B2 were streaked in MRS agar plates and incubated 48 h at 37 °C. Well-growth colonies were taken and cultured in MRS broth at 37 °C in oxygen low condition for 72 h. Later, culture was bathed in boiling water for 10 min, pH was neutralized with NaOH (0.1 M) and centrifuged at 10,000 rpm for 15 minutes at 4 °C. Supernatant were decanted in sterile tubes and stored at -20 °C.

4.3.4 Experimental treatments

LAB and CFS inhibition assay

Assay was performed with each LAB culture (B1 and B2) with the following ground beef treatments: Control (T) without inoculated bacteria, LAB inoculated (B), *E. coli* inoculated (E), *Salmonella* inoculated (S), LAB + *E. coli* (BE), LAB + *Salmonella* (BS), and LAB + *E. coli* + *Salmonella* (BES). Inoculation was made according to Sabike, Fujikawa & Edris (2015), taken ground beef portions, previously thawed, for each treatment and placed in sterile recipients; then, it was

inoculated with each bacterium culture at reason of 2 mL 100 g^{-1} with around 3 log CFU g^{-1} and mixed thoroughly with sterile steel spoon. In sterile glass bottles with a screw cap were placed 10 g of meat corresponding to each treatment by triplicate and placed at refrigeration temperature (4 °C).

CFS inhibition assay

CFS inhibition test was performed whit the following ground beef treatments: Control (T) without inoculation, *E. coli* inoculated (E), *Salmonella* inoculated (S), *E. coli* + CFS (CE), *Salmonella* + CFS (CS), and *E. coli* + *Salmonella* + CFS (CES). Ground beef inoculation was performed in the same manner as in previous test; 1mL of CFS were applied and mixing thoroughly, after taking 10 g of each treatment and placed on a glass sterile tubes with screw caps by triplicated. Then, treatments were storaged at constant fridge temperature (4 °C).

4.3.5 Bacterial counts

Bacterial cell counts were made according to Sabike et al. (2015). Samples were mixed with 90 mL of sodium chloride-peptone solution. After thoroughly mixing, the samples were 10-fold serial diluted with saline. Surfacing-plated method were performed by duplicate. Culture medium were depending on kind of bacteria was count: De Man, Rogosa and Sharpe (MRS) agar was used to LAB, eosin and methylene blue (EMB) agar for *E. coli*; and xylose lysine deoxycholate (XLD) agar for *Salmonella*. The counting times were 0, 1, 4, 8, 11 and 14 days of storage.

4.3.6 Statistical analysis

Results were analyzed with proc mixed model for repeated measures in the Statistical Analysis System (SAS Institute Inc., 9.4). Least squares mean were used to evaluate the effect of treatment at various times in the experiments.

4.4 Results

4.4.1 LAB inhibition

The results of the *E. coli* inhibition test in ground beef by LAB culture, B9 and B44, are shown in Figure 3. *E. coli* content of natural microbiota (TE) had a slight growth during the first 4 counts, and later a more accelerated growth was observed. The *E. coli* initial inoculated load (day 0) had no difference among all treatments ($p>0.05$). In day one, E treatment was showing a decrease in the population, may be due to the effect of temperature change and adaptation process; after that day, its growth was constant. The BE9 and BES9 treatments did show significant difference ($p<0.05$) in days 1 and 4, while in the following days there was no difference; also, no significant reduction ($p>0.05$) of inoculated *E. coli* was observed. On the other hand, no significant difference ($p>0.05$) between BE40 and BES40 treatments was observed; and significant reduction ($p<0.05$) of *E. coli* was observed since day 4 until day 11. The greatest *E. coli* reduction was observed at day 8 and 11, with an average difference of 2 log units; however, at day 14 the reductions were minimal.

