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

REGULACIÓN DE CITOCINAS SÉRICAS POR LA ADMINISTRACIÓN DE LECHES FERMENTADAS CON CEPAS ESPECÍFICAS DE *Lactobacillus* spp. O *Lactococcus* spp. EN UN MODELO MURINO ESTIMULADO CON LPS

Por:

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Los miembros del comité designado para la revisión de la tesis de Aline Reyes Díaz, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctor en Ciencias.

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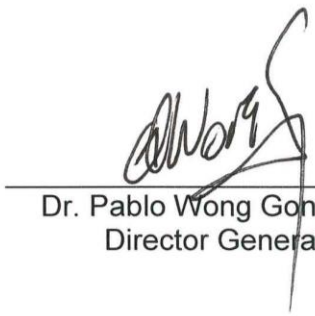

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TABLA DE CONTENIDO

RESUMEN

Los productos lácteos fermentados por bacterias ácido lácticas (BAL) son conocidos por sus efectos benéficos a la salud, ya que algunos de ellos contienen componentes bioactivos. La regulación del sistema inmune es uno de los beneficios de estos productos.

El objetivo principal de este estudio fue evaluar el efecto de leches fermentadas por cepas específicas de *Lactobacillus* (J20, J23, J25 o J28) y *Lactococcus* (NRRL B-50 571 o NRRL B-50 572) sobre la regulación de los niveles séricos de citocinas proinflamatorias (IL-1β, IL-6 y TNF-α) y antiinflamatorias (IL-10), en un modelo murino estimulado con lipopolisacárido (LPS). Se seleccionaron las cepas por sus propiedades tecnológicas y se determinaron las condiciones del proceso de fermentación de la leche. Una vez determinadas las mejores condiciones de fermentación, se administraron diferentes tratamientos preparados con cada cepa; a saber, leche fermentada (FM), leches fermentada pasteurizada (PFM) y fracciones <10 KDa de PFM (PFM10). Los tratamientos fueron administrados diariamente durante 4 semanas a ratas Wistar macho. Posteriormente, se indujo un proceso de inflamación aguda en los animales mediante la administración subcutánea (s.c.) de LPS y se determinaron los niveles de las citocinas 6 h posteriores a la inducción. Finalmente se identificaron los péptidos presentes en las leches fermentadas que podrían estar involucrados en la regulación de las citocinas séricas.

Los resultados evidenciaron que los tratamientos preparados con leches fermentadas (FM, PFM y/o PFM10) por cepas de *Lactobacillus* (J20, J23, J25 o J28) o *Lactococcus* (NRRL B-50 571 o NRRL B-50 572) regularon los niveles séricos de citocinas, ya que fueron capaces de disminuir los niveles de IL-6 y

TNF-α e incrementar los niveles de IL-10. Los resultados fueron dependientes de la cepa y el tratamiento administrado. También se observó que el efecto se mantuvo con la pasteurización de las leches fermentadas.

Los péptidos identificados por espectrometría de masas-masas se derivaron principalmente de las proteínas del suero de la leche, presentaron un peso molecular < 3 KDa y varios de ellos presentaron una carga neta positiva. Los péptidos cargados positivamente presentes en las leches fermentadas administradas pudieron haberse unido al LPS que presenta una carga neta negativa logrando con esto disminuir su efecto.

En conclusión, la administración de leches fermentadas por cepas específicas de *Lactobacillus* o *Lactococcus* mostró un efecto regulador de citocinas séricas en un modelo murino. Por lo anterior, las leches fermentadas estudiadas podrían tener potencial como alimentos funcionales.

Palabras clave: Leches fermentadas, citocinas, *Lactococcus*, *Lactobacillus*, lipopolisacárido, péptidos.

ABSTRACT

Dairy products fermented by specific lactic acid bacteria (LAB) are known for their health benefits, since some of them contain bioactive components. The regulation of the immune system is one of these health benefits when milk is fermented with specific strains. The objective of this study was to evaluate the effect of milk fermented by specific strains of *Lactobacillus* spp. (J20, J23, J25 or J28) or *Lactococcus* spp. (NRRL B-50 571 or NRRL B-50 572) on serum levels of pro-inflammatory (IL-1β, IL-6 and TNF-α) and anti-inflammatory (IL-10) cytokines in a lipopolysaccharide (LPS)-stimulated murine model. Specific strains were selected based on their technological properties. Additionally, milk fermentation conditions were established. Once fermentation conditions were established, different treatments were prepared and administered to male Wistar rats for four weeks. Treatments were as follows: fermented milk (FM), pasteurized fermented milk (PFM) and <10 KDa fractions of PFM (PFM10). Next, acute inflammation was induced in rats by subcutaneous (s.c.) injection of LPS and cytokines production was monitored at 6 h after injection of LPS. Finally, peptides present in fermented milk derived from dairy proteins that may be involved in cytokine regulation were identified. The results showed that fermented milk (FM, PFM and/or PFM10) by strains of *Lactobacillus* or *Lactococcus* regulated serum cytokine levels at 6 h after induction with LPS, since IL-6 and TNF levels were inhibited and IL-10 levels were increased in comparison to the control group. This effect was strain- and treatment-dependent. Also, the effect was present after pasteurization of fermented milk.

Mass spectrometric analysis revealed the presence of peptides mainly derived from whey proteins and they have a molecular weight < 3 KDa.

Additionally, positively charged peptides were identified, which could bind to the negatively charged LPS molecule thus reducing its toxic effect.

In conclusion, milk fermented with specific strains of *Lactobacillus* or *Lactococcus* presented a regulatory effect on cytokine serum levels in a murine model. Thus, these fermented milk show potential as functional food.

Keywords: Fermented milk, cytokines, *Lactococcus*, *Lactobacillus*, lipopolysaccharide, peptides.

INTRODUCCIÓN

Las bacterias ácido lácticas (BAL) son microorganismos en forma de cocos o bacilos gram-positivos, no esporulados, no móviles y ácido tolerantes, que comprenden los géneros *Lactococcus*, *Streptococcus* y *Lactobacillus,* entre otros (Claesson et al., 2007). Son reconocidos por sus habilidades fermentativas, por lo que son unos de los microorganismos más estudiados en el campo de los alimentos (D'Souza et al., 2012 Sharma et al., 2012; Steele et al., 2013).

La fermentación es considerada generalmente como una tecnología segura de conservación de alimentos que utliza BAL y puede categorizarse en dos grupos según la materia prima utilizada, como fermentación láctea y no láctea. La leche de diferentes mamiferos se puede utilizar en la fermentación láctea para la elaboración de diferentes productos. La leche de vaca, seguida de la leche de cabra y oveja son las materias primas más utilizadas para la elaboración de productos lácteos fermentados de mayor valor económico en todo el mundo (Widyastuti et al., 2014).

La leche es un alimento altamente perecedero y el propósito principal de la fermentación que utiliza BAL es prolongar el tiempo de vida útil así como preservar los componentes nutritivos de la leche (Gemechu, 2015). La leche en sí es uno de los hábitats naturales de las BAL; aunque, la presencia de las BAL en la fermentación láctea puede ser espontánea o mediante la inoculación de cultivos iniciadores (Wouters et al., 2002; Delavenne et al., 2012).

La fermentación de la leche que utiliza BAL, indudablemente da lugar a productos con propiedades organolépticas apreciadas. Las propiedades más importantes de las BAL son su habilidad para acidificar la leche y generar sabor y textura (Griffiths y Tellez, 2013; Kongo et al., 2013). De hecho, el sabor medio

ácido y agradable fresco, son características de los productos lácteos fermentados (Widyastuti, 2014). En general, la tecnología de la fermentación es relativamente simple y rentable, y las BAL se han establecido y usado en la producción industrial de productos lácteos fermentados (Mäyrä-Mäkinen y Bigret 2004).

El interés por desarrollar productos lácteos fermentados que confieran beneficios a la salud va en aumento (Shah, 2007; Ali, 2010; Panesar, 2011; Liu et al., 2011). Los productos lácteos fermentados por BAL por lo general contienen componentes bioactivos que se ha mostrado aportan varios beneficios a la salud (Griffiths y Tellez, 2013). La regulación del sistema inmune es uno de los beneficios de los productos lácteos. La mayoría de esos beneficios se han mostrado por la fermentación que emplea diferentes BAL como son las del género *Lactobacillus*, y sus efectos parecen ser dependientes de la cepa (Gourbeyre et al., 2011; Santiago-López et al., 2016). *Lactobacillus* puede llevar a cabo una respuesta inmune innata y adaptativa en el huésped por la unión a patrones de reconocimiento (PRR) expresados sobre las células inmunes y de muchos otros tejidos, incluyendo el epitelio intestinal. Los PRR reconocen estructuras moleculares conservadas conocidas como patrones moleculares asociados a microorganismos (PAMPs). El complejo PRR-PAMP induce la señal para la producción de citocinas, quimiocinas y otros efectores innatos (Wells, 2011).

Las citocinas son reguladores de la respuesta del huésped a la infección, de respuestas inmunes, inflamación y traumas (McDermott y Tschopp, 2007). Aunque en estado fisiológico las citocinas funcionan para promover la salud, algunas de ellas producidas en exceso pueden agravar las enfermedades (proinflamatorias), mientras que otras sirven para disminuir la inflamación (antiinflamatorias) (Dinarello, 2000). Por lo tanto, las citocinas pueden dividirse de acuerdo a su función. Algunas citocinas son principalmente factores de crecimiento de linfocitos, otras funcionan como proinflamatorias o antiinflamatorias, mientras que otras citocinas polarizan la respuesta inmune a antígeno (Dinarello, 2007).

El lipopolisacárido (LPS) de bacterias gram-negativas induce la producción de citocinas proinflamatorias. Después de la inyección o exposición, el LPS sistémico se une a la proteína de membrana CD14, formando un complejo que se une a su vez al complejo TLR-4/MD-2 sobre la superficie de macrófagos, neutrófilos y células endoteliales, que las activa y provoca que esas células produzcan varias citocinas proinflamatorias, como son factor de necrosis tumoral (TNF-α), interleucina 6 (IL-6) e interleucina 1β (IL-1β) (Fernández-Martínez et al., 2004).

Esas citocinas estimulan a macrófagos además de otras células, lo que da lugar a la activación de factores de transcripción, por ejemplo, al factor nuclear κB (NF-κB), entre otros mediadores, además de la iniciación de la cascada de señalización de las caspasas. Además, los mediadores antiinflamatorios, como IL-10 son inducidos y contribuyen a la modulación de respuestas sistémicas inflamatorias (Simpson et al., 1997; Aono et al., 1997; Harry et al., 1999; Hamada et al., 1999; Karima et al., 1999).

