Centro de Investigación en Alimentación y Desarrollo, A.C.

AISLAMIENTO Y CARACTERIZACIÓN DE BACTERIOCINAS PRODUCIDAS POR CEPAS DE *Lactobacillus spp.* AISLADAS DE QUESO COCIDO ARTESANAL MEXICANO

Por:

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TABLA DE CONTENIDO

RESUMEN

Los alimentos son susceptibles a contaminación por diferentes bacterias patógenas durante y posterior al procesamiento. Por lo tanto, es importante conocer nuevas alternativas naturales que ayuden a la conservación de estos. Las bacterias ácido lácticas (BAL) se han reportado como productoras de péptidos antimicrobianos tales como las bacteriocinas. Por lo anterior, el objetivo de este trabajo fue aislar y caracterizar bacteriocinas producidas por BAL nativas para su uso en la bioconservación de alimentos. Los resultados mostraron que los extractos obtenidos de la fermentación por *Lactobacillus spp*. en medio de cultivo presentaron actividad antimicrobiana contra bacterias patógenas indicadoras. Los extractos que presentaron la mayor actividad antimicrobiana ($p < 0.05$) obtenidos de *Lactobacillus fermentum* (J23 y J32), se separaron por ultrafiltración y se analizó la fracción ≤3 kDa por cromatografía líquida de fase reversa (RP-HPLC). Se evaluó la actividad antimicrobiana de las diferentes fracciones colectadas de RP-HPLC. La fracción F3 de J23 presentó actividad antimicrobiana contra *E. coli* y fue también inhibitoria contra *S. aureus, L. innocua, S. typhimurium* y *S. cholerae*. Por el contrario, las fracciones F1, F5 and F7, presentaron actividad antimicrobiana contra *S. aureus* y *L. innocua* pero no para el resto de los microorganismos indicadores. Similarmente, F1 obtenida de *Lactobacillus fermentum* J32, presentó actividad antimicrobiana contra *S. aureus* y *L. innocua,* pero no contra el resto de los microorganismos. Por otro lado, F3 and F4 de *Lactobacillus fermentum* J32 presentaron actividad antimicrobiana contra todos los microorganismos indicadores. De las fracciones de RP-HPLC colectadas J23 y J32, se identificaron las secuencias de 6 y 9 péptidos, respectivamente, por espectrometría de masas por trampa de iones (MS/MS). Posteriormente, se evaluó la capacidad antimicrobiana de las fracciones obtenidas de J23 y J32 en leche pasteurizada. Las cuentas microbianas de *S. aureus, L. innocua*, *E. coli y S. typhimurium,* fueron significativamente ($p < 0.05$) menores en la leche adicionada con las fracciones de J23 o J32 que en el grupo testigo (leche pasteurizada

inoculada con el patógeno indicador). Por lo que, las fracciones de J23 y J32 presentaron bacteriocinas inhibitorias para los patógenos indicadores evaluados. Las bacteriocinas producidas por *L. fermentum* J23 y J32 presentan un excelente potencial para su aplicación en la bioconservación de productos lácteos.

Palabras clave: Bacterias ácido lácticas, bacteriocinas, bioconservación, actividad antimicrobiana, patógenos

ABSTRACT

Foods are susceptible to contamination by different pathogenic bacteria during and after processing. Therefore, it is important to look for new natural alternatives to help the preservation of foods. Lactic acid bacteria (LAB) have been reported as producing antimicrobial peptides such as bacteriocins. Therefore, the objective of this work was to isolate and characterize bacteriocins produced by native BAL for use in food bioconservation. The results showed that the extracts obtained from the fermentation of Lactobacillus spp. presented antimicrobial activity against pathogenic indicator bacteria. The extracts that presented the highest antimicrobial activity obtained from Lactobacillus fermentum (J23 and J32) were treated by ultrafiltration and the fractions of less than 3 kDa were analyzed by reverse phase liquid chromatography (RP-HPLC). Antimicrobial activity of the differente fractions collected by RP-HPLC was evaluated. Fraction F3 from J23 had antimicrobial activity against *E. coli* and was also inhibitory against *S. aureus*, *L. innocua* and *S. typhimurium*. In contrast, fractions F1, F5 and F7, showed higher antimicrobial activity against *S. aureus* and *L. innocua* but not for the other indicators microorganisms. Similarly, F1 obtained from *Lactobacillus fermentum* J32 presented antimicrobial activity against *S. aureus* and *L. innocua*, but not against other microorganisms. In addition, F3 and F4 produced by Lactobacillus fermentum J32 showed antimicrobial activity against all microorganism indicators. RP-HPLC fractions from J23 and J32, contained 6 and 9 peptides, respectively, identified by ion trap mass spectometry (MS / MS). Microbial counts for *S. aureus, L. innocua*, *E. coli* and *S. typhimurium* were significantly lower ($p < 0.05$) in milk with fractions from J23 or J32 than the control (pasteurired milk inoculated with the indicator pathogen). Thus, fractions from J23 and J32 presented bacteriocins inhibitory agaist the indicator pathogens evaluated. Bacteriocins produced by L. fermentum J23 and J32 have excellent potential for their application in the preservation of dairy foods.

Key words: Bacteria acid lactic, bacteriocins, antimicrobial activity, pathogens

Capítulo 1

Integración general

INTRODUCCCIÓN

Las bacterias ácido lácticas (BAL) son un grupo de microorganismos de morfología variada que se caracteriza por sintetizar ácido láctico como su principal metabolito. Algunas BAL son utilizadas como cultivos iniciadores en la industria alimentaria debido a su capacidad para producir cambios estructurales y sensoriales deseables en una gran variedad de alimentos fermentados, tales como los derivados de productos lácteos, vegetales, productos cárnicos, productos de panadería y bebidas alcohólicas entre otros. Sin embargo, para que las BAL puedan ser utilizadas en los alimentos deben ser reconocidas como seguras, es decir, deben tener grado GRAS (Campos, 2002; Topisirovic et al., 2006; Parada et al., 2007).

Las BAL también juegan un papel importante en la bioconservación de los alimentos, debido a que son capaces de producir sustancias antimicrobianas como el diacetilo, peróxido de hidrógeno, acetaldehído, compuestos no proteicos de bajo peso molecular y las bacteriocinas, siendo estas últimas las que han despertado un mayor interés por la industria alimentaria por su eficacia para eliminar microorganismos patógenos (Arqués et al. 2011; Balciunas et al., 2013).

El uso de las bacteriocinas como bioconservantes se atribuye a sus características para inhibir diferentes microorganismos patógenos, su acción en amplios pHs y termoestabilidad. Las bacteriocinas son péptidos con actividad antimicrobiana, producidas por diferentes bacterias para inhibir el crecimiento de otros microorganismos patógenos como *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella spp*., *Escherichia coli*, entre otras (Ghanbari et al., 2008). Estos péptidos actúan sobre la membrana y pared celular (Karapetyan et al., 2010).

Existe una gran diversidad de bacteriocinas reportadas en la mayoría de los géneros bacterianos, incluso dentro de una misma bacteria podrían producirse diferentes tipos de bacteriocinas (Cintas et al., 2001).

Las bacteriocinas son sintetizadas en el ribosoma de las BAL con más de 60 aminoácidos y pueden ser péptidos moléculas alongadas o péptidos de moléculas globulares con un amplio rango de peso molecular (Naghmouchi, *et al*., 2007). La célula productora sintetiza una molécula que la inmuniza contra la propia bacteriocina. Las bacterias productoras de bacteriocinas se auto protegen de la toxicidad de estos compuestos (Kemperman *et al*., 2003). Así la bacteria puede seguir reproduciéndose y liberando compuestos antimicrobianos en el alimento, lo cual da estabilidad al producto y logra períodos de vida de anaquel más largos (Snyder y Worobo, 2013).

Actualmente, las bacteriocinas nisina y pediocina PA-1 tienen liciencia para su uso como bioconservantes en la industria alimentaria (Zacharoff et al., 2012). La nisina (aislada de *L. Lactis*) en su presentación comercial (Nisaplin®) ha demostrado ser efectiva en el control de *C. tyrobutyricum* (Vuyst y Vandamme, 1994). En el procesado de quesos, se aplica como control del desarrollo de microorganismos esporulados y productores de gas como los clostridios (incluso *C. Botulinum), L. monocytogenes y S. aureus* (Thomas et al., 2001). Por otro lado, pediocina PA-1 (*aislada de Pediococcus acidilactici*) se utiliza en forma de “Alta 2431“ (Quest®), se utiliza en empaques al vacío de salchichas tipo Viena, para el control de *L. monocytogenes* (Simha, et al., 2012).

Es de gran interés que BAL utilizadas de manera tradicional puedan sintetizar compuestos con actividad antimicrobiana contra microorganismos patógenos de interés en alimentos. De manera particular, el estudio de las bacteriocinas producidas por cepas nativas de quesos artesanales mexicanos es escaso, y solamente se han reportado las propiedades físicas y bioquímicas. Por lo anterior, este trabajo de investigación tiene como objetivo aislar y caracterizar bacteriocinas producidas por cepas de *Lactobacillus ssp*. para estudiar su capacidad de inhibir el crecimiento de patógenos específicos.

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HIPÓTESIS

Cepas aisladas de *Lactobacillus spp.* aisladas de queso Cocido artesanal mexicano producen bacteriocinas capaces de inhibir el crecimiento de patógenos específicos.

OBJETIVOS

Objetivo general

Aislar y caracterizar bacteriocinas producidas por cepas de *Lactobacillus spp.* para estudiar su capacidad de inhibir el crecimiento de patógenos específicos.

Objetivos particulares

1. Evaluar la capacidad antimicrobiana de extractos crudos (EC) obtenidos de cultivos de *Lactobacillus spp.* contra patógenos específicos y seleccionar aquellos con mayor capacidad.
2. Determinar la capacidad antimicrobiana asociada a bacteriocinas de los EC seleccionados y su resistencia a diferentes tratamientos.
3. Aislar y caracterizar bacteriocinas a partir de los EC seleccionados para evaluar su capacidad antimicrobiana contra patógenos específicos.
4. Evaluar la capacidad antimicrobiana de las bacteriocinas aisladas contra patógenos en leche pasteurizada.

INTEGRACIÓN DEL MANUSCRITO

La información de esta tesis está dividida en 4 capítulos, los cuales se muestran resumidos a continuación.

Capitulo 1. Bacteriocinas de bacterias ácido lácticas: Mecanismos de acción y actividad antimicrobiana contra patógenos en quesos.

Heredia-Castro, Priscilia Yazmín; Hérnández-Mendoza Adrian; González-Córdova, Aarón Fernando; Vallejo-Cordoba, Belinda.

En este capítulo se presenta una revisión sobre la evidencia cíentifica disponible sobre la aplicación de bacteriocinas en quesos. Se revisa las características de las bacteriocinas, clasificación y los mecanismos de acción que presentan. También, se menciona como es la actividad antimicrobiana de bacteriocinas contra patógenos en quesos y como es la actividad de BAL productoras de bacteriocinas *in situ* en quesos. Además, se describieron los aspectos que se deben considerar para la aplicación de bacteriocinas en quesos: 1) el efecto antimicrobiano de las bacteriocinas no solo actúa contra patógeno, sino puede afectar a la microflora natural, 2) tener en cuenta la carga microbiana del patógeno, 3) la producción de bacteriocinas se vincula con el crecimiento celular. Este capítulo, se encuentra en revisión por la Revista INTERCIENCIA.

Capítulo 2. Antimicrobial activity and partial characterization of bacteriocin-like inhibitory substances produced by *Lactobacillus* spp. isolated from artisanal Mexican cheese.

Priscilia Y. Heredia-Castro, Aarón F. González-Córdova, Adrián Hernández-Mendoza, Evelia Acedo-Félix, BelindaVallejo-Cordoba.

En este capítulo se evaluó la actividad antimicrobiana de diferentes cepas de *Lactobacillus spp.* aisladas de Queso Cocido contra diferentes microorganismos patógenos. Se comprobó que los extractos obtenidos de las cepas de *Lactobacillus spp.* eran de naturaleza proteíca. Además, tres cepas presentaron actividad antimicrobiana contra bacterias Gram positivas y Gram negativas, las cuales presentan gran potencial para su aplicación en alimentos. Este capítulo se encuentra publicado en la Revista Journal of Dairy Science.

Capítulo 3. Partial purification and characterization of bacteriocin produced by specific *Lactobacillus fermentum* isolated from artisanal Mexican Cheese.

Priscilia Yazmín Heredia-Castro, Adrian Hernández-Mendoza, Aarón Fernando González-Córdova, Carmen Estrada-Montoya, María de Jesús Torres-Lanez, Belinda Vallejo-Galland.

En este capítulo se presenta el aislamiento y la caracterización de las bacteriocinas producidas por *Lactobacillus fermentum* aisladas de queso Cocido. Los extractos de *Lactobacillus fermentum* J23 y J32, fueron caracterizados por RP- HPLC. Se colectaron fracciones de cada uno de los extractos y se evaluó la capacidad antimicrobiana de las mismas. Además, las estructuras químicas de los péptidos en las diferentes fracciones colectadas fueron identificadas por espectrometría de masas. Este capítulo se enviará a la revista International Journal of Dairy Technology.

Capítulo 4.

Effect of bacteriocin containing fractions produced by *Lactobacillus fermentum* on the inactivation of pathogens in pasteurized milk

Priscilia Yazmín Heredia-Castro, Jesús Sosa-Castañeda, Adrian Hernández-Mendoza, Aarón Fernando González-Córdova, María de Jesús Torres-Llanez, Belinda Vallejo-Galland.

En este capítulo se presenta el efecto de fracciones conteniendo bacteriocinas obtenidas de *Lactobacillus fermentum* contra diferentes patógenos en leche pasteurizada durante 15 días de almacenamiento a 4 C. Se pudo observar que las fracciones fueron efectivas en la reducción de la cuenta microbiana de *S. aureus, L. innocua*, *E. coli* y *S. typhimurium* en leche pasteurizada. Estas fracciones conteniendo bacteriocinas presentan un excelente potencial para su aplicación en la industria alimentaria. Este capítulo se enviará a la revista International Journal of Food Microbiology.

CONCLUSIONES

En este estudio, *Lactobacillus spp.* aislados de queso Cocido artesanal mexicano mostraron actividad antimicrobiana contra *S. aureus, L.innocua, E.coli y S. typhimurium* usando el método de difusión en disco. De las cepas estudiadas, J23 y J32 fueron las que presentaron mayor actividad antimicrobiana. De las fracciones obtenidas de *L. fermentum* J23 por RP-HPLC, la fracción F3 presentó mayor actividad antimicrobiana contra *E.coli* y las fracciones F5 y F7 mostraron mayor actividad antimicrobiana contra *S. aureus* y *L. innocua*. Las fracciones F3 y F4 obtenidas de *L. fermentum* J32, presentaron actividad antimicrobiana contra todos los microorganismos indicadores. Estos resultados sugieren que ambas cepas de *Lactobacillus fermentum* evaluadas produjeron al menos dos diferentes bacteriocinas. Además, las fracciones obtenidas de J23 y J32 utilizadas en leche pasteurizada, redujeron la cuenta microbiana de *S. aureus, L. innocua*, *E. coli y S. typhimurium.* Por todo lo anterior, las bacteriocinas producidas por *L. fermentum* J23 y J32 presentan gran potencial para ser utilizados como agentes antimicrobianos en productos lácteos. Se requieren más estudios para evaluar su efectividad como bioconservantes en quesos mexicanos.

Capítulo 2

Bacteriocinas de bacterias ácido lácticas: Mecanismos de acción y actividad antimicrobiana contra patógenos en quesos.

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Resumen.

Las bacterias ácido lácticas (BAL) son microorganismos que han sido utilizadas durante décadas por la industria, debido a que confieren características sensoriales y reológicas deseables en los productos lácteos. Ciertas BAL tienen la capacidad de conservar productos lácteos y esta característica en particular se debe a diferentes metabolitos, dentro de los cuales se encuentran las bacteriocinas. Las bacteriocinas son péptidos de origen ribosomal que actúan principalmente formando poros en la membrana celular de las bacterias causándoles la muerte. El uso de bacteriocinas como bioconservantes se debe a que puede inhibir diferentes microorganismos patógenos, es estable a diferentes pH y temperaturas. Debido a estas caracteristicas, la aplicación de bacteriocinas tienen una potencial aplicación en la industria alimentaria. Particularmente, los quesos presentan problemas frecuentes de contaminación por patógenos, por lo cual, una alternativa que se ha considerado es el uso de bacteriocinas, sin embargo, a pesar de que se ha reportado que su uso puede verse limitado, la evidencia indica que su aplicación en forma libre puede controlar la contaminación por patógenos. Además, se han propuesto estrategias para mejorar su actividad, como lo son el tratamiento térmico, la aplicación en forma de liposomas y películas. En conclusión, la utilización de bacteriocinas o BAL productoras de bacteriocinas en quesos podría ser viable para su utilización como control sanitario en la industria quesera.

Bacteriocinas de bacterias ácido lácticas: Mecanismos de acción y actividad antimicrobiana contra patógenos en quesos

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Resumen

Las bacterias ácido lácticas (BAL) son microorganismos que han sido utilizadas durante décadas por la industria, debido a que confieren características sensoriales y reológicas deseables en los productos lácteos. Ciertas BAL tienen la capacidad de conservar productos lácteos y esta característica en particular se debe a diferentes metabolitos, dentro de los cuales se encuentran las bacteriocinas. Las bacteriocinas son péptidos de origen ribosomal que actúan principalmente formando poros en la membrana celular de las bacterias causándoles la muerte. El uso de bacteriocinas como bioconservantes se debe a que puede inhibir diferentes microorganismos patógenos, es estable a diferentes pH y temperaturas. Debido a estas caracteristicas, la aplicación de bacteriocinas tienen una potencial aplicación en la industria alimentaria. Particularmente, los quesos presentan problemas frecuentes de contaminación por patógenos, por lo cual, una alternativa que se ha considerado es el uso de bacteriocinas, sin embargo, a pesar de que se ha reportado que su uso puede verse limitado, la evidencia indica que su aplicación en forma libre puede controlar la contaminación por patógenos. Además, se han propuesto estrategias para mejorar su actividad, como lo son el tratamiento térmico, la aplicación en forma de liposomas y películas. En conclusión, la utilización de bacteriocinas o BAL productoras de bacteriocinas en quesos podría ser viable para su utilización como control sanitario en la industria quesera.