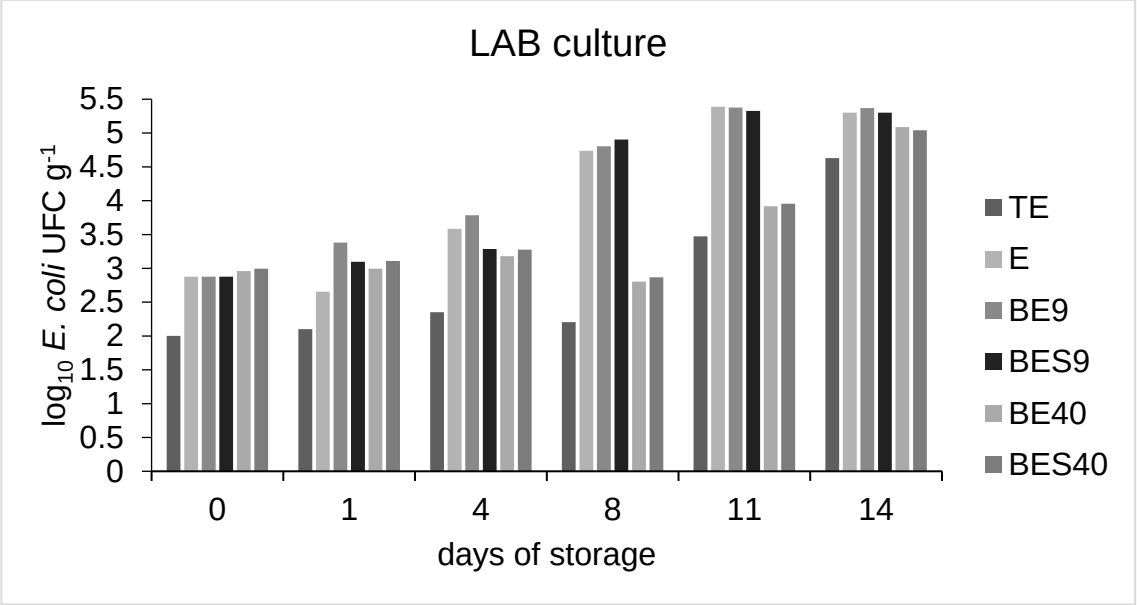

Figure 3. *E. coli* population in ground beef inoculated with cultures of *Lb. delbrueckii* (B9) and *L. lactis* (B40) at 4 °C for 14 days.

The *Salmonella* counts from meat inoculated with LAB are shown in Figure 4. Ground meat utilized did not show *Salmonella* contamination (TS), and initial *Salmonella* load of treatments were not significantly different ($p<0.05$). In all sampling days, *Salmonella* population reductions were observed with respect to S treatment. BS9 and BES9 treatments presented slight reductions; on the contrary, treatments with BS40 and BES40 reduced the *Salmonella* population by an average of 2 log units, in days 4, 8 and 11, later the reduction was smaller.