Las citocinas proinflamatorias aunque contribuyen a la defensa del huésped, cuando son liberadas en exceso pueden inducir desórdenes inmunopatológicos (Miettinen et al., 1996; Neumann et al., 2009). Se ha reportado que la administración oral de *Lactobacillus* puede modular el perfil de citocinas no sólo a nivel intestinal, sino también a nivel sistémico (Arribas et al., 2009; Baharav et al., 2004; Drago et al., 2010; Takata et al., 2011; Villena et al., 2012; Juarez et al., 2013). Aunque la mayoría de los efectos benéficos de las BAL requiere el contacto directo de la bacteria, algunos reportes han demostrado que moléculas liberadas por *Lactobacillus* durante la fermentación son capaces de modular el sistema inmune (Abdou et al., 2006; Granier et al., 2013; Agyei et al. 2016).

Las proteínas de la leche son una buena fuente de péptidos bioactivos, que pueden afectar positivamente la salud, incluyendo el sistema inmune, los cuales son conocidos como péptidos inmunomoduladores (Nongonierma and FitzGerald, 2015). Varios de esos péptidos llevan a cabo su función ya sea como péptidos contenidos en hidrolizados o como péptidos individuales

(Hernández-Ledesma et al., 2014). Algunos de estos péptidos pueden estimular o inhibir ciertas funciones del sistema inmune. Además, dependiendo de la dosis administrada, las condiciones experimentales y la función a evaluar, la misma sustancia puede mostrar actividades estimuladoras o inhibidoras. Esas actividades son reunidas en un término conocido como “inmunomodulación” (Werner et al. 1986). La inmunomodulación es necesaria para controlar las consecuencias de un sistema inmune desregulado y los péptidos bioactivos tienen el potencial como una alternativa para el manejo de enfermedades con éstas características (Agyei et al, 2016).

La inmunomodulación por péptidos bioactivos ocurre mediante la unión a receptores específicos, en consecuencia se promueven las respuestas inmunológicas y funciones celulares, concluyendo en la supresión o estimulación de respuestas inmunes especificas (activación y proliferación de linfocitos, producción de anticuerpos, expresión de citocinas) e inespecíficas (funciones de macrófagos, granulocitos y células *natural killer*) (Meisel, 2004; Gauthier et al. 2006). Estos péptidos son considerados entre los componentes más prometedores producidos por las BAL para ser explotados en la producción industrial de alimentos funcionales (Agyei et al., 2016).

Por lo anterior, este estudio tuvo como objetivo evaluar el potencial efecto inmunomodulador de leches fermentadas por diferentes cepas específicas de *Lactobacillus* o *Lactococcus* sobre los niveles séricos de citocinas proinflamatorias y antiinflamatorias (IL-1β, IL-6, TNF-α e IL-10) en un modelo murino estimulado con LPS y determinar los péptidos presentes en las leches fermentadas.

HIPÓTESIS

Leches fermentadas por cepas específicas de *Lactobacillus* spp. o *Lactococcus* spp. presentan un efecto regulador sobre los niveles séricos de citocinas en un modelo murino estimulado con LPS.

OBJETIVOS

Objetivo general

Estudiar el efecto regulador de leches fermentadas por cepas específicas de *Lactobacillus* spp. o *Lactococcus* spp. sobre los niveles séricos de citocinas en un modelo murino estimulado con LPS.

Objetivos particulares

1. Determinar las condiciones de fermentación y la población inicial del inóculo para la fermentación de la leche con las cepas bajo estudio.

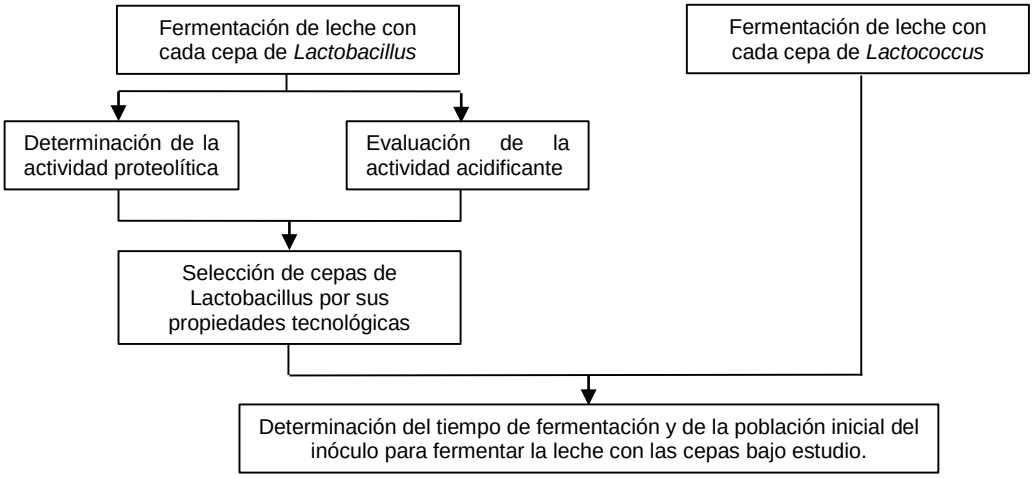
2. Evaluar la regulación de citocinas por la administración de leches fermentadas (sin pasteurizar, pasteurizadas y fracciones < 10 KDa) con cepas específicas de *Lactobacillus* en un modelo murino estimulado con LPS.

3. Evaluar la regulación de citocinas por la administración de leches fermentadas (sin pasteurizar, pasteurizadas y fracciones < 10 KDa) con cepas específicas de *Lactococcus* en un modelo murino estimulado con LPS.

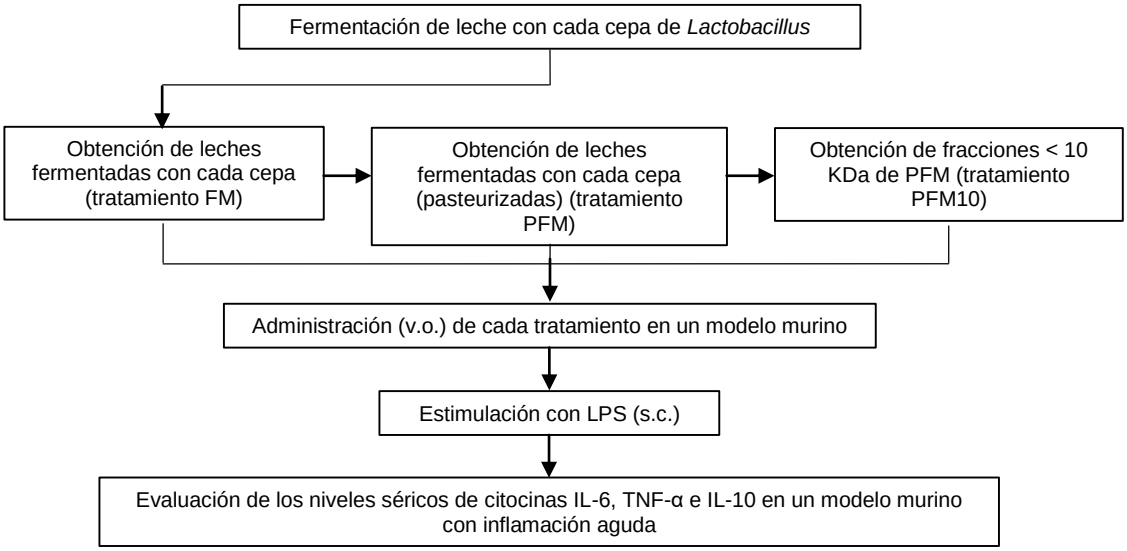
4. Identificar los péptidos presentes en las leches fermentadas bajo estudio.

METODOLOGÍA

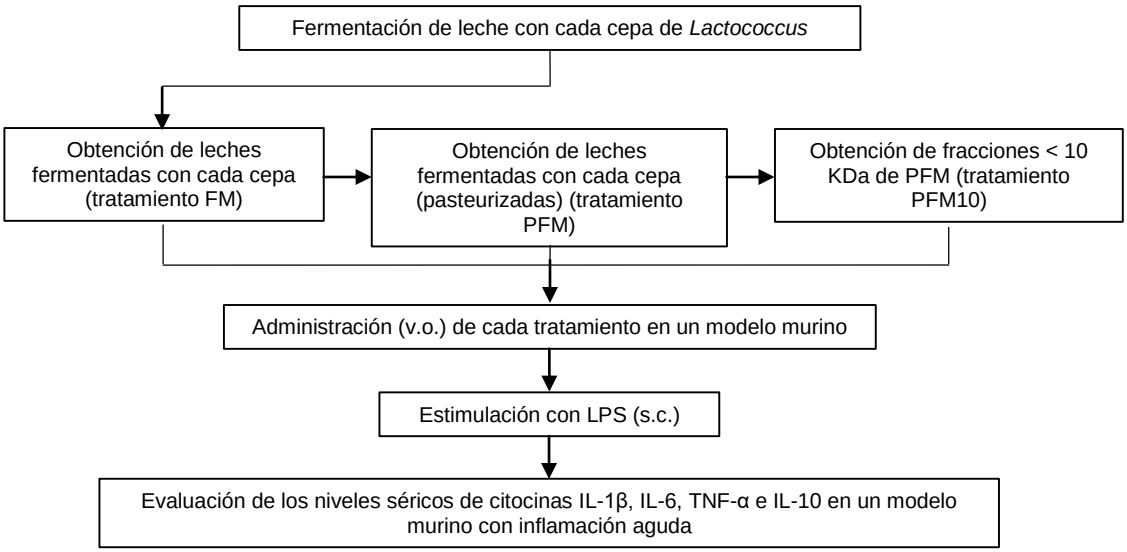
Etapa I. Determinación de las condiciones de fermentación y de la población inicial del inóculo para fermentar la leche con las cepas bajo estudio.



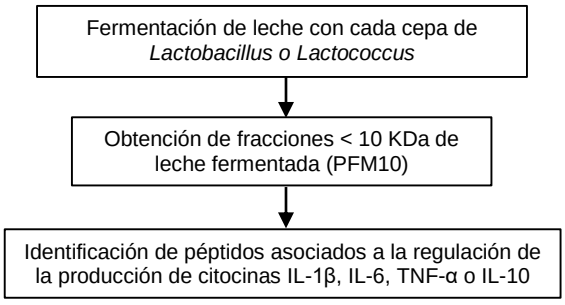
Etapa II. Evaluación del efecto regulador de leches fermentadas (sin pasteurizar, pasteurizadas y fracciones < 10 KDa) por cepas específicas de *Lactobacillus* en un modelo murino estimulado con LPS.



Etapa III. Evaluación del efecto regulador de leches fermentadas (sin pasteurizar, pasteurizadas y fracciones < 10 KDa) por cepas específicas de *Lactococcus* en un modelo murino estimulado con LPS.



Etapa IV. Identificación de los péptidos presentes en las leches fermentadas bajo estudio.