Introducción

Hoy en día, las BAL son ampliamente utilizadas en la industria alimentaria por su capacidad de conferir diferentes características sensoriales como textura, sabor y olor agradable a los alimentos fermentados (Parra Huertas, 2010; O'Bryan *et al.*, 2015a). Las BAL son un grupo de bacterias clasificadas como Gram positivas, no forman esporas, no presentan motilidad, pueden tener forma de cocos o bacilos y pueden ser microaerofílicos o anaerobios facultativos. Las BAL se caracterizan por producir ácido láctico durante el proceso de la fermentación como su principal metabolito (Monroy *et al.*, 2009; Siamansouri *et al.*, 2013). Se tiene conocimiento que desde el siglo XVIII, en África, Asia y Europa, ya se utilizaban los cultivos de BAL para fermentar la leche. En las últimas décadas, se ha explorado el potencial de las BAL como bioconservador natural de productos lácteos; y esto se debe a la producción de diversos metabolitos como el ácido láctico, peróxido de hidrógeno, diacetilo, dióxido de carbono (CO_2) y bacteriocinas (Siamansouri *et al.*, 2013; O'Bryan *et al.*, 2015b); siendo estas últimas las que han despertado mayor interés por parte de algunos investigadores. Dentro de las ventajas que se han considerado para la aplicación y uso de las bacteriocinas, se encuentran las siguientes: son consideradas como seguras para el consumo humano (grado GRAS por sus siglas en inglés); al biodegradarse no forman compuestos secundarios; no causan toxicidad en células eucariotas y presentan un espectro de inhibición más amplio que las producidas por bacterias Gram negativas. Además, su aplicación no solo se enfoca como bioconservador de alimentos, sino también en cosméticos y como tratamientos biomédicos contra infecciones

en la medicina humana y veterinaria (Dolz, 2008; López *et al.*, 2008; Beristain-Bauza *et al.*, 2012; Józefiak y Sip, 2013; Yang *et al.*, 2014). Aunque en un principio se pensaba que las bacteriocinas solo actuaban contra bacterias estrechamente relacionadas con la cepa productora, en años recientes este concepto ha cambiado, ya que se han encontrado bacteriocinas que pueden actuar contra cepas distanciadas filogenéticamente con la cepa productora (Dolz, 2008). En las últimas décadas, los estándares de calidad en la industria alimentaria cada vez son más exigentes. Particularmente, en los quesos se han encontrado una diversidad de microorganismos patógenos, debido a que la mayoria son elaborados con leche cruda, la composición que presenta es una fuente rica de nutrientes para los microorganismos y la manipulación en su proceso de elaboración. Se ha propuesto que las bacteriocinas o las BAL productoras de bacteriocinas pueden ser una alternativa natural para su conservación. Sin embargo, se ha reportado que su uso podría verse limitado debido a las características físicas y químicas del propio queso (Kousta *et al.*, 2010; Jeanson *et al.*, 2011; Aly *et al.*, 2012; Favaro *et al.*, 2015). Por otro lado, se han planteado dos modos de acción de las bacteriocinas, el bactericida y el bacteriostático, y se proponen varios tipos de mecanismos de acción de acción (Beristain-Bauza *et al.*, 2012; Józefiak y Sip, 2013). Por lo anterior, el objetivo de ésta revisión es actualizar la información disponible sobre las características generales de las bacteriocinas, su clasificación y su mecanismo de acción. Principalmente, se analizará la actividad antimicrobiana de las bacteriocinas y de las BAL productoras de bacteriocinas contra patógenos presentes en quesos

103 y se discutirá sobre las perspectivas a futuro de su aplicación como
104 conservador comercial.
105
106 **Generalidades y clasificación de bacteriocinas de BAL**
107 Las bacteriocinas se definen como péptidos de origen ribosomal que son
108 secretados al medio extracelular y tienen la capacidad de inhibir el crecimiento
109 de otros microorganismos (Monroy *et al.*, 2009; Beshkova y Frengova, 2012;
110 Mondragón Preciado *et al.*, 2013). Las bacteriocinas se han encontrado en la
111 mayoría de las bacterias y se estima que el 99% de ellas son capaces de
112 sintetizar cuando menos una bacteriocina (Dolz, 2008). Las mayoría de las
113 bacteriocinas de BAL son péptidos que contienen residuos de aminoácidos,
114 como la lisina, arginina e histidina, los cuales les confieren un carácter catiónico
115 (a un pH neutro), también contienen residuos de alanina, valina, leucina,
116 isoleucina, prolina, metionina, fenilalanina y triptófano, lo que les proporciona su
117 naturaleza hidrofóbica, además las bacteriocinas también son de carácter
118 anfipático (Diep y Nes, 2002; Yusuf, 2013). Las características generales de las
119 bacteriocinas se encuentran resumidas en la Tabla 1.
120 Las bacteriocinas han sido agrupadas en 5 tipos diferentes según varios
121 criterios de clasificación, como por ejemplo, organismos productores, pesos
122 moleculares, propiedades físicas, estructuras químicas, el modo de acción,
123 características genéticas y bioquímicas (Tabla 2). La clase I ó lantibióticos son
124 péptidos de muy bajo peso molecular (<5 kDa) y son resistentes a altas
125 temperaturas. La clase II son péptidos pequeños termoestables (<10 kDa) y
126 estos se encuentran divididos en 5 subclases (IIa, IIb, IIc, IId y IIe). La clase III

son péptidos termolábiles de alto peso molecular (> 30 kDa). La clase IV son péptidos grandes asociados con carbohidratos o lípidos y la clase V son péptidos con una estructura circular sin modificaciones postraduccionlaes (Nes *et al.*, 2007; Monroy *et al.*, 2009; Zacharof y Lovitt, 2012; Balciunas *et al.*, 2013).

Mecanismos de acción de las bacteriocinas

La mayoría de las bacteriocinas de las BAL inhiben el crecimiento las bacterias mediante la formación de poros en la membrana celular. Esto se debe a que las bacteriocinas están cargadas positivamente y facilitan su interacción con los fosfolípidos de la membrana celular que están cargados negativamente. La naturaleza anfipática de las bacteriocinas facilitan su distribución a lo largo de la superficie de la membrana celular de la bacteria diana (Cotter *et al.*, 2005; Nishie *et al.*, 2012; Yusuf, 2013). Las bacteriocinas cuando entran en contacto con la membrana celular, la mitad de su estructura donde se encuentra el N-terminal, que tiene forma de lámina, se une a la superficie de la bacteria, mientras que en su otra mitad donde se encuentra el C-terminal, que es la parte hidrofóbica con forma helicoidal, penetra hacia el interior hidrofóbico de la membrana celular, de tal manera que resulta en la pérdida de iones K, de energía en forma de ATP y en algunos casos, de aminoácidos y moléculas de bajo peso molecular. Las consecuencias de ello, son la disminución del potencial de membrana y escasa disponibilidad de las reservas energéticas de la célula, lo que conlleva a la disminución de la síntesis de ADN, ARN y proteínas, lo que finalmente desencadena la muerte de la célula (Vásquez *et al.*, 2009; Yusuf, 2013; Bemena *et al.*, 2014). Algunas bacteriocinas de clase I,

como la nisina, han demostrado tener un modo de acción dual, además, son capaces de causar la muerte celular rápida cuando se utiliza al lípido II para formar poros en la membrana (López *et al.*, 2008; Perez *et al.*, 2014). La naturaleza anfipática de las bacteriocinas de clase II hace fácil la inserción del péptido en la membrana de la bacteria diana, provocando su despolarización y la muerte (Drider *et al.*, 2006; Yusuf, 2013). Las bacteriocinas de clase III, como la lisostafina, pueden funcionar directamente en la pared celular de las bacterias diana Gram positivas, conduciendo la muerte y lisis de la bacteria (Lai *et al.*, 2002; Cotter *et al.*, 2005).

Actividad antimicrobiana de bacteriocinas contra patógenos presentes en quesos

En la actualidad, la nisina y pediocina PA-1 son las únicas bacteriocinas que se comercializan en la industria para la conservación de alimentos. La nisina fue la primer bacteriocina que se comercializó y sus efectos antimicrobianos son los más documentados, aunque en quesos la información es escasa (Sobrino-López y Martín-Belloso, 2008; Favaro *et al.*, 2015) (Tabla 3). La actividad de la nisina en quesos ha sido evaluada en de forma libre, es decir, aplicada directamente en los quesos. Se ha reportado que la nisina fue capaz de disminuir el conteo de *S. aureus* en queso fresco Minas sin afectar las propiedades fisicoquímicas, características sensoriales, aunque se vio desfavorecido el índice de maduración (Pinto *et al.*, 2011; Felicio *et al.*, 2015). Sin embargo, en queso fresco Hispánico, la combinación de ácido caprilico con

nisina y cinamaldehido controlo a *L. monocytogenes* y presentó bajo impacto en la flora natural del queso (Gadotti *et al.*, 2014).

El contenido de grasa del queso puede afectar la actividad antimicrobiana de la nisina debido a que la naturaleza anfipática de la nisina favorece la interacción de ésta con los lípidos afectando su actividad, sin embargo el NaCl puede incrementar su actividad (Chollet *et al.*, 2008). Tomando en cuenta lo anterior, algunos estudios se han enfocado en estrategias para aumentar la actividad de las bacteriocinas, por ejemplo, la nisina Z y nisina encapsuladas en forma de liposomas fueron eficientes al inhibir el crecimiento de *L. innocua* y *L. monocytogenes* en queso Cheddar y queso fresco Minas, respectivamente (Benech *et al.*, 2002; Malheiros *et al.*, 2012).

Por otro lado, otras estrategias como el uso de películas poliméricas y de caseinato de sodio adicionadas con enterocina y nisina en queso fresco Cottage y queso, controlaron a *L. monocytogenes* y *L. innocua* (Iseppi *et al.*, 2008; Cao-Hoang *et al.*, 2010). Otro estudio mostro que el tratamiento con calor más la adición de nisina presentó un efecto sinérgico contra *L. innocua* logrando eliminarla por completo después de 6 días de almacenamiento (Al-Holy *et al.*, 2012), mientras que el tratamiento con alta presión hidrostática aplicado en quesos es más eficiente cuando se incluye nisina como antimicrobiano (Lopez-Pedemonte *et al.*, 2003).

La actividad antimicrobiana de la nisina también ha sido probada en suero de queso, el cual es un sub-producto de la elaboración del mismo (Tabla 4). Se ha reportaron que la adición de nisina en suero de queso Feta redujo el conteo de *L. monocytogenes*, sin embargo modificó la flora microbiana normal

del suero (Samelis *et al.*, 2003). También, se ha reportado que la aplicación de bajas temperaturas, de campo eléctrico pulsado y utilización de MicroGARD™ (cultivo láctico pasteurizado; bioprotector natural) favorecen la acción antimicrobiana de la nisina contra *L. innocua* en suero liquido de queso a base de concentrado de proteína se suero (Gallo *et al.*, 2007a; Gallo *et al.*, 2007b; von Staszewski y Jagus, 2008).

Actividad antimicrobiana de BAL productoras de bacteriocinas *in situ* en diferentes quesos

Las BAL han sido utilizadas ampliamente por la industria en la elaboración de productos lácteos, debido a las ventajas tecnológicas que brindan al producto final (Parra Huertas, 2010). La capacidad antimicrobiana de las BAL productoras de bacteriocinas es un tema que en la actualidad ha tomado mayor importancia, sin embargo, son pocos los estudios que evalúan la capacidad antimicrobiana de las BAL productoras de bacteriocinas en la conservación de quesos (Tabla 5) (Sobrino-López y Martín-Belloso, 2008; Favaro *et al.*, 2015). Algunos estudios han reportado que la utilización de BAL productoras de bacteriocinas han sido eficientes para disminuir la concentración de *L. innocua* y *L. monocytogenes* en los quesos Manchego, madurado, fresco y Cottage (Rodriguez *et al.*, 1998; O'Sullivan *et al.*, 2006; Dal Bello *et al.*, 2012; Vera Pingitore *et al.*, 2012).

Otros estudios han demostrado que las BAL productoras de bacteriocinas son capaces de disminuir la concentración de esporas de *C. tyrobutyricum, C. beijerinckii* INIA 63 y otras esporas de especies de *Clostridium*

en queso Vidiago, queso semiduro, queso Kasseri y queso de oveja (Rilla *et al.*, 2003; Bogovič Matijašić *et al.*, 2007; Anastasiou *et al.*, 2009; Martínez-Cuesta *et al.*, 2010; Garde *et al.*, 2011). También, se ha reportado que aunque las BAL sean productoras de bacteriocinas *in vitro*, no garantiza que se mantenga su actividad en la elaboración de quesos (Nuñez *et al.*, 1997; Hamama *et al.*, 2002; Sarantinopoulos *et al.*, 2002). Sin embargo, otros estudios se han enfocado en combinar tratamientos antimicrobianos con las BAL productoras de bacteriocinas para mejorar la actividad antimicrobiana. Aly *et al.* (2012) reportaron que la aplicación de *Lc. lactis* subsp. *lactis* productora de nisina Z mejoró la actividad antimicrobiana en queso cuando fue combinada con un gel, así mismo, otro estudio demostró que el efecto combinado de alta presión y diferentes BAL productoras de las bacteriocinas (nisina A, nisina Z, lacticina 481, TAB 57, TAB 7, enterocina I, enterocina AS-48) disminuyeron el conteo de *L. monocytogenes* en queso (Arqués *et al.*, 2005), sugiriendo que el daño a la membrana ocasionado por la alta presión favorece la acción de las bacteriocinas en la membrana celular de las bacterias. Por otro lado, se ha reportado que *Lb. sakei* subsp. *sakei* 2a, productor de bacteriocinas, fue combinado con inulina como fuente de fibra. Los autores reportaron que la concentración de *L. monocytogenes* disminuyó en estos quesos, sugiriendo que las BAL productoras de bacteriocinas también son eficientes para elaborar quesos simbióticos. Así mismo, reportaron que *Lb. sakei* subsp. *sakei* 2a expresó los genes sakP y sakQ responsables de la síntesis de bacteriocinas en el queso. Sugiriendo la presencia de bacteriocinas en el queso simbiótico (Martinez *et al.*, 2015).

Otra opción que se ha considerado es la aplicación de BAL multiproductoras de bacteriocnas. Se ha reportado que la actividad antimicrobiana de la nisina en combinación con pediocina se incrementó contra *L. monocytogenes* (Rodríguez *et al.,* 2005). Izquierdo *et al.* (2009) utilizaron a *Ent. faecium* WHE 81 multiproductora de bacteriocinas en la suerficie de queso Munster contaminado con *L. monocytogenes* y reportaron que esta cepa erradicó casi por completo al patógeno. Sugiriendo que una BAL multiproductora de bacteriocinas tiene menos posibilidades de que el patógeno adquiera resistencia en comparación con las que producen una sola, además el efecto sinérgico entre bacteriocinas puede favorecer la actividad antimicrobiana. Sin embargo, se ha reportado que *L. lactis* CL2 productora de nisina y pediocina disminuyó el conteo de *L. monocytogenes*, *E. coli* y *S. aeurus* en queso, aunque su inhibición no fue mejor que una BAL productora solo de nisina, por lo que sugirieron que en el queso no se presentó un efecto sinérgico entre ambas bacteriocinas (Rodríguez *et al.,* 2005).

Por otro lado en uso de BAL productoras de bacteriocinas genéticamante modificadas también ha sido explorado. Buyong *et al.* (1998) insertaron el gen que codificaba para pediocina PA-1 a *Lc. lactis* subsp. *lactis* MM217 y observaron que la concentración de *L. monocytogenes* disminuyó significativamente en queso Cheddar*,* sugiriendo que el uso de estos microorganismos podría ayudar a disminuir los tratamientos químicos como control microbiológico. En otro estudio, McAuliffe *et al.* (1999) utilizaron a *L. lactis* DPC4275 transconjugante productora de lacticina 2147 y mostró que

inhibió el crecimiento de *L. monocytogenes* en un 99.9% en queso Cottage. Así mismo, Rodríguez *et al.,* 2005 reportaron que las cepas transformantes de *L. lactis* CL1 productora de pediocina y *L. lactis* CL2 productora de nisina y pediocina disminuyeron el conteo de *L. monocytogenes*, *E. coli* y *S. aeurus* en queso.