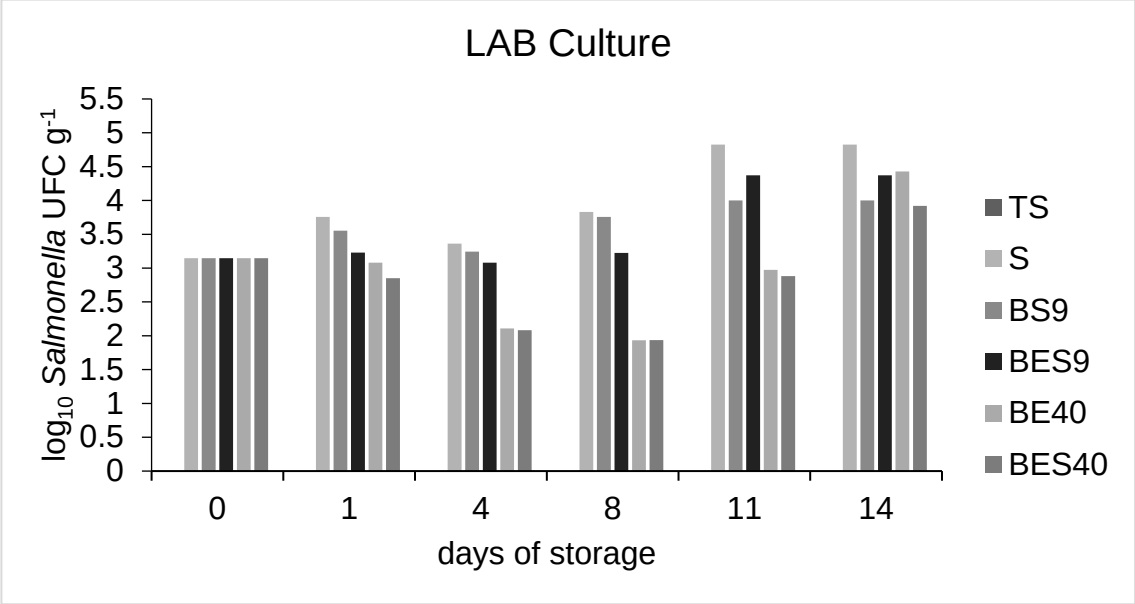

Figure 4. *Salmonella* population in ground beef inoculated with cultures of *Lb. delbrueckii* (B9) and *L. lactis* (B40) at 4 °C for 14 days.

4.4.2 CFS inhibition

The LAB CFS also reduced the population of *E. coli* as shown in the Figure 5. Native *E. coli* population (TE) had a growth since day zero to day 8 and afterwards it remained constant. Meanwhile, *E. coli* inoculated (E) had a slight growth on days 11 and 14. The reduction of *E. coli* was observed from day 1 in all treatments, the average daily reduction ($p<0.05$) was 0.6 log units; E40 treatment showed the greatest reduction (1 log unit) on day 11.

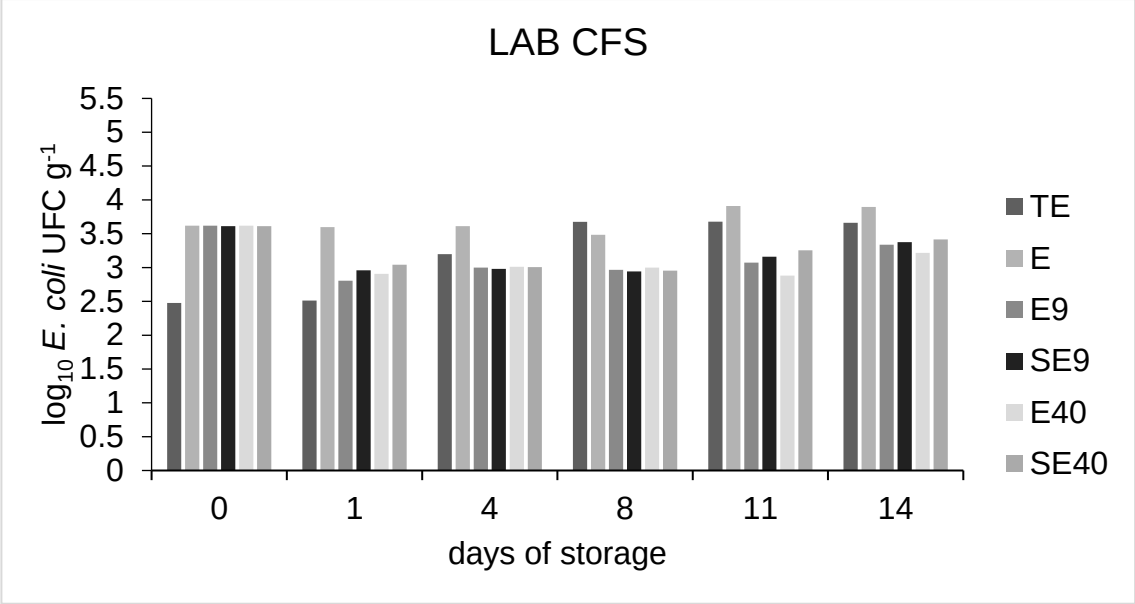

Figure 5. *E. coli* population in ground beef treated with CFS of *Lb. delbrueckii* (9) and *L. lactis* (40) at 4 °C for 14 days.