RESULTADOS Y DISCUSIÓN

Se evaluaron los niveles séricos de citocinas en ratas Wistar administradas con tratamientos preparados a partir de leches fermentadas por cepas específicas de *Lactobacillus* y su posterior estimulación con LPS.

La administración de leches fermentadas con J25 o J28 disminuyeron ($p < 0.05$) los niveles de la citocina proinflamatoria TNF-α. Por otro lado, los niveles de la citocina antiinflamatoria IL-10 aumentaron ($p < 0.05$) con la administración de las leches fermentadas con J20, J23 o J25.

Cuando se evaluó si el efecto regulador de las citocinas se mantenía con la inactivación de las cepas fermentativas por medio de la pasteurización, se encontró que las leches fermentadas (pasteurizadas) con J23, J25 o J28 disminuyeron ($p < 0.05$) los niveles de citocinas pro-inflamatorias, IL-6 y TNF-α, incluyendo J20 para esta última citocina. Además, las leches fermentadas (pasteurizadas) con J20, J23 o J25 incrementaron significativamente ($p < 0.05$) los niveles de la citocina antiinflamatoria, IL-10. En relación con las fracciones < 10 KDa de leches fermentadas (pasteurizadas) con J28, disminuyeron ($p < 0.05$) los niveles de IL-6 y TNF-α, incluyendo J20 para esta última citocina.

Por otra parte, también se evaluaron los niveles séricos de citocinas en ratas Wistar administradas con tratamientos preparados a partir de leches fermentadas por cepas específicas de *Lactococcus* y su posterior estimulación con LPS.

Los resultados mostraron que las leches fermentadas con NRRL B-50 571 o con NRRL B-50 572 disminuyeron ($p < 0.05$) los niveles de TNF-α e incrementaron los niveles de IL-10, respectivamente. La leche fermentada (pasteurizada) con NRRL B-50 571 y la fracción < 10 KDa de leche fermentada (pasteurizada) con NRRL B-50 572 disminuyeron ($p < 0.05$) los niveles de IL-6.

El decremento de IL-6 o TNF-α observado en animales administrados con fracciones < 10 KDa de leche fermentada por cepas de *Lactobacillus* o *Lactococcus*, sugieren un efecto inmunomodulador atribuible a los péptidos presentes en estas fracciones. Algunos reportes han mostrado que péptidos bioactivos, los cuales son liberadas por BAL durante la fermentación, son capaces de modular la producción de citocinas, y por lo tanto, modular el sistema inmune (Abdou et al., 2006; Granier et al., 2013; Agyei et al. 2016). Además, se ha reportado que los péptidos inmunomoduladores presentan pesos moleculares < 7 KDa, siendo más abundantes aquellos < 3 KDa (Reyes-Díaz et al., 2016).

En este estudio se identificaron los péptidos que podrían estar involucrados en la regulación de éstas citocinas, por medio del análisis de espectrometría de masas-masas. Los resultados mostraron que los péptidos presentaron un peso molecular < 3 KDa. Estos péptidos se derivaron principalmente de las proteínas del suero de la leche, particularmente a partir de lactotransferrina. Además varias secuencias peptídicas presentaron el aminoácido arginina en el extremo N-terminal y C-terminal. La literatura sugiere que arginina en el extremo de los péptidos bioactivos es una entidad dominante reconocida por receptores presentes en la superficie de macrófagos y linfocitos, los cuales incrementan su maduración y proliferación (Meisel and FitzGerald, 2003; Haque and Chand, 2008). Por lo anterior, varios de los péptidos identificados en las leches fermentadas administradas, podrían ser reconocidos por receptores de células inmunes y como consecuencia mostrar un efecto inmunomodulador.

Por otro lado, se calculó la carga neta de los péptidos identificados y varios de ellos presentaron una carga neta positiva, los cuales podrían unirse al LPS que está cargado negativamente y con esto disminuir el efecto tóxico del mismo en el organismo. LPS es el constituyente principal de la membrana externa de bacterias Gram-negativas y cuando es liberado a la circulación sanguínea provoca inflamación por la activación de monocitos y células endoteliales, lo cual puede ocasionar un choque séptico, incluso la muerte. Una

estrategia para contrarrestar este proceso es neutralizar el principal mediador de la actividad del LPS (lípido A), el cual presenta grupos fosforilo cargados negativamente, mediante moléculas de unión a LPS, como son péptidos cargados positivamente o proteínas (Van Amersfoort et al., 2003; Martínez-Sernández et al., 2016).

CONCLUSIONES

Los resultados mostraron que las leches fermentadas con las cepas de *Lactobacillus* o *Lactococcus* fueron capaces de disminuir la producción de citocinas proinflamatorias (IL-6 and TNF-α) e incrementar la producción de la citocina antiinflamatoria (IL-10) en un modelo murino de inflamación, a las 6 h posteriores a su inducción con LPS, lo que demuestra un potencial efecto inmunomodulador. El efecto fue dependiente de la cepa y se mantuvo cuando las leches fermentadas fueron sometidas a pasteurización posterior a la fermentación.

Por otro lado, el efecto regulador de las citocinas se observó cuando se administraron fracciones (< 10 KDa), por lo que este podría estar asociado a la presencia de los péptidos presentes en las leches fermentadas. Los péptidos identificados mostraron pesos moleculares < 3 KDa y la mayoría se generaron a partir de las proteínas del suero de la leche.

En conclusión, este estudio demostró que leches fermentadas por cepas específicas de *Lactobacillus* o *Lactococcus* fueron capaces de regular los niveles séricos de citocinas proinflamatorias y antiinflamatorias, en un modelo murino con inflamación inducida. La regulación de las citocinas podría atribuirse a la presencia de péptidos bioactivos producidos como resultado del metabolismo de las bacterias ácido lácticas durante la fermentación de la leche.

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INTEGRACIÓN DEL MANUSCRITO

La información de esta tesis está dividida en 4 capítulos, los cuales se resumen a continuación:

Capitulo I. Péptidos inmunomoduladores derivados de las proteínas de la leche.

Reyes-Díaz Aline; González-Córdova, Aarón Fernando; Hérnández-Mendoza Adrian; Vallejo-Cordoba, Belinda.

Para desarrollar este capitulo, se recopiló a partir de diferentes estudios, una lista de secuencias peptídicas derivadas de proteínas de la leche que han mostrado un efecto sobre el sistema inmune. Estas secuencias se describen estructural y fisicoquímicamente en este artículo de revisión. Los datos mostraron que la longitud de estos péptidos es muy variable (2-64 aminoácidos). Los aminoácidos más frecuentes en estas secuencias son prolina y ácido glutámico. En el extremo N-terminal destaca tirosina, en el extremo C-terminal lisina, así como arginina en ambos. Presentan pesos moleculares < 7 KDa, siendo más abundantes aquellos < 3 KDa. Son principalmente de carácter hidrofílico y presentan una diversidad de cargas a pH fisiológico, que oscilan entre -7 y +8 y. Estos resultados podrían ser clave para determinar el patrón estructural y a su vez la función de los péptidos inmunomoduladores, ya que al momento la relación estructura-función y los mecanismos por los cuales estos péptidos ejercen sus efectos finales no se han dilucidado por completo.

Este capitulo fue publicado en la Revista INTERCIENCIA:

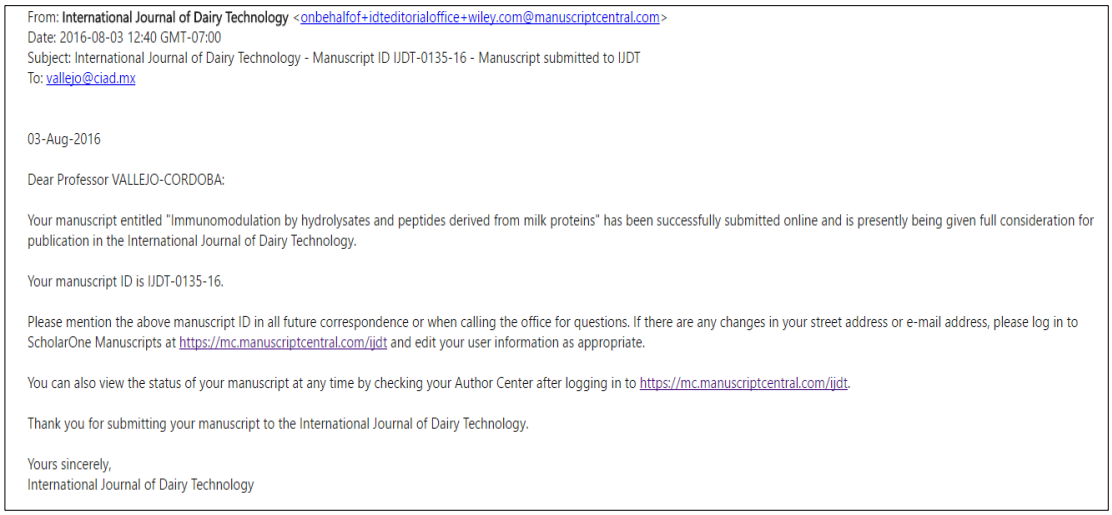
Reyes-Díaz A., González-Córdova A.F., Hernández-Mendoza A. y Vallejo-Cordoba B. 2016. Péptidos inmunomoduladores derivados de las proteínas de la leche. Interciencia, 41(2):84-91.

Capitulo II. Immunomodulation by hydrolysates and peptides derived from milk proteins

Reyes-Díaz Aline; González-Córdova, Aarón Fernando; Hérnández-Mendoza Adrian; Ricardo Reyes-Díaz; Vallejo-Cordoba, Belinda.

Este capítulo presenta una revisión sobre péptidos immunomoduladores derivados de proteínas lácteas, incluyendo hidrolizados, fracciones peptídicas y péptidos. Se describen las diferentes estrategias utilizadas para la producción de péptidos bioacivos. Se destaca el potencial de las proteínas de la leche como fuente de péptidos inmunomoduladores y se incluye información que evidencia las diversas funciones de dichos péptidos con el sistema inmune. La información recopilada mostró que péptidos con función inmunomoduladora en hidrolizados derivan tanto de caseína como de las proteínas del suero de la leche. Mientras que, las secuencias individuales de péptidos provienen principalmente de la caseína. Finalmente, se enfatiza la necesidad de generar información e identificar péptidos con función inmunomoduladora para entender el comportamiento de estas estructuras.

Este capítulo, se envió a la Revista International Journal of Dairy Technology para su revisión y posible publicación.