Conclusiones

Las bacteriocinas presentan diferentes mecanismos de acción que incluyen permeabilización de la membrana y daño al material genético, el cual desencadena la muerte del patógeno. Por otro lado, se ha cuestionado que la aplicación de bacteriocinas no garantiza su actividad debido a que depende en gran medida de la naturaleza de la matriz alimentaria (Sobrino-López y Martín-Belloso, 2008; Yusuf, 2013; Favaro *et al.*, 2015). Sin embargo, existen alternativas que favorecen la actividad antimicrobiana de las bacteriocinas, como el tratamiento térmico o altas presiones, la aplicación en forma de liposomas o películas. Por otro lado, ha sido cuestionada la eficacia de las BAL productoras de bacteriocinas aplicadas directamente en la elaboración de quesos, esto debido a que se ha mencionado que las colonias de BAL pueden quedar atrapadas en la matriz, lo que dificultaría la dispersión de las bacteriocinas y limitaría su actividad antimicrobiana (Jeanson *et al.*, 2011; Aly *et al.*, 2012). Sin embargo, existe evidencia que indica que la aplicación de BAL productoras de bacterianas en quesos es capaz de controlar la carga microbiana de patógenos, y que además existen alternativas para hacer más eficiente a la actividad antimicrobiana, como la adición de geles o la aplicación

de tratamientos de alta presión, aunque los estudios que se tienen al respecto son pocos. Por lo anterior, la evidencia indica que su aplicación tecnológica es favorable y podría ayudar a preservar los quesos de una manera natural y libre de compuestos químicos que muchas veces pueden ser tóxicos y perjudiciales para la salud. Adicionalmente, consideramos que se deberían de tomar en cuenta ciertos aspectos para una mejor aplicación de las bacteriocinas o de BAL productoras de bacteriocinas en quesos. Primero, el efecto antimicrobiano de las bacteriocinas no solo actúa encontrar el patógeno sino también puede afectar a la microflora natural presente en el queso, lo que podría ser desfavorable para el proceso de maduración del mismo. Segundo, se deberá de tomar en cuenta la carga microbiana del patógeno (a mayor carga microbiana mayor será la concentración de bacteriocinas necesaria para su eliminación). Tercero, la producción de bacteriocinas se vincula con el crecimiento celular, y está se puede verse afectada por diversos factores, como las sustancias químicas.

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502 **Tabla 1**. Características generales de las bacteriocinas.

Origen	-Natural; metabolitos extracelulares de cepas de bacterias Gram positivas y Gram negativas. - Se estima que el 99% de las bacterias son capaces de sintetizar cuando menos una bacteriocina.
Efecto en el organismo humano	-Seguro para el organismo humano; no tóxico, no carcinogénico, no alergénico, se inactivan por proteasas digestivas.
Espectro de acción	- Algunas bacteriocinas producidas principalmente por las BAL tienen amplio espectro de actividad antimicrobiana y actúan contra muchos microorganismos patógenos presentes en los alimentos. -El rango de actividad antimicrobiana de cada bacteriocina es diferente.
Modo de actividad	-Bactericida. -Bacteriostático. -Fungicida. Escasamente documentado.
Mecanismo de acción	- Permeabilización de la membrana. -Inhibición de DNA, RNA y biosíntesis de proteínas. -Lisis celular.
Estructura química	-Proteínas simples. -Glicoproteínas.

	-Lipoproteínas.
Peso molecular	-De unos pocos a unas docenas de kDa; usualmente menos de 10 kDa.
Número de AA en la molécula	-De 19 a 80; usualmente alrededor de 40.
Carácter	-Hidrofóbico. -Anfipático.
pI	-De 8.1 a 10.0
Localización de genes que codifican para las bacteriocinas	-Plásmidos. -Cromosomas. -Transposones; ambos (plásmidos y cromosomas).
Sensibilidad a enzimas	-Todas las bacteriocinas son sensibles a enzimas proteolíticas (pepsina, tripsina y pronasa).
Sensibilidad a temperaturas	-Compuestos termoestables; la mayoría de las bacteriocinas soportar de100-121 °C durante 15-30 min.
Sensibilidad a pH	-La mayoría de las bacteriocinas son estables en el rango de pH de 3.0 a 9.0

503 Adaptado de (Józefiak y Sip, 2013). BAL=Bacterias ácido lácticas; AA=aminoácidos; pI=punto isoeléctrico.

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508 **Tabla 2**. Clasificación las bacteriocinas.

Clasificación	Característica	Subcategoría	Ejemplo
Clase I ó lantibióticos	-Lantionina o péptidos que contienen β-lantioninato.	-Tipo A (moléculas lineales). -Tipo B (moléculas globulares).	-Nisina, subtilina, epidermina. -Mersacidina.
Clase II	-Clase heterogénea de péptidos termoestables pequeños.	-Subclase IIa (tipo de bacteriocina pediocina-antilisteria) -Subclase IIb (compuesto de dos pétidos) -Subclase IIc (otras bacteriocinas)	-Pediocina, enterocina, sakacina. -Plantaricina, lacticina F. -Lactococcina.
	-Grupo de péptidos lineales. - Degradación de proteínas grandes.	-Subclase IId -Subclase IIe	- Lacticina Q -Propionicina F
Clase III	-Péptidos grandes termolábiles.		-Helveticina J, millericina B.
Clase IV Clase V	-Péptidos cíclicos * -Péptidos de estructura circular		-Reutericina 6 -Enterocina AS-48 -Gasericina A

509 Adaptado de (Nes *et al.*, 2007; Monroy *et al.*, 2009; Balciunas *et al.*, 2013). * = asociados con lípidos o carbohidratos.

510 **Tabla 3.** Actividad antimicrobiana de bacteriocinas en quesos.

Tratamiento	Bacteriocina	Tipo de queso	Espectro de inhibición	Referencia
Encapsulación en liposomas	Nisina	Queso fresco Minas	*L. monocytogenes*	(Malheiros *et al.*, 2012)
Película polimérica	Enterocina 416K1	Queso fresco y suave	*L. monocytogenes*	(Iseppi *et al.*, 2008)
Encapsulación en liposomas	Nisina Z	Queso Cheddar	*L. innocua* *	(Benech *et al.*, 2002)
Bacteriocina libre + Calor	Nisina	Queso blanco	*L. innocua* *	(Al-Holy *et al.*, 2012)
Bacteriocina libre	Nisina	Queso Serro Minas	*S. aureus*	(Pinto *et al.*, 2011)
Película de caseinato de sodio	Nisina	Queso	*L. innocua* *	(Cao-Hoang *et al.*, 2010)
Bacteriocina libre	Nisina	Queso Minas	*S. aureus*	(Felicio *et al.*, 2015)
Ácido caprilico + cinamaldehido	Nisina	Queso fresco Hispano	*L. monocytogenes*	(Gadotti *et al.*, 2014)
Grasa + NaCl	Nisina	Queso	*K. rhizophila* 9341 *	(Chollet *et al.*, 2008)
Alta presión hidrostática	Nisina	Queso	*B. cereus* ATCC 9139	(Lopez-Pedemonte *et al.*, 2003)

511 *= Microorganismo indicador. L= *Listeria*; S= *Staphylococcus*. K= *Kocuria;* B= *Bacillus.*

Tabla 4. Actividad antimicrobiana de bacteriocinas de BAL en suero de queso.

Tratamiento	Bacteriocina	Tipo de suero	Espectro de inhibición	Referencia
Almacenamiento	Nisina	Suero de queso Feta	*L. monocytogenes*	(Samelis *et al.*, 2003)
Baja temperatura	Nisina	Concentrado de proteína de suero solida	*L. innocua* *	(Gallo *et al.*, 2007b)
Campo eléctrico pulsado	Nisina	Concentrado de proteína de suero solida	*L. innocua* *	(Gallo *et al.*, 2007a)
Microgard^{TM}	Nisina	Concentrado de proteína de suero solida	*L. innocua* *	(von Staszewski y Jagus, 2008)

*= Microorganismo indicador. L= *Listeria*.

519 **Tabla 5.** Actividad antimicrobiana de BAL productoras de bacteriocinas en quesos.

BAL	Bacteriocina	Tipo de queso	Espectro de inhibición	Referencia
Lc. lactis subsp. *lactis* MM217 geneticamente modificada	Pediocina PA-1	Queso Cheddar	*L. monocytogenes*	(Buyong *et al.*, 1998)
Lc. *lactis* subsp. *lactis* ESI 515	Nisina	Queso Manchego	*L*. *innocua* *	(Rodriguez *et al.*, 1998)
Lc. lactis DPC 4275 transconjugante	Lacticin 3147	Queso Cottage	*L. monocytogenes*	(McAuliffe *et al.*, 1999)
Lc. lactis subsp. *lactis* UL730	Nisina	Queso Moroccan	*S. aureus* J10	(Hamama *et al.*, 2002)
Ent. faecuim FAIR-E 198	Enterocina	Queso Feta	*Listeria*	(Sarantinopoulos *et al.*, 2002)
Lc. lactis ssp. *lactis* IPLA 729	Nisina Z	Queso Vidiago	*Cl. tyrobutyricum* CECT 4011	(Rilla *et al.*, 2003)
Lc. lactis TAB 50	Nisina A	Queso	*L. monocytogenes*	(Arqués *et al.*, 2005)
Lc. lactis TAB 26	Nisina Z			
Lc. lactis TAB 24	Lacticina 481			
Lc. lactis TAB 57	TAB 57			
Ent. faecium TAB 7	TAB 7			

Ent. faecalis TAB 52	Enterocina I			
Ent. faecalis INIA 4	Enterocina AS-48			
Lc. lactis CL1 y *Lc. lactis* CL2 transformantes	Pediocina	Queso	*L. monocytogenes; S. aureus; E. coli*	(Rodríguez *et al.*, 2005)
L. lactis DPC4275	Lacticina 3147	Queso madurado	*L. monocytogenes*	(O'Sullivan *et al.*, 2006)
Lc. lactis DPC4275	Lacticina 3147	Queso madurado	*L. monocytogenes* PKP1	(O'Sullivan *et al.*, 2006)
Lb. gasseri K7 (Rif^r)	Bacteriocina	Queso semiduro	*Cl. tyrobutyricum*	(Bogovič Matijašić *et al.*, 2007)
Ent. casseliflavus IM 416K1	Enterocina 416K1	Queso Cottage	*L. monocytogenes* NCTC 10888	(Iseppi *et al.*, 2008)
Ent. faecium WHE 81	Bacteriocinas	Queso Munster	*L. monocytogenes*	(Izquierdo *et al.*, 2009)
Streptococcus macedonicus ACA-DC 198	Mecedocina	Queso Kasseri	*Cl. tyrobutyricum* LMG 1285T	(Anastasiou *et al.*, 2009)
Lc. lactis IFPL 3593	lacticina 3147	Queso semiduro	*Cl. tyrobutyricum* CECT 4011	(Martínez-Cuesta *et al.*, 2010)

			Clostridium sp. A1	
			Clostridium sp. B2	
Lc. lactis subsp. *lactis* INIA 415	Nisina	Queso de	*Cl. beijerinckii* INIA 63	(Garde *et al.*, 2011)
	Lacticina 481	oveja		
Ent. mundtii CRL35 y *Ent. faecium* ST88Ch	Bacteriocinas	Queso fresco	*L. monocytogenes*	(Vera Pingitore *et al.*, 2012)
Lc. Lactis 29FL4	Nisina Z	Queso	*L. monocytogenes*	(Dal Bello *et al.*, 2012)
Lc. Lactis 32FL3	Lacticina 481	Cottage		
Lc. Lactis 32FL1	Lacticina 481			
Lc. Lactis 40FEL3	Nisina A			
Lc. lactis subsp. *Lactis*	Nisina Z	Queso	*Lb. sakei* ATCC15521 *	(Aly *et al.*, 2012)
Lb. sakei subsp. *sakei* 2a	Expresión de	Queso	*L. monocytogenes*	(Martinez *et al.*, 2015)
	los genes sakP	simbiótico		
	y sakQ			

*= Microorganismo indicador; Lc= *Lactococcus*; Lb= *Lactobacillus*; Ent= *Enterococcus*; Cl= *Clostridium*; L= *Listeria*; E= *Escherichia*; S= *Staphylococcus*; Lb= *Lactobacillus*.

Capítulo 3

Antimicrobial activity and partial characterization of bacteriocin-like inhibitory substances produced by *Lactobacillus* spp. isolated from artisanal Mexican cheese.

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Resumen

Los extractos crudos de *Lactobacillus fermentum* mostraron una alta actividad antimicrobiana frente a *Staphylococcus aureus, Listeria innocua*, *Escherichia coli* y *Salmonella typhimurium*. Se observó la inactivación completa de la actividad antimicrobiana después del tratamiento de los extractos crudos con proteinasa K, pronasa, papaína, tripsina y la lisozima, confirmando su naturaleza proteica. Sin embargo, la actividad antimicrobiana se disminuye en algunos de los extractos crudos cuando fueron tratados con ∝- amilasa lo cual indico que estuvieron involucrados carbohidratos. La actividad antimicrobiana de los extractos crudos fue estable a 65 ° C durante 30 min, sobre un amplio rango de pH (2-8), y con cloruro de potasio, citrato de sodio, etanol y butanol. Sin embargo, la actividad antimicrobiana se perdió después de calentar a 121 ° C durante 15 min, con la adición de metanol o Tween®80. Catorce de los dieciocho *Lactobacillus spp*., mostraron actividad antimicrobiana contra diferentes microorganismos y doce presentaron sustancias similares a bacteriocina (BLS). Los parámetros, tiempo de generación (GT) y tasa de crecimiento (μ) indicaron que la actividad antimicrobiana de tres de los extractos crudos fueran eficaces contra los cuatro microorganismos indicadores. Uno de los extractos crudos mostró una inhibición no sólo contra bacterias Gram positivas, sino también contra bacterias Gram negativas. BLS producido por esta cepa de *Lactobacillus* específica mostró potencial para su aplicación como bioconservante en alimentos.

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Antimicrobial activity and partial characterization of bacteriocin-like inhibitory substances produced by *Lactobacillus* spp. isolated from artisanal Mexican cheese

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ABSTRACT

Lactobacillus spp. from Mexican Cocido cheese were shown to produce bacteriocin-like substances (BLS) active against *Staphylococcus aureus*, *Listeria innocua*, *Escherichia coli*, and *Salmonella typhimurium* by using the disk diffusion method. Crude extracts of *Lactobacillus fermentum* showed strong inhibitory activity against *Staph. aureus*, *L. innocua*, *E. coli*, and *Salmonella choleraе*. Complete inactivation of antimicrobial activity was observed after treatment of crude extracts with proteinase K, pronase, papain, trypsin, and lysozyme, confirming their proteinaceous nature. However, antimicrobial activity was partly lost for some of the crude extracts when treated with α-amylase, indicating that carbohydrate moieties were involved. The antimicrobial activity of the crude extracts was stable at 65°C for 30 min over a wide pH range (2–8), and addition of potassium chloride, sodium citrate, ethanol, and butanol did not affect antibacterial activity. However, antimicrobial activity was lost after heating at 121°C for 15 min, addition of methanol or Tween 80. Fourteen out of 18 *Lactobacillus* spp. showed antimicrobial activity against different test microorganisms, and 12 presented bacteriocin-like substances. Generation time and growth rate parameters indicated that the antimicrobial activity of crude extracts from 3 different strains was effective against the 4 indicator microorganisms. One of the crude extracts showed inhibition not only against gram-positive but also against gram-negative bacteria. Bacteriocin-like substances produced by this specific *Lactobacillus* strain showed potential for application as a food biopreservative.

Key words: bacteriocin, lactic acid bacteria, antimicrobial activity

INTRODUCTION

Lactic acid bacteria (**LAB**) have been used in different foods for the production of organic substances that contribute to the sensory attributes and the preservation of food (Parada et al., 2007). The preservative properties are based on the antimicrobial metabolites such as organic acids, hydrogen peroxide, diacetyl, reuterin, antifungal peptides, and bacteriocins (Ponce et al., 2008; Arqués et al., 2011; Stoyanova et al., 2012; Ghanbari et al., 2013). Bacteriocins are ribosomally synthesized peptides with antimicrobial activity produced by bacteria such as LAB (*Lactococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Enterococcus*, and *Leuconostoc*) that are of particular interest due to their potential use as biopreservatives in the food industry (Todorov et al., 2011; Balciunas et al., 2013). The interest in the application of bacteriocins in food preservation has increased in the last years due their nontoxicity, sensitivity to proteases, general pH and heat stability, and antimicrobial effect against species of gram-positive bacteria, such as *Staphylococcus aureus*, *Clostridium tyrobutyricum*, *Listeria innocua*, *Listeria monocytogenes*, and *Bacillus cereus*. Additionally, a few bacteriocins have activity against gram-negative bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Helicobacter pylori* NCIPD 230, and *Campylobacter jejuni* (Gálvez et al., 2007; García et al., 2010; Todorov et al., 2010; Reis et al., 2012). However, only nisin (*Lactococcus lactis*) and pedocin PA-1 (*Pediococcus acidilactici*) are approved and permitted for preservation of foods (Parada et al., 2007; Pinto et al., 2011; Biscola et al., 2013). The factors affecting the bacteriocin activity in different food systems are the composition (proteins and lipids), enzymatic degradation, manufacturing process (high temperatures), physical properties, such as pH and

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additives present (Gálvez et al., 2007; Parada et al., 2007; Hartmann et al., 2011; Gao et al., 2013). Thus, the successes of using bacteriocins in the food industry for the control of different pathogens are necessary to demonstrate the antimicrobial activity of bacteriocins by in vitro studies and in food systems (Gálvez et al., 2008; Biscola et al., 2013). In dairy products, the antimicrobial effect of different bacteriocins against gram-positive bacteria has been studied, but few reports are available on the effect against gram-negative bacteria. Thus, demand for natural antimicrobial compounds has brought interest in new bacteriocins able to enhance dairy safety.

The aim of the present work was to screen for BLS production in wild *Lactobacillus*, isolated during the manufacture of artisanal Mexican Cocido cheese, with bioactivity against gram-positive and gram-negative bacteria. Mexican Cocido cheese is a semi-soft *pasta filata* cheese variety typical of the northwest of Mexico. It is made from raw milk using traditional techniques, which include acidification by using cheese whey from a previous cheese batch and renneting. A typical stage of the manufacturing is the process of cooking the curd until it reaches a dough-like consistency. Then it is placed into molds where it is shaped and cooled.