On the other hand, the population of *Salmonella* was also reduced by action of LAB CFS, as shown in the Figure 6. The ground meat used did not present contamination by *Salmonella* (TS). Population of *Salmonella* inoculated treatment (S) remained constant over time. A reduction ($p<0.05$) of the population with respect to S treatment was observed every day. On day one sampling date, the greatest reduction was observed, treatments S9 and S40 reduced on average 1.5 log units, while treatments where there was presence of *E. coli*, SE9 and SE40, reduction was on average 0.6 log units. In the following days, reductions were observed in the *Salmonella* population, although it was in a smaller proportion.

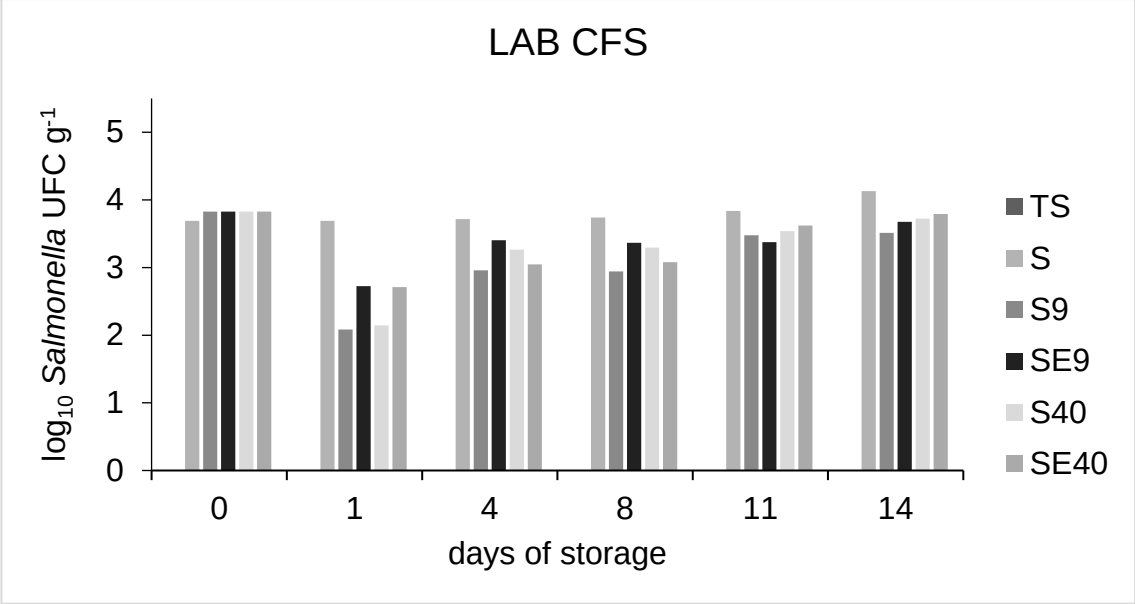

Figure 6. *Salmonella* population in ground beef treated with CFS of *Lb. delbrueckii* (9) and *L. lactis* (40) at 4 °C for 14 days.

4.5 Discussion

The results obtained in this study show that LAB strains used managed to reduce the population of the reference bacteria in raw ground beef. According to Simova, Beshkova, Angelov, & Dimitrov (2008) the antagonistic effect observed in *Lb. delbrueckii*, against psychrophilic as well as coliforms bacteria, is mainly due to the action of hydrogen peroxide produced under aerobic growth conditions; however, although several authors indicate that hydrogen peroxide is effective in reducing the growth of *E. coli* and *Salmonella* (Senne & Gilliland, 2003; Simova et al., 2008), some authors indicate that under special conditions these bacteria can produce enough catalase that protects them from the reduction effect of peroxide (Finn & Condon, 1975; Humphrey, Slater, McAlpine, Rowbury, & Gilbert, 1995; Watson & Schubert, 1969); This could explain weak population reduction of *E. coli* and *Salmonella* observed; however, a reduction was observed when the CFS of *Lb. delbrueckii* was used; and considering that hydrogen peroxide loses effectiveness when is heated, it is considered that there are other antimicrobial agents, such as bacteriocins (Boris, Jimenez-Diaz, Caso, & Barbes, 2001, Toba, Yoshioka, & Itoh, 1991).