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Capítulo III. Milk fermented by specific *Lactobacillus* strains regulate the serum levels of inflammatory cytokines on a LPS-stimulated murine model

Aline Reyes-Díaz; Verónica Mata-Haro, Jesús Hernández, Aarón F. González-Córdova; Adrián Hernández-Mendoza; Ricardo Reyes-Díaz, María de Jesús Torres-Llanez and Belinda Vallejo-Cordoba

En este capitulo se evaluó el efecto regulador de leches fermentadas por cepas específicas de *Lactobacillus* spp. (J20, J23, J25 o J28) en los niveles séricos de citocinas en un modelo murino inducido con lipopolisacárido (LPS). Se demostró que las leches fermentadas (con o sin pasteurización posterior a la fermentación) por estas cepas disminuyeron la concentración sérica de citocinas proinflamatorias (IL-6 y TNF-α) e incrementaron la concentración de la citocina antiinflamatoria (IL-10). Además se evidenció que los efectos observados se mantuvieron con la pasteurización de las leches fermentadas. Por otra parte, también se demostró la disminución de estas citocinas proinflamatorias en animales administrados con las fracciones (< 10 KDa) de leches que fueron fermentadas por dos de estas cepas (J20 y J28). El efecto regulador de leches fermentadas con las cepas J20, J23 y J25 se relacionó con los componentes presentes en la leche. Por otro lado, la disminución en los niveles de citocinas proinflamatorias, mostrados con fracciones de leches fermentadas por J20 y J28, se relacionó con los componentes < 10 KDa

presentes en la leche fermentada y ese efecto podría asociarse a los péptidos presentes. Finalmente, se identificaron los péptidos que regularon la producción de citocinas séricas, los cuales se derivaron principalmente de las proteínas del suero de la leche y presentaron pesos moleculares < 3 KDa en su mayoría. Estos péptidos presentaron una carga neta positiva debido a la presencia de arginina y lisina en los extremos, por lo que podrían unirse a la molécula de LPS que posee una carga neta negativa y en consecuencia disminuir los efectos producidos durante la inflamación inducida por LPS.

En conclusión las leches fermentadas con las cepas específicas de *Lactobacillus* estudiadas se proponen como nuevos alimentos funcionales para la prevención de transtornos inflamatorios sistémicos.

Capítulo IV. Regulation of lipopolysaccharide-induced serum cytokines in a murine model by milk fermented with *Lactococcus lactis*

Aline Reyes-Díaz; Verónica Mata-Haro, Jesús Hernández, Aarón F. González-Córdova; Adrián Hernández-Mendoza; Ricardo Reyes-Díaz and Belinda Vallejo-Cordoba.

En este capítulo se evaluó el efecto regulador de leches fermentadas por dos cepas específicas de *Lactococcus lactis* (NRRL B-50 571 o NRRL B-50 572) sobre los niveles séricos de citocinas IL1-β, IL-6, TNF-α e IL-10, en un modelo murino estimulado con lipopolisacárido (LPS). Se administraron tres tratamientos de leches fermentadas por cada cepa; a saber, leche fermentada (FM), leche fermentada pasteurizada (PFM) y la fracción < 10 KDa de leche PFM (PFM10). Los resultados mostraron que el tratamiento FM con la cepa NRRL B-50 571 disminuyó los niveles de TNF-α, el tratamiento FM con la cepa NRRL B-50 572 incrementó los niveles de IL-10 y los tratamientos PFM o PFM10 con las cepas NRRL B-50 571 o NRRL B-50 572, respectivamente, disminuyeron los niveles de IL-6 a las 6 h después de la estimulación con LPS. Los efectos mostrados sobre la producción de citocinas por la administración de

la leche fermentada podrían relacionarse a la presencia de péptidos resultantes de la hidrólisis de proteinas. Los péptidos presentaron en su mayoría pesos moleculares < 3 KDa y se derivaron principalmente de las proteínas del suero. Estos péptidos presentaron una carga neta positiva debido a la presencia de arginina y lisina en los extremos, por lo que podrían unirse a la molécula de LPS que posee una carga neta negativa y en consecuencia disminuir los efectos producidos por LPS. Por lo tanto, las leches fermentadas por estas cepas de *L. lactis* fueron efectivas para regular los niveles séricos de citocinas inducidas con LPS y se han propuesto como candidatos potenciales para la elaboración de nuevos productos funcionales capaces de modular la respuesta sistémica con un potencial efecto inmunomodulador.

CAPÍTULO I

Péptidos inmunomoduladores derivados de las proteínas de la leche.

PÉPTIDOS INMUNOMODULADORES DERIVADOS DE LAS PROTEÍNAS DE LA LECHE

ALINE REYES-DÍAZ, AARÓN F. GONZÁLEZ-CÓRDOVA, ADRIÁN HERNÁNDEZ-MENDOZA y BELINDA VALLEJO-CORDOBA

RESUMEN

Las proteínas de la leche son objeto de numerosas investigaciones debido al aporte que presentan en beneficio a la salud como fuente de péptidos bioactivos. La caracterización de los péptidos es un paso primordial e importante para poder entender cómo llevan a cabo su función. En este trabajo se recopiló, a partir de diferentes estudios, una lista de secuencias peptídicas derivadas de proteínas de la leche que han mostrado un efecto sobre el sistema inmune. Estas secuencias se describen por su longitud y composición de aminoácidos, así como fisicoquímicamente. Los datos muestran que su longitud comprende entre 2 y 64 aminoácidos. Los aminoácidos más frecuentes en estas secuencias son prolina y ácido glutámico. Destacan tirosina y lisina en el extremo N-terminal y C-terminal, respectivamente, y arginina en ambos extremos. Estos péptidos presentan pesos moleculares <7kDa, aunque son más abundantes aquellos <3kDa. Asimismo, ostentan una diversidad de cargas a pH fisiológico, que oscilan entre -7 y +8, siendo principalmente de carácter hidrofílico. El análisis de la información recopilada en esta revisión podría ser de importancia para determinar el patrón estructural y a su vez la función de los péptidos inmunomoduladores, ya que al momento la relación estructura-función y los mecanismos a través de los que estos péptidos ejercen sus efectos finales no han sido completamente elucidados.

La leche es un alimento rico en proteínas, las cuales están agrupadas principalmente en caseínas y proteínas del suero. La familia de las caseínas representa aproximadamente el 80% de la masa de las proteínas e incluye varios tipos de caseínas: α_{s1}, α_{s2}, β y κ, que forman complejos de micelas en la fase acuosa de la leche. Las proteínas del suero representan el 20% restante y entre ellas se encuentran β-lactoglobulina (no presente en la leche humana), α-lactoalbúmina, seroalbúmina, inmunoglobulinas, lactoferrina y transferrina, entre las más importantes (Sun y Jenssen, 2012).

Las proteínas de la leche han sido objeto de numerosas investigaciones debido al aporte que presentan en beneficio a la salud como fuente de péptidos con diferente bioactividad, como son los péptidos inmunomoduladores, que se han obtenido en gran parte a partir de estas proteínas (Dziuba *et al.*, 2009; Plaisancié *et al.*, 2013). La bioactividad específica de los péptidos bioactivos depende intrínsecamente de su biodisponibilidad, que está íntimamente relacionada con sus propiedades estructurales y fisicoquímicas (Phelan *et al.*, 2009). Agyei y Danquah (2012) mencionan que existe desconocimiento en relación al mecanismo de acción concreto por el cual los péptidos inmunomoduladores ejercen sus efectos finales. Esta falta de información persiste a la fecha, y podría atribuirse a la falta de la caracterización estructural de los péptidos involucrados.

Con estos antecedentes que ponen de manifiesto la falta de información más amplia y precisa para definir un patrón estructural que represente a los péptidos inmunomoduladores, el enfoque de esta revisión comprende la descripción de una lista de secuencias reportadas de aminoácidos con efecto

PALABRAS CLAVE / Péptidos Inmunomoduladores / Proteínas de la Leche / Secuencia de Aminoácidos /

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sobre el sistema inmune, en relación a su longitud y composición, así como fisicoquímicamente. Se discute también la dinámica sugerida para estas secuencias en el organismo con base en datos reportados en la bibliografía para péptidos bioactivos. Con los datos presentados se espera contribuir con información que permita conjuntar las características de la variedad de péptidos inmunomoduladores que a la fecha han sido reportados y así dar un primer paso para orientar estudios futuros en búsqueda de evidencias que aclaren la relación estructura-función de los péptidos inmunomoduladores.

Capacidad Inmunomoduladora de Péptidos Derivados de Proteínas Lácteas

Los péptidos bioactivos obtenidos a partir de proteínas de la leche son derivados de la hidrólisis derivada de diferentes métodos de proteólisis. Pueden ser producidos *in vivo*, mediante digestión gastrointestinal (la mucosa del intestino expresa al menos 15 peptidasas), así como por enzimas derivadas de la microbiota humana (Kilara y Panyam, 2003; Korhonen y Pihlanto, 2003; Hartmann y Meisel, 2007). La producción *in vitro* involucra la liberación de péptidos por la adición de enzimas (tripsina, pepsina o quimosina) o por la actividad metabólica de bacterias ácido lácticas (BAL) probióticas u otros microorganismos (*Lactobacillus helveticus, L. delbrueckii*, *Saccharomyces cerevisiae*) (Hebert *et al.*, 2010; Espeche Turbay *et al.*, 2012; Chatterton *et al.*, 2013; Juillerat-Jeanneret *et al.,* 2011). Gill *et al.,* (2000) señalan que la leche de vaca contiene de forma natural algunos péptidos con actividad inmunogénica en estado preformado o cerca de ser formados, que no requieren ninguna o una mínima modificación gástrica para ser biológicamente activos.

No obstante, a pesar del esfuerzo de algunos trabajos por aportar nuevas alternativas para mejorar la salud en este aspecto, varios de ellos reportan el efecto de un conjunto de elementos contenidos en hidrolizados completos o fracciones derivados de proteínas lácteas (Li y Mine, 2004; Mercier *et al.*, 2004; Prioult *et al.*, 2004; Saint-Sauveur *et al.*, 2009; Maldonado Galdeano *et al.*, 2011; LeBlanc *et al.*, 2002; Vinderola *et al.*, 2007) y muy pocos han logrado demostrar la actividad inmunomoduladora de péptidos individuales específicos o la combinación de algunos de ellos. Cabe la posibilidad de que solo algunos y no el conjunto de péptidos reportados en esos hidrolizados o fracciones pudieran ser los responsables del efecto observado (Tellez *et al*, 2010).

El mecanismo de acción por medio del cual estas secuencias actúan sobre el sistema inmune no esta totalmente explicado. Se sabe (Kayser y Meisel, 1996) que interactúan con el tejido linfoide asociado a la mucosa intestinal (GALT), de manera que atraviesan el epitelio intestinal y entran a la circulación, o se unen directamente a receptores de la superficie celular intraepitelial específica. Tal interacción desencadena diferentes funciones fisiológicas a través de la señalización celular, al suprimir o estimular ciertas respuestas que involucran tanto al sistema inmune innato como al adaptativo (Gill *et al.*, 2000; Hancock y Sahl, 2006; Oelschlaeger, 2010). El efecto culmina en la regulación de respuestas inmunes específicas (activación y proliferación de linfocitos, síntesis de anticuerpos, expresión de citocinas) y/o inespecíficas (actividad fagocítica de macrófagos, funciones de células granulocíticas y *natural killer*) (LeBlanc *et al.*, 2002; Danquah y Agyei, 2012).