MATERIALS AND METHODS

Strains, Media, and Culture Conditions

All 18 wild *Lactobacillus* strains were obtained from the culture collection of the Dairy Laboratory at the Food Research and Development Center, A.C. (CIAD, A.C., Hermosillo, Sonora, Mexico). These strains were isolated during the making of artisanal Mexican Cocido cheese in Hermosillo, Sonora, Mexico. Briefly, at least 4 colonies were randomly picked from de Man, Rogosa, and Sharpe (MRS; BD Difco, Sparks, MD) agar count plates from different samples collected from cheese milk, whey, curd, and cheese. After purification, colonies were stored at −80°C in in (80%, vol/vol) glycerol until further characterization (González et al., 2007).

Prior to each experiment, *Lactobacillus* strains were subcultured in 10 mL of MRS at 37°C (pH 6.5). Three consecutive cultures (1% inoculum) were prepared and incubated for 24, 20, and 16 h, respectively. The last culture was used as the inoculum for all the experiments (fresh culture).

Staphylococcus aureus ATCC 29213, *L. innocua* ATCC 33090, *S. typhimurium* ATCC 14028, and *E. coli* ATCC 25922 were cultured in brain heart infusion broth (BHI; BD Difco, Sparks, MD). Three subcultures were inoculated at 1% and incubated at 37°C. The first subculture was incubated for 24 and 16 h, and the last subculture was incubated for 8 h.

Molecular Identification of LAB

Lactic acid bacteria were grown in MRS broth for 18 h. Subsequently, 1 mL of sample was taken for DNA extraction using the commercial protocol PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA). The method of amplification was based in the protocol of MicroSEQ Identification Systems (Applied Biosystems). The PCR cycles were carried out in a thermocycler endpoint (Eppendorf, Hamburg, Germany). Each PCR product was purified with ExoSAP-IT (Affymetrix, Santa Clara, CA) and then sequenced based on the MicroSeq protocol. Sequencing reactions were purified using the BigDye Xterminator protocol (Applied Biosystems). The purified samples were examined in a 3500 Genetic Analyzer (Applied Biosystems). The sequences obtained were analyzed in the MicroSeq software v2.0 (Applied Biosystems), and compared with the library of MicroSeq Bacterial 500 (Applied Biosystems) to determine the identity of the strains by their similarity to reference strains.

Preparation of CE

Lactobacillus strains were grown in MRS broth and incubated for 16, 18, 24, and 72 h at 37°C in water bath shaker (Lab-Line Instruments Inc., Melrose Park, IL). Aliquots of culture medium were centrifuged at 4,000 × *g* for 20 min, at 4°C. The crude extracts (CE) were adjusted to pH 6.5 with a 1 *N* NaOH, treated with catalase enzyme (1,000 U/mL) for 1 h at 25°C, and filter sterilized (0.22 μm, Millex-GV, Millipore SpA, Milan, Italy). The untreated and treated CE were stored at 4°C (Karapetyan et al., 2010).

Antimicrobial Activity Determination

Antibacterial activity was tested against *Staph. aureus* ATCC 29213, *L. innocua* ATCC 33090, *S. typhimurium* ATCC 14028, and *E. coli* ATCC 25922 by the disk diffusion method. The filter-sterilized CE were used to evaluate antimicrobial activity. An aliquot of 20 μL supernatant was applied on a sterilized cellulose disk (6 mm) placed on a BHI soft agar plate (0.8% wt/vol) inoculated with each pathogen (10^7 cfu/mL). Plates were incubated at 37°C for 24 h, and the diameters of the inhibition zones around the disk were measured (Nespolo and Brandelli, 2010). The CE with inhibition zones larger or equal to 6 mm were selected (Ponce et al., 2008).

Effect of Enzymes on Antimicrobial Activity of CE

The CE were evaluated to find out if the inhibition was due to bacteriocin-like inhibitory substances (BLS). Thus, CE were treated with papain (30 U/mg), proteinase K (30 U/mg), pronase E (3.5 U/mg), lysozyme (35,000 U/mg), α-amylase (30 U/mg), and pepsin (250 U/mg) (all from Sigma-Aldrich, St. Louis, MO) at a concentration of 1 mg/mL. Enzymes were dissolved (0.2%, wt/vol) in 0.05 mol/L Tris, 5 mmol/L $CaCl_2$ buffer (pH 7). For pepsin testing, the enzyme was dissolved in citrate buffer (pH 3). Enzyme solutions were filter sterilized (0.22 μm, Millex-GV, Millipore SpA). The CE were incubated with the different enzyme solutions at 37°C for 2 h, after which the retention of BLS in treated samples was determined by the disk diffusion method previously described (Todorov and Dicks, 2006).

Effect of pH, Temperature, Organic Solvents, and Additives on Antimicrobial Substances

The effect of pH on the antimicrobial substance was tested by adjusting the pH of the CE to values ranging from 2 to 8 (at increments of 1 pH unit) with sterile 1 *N* NaOH or 1 *N* HCl. Incubation was for 30 min at 30°C. The samples were tested for antimicrobial activity by using the disk diffusion method. The effect of temperature on the activity of antimicrobial substances was tested by heating the cell CE at 65, 75, 100, or 121°C. Residual antimicrobial activity was tested by the well diffusion method after subjecting the CE to the different temperatures for 30 min. The effect of organic solvents and additives on the activity of antimicrobial substances was tested by adding 10% (vol/vol, final concentration) butanol, methanol, and ethanol to CE and 1% (wt/vol, final concentration) potassium chloride, sodium citrate, or Tween 80 to CE. Antimicrobial activity of CE treated with additives was evaluated by using disk diffusion method at 37°C for 5 h. Untreated CE served as controls (Biscola et al., 2013; Ghanbari et al., 2013).

Growth of Indicator Microorganisms in the Presence of Different CE

A 20-μL aliquot of CE filter sterilized supernatant (pH 6.5) was added to 150 mL of BHI culture containing different pathogens, namely, *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua*. Then, mixtures were incubated for 24 h. Optical density readings at 600 nm were recorded every 2 h. Growth data were adjusted with the modified Gompertz equation (Chowdhury et al., 2007):

$$\text{Log n} = A \times \text{EXP}\{-\text{EXP}[-B\times(t)]\}.$$

This equation was applied to growth on indicator microorganisms with different CE. Log n = decimal logarithm of microbial counts [Log (abs)] at time *t*, A = average of the logarithmic increase [Log (abs)], B = relative growth rate at time (h^{-1}), and *t* = time (h).

Statistical Analysis

The experiments followed a complete randomized design with 3 replicates per treatment: antimicrobial activity at 24, 48, and 72 h, CE with enzymes, pH, temperature, organic solvents, and additives. Growth experiments were carried out in duplicate. Data were analyzed by one-way ANOVA with a significance level of 0.5%. Means were analyzed by the comparison test of Tukey-Kramer with a significance level of 0.05%. For statistical analyses, the NCSS statistical software version 2007 (NCSS LLC, Kaysville, UT) was used.

RESULTS AND DISCUSSION

Molecular Identification

Lactobacillus strains were identified molecularly by comparing the nucleotide sequences of the 16Sr gene (500 bases) with other sequences deposited in the database of MicroSEQ bacteria. The MicroSEQ protocol states that a 97% homology with ATCC strains is enough to determine the molecular identification of bacteria. Thus, most strains were identified as *Lactobacillus fermentum* with >99% homology with *Lb. fermentum* ATCC 14931. Only J25 and J33 were identified as *Lactobacillus plantarum* and *Lactobacillus paracasei tolerans* with >99% homology to ATCC 14917 and ATCC 25599, respectively, and strains J26, J27, J31, J34, J36, and J37 were identified as *Lactobacillus pentosus* with >99% homology to ATCC 8041 (Table 1).

Antimicrobial Activity of CE from Lactobacillus spp. Against Staph. aureus, E. coli, S. typhimurium, and L. innocua

Antimicrobial activity by LAB may be due to different metabolites such as organic acids and hydrogen peroxide and could be erroneously attributed to the production of bacteriocin-like compounds. In this study, the CE were adjusted to pH 6.5 and treated with catalase to eliminate the effect of acid or hydrogen peroxide. Thus, the inhibitory activity of the CE was not due to the production of these compounds. The CE obtained from *Lactobacillus* spp. at 16 and 18 h did not show antimicrobial activity by the disk diffusion

Table 1. Molecular identification of lactic acid bacteria (LAB)[1]

Strain	Identified LAB	% Similarity
J10	*Lactobacillus fermentum*	99.72 (ATCC 14931)
J20	*Lactobacillus fermentum*	99.78
J23	*Lactobacillus fermentum*	99.78
J24	*Lactobacillus pentosus*	100 (ATCC 8041)
J25	*Lactobacillus plantarum*	100
J26	*Lactobacillus pentosus*	100
J27	*Lactobacillus pentosus*	100
J28	*Lactobacillus fermentum*	99.64
J29	*Lactobacillus fermentum*	99.77
J30	*Lactobacillus fermentum*	99.62
J31	*Lactobacillus pentosus*	100
J32	*Lactobacillus fermentum*	99.81
J33	*Lactobacillus paracasei tolerans*	99.99 (ATCC 25599)
J34	*Lactobacillus pentosus*	100
J35	*Lactobacillus fermentum*	99.78
J36	*Lactobacillus pentosus*	100
J37	*Lactobacillus pentosus*	99.99
J38	*Lactobacillus fermentum*	99.68

[1]Results are based on 16Sr gene (500 bases) deposited in the database of MicroSEQ.

method. Nevertheless, most of the CE obtained at 24, 48, and 72 h presented antimicrobial activity against *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua*, except for extracts produced by *Lb. pentosus* strain J24, J26, J27, and J34 (Figure 1). However, CE obtained at 72 h showed the largest antimicrobial activity for each strain ($P < 0.05$); therefore, this time was used for the rest of experiments. The largest antimicrobial activity of bacteriocins at 72 h as shown in this study is in agreement with previous studies (McClerren et al., 2006; Basurto-Cadena et al., 2012). In fact, authors reported minimum antimicrobial activity at 24 h and maximum antimicrobial activity after 60 h for a *Bacillus subtilis* strain (Basurto-Cadena et al., 2012), suggesting that the antagonistic activity of proteases was lost in the first 24 h, not affecting the activity of the bacteriocins in the rest of the time. Also, McClerren et al. (2006) reported that *Bacillus halodurans* produced the bacteriocin halocuracin after 90 h of incubation. It has been reported that several mechanisms may be responsible for the decrease of bacteriocin activity of which protein aggregation, proteolytic degradation (proteases), and bacteriocin adsorption to the cells were reported (De Vuyst et al., 1996).

Among the 14 strains that presented antimicrobial activity against indicator microorganisms, *Lb. fermentum* strain J23 presented the highest antimicrobial activity against all tested microorganisms. On the other hand, *Lb. fermentum* strains J20, J28, and J32 presented the highest antimicrobial activity against *E. coli* and *S. typhimurium*. Simova et al. (2009) reported that CE of *Lactobacillus rhamnosus* obtained at 20 h presented antimicrobial activity against *L. innocua*, *Staph. aureus*, and *E. coli*, whereas *Lactobacillus bulgaricus* presented activity against *Staph. aureus*, *E. coli*, and *S. typhimurium*. However, the antimicrobial activity reported by these authors was lower than the antimicrobial activity found in the present study. The effect of the CE against different test pathogens is very important, particularly against *Staph. aureus* and *E. coli*, because these pathogens have been previously found in Fresco cheese manufactured with raw milk (Torres-Llanez et al., 2006). *Staphylococcus aureus* has been described as a common contaminant of dairy products and was detected in milk and artisanal Mexican cheeses (Torres-Llanez et al., 2006).

Effect of Enzymes on Antimicrobial Substances Against Staph. aureus, E. coli, S. typhimurium, and L. innocua

The CE from *Lactobacillus* spp. were treated with different enzymes to verify the proteinaceous nature of the inhibitory substances (Table 2). Determination of the proteinaceous nature of the inhibitory substances confirmed that the antimicrobial agents produced by *Lactobacillus* spp. were bacteriocin-like substances (BLS), except for those from *Lb. fermentum* strains J29 and J30. According to Ghanbari et al. (2013), the inactivation of antimicrobial activity by the action of proteolytic enzymes was an indication of the proteinaceous nature of BLS.

Additionally, BLS from *Lb. fermentum* strains J10, J20, J25, J28, and J32 and *Lb. pentosus* strain J36 were inhibited by α-amylase, indicating that the bacteriocins produced by *Lactobacillus* may be glycoproteins (carbohydrate moiety), which require both the glyco and the protein portion of the molecule for the activity.

This indicates that probably these bacteriocins belong to group IV in their classification, which contains carbohydrates and lipids in the molecular structure (Todorov et al., 2011). However, this fact needs to be confirmed after bacteriocin purification and structure determination. Several glycoprotein bacteriocins have been reported such as *Enterococcus faecium* DB1 and *Lactobacillus brevis* DF01, which produced bacteriocins sensitive to α-amylase (Seo et al., 2014). Other bacteriocins reported were leuconocin S produced by *Leuconostoc paramesenteroides* and carnocin 54 produced by *Leuconostoc carnosum* (Todorov et al., 2010).

Effect of Temperature, pH, Organic Solvents, and Additives on Antimicrobial Substances Against Staph. aureus, E. coli, S. typhimurium, and L. innocua

Temperature Effect. The effect of temperature on the antimicrobial activity of CE against *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua* is shown in Table 3. In general, the antimicrobial activity was stable at 65°C for 30 min and was most unstable at 121°C for 15 min. The CE from J20, J23, J25, J28, J32, J37, and J38 showed the most stability to heat treatment, retaining 100% of antimicrobial activity after 30 min at 65 or 75°C. However, antimicrobial activity decreased to some extent at 100°C for 30 min because the residual activity of the CE was around 80 to 90% (Table 3). This observation indicates that these bacteriocins may be used as biopreservatives in foods that are subjected to pasteurization, as is the case for dairy foods. Similar results were reported for Pringsulaka et al. (2012), bacteriocins produced by *Weissella cibaria* N23 were stable after subjecting them to 60 to 100°C for 20 min. These bacteriocins retained 50% of the antimicrobial activity at 100°C for 30 min. Conversely, bacteriocins produced by *Lb. bulgaricus* BB18 and *Lb. lactis* BCM5 showed high stability to heat treatment, retaining the antimicrobial activity after 60 min at 100°C (Simova et al., 2009). It has been reported that bacteriocin stability to heat treatment produced by different strains may be attributed to differences in ecological and environmental adaptation (Ponce et al., 2008).

pH Effect. The CE produced by *Lactobacillus* spp. were inhibitory against *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua* at a wide pH range (2.0–8.0; Table 3). Similar results were reported where antibacterial activity of different LAB strains presented a wide pH range (4.0–8.0) (Mezaini et al., 2009). Bacteriocin ALP57 from *Pediococcus pentosaceus* and bacteriocin ALP7 from *E. faecium* maintained antimicrobial activity against different strains at pH range from 2 to 8, but lost activity at pH 12 (Pinto et al., 2009). This is in agreement with the fact that the highest antimicrobial activity was found at the acid or neutral pH (Ponce et al., 2008). Bacteriocin stability in a wide pH range, as was shown in this study, is of technological importance

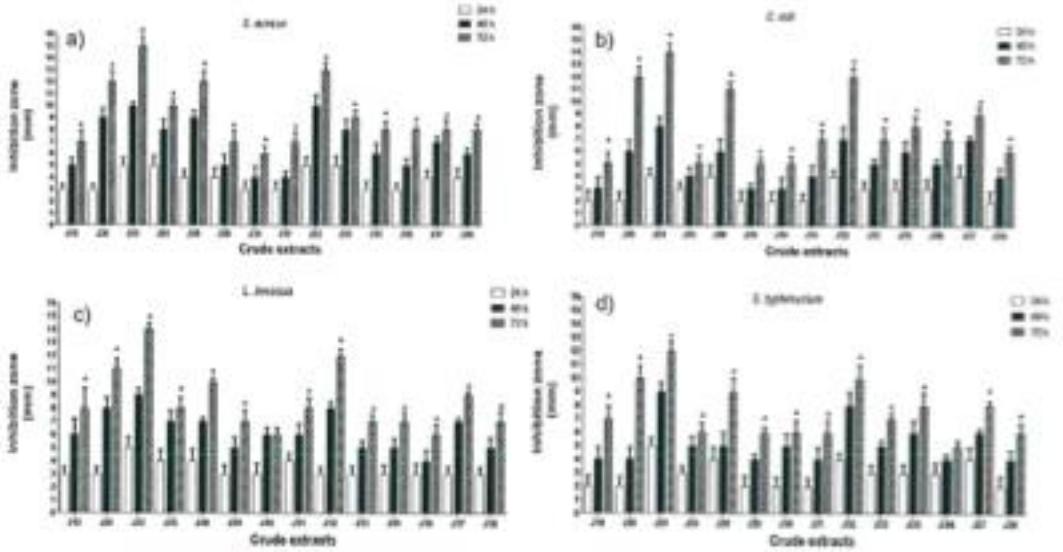

Figure 1. Antimicrobial activity of crude extracts obtained from different *Lactobacillus* against indicator strains. *Indicates significant differences between 24, 48, and 72 h for each strain ($P < 0.05$). Error bars indicate 95% confidence interval.

Table 2. Effect of enzymes on the residual antimicrobial activity (%) of crude extracts from different *Lactobacillus* strains on indicator microorganisms[1]

	Strain															
Enzyme	J19	J20	J23	J25	J28	J29	J30	J31	J32	J33	J35	J36	J37	J38
Papain	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	83 ± 0.9	76 ± 0.8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Pepsin	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	67 ± 0.6	98 ± 0.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Protease E	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	56 ± 0.9	89 ± 0.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Proteinase K	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	88 ± 0.5	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
α-Amylase	15 ± 0.4	65 ± 0.18	100 ± 0	22 ± 0.8	25 ± 0.6	100 ± 0	10 ± 0	100 ± 0	80 ± 0.9	100 ± 0	100 ± 0	75 ± 0.9	100 ± 0	100 ± 0

[1]Enzyme concentration 1 mg/mL. Results represent the mean value of triplicates ± SD.