On the other hand, *L. lactis* presented a greater population reductive effect for both bacteria, because the conditions in which the experiment was presented were aerobic, it is assume the production and presence of hydrogen peroxide in the meat as well as in the CFS (Duwat et al., 2001), and considering the effectiveness reduction of hydrogen peroxide due to the thermal treatment of CFS, it was considered that the effect of population reduction was due to other substances, like bacteriocins. *L. lactis* is characterized as a bacteriocin-producing bacterium; some strains can synthesize nisin (Kelly, Asmundson, & Huang, 1996), lactococcin (Holo, Nilssen, & Nes, 1991), lacticin, lactocyclin (Zendo, Ishibashi and Sonomoto, 2014), among others. Therefore, it is feasible that the meat native strain used in this experiment, produces some bacteriocin or like-bacteriocin substance.

This could give the possibility of being used as a bio-preservative of ground beef; however, the population reduction of *E. coli* and *Salmonella* were moderated. The observed reduction range was of 0.5 to 2 log units. These results can be compared with those obtained by Smith et al. (2005), where the reduction of the population of *E. coli* and *Salmonella* were observed in range of 2 to 5 log units. This difference can be attributed to initial LAB concentration (7 log CFU g^{-1}), that was 2 log unit higher than reference bacteria (5 log CFU g^{-1}). The effectiveness of using live LAB to reduce bacterial contamination and to improve the meat microbiological quality was demonstrated (Buncic et al., 2014). However, some studies indicate that these bacteria can also impair quality and reduce meat shelf-life (Pothakos, Devlieghere, Villani, Björkroth, & Ercolini, 2015). Some LAB genera in meat, like those used in this study, can generate gas, polysaccharide slime (Hamasaki, Ayaki, Fuchu, & Sugiyama, 2003; Lücke, 2000) and its activity could cause rarely odor, color change (Gram et al., 2002) and biofilm production (Gómez et al., 2016). While the latter have been considered as bio-protectors that prevent colonization by pathogenic bacteria (Gómez et al., 2016), some studies indicate that moderate concentrations of *Lb. delbrueckii* (5.3 log CFU g^{-1}) (Hovhannisyan et al. 2016) and *L. lactis* (Jones, Hussein, Zagorec, Brightwell, &

Tagg, 2008) manage to reduce the population of *E. coli* to 3 log units in a period of three days without altering the organoleptic characteristics. Although several authors indicate that the characteristics described above are positive for the implementation of said LAB in bio-conservation protocols, in ground beef, the effect is not immediate. USDA recommends to eat ground meat immediately after the purchase and not be stored in refrigeration for more than two days; taking this into account, the use of lactic cultures is outdated, the lactic culture must adapt and then produce antagonism factors. That effect was observed until day 4, while the CFS reduction effect was observed since first day and was reduced along the time; similar results were reported by Ahn, Kim, & Kim, (2017) and Roldán et al. (2011).

In addition, it should be considered that some aspects of the control system are specific for each group of pathogens. Meat pathogens such as *Salmonella* and *E. coli* are controlled by measures applied during the first phases of the production chain (farm), in addition to the optimization of slaughterhouse hygiene. Unlike others pathogens, such as *Listeria monocytogenes*, what are better controlled during the later stages of processing and storage (Kopper et al., 2014). This opens the possibility of using LAB cultures as probiotics to reduce animal pathogen population and contamination at slaughter time.

The spoilage processes even take place in meat at refrigeration temperature, and depends on the associations with other bacteria, such as those of Enterobacteriaceae genus that can grow at refrigeration temperatures; these results mean that variation in the refrigeration temperature can favor pathogens growth (Bruhn et al., 2004; Ross, Ratkowsky, Mellefont, & McMeekin, 2003), so that the lactic cultures could also be used to protect the meat in cases of cold chain rupture.

4.6 Conclusions

Cell-free supernatant of lactic acid bacteria showed more effective and faster reduction effect compared to lactic acid bacteria cultures, but its effect was reduced along time.

Based on population reduction of reference bacteria, *Lactococcus lactis* is more effective than *Lactobacillus delbrueckii.*

4.7 Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be regarded as a potential conflict of interest.

4.8 Acknowledgements

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