Existe escaza evidencia que muestre las vías de señalización que podrían estar involucradas para que los péptidos puedan llevar a cabo su efecto inmunomodulador. Un mecanismo implicado es la vía de señalización por AMPc (adenosín monofosfato cíclico), que se sabe actúa como segundo mensajero al activar la PKA (proteína cinasa A) y/o la Epac-1 (proteína de intercambio activada directamente por AMPc). El AMPc ha mostrado efectos inhibidores sobre varias funciones en macrófagos alveolares al inhibir la fagocitosis (Aronoff *et al.*, 2004) y la producción de mediadores inflamatorios (Rowe *et al.*, 1997). Otra vía involucra la estimulación de la proteína cinasa activada por mitógenos (MAPK) y el factor nuclear NF-κB, ya que posterior a la adición de glicomacropéptido (GMP) bovino en una línea celular de monocitos tumorales y otra de monocitos normales, se incrementó la síntesis de citocinas proinflamatorias como TNF-α, IL-1β e IL-8 (Requena *et al.*, 2009).

La evidencia obtenida hasta el momento ha demostrado el efecto sobre el sistema inmune de un conjunto de secuencias que son derivados de proteínas de origen lácteo, las cuales se detallan en la Tabla I, donde se observa que los péptidos inmunomoduladores reportados derivan casi en su totalidad de la caseína (proteína) de la leche (22/23 secuencias, equivalentes al 96%), los cuales se distribuyen como sigue: α_{s1} 22%, α_{s2} 4%, β 61% y κ 9%. La secuencia restante corresponde a un péptido derivados de proteínas del suero (una secuencia, equivalente al 4%, que corresponde al péptido lactoferricina o LF).

La información anterior denota que existe escasa información sobre péptidos individuales derivados de proteínas del suero de la leche con efecto inmunomodulador. Sin embargo, se ha reportado el efecto de estas proteínas sobre el sistema inmune cuando presentan su estructura completa o de hidrolizados de las mismas que contienen un conjunto de péptidos que podrían presentar dicha actividad de forma individual (Mercier *et al.*, 2004; Prioult *et al.*, 2004; Saint-Sauveur *et al.*, 2009; Tellez et al, 2010; Maldonado Galdeano *et al.*, 2011; LeBlanc *et al.*, 2002; Vinderola *et al.*, 2007).

Características estructurales y fisicoquímicas de péptidos inmunomoduladores

Características estructurales

Como se muestra en la Tabla I, los valores de la longitud de los péptidos inmunomoduladores es muy amplio y variable (2-64 aminoácidos), siendo los más frecuentes aquellos con 6-7 aminoácidos en su secuencia. El hecho que estas secuencias presenten longitudes de cadena muy variadas sugiere que los péptidos inmunomoduladores pueden tomar diferentes rutas de transporte en el epitelio intestinal una vez que son ingeridos, de lo cual dependerá su biodisponibilidad, así como el mecanismo de acción, que culminará en una bioactividad específica sobre el sistema inmune.

En relación a la composición de aminoácidos presentes en las proteínas, todos se encuentran distribuidos en el conjunto de las secuencias inmunomoduladoras, con al menos dos residuos en la secuencia que lo contiene. Esto resalta el aporte nutricional en adición a su actividad biológica. De ahí radica la importancia de incorporarlos a la dieta. Swaisgood (1992) describió la composición de aminoácidos contenidos en las principales proteínas de la leche y reportó la abundancia de los residuos prolina (Pro), ácido glutámico (Glu), glutamina, leucina (Leu) y lisina (Lys). De éstos, Pro y Glu son los aminoácidos más frecuentes en los péptidos inmunomoduladores aquí enlistados, con el 13,5 y 12,4% de residuos, respectivamente, en el total de las secuencias.

Pro está presente en prácticamente todas las secuencias de este estudio, excepto en las dos secuencias más cortas que corresponden a un di- y un tripéptido. En un estudio, Dziuba *et al.* (2009) realizaron una proteólisis simulada con herramientas informáticas de proteínas de la leche. Los resultados

TABLA I
EFECTO ESPECÍFICO DE PÉPTIDOS INMUNOMODULADORES DERIVADOS DE PROTEÍNAS DE LA LECHE

Nº	Proteína	Péptido	Enzima	Efecto inmune	Referencias
1	α_{s1}-CN (1-23) Isracidina (N-terminal)	RPKHPIKHQGLPQEV LNENLLRF	Q	Protección contra infección por *Staphylococcus aureus* en ratón. ↑ respuesta fagocítica en ratón infectado con *Candida albicans* Protege a vacas y carnellos contra mastitis.	Lahov y Regelson, 1996 Minkiewicz *et al.*, 2000
2	α_{s1}-CN (17-33)	NENLLRFFVAPFPEVFG	BAL	Inhibe a la enzima MMP-9 en células HT-29 y SW480, que se expresa durante el desarrollo de enfermedades inflamatorias de colon.	Juillerat-Jeanneret *et al.*, 2011 Chatterton *et al.*, 2013
3	α_{s1}-CN (59-79) Caseinofosfopéptido	QMEAESISSSEEIVPN SVEQK	T	↑ Producción de IgG en células de bazo de ratón. ↑ IgA sérico e intestinal en ratón.	Hata *et al.*, 1998
4	α_{s1}-CN (143-149)	AYFYPEL	NR	↑ Expresión del gen MUC5AC y la secreción de mucina en células HT29-MTX, proporcionando un efecto de barrera intestinal.	Martínez-Maqueda, 2013
5	α_{s1}-CN (144-149)	YFYPEL	NR	↑ Expresión del gen MUC5AC y la secreción de mucina en células HT29-MTX.	Martínez-Maqueda, 2013
6	α_{s2}-CN (1-32)	KNTMEHVSSSEESIISQE TYKQEKNMAINPSK	T	Efecto estimulador sobre células del bazo.	Hata *et al.*, 1999 Otani *et al.*, 2000
7	β-CN (1-25) Caseinofosfopéptido	RELEELNVPGEIVES LSSSEESITR	T	↑ Proliferación de células del bazo y timocitos de placas de Peyer en ratón. ↑ Producción de inmunoglobulina en células de bazo de ratón.	Hata *et al.*, 1998
8	β-CN (1-28) Caseinofosfopéptido	RELEELNVPGEIVESLS SSEESITRINK	T	↑ Proliferación de linfocitos. ↑ Niveles IgA en cultivos celulares.	Otani *et al.*, 2000
9	β-CN humana (54-59)	VEPIPY	T	↑ Fagocitosis de en células peritoneales murinas.	Parker *et al.*, 1984 Gattegno *et al.*, 1988
10	β-CN (60-66) β-casomorfina-7	YPFPGPI	NR	↓ Proliferación de linfocitos a bajas concentraciones. ↑ Proliferación de linfocitos a altas concentraciones.	Kayser y Meisel, 1996
11	β-CN (63-68)	PGPIPN	NR	↑ Formación de anticuerpos. ↑ Fagocitosis de macrófagos murinos.	Migliore-Samour y Jolles, 1988
12	β-casomorfina (60-70)	YPFPGPIPNSL	NR	↓ Proliferación de linfocitos. ↑ Resistencia a infección por *Klebsiella pneumoniae* en ratón.	Migliore-Samour y Jolles, 1988
13	β-CN (98-105)	VKEAMAPK	T	Inhibe a lipooxigenasa, aumentando la permeabilidad vascular.	Rival *et al.*, 2001 Chatterton *et al.*, 2013
14	β-CN (94-123)	GVSKVKEAMAPKHKE MPFPKYPVEPFTESQ	NR	↑ Expresión de los genes MUC2 y MUC4 y la secreción de mucina en células HT29-MTX. ↑ Número de células de Paneth y células calciformes (células de Goblet) en un modelo murino proporcionando un efecto de barrera intestinal.	Plaisancié *et al.*, 2013 Chatterton *et al.*, 2013
15	β-CN (169-176)	KVLPVPQK	NR	Inhibe a lipooxigenasa, enzima relacionada a procesos inflamatorios.	Rival *et al.*, 2001 Chatterton *et al.*, 2013
16	β-CN (170-176)	VLPVPQK	T	Inhibe a lipooxigenasa, enzima relacionada a procesos inflamatorios.	Rival *et al.*, 2001 Chatterton *et al.*, 2013
17	β-CN (177-183)	AVPYPQR	T	Inhibe a lipooxigenasa, enzima relacionada a procesos inflamatorios.	Rival *et al.*, 2001 Chatterton *et al.*, 2013
18	β-CN (191-193)	LLY	T	↑ Formación de anticuerpos. ↑ Fagocitosis. ↑ Proliferación de células T dependiente de antígeno.	Berthou *et al.*, 1987 Gill *et al.*, 2000 Clare y Swaisgood, 2000
19	β-CN (192-209)	LYQEPVLGPVRGPFPIIV	P-Q	Modula la proliferación de linfocitos. Efecto mitogénico sobre nódulos linfáficos y células de bazo en rata.	Coste *et al.*, 1992
20	β-casocinina-10 (193-202)	YQEPVLGPVR	NR	↑ Proliferación de linfocitos en rata a altas concentraciones.	Kayser y Meisel, 1996
21	κ-CN (38-39)	YG	NR	↑ Proliferación de linfocitos.	Kayser y Meisel, 1996 Gill *et al.*, 2000 Otani *et al.*, 2000
22	κ-CN (106-169) Glicomacropéptido	MAIPPKKNQDKTEIPTINTIAS GEPTSTPTTEAVESTVATLED SPEVIESPPEINTVQVTSTAV	Q	↑ Secreción de TNF, IL-10. Normaliza la expresión de IL-1β, IL-17, IL-23, IL-6, TGF-β e IL-10. Presenta actividad antiinflamatoria relacionada con linfocitos Th1 yTh17.	Requena *et al.*, 2009; 2010
23	LF (17-41)	FKCRRWQWRNKKLG APSITCVRRAF	P	↑ Actividad fagocítica de neutrófilos humanos. ↓ Respuesta a IL-6 cuando se estimula con LPS. ↑ Liberación de IL-8 de leucocitos.	Miyauchi *et al.*, 1998 Clare y Swaisgood, 2000

Los símbolos aquí utilizados para denotar las enzimas son Q: quimosina, BAL: enzimas derivadas de bacterias ácido-lácticas, T: tripsina, NR: no reportado o no se utilizaron enzimas debido a la utilización de péptidos sintéticos, P: pepsina.

evidenciaron que los péptidos con actividad inmunomoduladora, además de péptidos con otras bioactividades, que contienen Pro dentro de su secuencia son hidrolizados por prolin-oligopeptidasas. En referencia con lo anterior, se puede inferir que hay una gran probabilidad de que los péptidos inmunomoduladores sufran degradación por efecto de enzimas digestivas cuando son ingeridos vía oral. Esto podría suponer la posibilidad de que el mecanismo de transporte de estos péptidos es el seguido por péptidos pequeños, los cuales son transportados mediante transportadores expresados en los enterocitos. Estos datos deben considerarse para el diseño de péptidos bioactivos cuando intervengan enzimas digestivas durante su estudio. En el orden de las ideas anteriores que resaltan la importancia de Pro como elemento de importancia para efectuar una actividad biológica, se encontró que péptidos sintéticos con una longitud de al menos 15 residuos de aminoácidos, con una Pro en la posición 6 estimuló la liberación de IL-1 y TNF-α en monocitos. La sustitución de Pro en esa posición por otro residuo convirtió al péptido en inactivo (López-Moratalla *et al.*, 1994).