Table 3. Effect of temperature, pH, organic solvents, and additives on residual antimicrobial activity (%) of crude extracts from different *Lactobacillus* strains (J10 to J38) on indicator microorganisms[1]

		Strain														
Treatment	Concentration	J10	J20	J23	J25	J28	J29	J30	J31	J32	J33	J35	J36	J37	J38
Temperature/time																
40°C/30 min		95 ± 0.28	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	98 ± 0.26	100 ± 0	79 ± 0.19	100 ± 0	67 ± 0.28	100 ± 0	100 ± 0
75°C/30 min		86 ± 0.14	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	93 ± 0.17	100 ± 0	58 ± 0.13	89 ± 0.19	63 ± 0.19	100 ± 0	100 ± 0
100°C/30 min		22 ± 0.09	85 ± 0.19	90 ± 0.12	75 ± 0.17	89 ± 0.19	89 ± 0.5	69 ± 0.38	88 ± 0.12	16 ± 0.56	49 ± 031	0 ± 0	95 ± 0.17	90 ± 0.23
121°C/15 min		0 ± 0	35 ± 0.21	37 ± 0.23	17 ± 0.09	28 ± 0.87	0 ± 0	15 ± 0.74	0 ± 0	0 ± 0	0 ± 0	0 ± 0	22 ± 0.15	0 ± 0.04
pH																
2		100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
3		100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
4–8		100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Organic solvent																
Butanol	10% (vol/vol)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Ethanol	10% (vol/vol)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Methanol	10% (vol/vol)	95 ± 0.48	98 ± 0.32	95 ± 0.59	90 ± 0.25	96 ± 0.32	91 ± 0.44	95 ± 0.49	96 ± 0.33	86 ± 0.21	95 ± 0.32	0.91 ± 0.15	98 ± 0.16
Additive																
Potassium chloride	1% (wt/vol)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Sodium citrate	1% (wt/vol)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Tween 80	1% (wt/vol)	0 ± 0	23 ± 0.79	18 ± 0.62	0 ± 0	10 ± 0.24	0 ± 0	11 ± 0.24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	16 ± 0.49	13 ± 0.29

[1]Results represent the mean value of triplicate ± SD.

for the dairy industry, because they may be used in different foods, such as fermented milk products.

Organic Solvent and Additive Effects. The CE remained inhibitory to *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua* after 30 min of treatment with substances such as sodium citrate and potassium chloride (Table 3). Parada et al. (2007) reported that the bacteriocin lactocin 705 against *L. monocytogenes* lost activity in the presence of sodium chloride, sodium citrate, ascorbic acid, and sodium lactate. Although the addition of anionic compounds did not affect antibacterial activity against test microorganisms, the addition of Tween 80 decreased their activity (Table 3). Additionally, CE produced by *Lactobacillus* spp. presented antimicrobial activity against *Staph. aureus*, *E. coli*, *S. typhimurium*, and *L. innocua* in the presence of different organic solvents (Table 3). Similarly, lactocin RN78 and plantaricin LC74 bacteriocins produced by *Lactobacillus* spp. maintained antimicrobial activity after treatments with organic solvents such as butanol, ethanol, and methanol. The fact that bacteriocins were stable with organic solvents indicated that they were soluble in them (Ghanbari et al., 2013).

Growth Inhibition of Staph. aureus, E. coli, S. typhimurium, and L. innocua in the Presence of Different CE

Microbial growth parameters growth rate (μ) and generation time (gt) showing inhibition of indicator microorganisms by the different CE containing BLS are summarized in Tables 4, 5, 6, and 7. Coefficients of determination ($R^2 = 0.95$ to 0.99) indicated that the Gompertz model was able to describe microbial growth accurately ($P < 0.05$). Of the 12 CE that presented antimicrobial activity and contained BLS, *Lb. fermentum* strain J23 showed the longest gt and the lowest μ compared with the controls (Tables 4, 5, 6 and 7). Thus, the CE from J23 had the greatest inhibitory activity against the 4 test microorganisms and may be the one with the largest concentration of BLS. The CE from J20 and J32 showed the next longest gt and the next lowest μ compared with the controls, for *Staph. aureus* and *Salmonella*, respectively (Tables 5 and 7). Therefore, CE from *Lb. fermentum* strains J20 and J32 had the greatest inhibitory activity against these pathogens after *Lb. fermentum* strain J23. Growth inhibition of the indicator microorganism by these 3 CE is shown in Figure 2. It is important to note that CE from J23 inhibited growth of not only gram-positive bacteria but also of gram-negative bacteria. Thus, these results indicated that CE from *Lb. fermentum* strain J23 contained BLS with a wide antimicrobial spectrum.

Table 4. Microbial growth parameters, derived from modified Gompertz equation, of *Escherichia coli* and *Lactobacillus* spp.[1]

Item	R^2	μ (abs/h)[2]	Generation time (h)
E. coli	0.98	0.20 ± 0.05^{a}	1.26 ± 0.23^{a}
J10	0.98	0.19 ± 0.00^{a}	1.33 ± 0.24^{a}
J20	0.98	0.09 ± 0.003^{b}	2.56 ± 0.23^{b}
J23	0.98	0.08 ± 0.004^{b}	2.65 ± 0.46^{b}
J25	0.98	0.10 ± 0.06^{b}	$2.05 \pm 0.42^{a,b}$
J28	0.98	0.08 ± 0.005^{b}	2.54 ± 0.67^{b}
J31	0.99	0.10 ± 0.04^{b}	2.37 ± 0.31^{b}
J32	0.95	0.10 ± 0.07^{b}	1.63 ± 0.42^{b}
J33	0.97	0.11 ± 0.08^{b}	$1.93 \pm 0.18^{a,b}$
J35	0.98	0.13 ± 0.06^{b}	$1.99 \pm 0.11^{a,b}$
J36	0.97	0.12 ± 0.03^{b}	1.36 ± 0.14^{a}
J37	0.98	0.09 ± 0.006^{b}	2.30 ± 0.52^{b}
J38	0.97	0.12 ± 0.06^{b}	$1.80 \pm 0.31^{a,b}$

[a–b]Different superscripts within a column indicate significant differences ($P < 0.05$).

[1]Data represent the mean values of duplicates ± SD.

[2]μ = growth rate, measured in absorbance units per hour.

CONCLUSIONS

Screening of *Lactobacillus* spp. isolated and identified in artisanal Mexican Cocido cheese for antimicrobial substances showed that they presented bacteriocin-like substances not only against gram-positive bacteria but also against gram-negative bacteria. This is an important finding since bacteriocins from LAB are not usually effective against gram-negative bacteria and rather present a narrow antimicrobial spectrum. Studies are undergoing to fully characterize the bacteriocin-like substances produced by *Lb. fermentum* strains J20, J23, and J32 and evaluate their antimicrobial activity in dairy foods.

Table 5. Microbial growth parameters, derived from modified Gompertz equation, of *Staphylococcus aureus* and *Lactobacillus* spp.[1]

Item	R^2	μ (abs/h)[2]	Generation time (h)
Staph. aureus	0.99	0.19 ± 0.06^{a}	1.34 ± 0.34^{a}
J10	0.99	0.18 ± 0.04^{a}	1.36 ± 0.27^{a}
J20	0.97	0.06 ± 0.008^{b}	2.79 ± 0.33^{b}
J23	0.98	0.05 ± 0.002^{b}	4.18 ± 0.45^{b}
J25	0.99	0.17 ± 0.08^{a}	1.35 ± 0.31^{a}
J28	0.98	0.08 ± 0.004^{b}	1.79 ± 0.36^{a}
J31	0.97	0.12 ± 0.05^{a}	2.08 ± 0.26^{a}
J32	0.96	0.08 ± 0.06^{b}	2.63 ± 0.54^{b}
J33	0.96	0.17 ± 0.09^{a}	2.55 ± 0.29^{b}
J35	0.97	0.16 ± 0.05^{a}	2.79 ± 0.42^{b}
J36	0.97	0.18 ± 0.04^{a}	1.42 ± 0.11^{a}
J37	0.99	0.09 ± 0.009^{b}	2.42 ± 0.22^{b}
J38	0.96	0.16 ± 0.07^{a}	1.36 ± 0.26^{a}

[a–b]Different superscripts within a column indicate significant difference ($P < 0.05$).

[1]Data represent the mean value of duplicates ± SD.

[2]μ = growth rate, measured in absorbance units per hour.

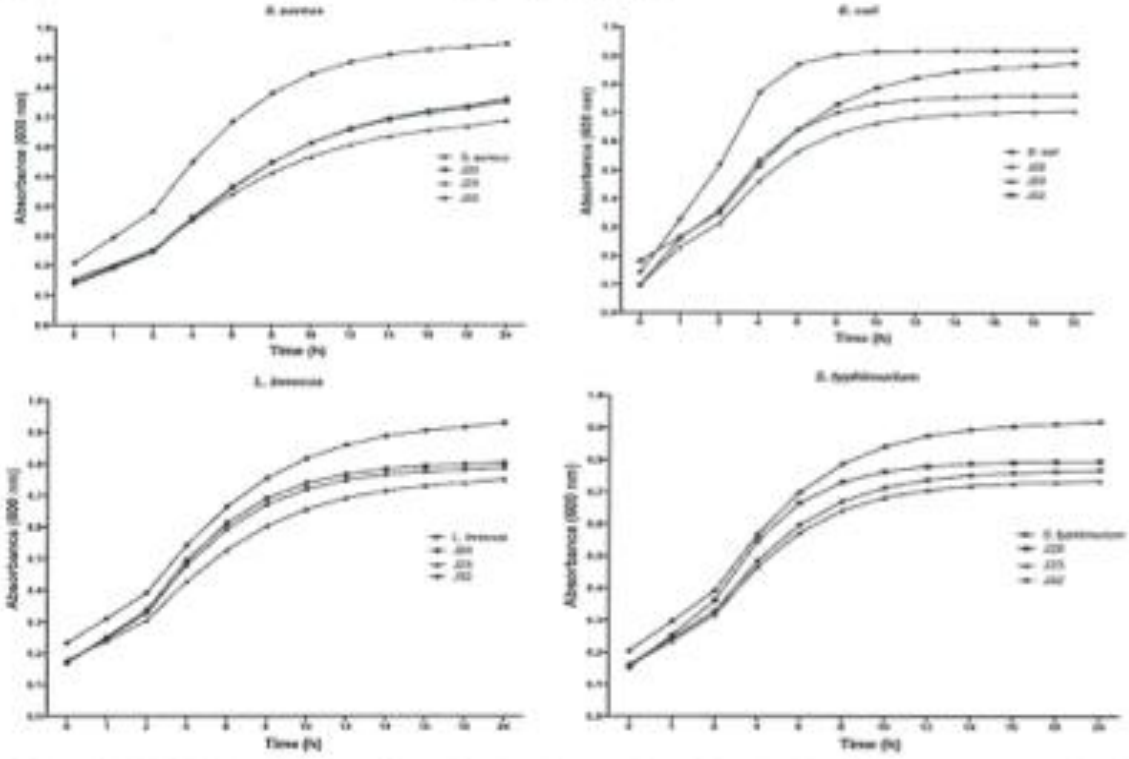

Figure 2. Growth inhibition of indicator microorganisms by crude extracts from different *Lactobacillus* strains. Parameters were derived from modified Gompertz equation.

Table 6. Microbial growth parameters, derived from modified Gompertz equation, of *Listeria innocua* and *Lactobacillus* spp.[1]

Item	R^2	μ (abs/h)[2]	Generation time (h)
L. innocua	0.99	0.19 ± 0.03[a]	1.25 ± 0.22[a]
J10	0.96	0.18 ± 0.00[a]	1.63 ± 0.19[b]
J20	0.97	0.09 ± 0.006[b]	2.80 ± 0.24[d]
J23	0.98	0.09 ± 0.005[b]	2.15 ± 0.37[a]
J25	0.99	0.18 ± 0.00[a]	2.02 ± 0.34[d]
J28	0.98	0.10 ± 0.008[b]	2.16 ± 0.28[a]
J31	0.99	0.12 ± 0.00[a]	1.72 ± 0.18[a]
J32	0.96	0.10 ± 0.00[b]	2.16 ± 0.36[a]
J33	0.97	0.19 ± 0.00[a]	1.2 ± 0.11[a]
J35	0.98	0.11 ± 0.07[a]	1.63 ± 0.19[b]
J36	0.99	0.19 ± 0.09[a]	1.63 ± 0.25[b]
J37	0.98	0.09 ± 0.008[b]	1.78 ± 0.42[a]
J38	0.99	0.18 ± 0.09[a]	1.26 ± 0.37[a]

[a–d]Different superscripts within a column indicate significant difference ($P < 0.05$).
[1]Data represent the mean value of duplicate ± SD.
[2]μ = growth rate, measured in absorbance units per hour.

Table 7. Microbial growth parameters, derived from modified Gompertz equation, of *Salmonella typhimurium* and *Lactobacillus* spp.[1]

Item	R^2	μ (abs/h)[2]	Generation time (h)
S. typhimurium	0.99	0.20 ± 0.06[a]	1.25 ± 0.15[a]
J10	0.97	0.15 ± 0.00[b]	1.6 ± 0.15[b]
J20	0.99	0.09 ± 0.003[c]	2.80 ± 0.28[d]
J23	0.99	0.054 ± 0.003[d]	2.76 ± 0.16[c]
J25	0.98	0.19 ± 0.04[a]	1.26 ± 0.11[a]
J28	0.99	0.09 ± 0.004[c]	2.60 ± 0.22[c]
J31	0.96	0.14 ± 0.07[b]	1.59 ± 0.12[b]
J32	0.99	0.08 ± 0.004[c]	2.69 ± 0.17[c]
J33	0.99	0.14 ± 0.04[b]	1.59 ± 0.30[b]
J35	0.98	0.15 ± 0.07[b]	1.62 ± 0.35[b]
J36	0.96	0.18 ± 0.01[a]	1.26 ± 0.18[a]
J37	0.99	0.09 ± 0.003[c]	2.52 ± 0.19[c]
J38	0.97	0.15 ± 0.08[b]	1.26 ± 0.25[a]

[a–d]Different superscripts within a column indicate a significant difference ($P < 0.05$).
[1]Data represent the mean values of duplicates ± SD.
[2]μ = growth rate, measured in absorbance units per hour.

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Capítulo 4

Partial purification and characterization of bacteriocins produced by Lactobacillus fermentum from artisanal Mexican cheese.

Capítulo 4. Partial purification and characterization of bacteriocins produced by *Lactobacillus fermentum* from artisanal Mexican Cheese.

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Resumen

El objetivo de este trabajo fue aislar y caracterizar las bacteriocinas producidas por *Lactobacillus fermentum* aisladas de queso Cocido. Los extractos de *Lactobacillus fermentum* J23 y J32, fueron caracterizados por RP- HPLC en una columna C18. La fracción F3 presentó la mayor actividad antimicrobiana contra *E. coli* ($p < 0.05$) y fue también inhibitoria contra *S. aureus, L. innocua* y *S. typhimurium*. Por el contrario, las fracciones F1, F5 and F7, presentaron mayor actividad antimicrobiana contra *S. aureus* y *L. innocua* pero no para el resto de los microorganismos indicadores. Similarmente, F1 obtenida de *Lactobacillus fermentum* J32, presentó actividad antimicrobiana contra *S. aureus* y *L. innocua,* pero no contra el resto de los microorganismos. Por otro lado, F3 and F4 producidas por *Lactobacillus fermentum* J32 presentaron actividad antimicrobiana contra todos los microorganismos indicadores. Estos resultados sugieren que ambas cepas de *Lactobacillus fermentum* evaluadas produjeron al menos dos diferentes bacteriocinas. De las fracciones de RP-HPLC colectadas para J23 y J32, se identificaron las secuencias de 6 y 9 péptidos, respectivamente, por espectrometría de masas por trampa de iones (MS/MS). El espectro inhibitorio de las bacteriocinas mostraron un amplio rango de actividad antimicrobiana contra patógenos que podrían estar presentes en alimentos. Las bacteriocinas y sus cepas productoras podrían tener aplicación como bioconservantes

Partial purification and characterization of bacteriocins produced by specific *Lactobacillus fermentum* strains isolated from artisanal Mexican Cocido Cheese

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Abstract

The aim of this work was to partially purify and characterize bacteriocins produced by *Lactobacillus fermentum* isolated from artisanal Mexican Cocido cheese. Bacteriocins isolated from the cell-free supernatant of *Lactobacillus fermentum* J23 and J32 were partially purified by reversed phase-HPLC (RP-HPLC) on a C_{18} column. The crude extracts obtained from J23 or J32 showed activity against all indicator pathogenic strains tested. For J23, one of the purified fractions (F3) presented the highest ($p < 0.05$) activity against *E.coli* and was also inhibitory against *S. aureus, L. innocua, S. typhimurium and S. cholerae.* On the other hand, F1, F5 and F7, presented antimicrobial activity against *S. aureus* and *L. innocua* but not for the rest of the indicator microorganisms. Similarly, F1 from *Lactobacillus fermentum* J32, presented antimicrobial activity against *S. aureus* and *L. innocua,* but not against the rest of the microorganisms. On the other hand, F3 and F4 produced by *Lactobacillus fermentum* J32 presented antimicrobial activity against all indicator microorganisms. These results suggested that both *Lactobacillus fermentum* strains tested produced at least two different bacteriocins. Six and nine peptides for J23 and J32, respectively, were sequenced for the different RP-HPLC collected fractions by using ion trap mass spectrometry (MS/MS). The inhibitory spectrum of these bacteriocins showed a wide range of activities against similar bacterial strains, food-spoilage and food-borne pathogens. The bacteriocin and its producing strain may find application as bio-preservatives and food-borne pathogens in food products.

Keywords: bacteriocin, lactic acid bacteria; antimicrobial activity, microorganism pathogens, purification

Introduction

LAB produce the antimicrobial substances lactic acid, hydrogen peroxide, diacetyl, acetoin as well as small heat-stable inhibitory peptides known as bacteriocins and have considerable advantage in the competition with other micro-organisms, including pathogens and other harmful bacteria (Simova et al., 2009). Bacteriocins of lactic acid bacteria (LAB) are antimicrobial peptides that are synthesized ribosomally and secreted by bacteria. Also, secondary metabolites are secreted to inhibit the growth of competitive bacterial strains (Nirmala et al. 2001; Cleveland et al. 2001; Mataragas et al. 2002; Halami et al. 2005; Drider et al. 2006; Kjos et al. 2010; Hwanhlem et al. 2013). Most bacteriocins are small, basic (a net positive charge at neutral or slightly acidic pH) and amphiphilic in nature and vary in spectrum and mode of activities. Bacteriocins also display different molecular structures, molecular masses, thermo stabilities, pH ranges of activity and genetic determinants (Nes et al. 1996; Cintas et al. 2001; Parada et al. 2007; Javed et al. 2011; Zacharof and Lovitt 2012).

The bacteriocins of some bacteria inhibit growth of closely related microbes, while others inhibit a much wider range of microorganisms, including food-borne pathogens and spoilage microorganisms such as *Listeria monocytogenes,*

Bacillus cereus, Staphylococcus aureus and *Clostridium tyrobutyricum* (Gálvez et al. 2008).

In the last 25 years, intensive research into the bacteriocins produced by LAB has been undertaken with the aim of improving the microbial quality and safety of fermented products (de Vuyst and Leroy 2007). Bacteriocins produced by the so-called “dairy strains” are particularly worthwhile to investigate since their effect on pathogenic microorganisms offers important potential for expanding the range of healthful dairy foods (Simova et al., 2009).

Basically, there are two ways of bacteriocin application to dairy foods: either directly as a purified compound or as a crude bacterial fermentate, or indirectly via the bacteriocin-producing organism. Both ways have advantages and disadvantages, which largely depend on the particular food and the intended function of the bacteriocin. The *in situ* production of a bacteriocin by living bacteria within a food matrix is compelling especially in processes involving fermentations, such as the production of fermented sausages, vegetables, sourdough, or dairy products (Hammami et al., 2010). However, the success of such an applications requires a producer strain that is able to survive within the particular environment, that does not have a negative impact on product taste or quality, and that produces the respective antimicrobial substance in sufficient amounts to inhibit the target organism (Galvez, et al., 2008; Settanni and Corsetti, 2008).

In previous work, we reported on the screening for BLS production by wild *Lactobacillus*, isolated during the manufacture of artisanal Mexican Cocido cheese, with bioactivity against gram-positive and gran-negative bacteria

(Heredia-Castro, et al. 2015). Fourteen out of 18 *Lactobacillus* spp. showed antimicrobial activity against different test microorganisms, and 12 presented bacteriocin-like substances. However, generation times and growth rates indicated that the antimicrobial activity of crude extracts from three different strains was effective against four indicator microorganisms. In fact, one the crude extracts showed inhibition not only against gram-positive but also against gram-negative bacteria (Heredia-Castro, et al., 2015). Thus, it would be of interest to study the two most promising strains. Thus, in this work, we report on the isolation and characterization of the bacteriocins produced by two of of *Lactobacillus fermentum*.

2. Materials and Methods

2.1 Strains, media and culture conditions

The strains *Lactobacillus fermentum* J23 and J32 were isolated from artisanal Mexican Cocido cheese. Strains were cultured in 10 mL of MRS broth at pH 6.5 (MRS, BD Difco™, Sparks, MD, USA). Two subcultures were inoculated at 1% and incubated at 37 °C. The first subculture was incubated for 24 h and the last subculture was incubated for 72 h. The indicator microorganisms: *Staphylococcus aureus* (*S. aureus*) ATCC 29213, *Listeria innocua* (*L. innocua*) ATCC 33090, *Salmonella typhimurium* (*S. typhi*) ATCC 14028, *Salmonela cholerae* (*S. cholerae*) ATCC 10708 and *Escherichia coli* (*E. coli*) ATCC 25922 were cultued in Brain Heart Infusion broth (BHI, BD Difco™, Sparks, MD, USA). Two subcultures were inoculated at 1% and incubated at 37 °C. The first

subculture was incubated for 24 h and the last subculture was incubated for 12 h (Messi et al., 2003).

2.2. Crude Extract Preparation

Cultures were grown in MRS broth (MRS, Difco™, Sparks, MD, USA) and incubated for 72 h at 30°C in a water bath shaker (Lab-Line Instruments Inc., Melrose Park, IL). Aliquots of culture medium were centrifuged at 4,000 x g for 20 min, at 4°C. The cell free supernatants were adjusted to pH 6.5 with a 1 N NaOH solution and treated with catalase enzyme (1000 U/mL) for 1 h at 25 °C to prevent the inhibitory effect of organic acids and hydrogen peroxide. Then, the treated supernatants were filter sterilized (0.22 µm, Millex-GV, Millipore SpA, Italy) and stored at 4 °C (Karapetyan et al., 2010). Before HPLC analysis, filtered and treated supernatants were ultrafiltered through 3-kDa-cutoff membranes (Pall Life Sciences, Port Washington, NY) at 9,800 x g for 6 min (J2-21 rotor; Beckman Coulter Inc.). Permeates were collected, filtered through a 0.45 µm disposable hydrophilic filter and subjected to reverse phase (RP) HPLC analyses.

2.3 Isolation of Bacteriocin Containing Fractions (BCF) by Reversed-Phase HPLC

Bacteriocin containing fractions (BCF) were obtained from filtered permeates by reversed-phase (RP) HPLC (1100 series; Agilent Technologies Japan Ltd., Tokyo, Japan). Separation was carried out with a Zorbax 300 Extend-C18 (250 x 4.6 mm, 5 µm particle size, 300-A pore size) from Agilent Technologies (Agilent

Technologies Japan Ltd., Tokyo, Japan) with a solvent flow rate of 0.25 mL/min. Once the column was equilibrated with solvent A (0.04% TFA in water), 20 µL of the sample were injected. BCF were eluted with an increasing gradient of solvent B (0.03 % TFA in acetonitrile) from 0 to 100 % in solvent A for 70 min. BCF monitored at 214 nm were collected from 15 chromatographic runs, freeze dried and resuspended in 1.5 mL of distilled water for antimicrobial activity determination and mass spectrometry analysis.

2.4 Analysis of BCF by Tandem Mass Spectrometry

Mass spectrometry analysis was performed using a 1100 Series LC/MSD Trap (Agilent Technologies Inc., Waldbronn, Germany) equipped with an electrospray ionization source (LC-ESI-MS). The conditions for MS detection were those as described by Rodriguez-Figueroa et al., 2011. The total ion chromatograms were taken in a mass range from m/z 50 to 2500 mass/charge (m/z). The molecular mass was calculated from m/z value of the ion peak with the highest intensity detected from each BCF.

2.5 Antimicrobial activity determination of BCF

Antimicrobial activity was tested against indicator microorganisms *S. aureus* ATCC 29213, *L. innocua* ATCC 33090, *S. typhi* ATCC 14028, *S. cholerae* ATCC 10708 and *E. coli* ATCC 25922. BCF collected from RP HPLC were used to evaluate antimicrobial activity by the disk diffusion method. An aliquot of 20 µL fractions was applied on a sterilized cellulose disk (6 mm) placed on a Mueller Hinton soft agar plate (0.8% w/v) inoculated with each pathogen (10^7 CFU/mL).

Plates were incubated at 37 °C for 24 h, and the diameters of the inhibition zones around the disk were measured (Nespolo et al., 2010). Fractions that presented inhibition zones larger or equal to 6 mm were selected (Ponce et al., 2008).

2.5 Statistical analysis

The experiments followed a complete randomized design with two replicates per treatment. Data were analyzed by one-way ANOVA with a significance level of 0.5 %. Means were analyzed by the comparison test of Tukey-Kramer with a significance level of 0.05 %. For statistical analysis, the NCSS statistical software version 2007 (NCSS, LLC, Kaysville, UT) was used.

3. Results and discussion

Permeats of crude extracts (< 3 kDa) from Lactobacillus fermentum J23 and J32 presented several peaks at 214 nm (Figures 1 and 2). BCF (F1, F3, F5 and F7) collected for *Lactobacillus fermentum* J23 depicted in Figure 1, showed antimicrobial activity against the indicator strains (Figure 3). F3 had significantly (p <0.05) higher antimicrobial activity against *E.coli* than against *Salmonella thypimurium* or *cholerae* (Figure 3). Also F3 had a significantly (p <0.05) higher antimicrobial activity against *E.coli* than the crude extract (Figure 3). On other hand, fractions F1, F5 and F7 only showed antimicrobial activity against *S. aureus* and *L. innocua* and this activity was significantly higher (p<0.05) than that of the crude extract. Data suggest that *Lactobacillus fermentum* J23 produced at least two different bacteriocins. These results are partially in

agreement with those reported by Zapata et al. (2009), who found that the bacteriocin produced by *Lactobacillus plantarum* inhibited not only Gram positive bacteria, but also Gram negative bacteria such as *E. coli* and *Salmonella*. It has been reported that different spectrum of inhibitory action may be obtained depending on the bacteriocin producing strain, the indicator stain, and also the method used for bacteriocin detection (Drider et al., 2006).

BCF (F1, F3 and F4) from *Lactobacillus fermentum* J32 showed antimicrobial activity against different indicator strains (Figure 4). F1 presented antimicrobial activity against *S. aureus* and *L. innocua,* but not against *E. coli, S. typhimurium or S. cholera* (Figure 4). On the other hand, F3 and F4 presented antimicrobial activity against all indicator strains, but this activity was significantly ($p<0.05$) lower for F4 than for the crude extract for all indicator strains (Figure 4). Thus, these data suggest that *Lactobacillus fermentum* J32 also produced at least two different bacteriocins.

The active fractions obtained after RP chromatography were analyzed by tandem mass spectrometry. Six and nine peptides were identified for J23 and J32, respectively and their molecular weight was calculated (Table 1 and 2). Similarly, bacteriocins with molecular masses ≤3 kDa were reported for *E. faecium* (Chen 2007). In recent years, there has been a large number of reports dealing with the antimicrobial activity, purification and characterization of bacteriocins produced by LAB. However, because of the potencial use of bacteriocins from lactic acid bacteria as food preservatives, it is of great importance to gain insight into their chemical structure. Although a substantial number of different bacteriocins have been described, their primary structure is

still lacking and only a few have been described in detail at the molecular level. To the best of our knowledge, this is the first report on the structural characterization of bacteriocins produced by *Lactobacillus fermentum*. The structural characterization of the bacteriocins as presented in this work, may provide valuable information for further studies on the efficient production of these peptides for industrial applications.

4. Conclusions

The bacteriocins produced by *Lactobacillud fermentum* J23 and J32*,* inhibited growth of a range of Gram positive and Gram negative bacteria associated to food poisoning such, such as *E. coli*, *S. aureus*, *L. innocua* and *Salmonella*. These results may be considered a starting point which may lead to study their uses in different food matrices.

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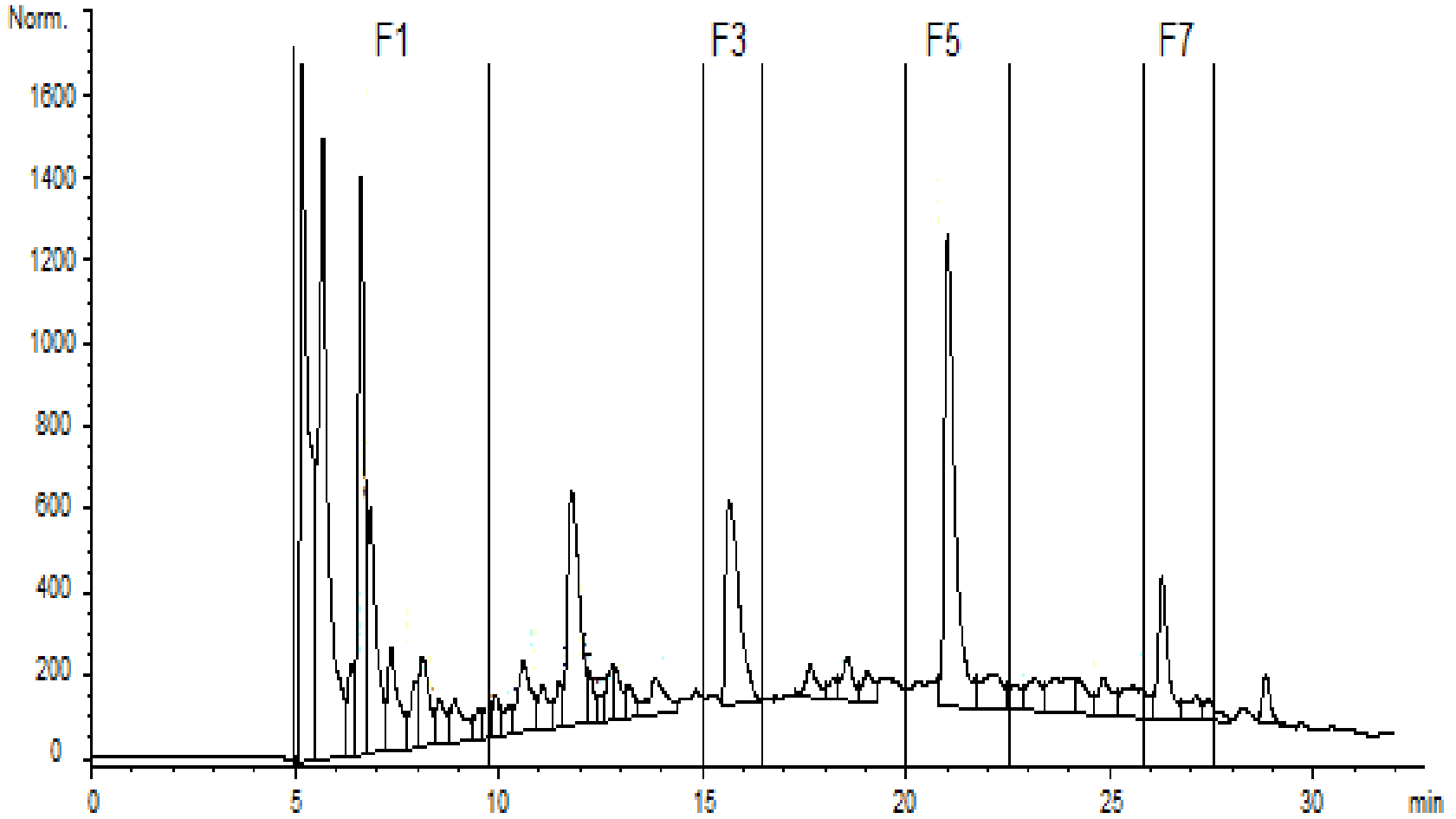
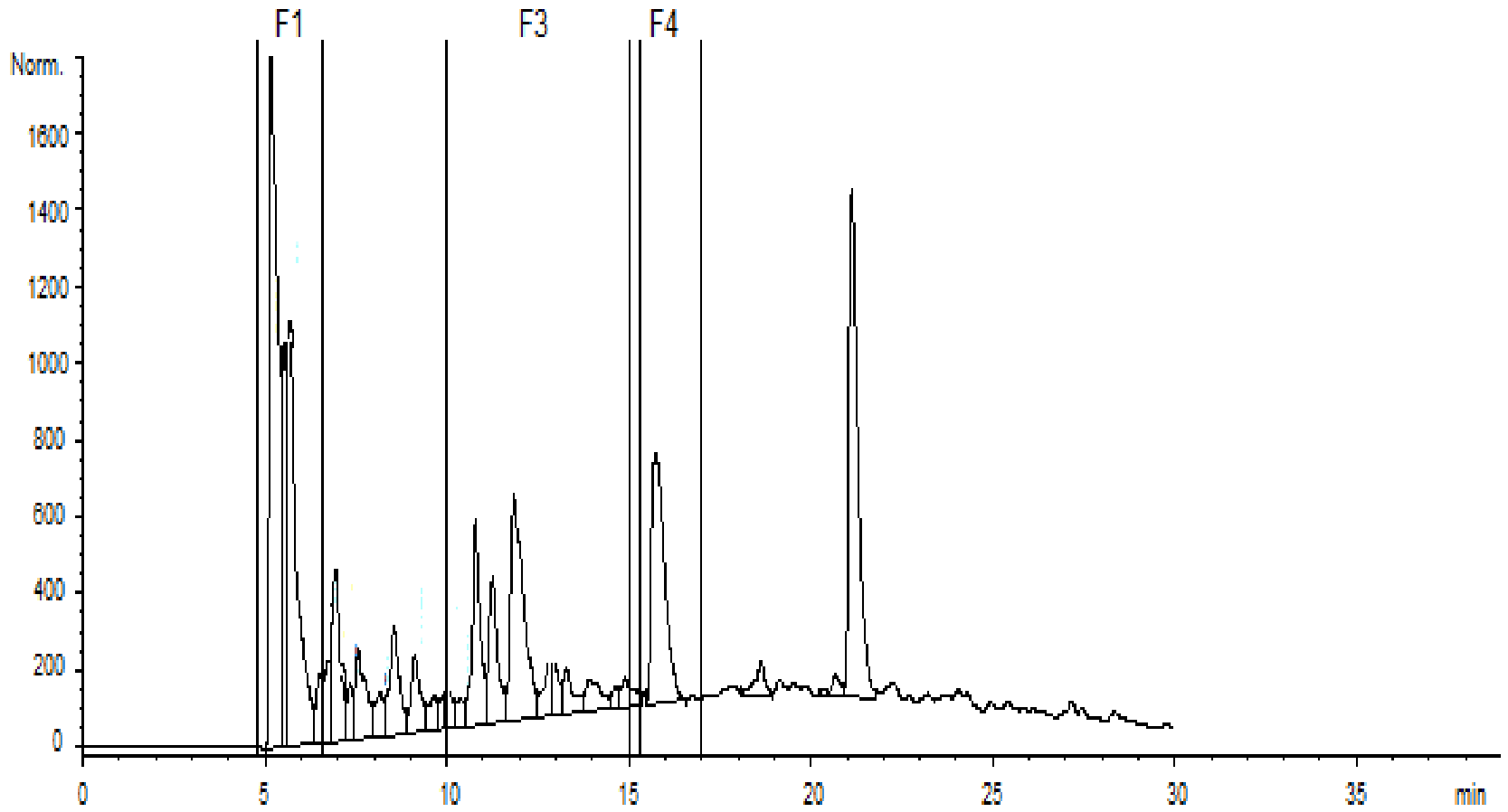


303

304 **Figure 1.** Fractionation by RP-HPLC of the permeate (< 3 kDa) obtained from the crude extract of *Lactobacillus*
305 *fermentum* J23.