Glu también podría ser un aminoácido clave en la estructura de los péptidos inmunomoduladores, pues se encontró que al formar parte de diferentes glicopéptidos sintéticos puede estimular la producción de células formadoras de anticuerpos en el bazo de ratón, o si se cambian las condiciones de su estructura puede actuar como un inmunosupresor (Kondratenko *et al.*, 2006). Bajo el mismo enfoque que resalta la importancia del Glu en péptidos imunomoduldores, López-Moratalla *et al.* (1994) demostraron el efecto sobre el sistema inmune de secuencias con aminoácidos como valina, Leu, isoLeu, glicina, alanina o Lys en la posición 2, y Glu o ácido aspártico (Asp) en la posición 11. La sustitución de residuos en la posición 2 y 11 por otros residuos aminoacídicos disminuyeron las propiedades inmunomoduladoras descritas, o llevó a la pérdida de la actividad en conjunto. Glu tiene importancia fisiológica y metabólica, pues es crítico para la función celular y un importante neurotransmisor, además de actuar como precursor para la biosíntesis de glutatión, arginina (Arg) y Pro (Sapolsky, 2005; Okubo *et al.*, 2010).

Uno de los aminoácidos más frecuente en estas secuencias es serina (Ser). Aunque solo ocho de las 23 secuencias lo contienen en su estructura, está presente en elevada proporción en las mismas. Al momento no hay estudios que respalden su importancia en los péptidos bioactivos; no obstante, esta revisión resalta un patrón estructural en la región interna de las secuencias indicadas con los números 3, 6, 7 y 8 en la Tabla I, donde sobresalen las secuencias repetidas SSSEEXI y ESXSSSEEX, (las letras S, E e I corresponden a residuos de Ser, Glu e isoLeu, y la X corresponde a Leu, isoLeu o Ser). Esta información debería estudiarse a profundidad para conocer su relación con el efecto inmunomodulador de estas secuencias.

También puede observarse en las secuencias reportadas en la Tabla 1, que en su extremo amino-terminal destaca el aminoácido tirosina (Tyr), y Lys en el extremo carboxilo-terminal, así como Arg en ambos extremos. Los extremos N-terminal y C-terminal son importantes, pues existe evidencia que sugiere que aminoácidos como Arg en la región amino- o carboxilo-terminal de péptidos bioactivos es la entidad dominante que reconocen receptores de la superficie de linfocitos y macrófagos, promoviendo su maduración y proliferación (Meisel y FitzGerald, 2003; Haque y Chand, 2008, Phelan *et al.*, 2009).

Propiedades fisicoquímicas

La forma de transporte de los péptidos bioactivos a nivel intestinal depende no solo de sus características estructurales, sino también de sus propiedades fisicoquímicas. La descripción fisicoquímica para los péptidos inmunomoduladores de esta revisión se apoyó en herramientas informáticas del servidor Expasy (http://www.expasy.org), como son ProtParam y SAPS (Gasteiger *et al.*, 2005), para lo cual se contempló la ocurrencia de frecuencia de parámetros como el peso molecular (PM), número de residuos con carga negativa, número de residuos con carga positiva, promedio de hidropaticidad, punto isoeléctrico teórico (pI) y vida media (VM) (Tabla II).

Con una relación directamente proporcional a la longitud, los PM de los péptidos inmunomoduladores incluidos en esta revisión son variables y en general corresponden a estructuras <7kDa, siendo la mayoría (18 de 23) de las secuencias <3kDa; algunas (4/23) oscilan entre los 3-4 kDa y la secuencia correspondiente a GMP es de 6-7kDa. El intervalo de tamaños de los péptidos bioactivos que han sido estudiados en general van de di, tri y oligopéptidos a polipéptidos de alto PM (Hernández-Ledesma *et al.*, 2005). En un estudio donde se utilizó un modelo de digestión gastrointestinal *in vitro* se evaluó la capacidad de sobrevivencia de fracciones peptídicas de diferentes PM. Los resultados mostraron que péptidos con PM>3kDa fueron más fáciles de digerir que aquellos <3kDa (Chen y Li, 2012). Se ha reportado que los péptidos más pequeños son transportados intactos a la circulación a través de enterocitos, mediante transportadores de péptidos expresados en intestino como por ejemplo PepT1 (Matsui *et al.*, 2002,

TABLA II
RESUMEN DE PARÁMETROS FISICOQUÍMICOS DE PÉPTIDOS INMUNOMODULADORES

Nº de secuencia	PM (Da)	pI	Residuos (-)	Residuos (+)	VM (h)	Promedio de hidropaticidad
1	2764,2	9,99	Glu (2)	Arg (2), Lys (2)	1	-0,987
2	1996,3	4,53	Glu (2)	Arg (1)	1,4	0,406
3	2321,5	3,98	Glu (5)	Lys (1)	0,8	-0,781
4	902,0	4,0	Glu (1)	(0)	4,4	0,100
5	830,9	4,0	Glu (1)	(0)	2,8	-0,183
6	3669,0	5,63	Glu (5)	Lys (4)	1,3	-1,238
7	2803,0	4,12	Glu (7)	Arg (2)	1	-0,596
8	3158,4	4,36	Glu (7)	Arg (2), Lys (1)	1	-0,636
9	716,8	4	Glu (1)	(0)	100	0,117
10	789,9	5,52	(0)	(0)	2,8	0,114
11	593,6	5,96	(0)	(0)	>20	-0,700
12	1201,3	5,52	(0)	(0)	2,8	-0,287
13	873,0	8,56	Glu (1)	Lys (2)	100	-0,400
14	3417,9	8,34	Glu (4)	Lys (5)	30	-0,887
15	908,1	10,0	(0)	Lys (2)	1,3h	-0,287
16	779,9	8,72	(0)	Lys (1)	100	0,229
17	829,9	8,79	(0	Arg (1)	4,4	-0,229
18	ND	ND	(0)	(0)	ND	ND
19	1994,4	6,0	Glu (1)	Arg (1)	5,5h	0,667
20	1157,3	6,0	Glu (1)	Arg (1	2,8h	-0,420
21	ND	ND	(0)	(0)	ND	ND
22	6707,4	4,04	Asp (2), Glu (8)	Arg (3)	30	-0,370
23	3108,7	11,84	(0)	Arg (5), Lys (3)	1,1	-0,370

PM: peso molecular, pI: punto isoeléctrico, VM: vida media, ND: no disponible.

Foltz *et al.*, 2007), mientras que los oligopéptidos pueden ser absorbidos por transporte pasivo a través de regiones hidrofóbicas de la membrana epitelial o por vía paracelular a través de uniones estrechas (Fei *et al.*, 1994; Satake *et al.*, 2002; Shimizu, 2004; Darewicz *et al.*, 2011). La vía paracelular no es una ruta de transporte degradativa al mantener los péptidos transportados intactos y la modulación de la estructura de las uniones estrechas por sustancias de los alimentos facilita el transporte paracelular (Tsukita *et al.*, 2001). Además, los oligopéptidos también pueden ser transportados por transcitosis (transporte transcelular mediado por vesículas) (Shen *et al.*, 1992) y en algunos casos no es necesario que los péptidos sean absorbidos para ejercer sus propiedades biológicas, como sucede con algunos péptidos que se unen a colesterol y actúan en el tracto gastrointestinal (Wang y González de Mejía, 2005). Cabe resaltar que la bioactividad de péptidos demostrada en estudios *in vitro* no se traduce generalmente en efectos en estudios *in vivo*, ya que pueden influir condiciones relacionadas con la absorción, biodisponibilidad y susceptibilidad de los péptidos a la degradación por enzimas fisiológicas en fragmentos inactivos (Hernández-Ledesma *et al.*, 2005). En este sentido, se ha reportado que la bioactividad *in vitro* de péptidos con menor PM representa el mayor grado lo observado *in vivo* (Qian *et al.* 2011).

Los péptidos que han sido señalados como inmunomoduladores muestran una diversidad de cargas a pH fisiológico que oscilan entre -7 y +8, ubicándolos como péptidos catiónicos, neutros y aniónicos. Esto se debe a la variación en la presencia de Arg y Lys (residuos cargados positivamente), en relación con Asp y Glu (residuos cargados negativamente) en su secuencia. Estos péptidos presentan mayoritariamente una carga neta de 0 (30% de las secuencias) y en menor proporción presentan cargas totales de -1 y +1 (21 y 17%, respectivamente) y -7, -5, -4, +2 y +8 distribuidas para el resto.

Hasta el momento el efecto de la carga en relación a la función de los péptidos inmunomoduladores no ha sido descrito a profundidad. En un estudio en que se evaluó a la lactoferricina (LF), péptido catiónico de 25 aminoácidos aislado después de la escisión gástrica de la lactoferrina, una enzima multifuncional transportadora de hierro (Haug y Svendsen, 2001; Richardson *et al.*, 2009), se reportó que los residuos de cisteína en su estructura generaron un puente disulfuro que une la región N-terminal cargada positivamente y la región C-terminal del péptido, aunado a su alto contenido en aminoácidos básicos que le dan una carga de +7,84 a pH 7,0. Estas características podrían estar relacionadas con su función inmunomoduladora. Además, en la bibliografía se reporta el alcance de secuencias con carácter catiónico, que permite su interacción con fosfolípidos aniónicos de la membrana bacteriana o de otros patógenos, permitiendo la interacción péptido-membrana previo a la actividad antimicrobiana (Hancock y Patrzykat, 2002; Hancock y Rozek, 2002). El pH del medio donde se encuentre el péptido es también esencial para determinar sus propiedades ácido-base, aspecto importante pues de ello dependen las propiedades químicas y la funcionalidad biológica de los péptidos.