306

307 **Figure 2.** Fractionation by RP-HPLC of the permeate (< 3 kDa) obtained from the crude extract of *Lactobacillus*
308 *fermentum* J32.

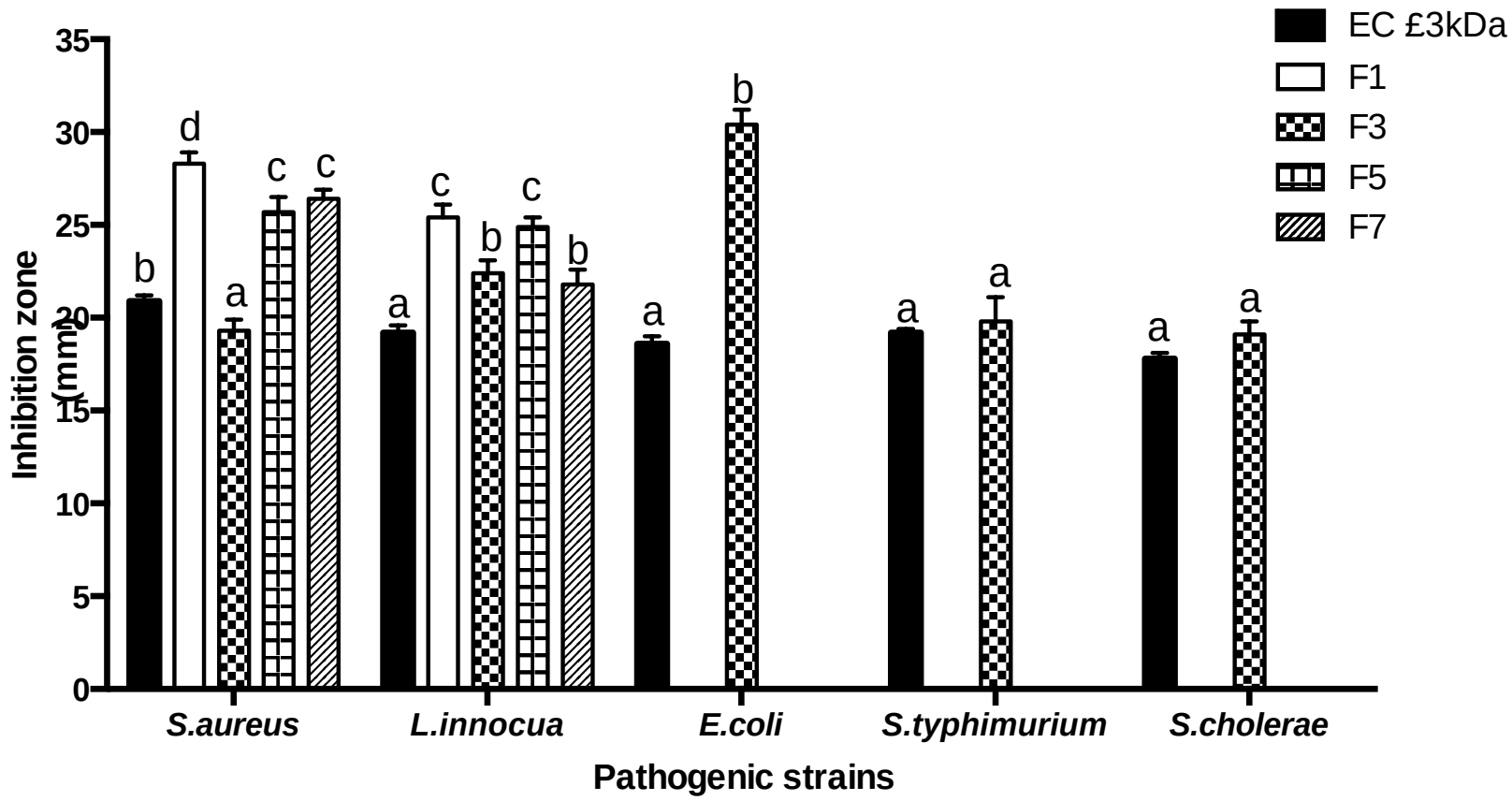
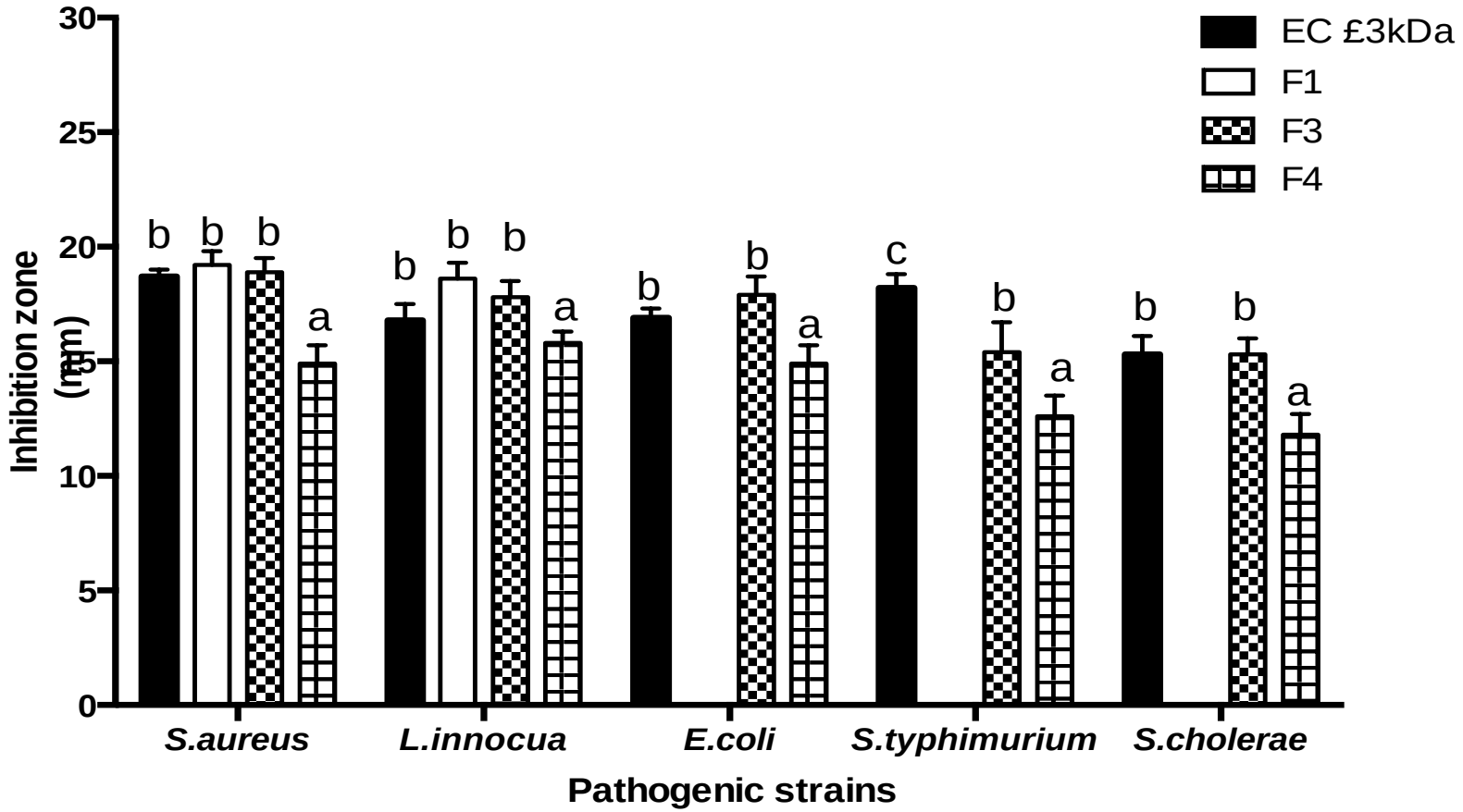


Figure 3. Antimicrobial activity of each RP-HPLC fraction from *Lactobacillus fermentum* J23.
** Inhibition zone (mm) ± SD. The antibacterial activity was determined by the disk diffusion method.
** Different literals among columns indicate significant differences($p<0,05$).



313

314 **Figure 4.** Antimicrobial activity of each RP-HPLC fraction from *Lactobacillus fermentum* J32.
315 ** Inhibition zone (mm) ± SD. The antibacterial activity was determined by the disk diffusion method.
316 ** Different literals among columns indicate significant differences(p<0,05).
317

Table 1. Identification of bacteriocins contained in RP-HPLC fractions from J32 with antimicrobial activity.

Fraction	Theoretical mass	Ion for MS/MS (m/z)	Sequence
F1	2200.2	366.7 (6+)	CTPRGCFGASVVKVVPSFMTD
	2262.8	343.8 (6+)	KSSSYWPVSMWYMTESS
F3	2161.8	360.3 (6+)	AGVDL/IECYWWWKVVVYGS
	2035.8	678.6 (3+)	AAYGAVAGTCMVHHHHHTM
F4	2201.2	550.3 (4+)	ASVKCRVVFMSKVVSGHVVVVA
	2171.7	723.9 (3+)	TSKSWWSL/IAHYDAHSVVVV
	2126.4	354.4 (6+)	APADARSSSAHSSSWSWWM
	2153.0	430.6 (5+)	AASEDSVSVSVSVSVSFFY
	2116.0	2116.0 (1+)	ATSSSSYHVSSSNKSSMQHC

Table 2. Identification of bacteriocins contained in RP-HPLC fractions from J23 with antimicrobial activity.

Fraction	Theoretical mass	Ion for MS/MS (m/z)	Sequence
F1	2168.8	2168.8 (1+)	TMSSSSSSVVHAVVSSSSSSSSS
F3	2056.2	685.4 (3+)	ASSDVVVFEVVVVVRCSSSS
F5	2135.1	711.7 (3+)	CSFMSKVSMWVVSSSSSSKS
	2140.9	2140.9 (1+)	SSKYCVVSSSSEL/IQMVCRH
F7	2014.8	503.6 (4+)	CCAASSPMNVSSVVVQMSSGA
	2006.0	501.5 (4+)	GPWSSPMNVSSVVVQMSSGA

Capítulo 5

Effect of bacteriocin containing fractions produced by *Lactobacillus fermentum* on the inactivation of pathogens in pasteurized milk

Capítulo 5. Effect of bacteriocin containing fractions produced by *Lactobacillus fermentum* on the inactivation of pathogens in pasteurized milk.

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Resumen

Se evaluó la actividad antimicrobiana de fracciones que contienen bacteriocinas (BCF) obtenidas de *Lactobacillus fermentum* J23 y J32 contra patógenos indicadores en leche descremada y semi-descremada pasteurizada. Para esto, se determinaron las cuentas de células viables de los patógenos indicadores después de la inoculación de la leche pasteurizada (10^7 cfu/mL) a 37 C por 12 h y a 4 C por 15 días de almacenamiento. Las cuentas de células de *S. aureus*, *L. innocua*, *E.coli* y S*. typhimurium* en leche con BCF fueron significativamente más bajas que la del testigo (P <0,05). BCF de J23 y J32 presentaron actividad antimicrobiana contra los patógenos después de 4 h de incubación a 37 C. Además, las cuentas de células fueron indetectables en la leche descremada con BCF de J23 para *S. aureus* y *E. coli* después de 15 días de almacenamiento a 4 C. La actividad antimicrobiana de BCF de J23 y J32 contra patógenos Gram positivos y Gram negativos en la leche descremada pasteurizada fue significativamente mayor (P <0.05) que en la leche semi-descremada. Los resultados presentados en este estudio sugirieron que BCF de J23 y J32 son bacteriocinas con un amplio espectro inhibitorio en la leche. Por lo tanto, BCF de J23 y J32 podrían ser utilizadas en el control de patógenos durante la elaboración de productos lácteos y presentan un gran potencial como bioconservantes en alimentos.

Effect of bacteriocin containing fractions produced by *Lactobacillus fermentum* on the inactivation of pathogens in pasteurized milk

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Abstract

Antimicrobial activity of bacteriocin containing fractions (BCF) obtained from *Lactobacillus fermentum* J23 and J32 against pathogens in milk was evaluated. Effect of milk fat on the activity of BCF from J23 and J32 against *S. aureus*, *L. innocua*, *E. coli* and *S. typhimurium* in pasteurized skim and low fat milk was evaluated. Changes of pathogens viable cell counts after milk inoculation (10^7 CFU/mL) during incubation at 37 °C for 12 h and storage at 4°C for 15 d were determined. Cell counts of the different pathogens in milk with BCF were significantly lower than the control ($P < 0.05$). BCF from J23 and J32 were bactericide against all the pathogens tested after 4 h of incubation at 37 °C. Cell counts were undetectable in skim milk with BCF from J23 for *S. aureus* and *E. coli* after 15 d of storage at 4 °C. Antimicrobial activity of the BCF from J23 and J32 against Gram positive and Gram negative pathogens in pasteurized skim milk was significantly higher ($P < 0.05$) than in low fat milk. The results presented in this study suggested that BCF from J23 and J32 were bacteriocins with a broad inhibitory spectrum in milk. Thus, BCF from J23 and J32 could be used in the control of pathogens during the manufacture of dairy products and present great potential as food biopresevatives.

Keywords: Bacteriocin, lactic acid bacteria, antimicrobial activity, milk, dairy products

1. Introduction

Bacteriocins are peptides synthesized within ribosomes and released into the extracellular medium by Gram positive and Gram negative microorganisms, although, those produced by lactic acid bacteria (LAB) have received greater attention, due to their high potencial for application in the food industry as natural antimicrobial agents (da Silva et al., 2015). The use of bacteriocins to control foodborne pathogens and spoilage bacteria has been reported in variety of foods including dairy products (Arqués et al., 2011). Bacteriocins and bacteriocin producing strains have been used in dairy products to control *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes* (Han et al., 2013). Bacteriocins of LAB are generally recognized as safe (GRAS) and their use to preserve foods including dairy products, meat and vegetables has been reported (Gao et al., 2013). However, the capacity of LAB to produce bacteriocins and other antimicrobial molecules, and the bacteriocins activity in food matrices may be affected by several factors such as chemical composition, the use of food additives and the physical conditions of food (Cleveland et al., 2001; Bizani et al., 2008). Several authors have reported the good potential of bacteriocins to inhibit Gram-positive bacteria both in model and in real food systems, such as cheese, meat and ready-to-eat vegetables (Jamuna et al., 2009; Loessner et al., 2003; Molinos et al., 2005). Nisin was the first characterized bacteriocin and it is produced by *Lactococcus lactis,* it has a antimicrobial spectrum against Gram positive pathogens associated with foods (Kim et al., 2008). The use of nisin in over 50 countries, mainly in the European Union, is allowed as at food preservative (Jones et al., 2005). In fact, nisin, and

in particular the natural variant Z, is commonly used in various food products in order to increase their microbiological safety, due to its high stability (de Arauz et al., 2009). Recent studies have shown that the nisin spectrum of activity may also be extendend to Gram negative bacteria by using it in combination with other antimicrobial agents (Kim et al., 2008). The activity against Gram negative bacteria was not frequently reported in bacteriocins from LAB. There have been few studies reported on the effects of chemical composition and processing on the activity of bacteriocins against Gram negative bacteria in milk products (Gao et al., 2013). The potencial application of bacteriocins against Gram negative bacteria through their synergistic effects with other antimicrobials has gained increased interest (Rodríguez et al., 2005).

L. fermentum J23 and J32 were isolated from artisanal Mexican cheese, selected by their antimicrobial properties against Gram positive and Gram negative bacteria. Thus, the objetive of the present work was to evaluate the antimicrobial capacity of *L. fermentum* J23 and J32 agaisnt *S. aureus*, *L. innocua*, *S. typhimurium* and *E. coli* in pasteurized skim and low fat milk.

2. Materials and methods.

2.1 Culture and growth conditions

Staphylococcus aureus (*S. aureus*) ATCC 29213, *Listeria innocua* (*L. innocua*) ATCC 33090, *Salmonella typhimurium* (*S. typhimurium*) ATCC 14028 and *Escherichia coli* (*E. coli*) ATCC 25922 were cultured in BHI (Brain Heart Infusion broth, BD Difco™, Sparks, MD, USA). Three subcultures were inoculated at 1% and incubated at 37 °C. The first subculture was incubated for 24 and 16 h and

the last subculture was incubated for 8 h. *Lactobacillus* strains were subcultured in 10 mL of MRS at 37 °C (pH 6.5). Three consecutive cultures (1% inoculum) were prepared and incubated for 24, 20 and 16 h, respectively. The last culture was used as the inoculum for all the experiments (fresh culture).