Continuando con la descripción de las propiedades fisicoquímicas de los péptidos inmunomoduladores, se predijo el índice de hidropatía. La herramienta informática empleada para ello fue ProtScale (http://web.expasy.org/protscale), la cual obtiene la polaridad relativa de cada aminoácido, que se ha determinado experimentalmente midiendo el cambio de energía libre al trasladar un residuo dado de un solvente hidrofóbico al agua. La energía libre de transferencia varía desde muy exergónica para residuos cargados o polares a muy endergónica para aminoácidos con cadenas laterales aromáticas o alifáticas, y finalmente el programa suma las energías libres de transferencia para dichos residuos. La verificación se realizó en conjunto para 21 secuencias de la Tabla I, de acuerdo a la escala de Kyte-Doolittle (1982), que considera a las regiones de las secuencias analizadas con índice >0 en el promedio de hidropatía como hidrofóbicas, y aquellas con valor negativo como hidrofílicas.

La información obtenida de la Tabla II para cada péptido individual muestra que el 72% de las secuencias son hidrofílicas, siendo el restante de carácter hidrofóbico. No fue posible la verificación de dos secuencias, al no contar con la longitud mínima de cinco aminoácidos que el programa requiere. El hecho que los péptidos inmunomoduladores sean mayoritariamente de carácter hidrofílico implica una baja afinidad por las membranas de células u organelos subcelulares, por lo que este podría ser un dato útil para inferir el mecanismo de transporte de cada péptido.

Dentro de las características fisicoquímicas se incluyen también el punto isoeléctrico y la vida media, datos que pueden aportar una idea sobre la estabilidad del péptido. El punto isoeléctrico es un parámetro a considerar al momento de verificar el comportamiento de los péptidos en solución. En el punto isoeléctrico de la proteína la solubilidad generalmente aumenta con la hidrólisis, ya que es principalmente el resultado de la reducción en peso molecular y del aumento en el número de grupos polares (Slattery y Fitzgerald, 1998; Caessens *et al.*, 1999). Por otro lado, la vida media indica el tiempo que toma a la mitad de la cantidad de proteína presente para desaparecer de una célula (Gasteiger *et al.*, 2005). En el presente trabajo, el cálculo se hace para estudios en humanos e *in vitro*. ProtParam estima la vida media contemplando el aminoácido N-terminal de la secuencia a investigar. Se ha demostrado que la identidad del residuo N-terminal de una proteína juega un papel importante para determinar su estabilidad *in vivo*, al participar en los procesos de degradación proteolítica vía ubiquitina (Gasteiger *et al.*, 2003), para lo que se han creado proteínas beta-galactosidasa por mutagénesis sitio-dirigida con diferentes aminoácidos en la región N-terminal. De este modo, las proteínas diseñadas presentaron diferentes tiempos de vida *in vivo* de más de 100h y al menos 2min, dependiendo de la naturaleza del aminoácido en la región N-terminal y del modelo experimental (levadura *in vivo*, reticulocitos de mamífero *in vitro*, *E. coli in vivo*). Así, se puede ordenar el conjunto de aminoácidos individuales respecto a la vida media que ellos confieren cuando están presentes en la región N-terminal de una proteína (Bachmair *et al.*, 1986; Gonda *et al.* 1989; Tobías *et al.*, 1991).

Conclusiones

Esta revisión describe la longitud y composición de secuencias lineales de aminoácidos con actividad inmunomoduladora demostrada en la bibliografía disponible, así como sus propiedades fisicoquímicas. La información muestra que las proteínas de la leche, principalmente las caseínas, dan lugar a la gran mayoría de péptidos inmunomoduladores estudiados a la fecha. Los péptidos inmunomoduladores revisados presentan una longitud de cadena muy variada, sugiriendo las diversas rutas de transporte que pueden tomar en el epitelio intestinal. Muestran un número elevado de residuos de Pro y Glu, aminoácidos que podrían ser clave en estas secuencias para realizar su función. La abundancia de Pro en estas secuencias sugiere una gran probabilidad de que los péptidos inmunomoduladores sufran degradación por efecto de enzimas digestivas cuando son ingeridos vía oral. Presentan secuencias repetidas ricas en Ser, mientras que Tyr y Lys están presentes en mayor proporción en los

extremos N-terminal y C-terminal, respectivamente. Se confirmó la presencia de Arg en la mayoría de estas secuencias en el extremo N-terminal o C-terminal, sugiriendo el reconocimiento de estos péptidos por receptores específicos de membrana. Los pesos moleculares corresponden a estructuras <7kDa, aunque los de mayor incidencia son aquellos <3kDa. En base a la evidencia existente se infiere que estas secuencias muestran una disminución en la probabilidad de sufrir degradación intestinal para péptidos <3kDa. Las cargas que presentan se encuentran en un intervalo entre -7 y +8. Mayoritariamente son de carácter hidrofílico, dato que sugiere una baja afinidad por las membranas de células u organelos subcelulares. Estos datos son útiles para inferir en el mecanismo de transporte, por consiguiente su biodisponibilidad, así como el mecanismo de acción, que culminará en una bioactividad específica sobre el sistema inmune. Con esta información es posible asumir que la existencia de una gran diversidad de péptidos inmunomoduladores se debe a su variada estructura química, que le confiere una diversidad de propiedades fisicoquímicas. En respuesta a ello, los mecanismos de acción son variados. Esto hace a los péptidos inmunomoduladores difíciles de estudiar, pero al mismo tiempo más interesantes. Cabe recordar que los datos fisicoquímicos de cada secuencia se obtuvieron como una etapa predictiva empleando herramientas informáticas para su análisis; por lo tanto, la información relacionada con la dinámica de estas estructuras debe ser confirmada mediante estudios *in vitro* e *in vivo* para poder soportarla, ya que dependerá de la naturaleza del modelo de estudio, la dosis, la duración del tratamiento, la genética y el estado fisiológico, entre otros. El avance logrado en el entendimiento de estas secuencias representa información que debería ser importante como un primer paso en los diseños experimentales.

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IMMUNO-MODULATING PEPTIDES OBTAINED FROM MILK PROTEINS

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SUMMARY

Milk proteins are the subject of numerous investigations, as they represent a supply of bioactive peptides beneficial to health. The characterization of the peptides is a fundamental step in order to understand how they exert their function. The present paper gathers, from a number of different studies, a list of peptidic sequences derived from milk proteins that have shown to have effect on the immune system. These sequences are described as to their length and aminoacid composition, as well as physical-chemical properties. The data shows that their length comprises 2 to 64 aminoacids. The most frequent ones are proline and glutamic acid. Tyrosine and lysine are present in in the extreme N-terminal and C-terminal, respectively, while arginine is at both extremes. These peptides have molecular weights <7kDa, although those <3kDa are most abundant. Also, their charges differ widely at physiological pH, between -7 and +8, being mainly of hydrophyllic character. The analysis of the gathered information in the present review could be of importance for the determination of the structural pattern and in turn the function of immuno-modulating peptides, as at present the structure-function relationships and the mechanisms employed by this peptides to exert the final effects are not completely elucidated.

PEPTÍDEOS IMUNOMODULADORES DERIVADOS DAS PROTEÍNAS DO LEITE

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RESUMO

As proteínas do leite são objeto de numerosas investigações devido ao aporte que apresentam em benefício para a saúde como fonte de peptídeos bioativos. A caracterização dos peptídeos é um passo primordial e importante para poder entender como realizam sua função. A partir de diferentes estudos, neste trabalho foi elaborada uma lista de sequências peptídicas derivadas de proteínas do leite que têm mostrado um efeito sobre o sistema imune. Estas sequências são descritas por seu comprimento e composição de aminoácidos, assim como características físico-químicas. Os dados mostram que seu comprimento compreende entre 2 e 64 aminoácidos. Os aminoácidos mais frequentes nestas sequências são prolina e ácido glutâmico. Destacam-se tirosina e lisina no extremo N-terminal e C-terminal, respectivamente, e arginina em ambos os extremos. Estes peptídeos apresentam pesos moleculares <7kDa, ainda que são mais abundantes aqueles <3kDa. Da mesma forma, ostentam uma diversidade de cargas a pH fisiológico, que oscilam em entre -7 e +8, sendo principalmente de carácter hidrofílico. A análise da informação recolhida nesta revisão poderia ser de importância para determinar o padrão estrutural e por sua vez a função dos peptídeos imunomoduladores, já que no momento a relação estrutura-função e os mecanismos através dos quais estes peptídeos exercem seus efeitos finais não têm sido completamente elucidados.

CAPÍTULO II

Immunomodulation by hydrolysates and peptides derived from milk proteins

Immunomodulation by hydrolysates and peptides derived from milk proteins

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Abstract

Milk protein-derived peptides have shown immunomodulatory properties either singly or in a mixture. However, sufficient information on the behavior of these compounds is lacking, complicating their further study. This review summarizes current knowledge on immunomodulatory milk protein-derived peptides, including hydrolysates, peptide fractions and single peptides, based on the existing scientific literature. The evidence shows that hydrolysates derived from both casein and whey proteins exhibit an immunomodulatory effect. With regard to single peptides, their immunomodulatory effect may be mainly attributed to the protein sequences that are derived from caseins. The identification of peptides and their sequences in hydrolysates, as well as their characterization, is necessary in order to increase knowledge on these compounds.

Keywords: Immunomodulatory peptides, milk proteins, hydrolysates, lactic acid bacteria.

Introduction

Food proteins are a nutritious source of amino acids and also form the precursors of biologically active peptides. Bioactive peptides are defined as fragments released from proteins; these interact with specific receptors and stimulate or inhibit certain effects on the immune system, resulting in an overall positive impact on human health (Muro et al. 2011; Choi et al. 2012; De Gobba et al. 2014; O'Keeffe and FitzGerald, 2015). Although proteins engage in a wide array of activities, in most cases protein hydrolysates containing peptides or single peptides have displayed better bioactivity than their parent proteins (Lönnerdal, 2003).

Several reviews have described the different biological effects of food protein-derived peptides (Hartmann and Meisel, 2007; Udenigwe and Aluko, 2012; Agyei and Danqhua, 2012). Particularly, immunomodulatory activity has been shown in the hydrolysates of major milk proteins (Gill et al, 2000; Brandelli et al., 2015; Hsieh et al 2015; Nongonierma and Fitzgerald 2015; Park and Nam, 2015). However, the evidence for this bioactivity is limited, as few studied have addressed this topic. Research has been subsequently focused on *in vitro* studies, in which the effect of hydrolysates on cells of the immune system is observed, as well as *in vivo* studies, in which processes of enzymatic degradation, absorption and transport of hydrolysates and peptides to specific receptors are monitored. However, the molecular mechanisms by which bioactive peptides exert their immunomodulatory effect are not yet well-defined. Each of these peptides has a unique structure that could differentially affect downstream immunological responses and cellular functions (Hernández-Ledesma et al. 2014).