2.2 Preparation of crude extracts (CE)

L. fermentum J23 *and* J32 were grown in MRS broth and incubated for 16, 18, 24 and 72 h at 37 °C in a shaked water bath (Lab-Line Instruments, INC, Melrose Park, IL). Aliquots of culture media were centrifuged at 4,000 g for 20 min, at 4°C. The CE were adjusted to pH 6.5 with a 1 N NaOH, treated with catalase enzyme (1000 U/mL) for 1 h at 25 °C and filter sterilized (0.22 µm, Millex-GV, Millipore SpA, Italy). The untreated and treated CE were stored at 4 °C (Karapetyan et al., 2010).

2.3 Effect of bacteriocins containg fractions (BCF) of J23 and J32 in milk against different pathogens.

Commercial skim and low fat dry milk were reconstituted at 10% (v/v) and pasteurized at 65 °C for 30 min. Milk samples with 1% BCF from J23 or J32 were inoculated with each pathogen (10^7 CFU/mL). Milk inoculated with the indicator pathogens was used as a control. BCF with antimicrobial activity from J23 or J32 were collected by RP-HPLC as reported by Heredia-Castro et al., 2015b. Milk bacterial counts were determined after inoculation, at 2-h intervals during the first 12h of incubation at 37 °C, and at 3-d intervals for up to 15 days during storage at 4 °C.

120

121 **2.4 Microbiological analysis**

122 *S. aureus* counts were determined on duplicate plates of Baird-Parker agar base
123 (BD Difco™, Sparks, MD, USA) supplemented with EY Tellurite Enrichment (BD
124 Difco™, Sparks, MD, USA). *L. innocua* counts were determined on duplicates
125 plates of PALCAM Listeria agar (BD Difco™, Sparks, MD, USA). *E. coli* counts
126 on duplicate plates of McConkey agar (BD Difco™, Sparks, MD, USA). The
127 bacteria counts were incubated at 37 °C for 48 h.

128

129 **2.5 Statistical analysis**

130 The experiments followed a complete randomized design with three replicates
131 per treatment. Growth data was carried out in duplicates. Data was analyzed by
132 one-way ANOVA with a significance level of 0.5 %. Means were analyzed by the
133 comparison test of Tukey-Kramer with a significance level of 0.05 %. For
134 statistical analyses, the NCSS statistical software version 2007 (NCSS, LLC,
135 USA) was used.

136

137 **3. Results**

138 **3.1 Effect of BCF from J23 and J32 on the growth of Gram negative and**
139 **Gram positive indicator pathogens in pasteurized milk.**

140 The inhibitory effect of BCF from J23 and J32 on Gram positive and Gram
141 negative pathogens in milk are shown in Figure 1. Counts of the different
142 pathogens in milk were significantly reduced by the treatments ($P < 0.05$). After
143 4 h, antimicrobial activity of BCF from J23 or J32 was bacteriostatic for all the

144 pathogens. After 12 h, there was a log count (ufc/mL) reduction of 5.5 to 7 for all
145 the indicator pathogens with respect to the control. Similar results were obtained
146 for *S. aureus* in milk with nisin added. (Kim et al., 2008).
147
148 **3.2 Effect of BCF from J23 and J32 on growth of Gram negative and Gram**
149 **positive indicator pathogens during storage of pasteurized milk.**
150 The effect of BCF from J23 and J32 on growth of Gram positive and Gram
151 negative pathogens in pasteurized skim milk and low fat milk during storage at 4
152 C are shown in Figure 2 and 3. Cell counts of the pathogens (*S. aureus*, *L.*
153 *innocua*, *E. coli* and *S. typhimurium*) in pasteurized milk without BCF increased
154 3 log cycles after one day storage at 4 °C. The addition of 1% of BCF from J23
155 or J32, decresead cell counts significantly ($P < 0.05$) since one day of storage
156 (Figure 2 and 3). The addition of BCF from J23 in skim and low fat milk,
157 decreased *S. aureus* counts by 2.76 and 2. 29 log cfu/mL respectively after 3 d.
158 Furthermore, after 15 d, cell counts were undetectable in skim milk and
159 decreased 5.13 log cfu/mL with respect to the control. This results suggests that
160 the mode of action of BCF from J23 against *S. aureus* is bactericide. Similar
161 bactericide effect by BCF from J23 and J32 in skim and low fat milk on *E. coli*,
162 was observed after 3 d since counts decreased by 3.4 and 2.58 log cfu/mL,
163 respectively. Also, after 15 d, cell counts were undetectable in skim milk and
164 decreased 5.86 log cfu/mL in low fat milk. Effects of BCF from J23 and J32 in
165 skim and low fat milk, decreased *L. innocua* counts by 3.01 and 2.69 log cfu/mL
166 after 3 d. Also, after 15 d, cell counts decreased by 5.43 and 4.74 log cfu/mL in
167 skim and low fat milk, respectively. Effects of BCF from J23 and J32 in skim and

low fat milk, decreased *S. typhimurium* by 5.91 and 4.61 log cfu/mL after 3 d. Also, after 15 d, cells counts decreased by 6.21 and 5.61 log cfu/mL in skim and low fat milk, respectively. The BCF of J23 and J32 had a broad and significantly antimicrobial activity against *S. aureus*, *L. innocua*, *E.coli*, and *S. typhimurium*. In general, it was observed that BCF from J23 and J32 presented significantly ($P < 0.05$) higher antimicrobial activity in skim milk than in low fat milk. It has been reported that bacteriocins may be adsorbed onto milk fat globules and as a result, the amount of bacteriocins available to react with the cell membrane of the target organism may be reduced (Bhatti et al., 2004).

4. Conclusions

The results presented in this study suggested that BCF from J23 and J32 contained bacteriocins with a broad inhibitory spectrum in milk. Therefore, BCF may have potencial application for inhibiting growth of Gram positive and Gram negative pathogens during the manufacture and storage of dairy products..

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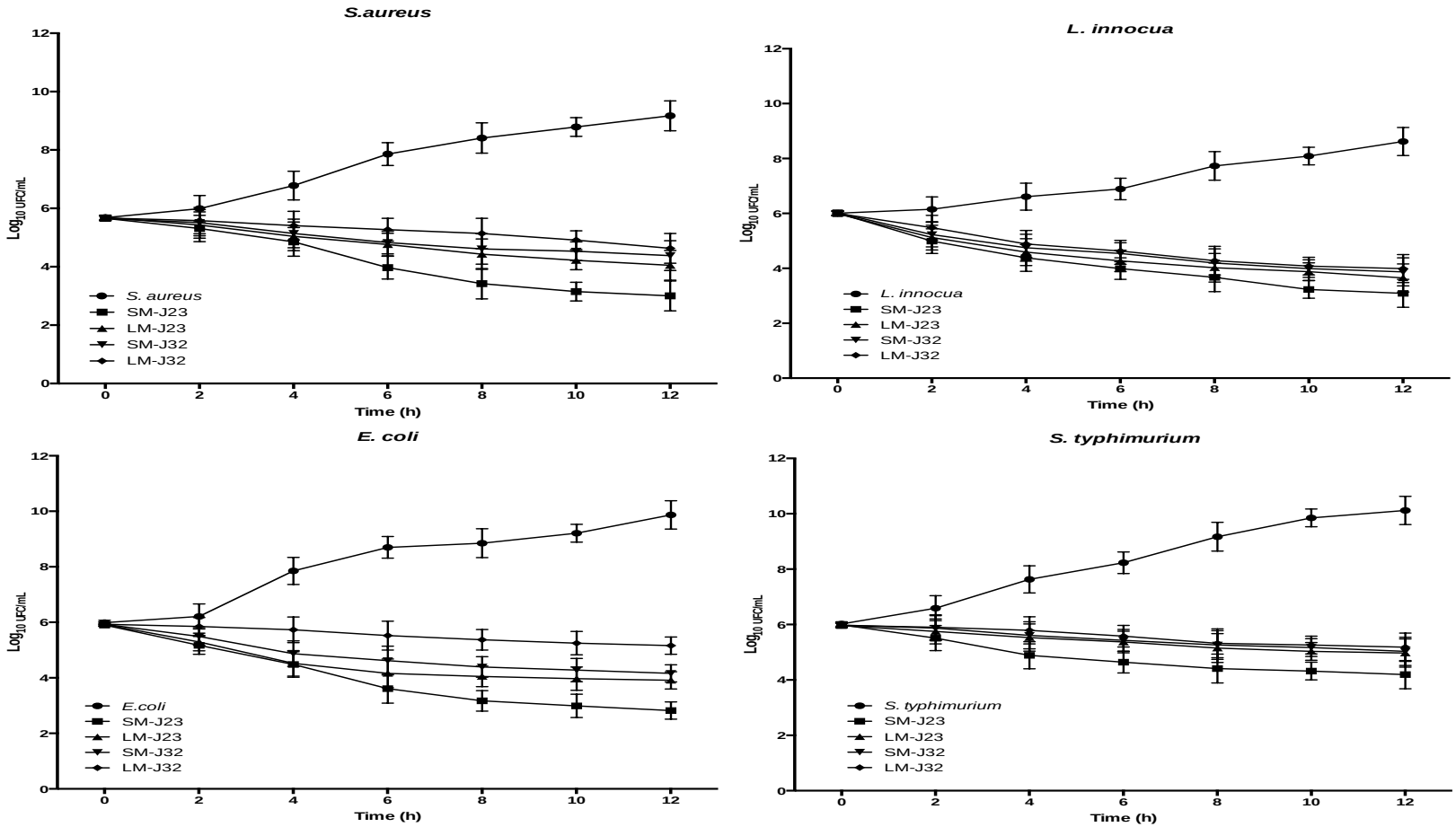

246

247 **Figure 1.** Effect of the BCF from J23 and J32 on growth of Gram negative and Gram positive pathogens in pasteurized

248 milk after incubation at 37 °C for 12 h. Skim milk (SM). Low fat milk (LM).

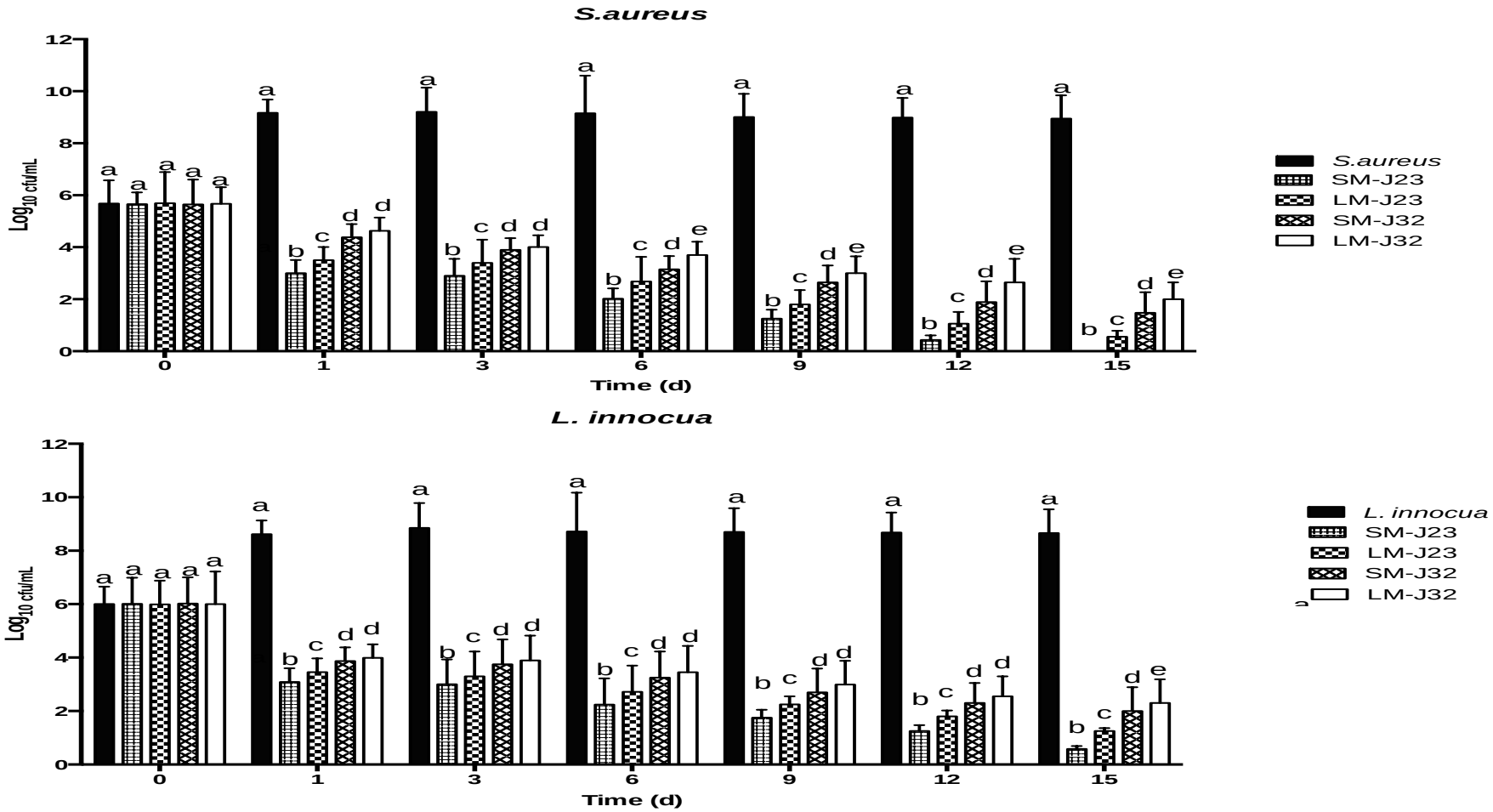

Figure 2. Inhibitory effect of the crude extracts J23 and J32 on the growth of Gram positive pathogens in milk pasteurized during storage at 4 °C for 15 days. Skim milk (SM). Low fat milk (LM). Different literal indicates significant by each time (days) ($P < 0.05$). Error bars indicate 95% confidence interval.

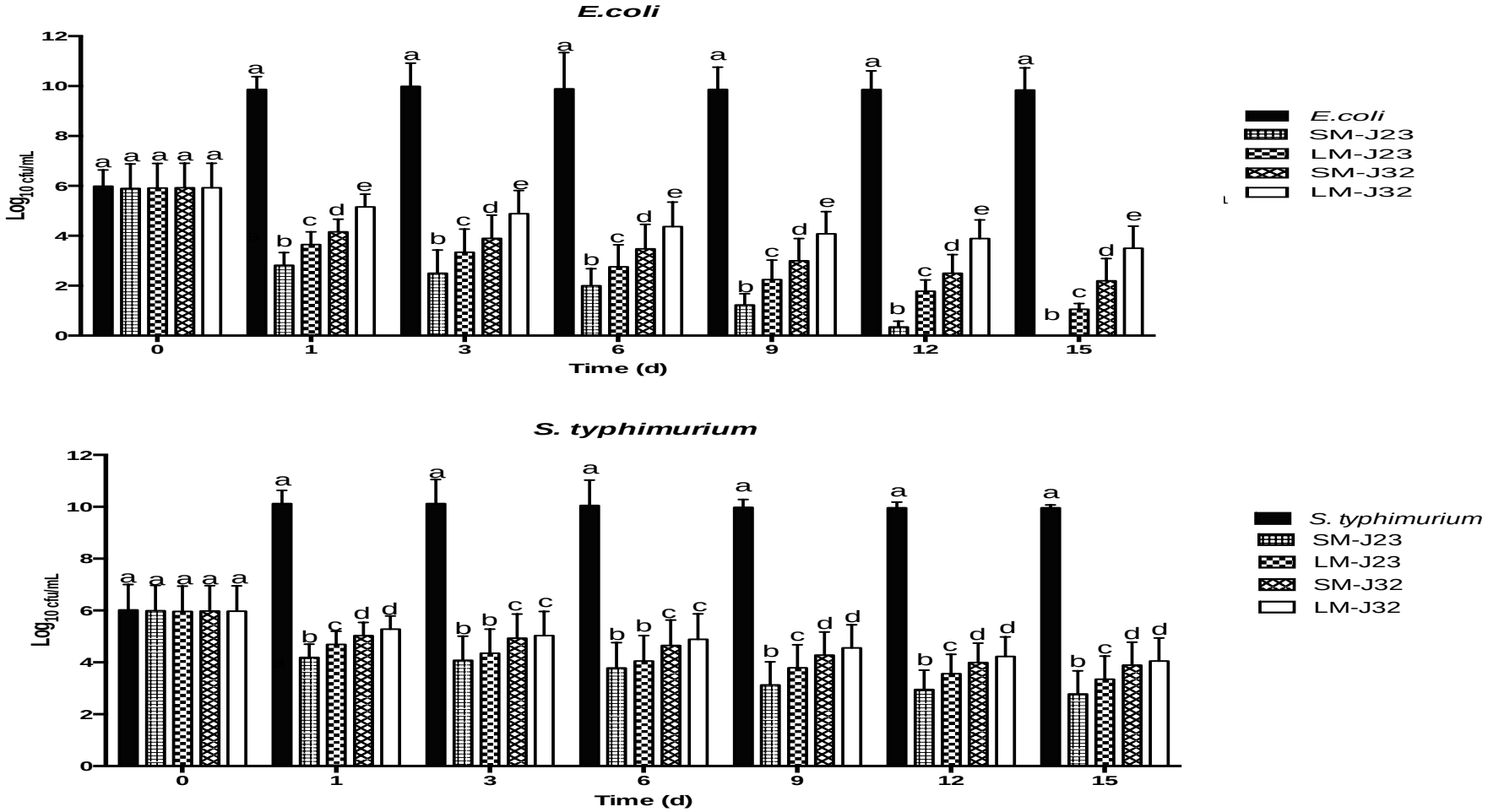

Figure 3. Inhibitory effect of the crude extracts J23 and J32 on the growth of Gram negative pathogens in milk pasteurized during storage at 4 °C for 15 days. Skim milk (SM). Low fat milk (LM). Different literal indicates significant by each time (days) ($P < 0.05$). Error bars indicate 95% confidence interval.

06