Therefore, it is necessary to gather existing information and identify the specific sequences that have demonstrated immunomodulatory effects. Due to the fact

that the immunomodulatory peptides previously reported on are mostly derived from milk proteins, the aim of this article consists in reviewing the available scientific literature on milk-derived peptides. This review includes and describes the existing evidence on the immunomodulatory effect of hydrolysates, peptide fractions and single peptides, which have been experimentally proven, in addition to identifying the specific milk proteins that are sources of these bioactive products.

Production of bioactive peptides

Peptides remain inactive while encrypted in their native protein but may be released by specific enzymes that break down peptide bonds in a process called proteolysis (Li-Chan, 2015). Hence, hydrolysis of proteins is an important process for the liberation of potent bioactive peptides (Nagpal et al, 2011). In order to study this process, different strategies may be employed, for example the simulation of gastrointestinal processes, proteolysis by microbial fermentation or other *in vitro* techniques using specific proteinases (Simone et al., 2009).

During the gastrointestinal process after oral ingestion, proteins are digested in the stomach by pepsin under acidic conditions. Then, digestion products are hydrolyzed by pancreatic enzymes, such as trypsin, chymotrypsin and peptidases, which are derived from the intestinal brush border membranes, as well as released by other enzymes of the human microbiota (Saavedra et al. 2013). Finally, to exert their physiological effects *in vivo*, bioactive peptides must reach their target sites at the luminal side of the intestinal tract or at specific peripheral organs, following their absorption (Segura-Campos et al., 2011; Wada and Lönnerdal, 2014). In this regard, gastrointestinal enzymatic processes have been simulated to resemble the normal human digestion of

proteins and evaluate the possibility of bioactive peptide release after normal consumption of food proteins (Udenigwe and Aluko, 2012).

Concerning proteolysis by microbial fermentation, it has been reported that the metabolic activity of lactic acid bacteria (LAB) can generate *de novo* bioactive peptides from milk, via the enzymatic degradation of parent proteins (Hayes et al., 2007). Production of bioactive peptides achieved by the fermentation/ripening process of protein substrates or precursors may be performed using several microorganisms, mainly bacteria, e.g., *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus delbrueckii* subsp. *lactis*. These microorganisms have cell envelope-associated proteases that are responsible for the breakdown of proteins and consequently the release of peptides (Hebert et al. 2008; Kamau et al. 2010; Espeche Turbay et al. 2012). However, the presence of other biomolecules in fermented products may also exert biological effects. For example, other components originating from the bacteria (peptidoglycans, lipoteichoic acid, bacterial DNA, exopolysaccharides) or derived from bacterial synthesis and fermentation processes may be present in the fermented medium (lactic acid, oligosaccharides, gamma aminobutyric acid, growth factors, hormones and/or bacteriocins) and exhibit an additional effect. Hence, it is inconclusive as to whether the observed bioactivities are due to the peptides that are specifically released during fermentation (Abdou et al., 2006; Granier et al., 2013; Agyei et al. 2016).

Proteolysis carried out *in vitro* involves the use of single or multiple specific or nonspecific proteases to hydrolyze proteins and release peptides of interest. This technique produces hydrolysates with predictable peptide fractions, due to the specificity of enzymes in cleaving certain bonds of the protein chain (Kamau et al. 2010). Post-hydrolysis processing is used to isolate bioactive peptides from a complex mixture of other inactive molecules (Wang and Gonzalez De

Mejia 2005; Aluko, 2008). In addition to this, bioactive peptides may be purified from protein hydrolysates by different separation techniques and then assayed to confirm their bioactivity. Lastly, physiologically active peptides may be chemically synthesized to confirm the biological properties associated with a specific amino acid sequence (Clare and Swaisgood, 2000).

In addition to the above, informatics tools are helpful to optimize the production of bioactive peptides and to conduct different studies on peptides. Such tools enhance the understanding of different mechanisms of interaction between receptors and bioactive peptides. Furthermore, these may simplify the production of peptides and predict which peptides may be obtained from known food proteins by performing *in silico* studies prior to wet-laboratory synthesis (Agyei and Danquah, 2012).

Milk proteins: a source of bioactive peptides

Although potential bioactive peptides have been identified in several animal or plant proteins belonging to the current human diet, milk proteins are recognized as a major source of bioactive peptides (Hafeez et al. 2014). In fact, in a study carried out by Dziuba et al., (2009), the function of milk proteins as bioactive peptide precursors was evaluated in a computer-aided proteolysis simulation. The occurrence of bioactive motifs and their associated effects are listed at following, in order of greater to lesser frequency: antihypertensive, dipeptidyl peptidase IV inhibition, opioid, opioid antagonism, antioxidative, antithrombotic, immunomodulatory, bonding and transporting of metals and metal ions, antibacterial, antiviral, and contraction of smooth muscles. However, some peptides are known to display multi-functional properties (Saavedra et al. 2013).

Immunomodulatory properties of milk-derived peptides

An adequate functioning of the immune system is essential to maintain health. However, strategies to effectively modulate the immune response have not been well-explored. Although different drugs exist to slow the progression of specific diseases in humans, their side-effects may sometimes outweigh their benefits (Nongonierma and Fitzgerald, 2015). In this context, some peptides have been shown to stimulate or to inhibit certain functions of the immune system, depending on the dose administered, the experimental conditions and their functioning in the body. As a whole, such activities may be labeled as “immunomodulation” (Werner et al. 1986). Immunomodulation is necessary to control the consequences of a deregulated immune system, and bioactive peptides represents a potential alternative for disease management, as they have low toxicity and do not tend to accumulate in body tissues (Gokhale and Satyanarayanajois, 2014; Agyei et al, 2016).

Immunomodulation via bioactive peptides occurs when peptides bind to a specific receptor and downstream immunological responses and cellular functions are consequently promoted, concluding in the suppression or stimulation of either specific (lymphocyte activation and proliferation, antibody production, cytokine expression) and/or non-specific (functioning of macrophages, granulocytes and natural killer cells) immune responses (Meisel, 2004; Gauthier et al. 2006).

Studies on hydrolysates that contain immunomodulatory peptides as well as single sequences of immunomodulatory peptides are considered in this review. First, the hydrolysates and peptide fractions are described, and then, the single sequences are presented. Both groups are summarized in Tables 1 and 2, respectively.

The immunomodulatory action of casein hydrolysates from bovine milk and their effects on the proliferation of spleen lymphocytes and rabbit Peyer's patch cells

of mice have been evaluated. Pancreatin and trypsin hydrolysates of α_{s1}-casein and β-casein significantly inhibited proliferative responses; pancreatin and trypsin hydrolysates of κ-casein also showed an inhibitory effect (Otani and Hata, 1995). Meanwhile, Sutas et al. (1996) found that peptides derived from the pepsin/trypsin hydrolysis of k-casein significantly stimulated the mitogen-induced proliferation of human lymphocytes. However, in this same study, k-casein hydrolyzed by L. casei GG-derived enzymes showed a consistent suppressive effect on lymphocyte proliferation. In addition, the effect of glycomacropeptide (GMP), a fragment of 64 aminoacids derived from casein, on human peripheral blood monocytes has been tested; the hydrolysis of GMP by pepsin resulted in higher proliferative and phagocytic activities. This indicates that the pepsin-hydrolysated fragments of GMP enhanced immunostimulatory activities (Li and Mine, 2004).

Regarding whey proteins, it has been shown that α-La hydrolysate enhanced the humoral immune response in mice; this effect involves the modulation of the activities of both B lymphocytes and T helper cells (Bounous, 1981; Bounous and Kongshavn, 1985). In addition, Miyauchi et al. (1997) evaluated a hydrolysate of bovine lactoferrin. The results showed a stimulatory effect on B cells and immunoglobulin, as well as increased production of Peyer's patch cells, suggesting that the use of the lactoferrin hydrolysate is beneficial for enhancing mucosal immunity. Furthermore, the immunomodulatory properties of the enzymatic hydrolysates (trypsin/chymotrypsin) of commercial whey protein were evaluated, in which it was found that hydrolysates significantly increased the proliferation of lymphocytes (Mercier et al. 2004). Whey proteins have been evaluated as a whole complex and also after their hydrolysis. For example, another report has shown that both whey protein and its peptide fractions, obtained with trypsin/chymotrypsin, modulated the immune system when administered to mice (Saint-Sauveur et al. 2009). It is also known that β-

Lactoglobulin is the most abundant whey protein and is a carrier of small, hydrophobic molecules, including retinoic acid, which is a potential modulator of lymphocyte responses (Guimont et al. 1997). For instance, Elitsur et al. (1997) found that trans-retinoic acid has activity to potentially enhance the mitogen-stimulated proliferation of lymphocytes (LPL) derived from human colonic lamina propria. This effect was not seen when lymphocytes preparations were depleted of macrophages, suggesting that trans-retinoic acid might affect accessory cells during lymphocyte proliferation.

Beyond the addition of specific or unspecific enzymes, evidence on the activity of peptides derived from milk fermented by LAB continues to increase. For example, *Lactobacillus paracasei* NCC2461 may help to prevent allergy to milk intake in mice by inducing oral tolerance to β-lactoglobulin. Likewise, it was demonstrated that the addition of *L*. *paracasei* resulted in hydrolyzed peptides and induced tolerance to β-lactoglobulin, thereby suppressing lymphocyte proliferation, stimulating interleukin-10 (IL-10) production and down-regulating IFN-γ and IL-4 secretion (Prioult et al. 2004).

Furthermore, the effects of a probiotic fermented milk and a bacteria-free supernatant of probiotic fermented milk on the reconstitution of the intestinal mucosa and the stimulation of local and systemic immunity were evaluated, as a part of a re-nutrition diet considering a murine model of non-severe protein-energy malnutrition (Galdeano et al. 2011). These treatments improved the intestinal microbiota and increased the number of IgA cells, macrophages and dendritic cells. The production of different cytokines (IFN-γ, TNF-α, IL-12) by these cells and the phagocytic activity in the peritoneum and spleen also increased (Galdeano et al. 2011). Similarly, the effect of peptides containing nonbacterial fractions of milk fermented by *Lactobacillus helveticus* on the growth of the humoral immune system were evaluated; these fractions significantly increased intestinal IgA-producing cells in mice (LeBlanc et al.

2002). Correspondingly, it was found that these fractions also modulated mucosal immunity and resulted in an increase in the number of IL-10, IL-2 and IL-6 cells. Particularly, IL-6 was affected in the epithelial cells of the small intestine (Vinderola et al. 2007).

While the immunomodulatory effects of hydrolysates and peptide fractions derived from milk proteins have thus far been reviewed, some research studies have also indicated that single peptides are capable of exerting a beneficial effect on the immune system. Casein, for example, has been evaluated for its capacity to liberate immunomodulatory peptides. These findings have revealed that α_{S1}-casein, following chymosin hydrolysis, releases the N-terminal segment (1–23), which protects mice against *Candida albicans* by stimulation of both phagocytosis and an immune response (Lahov and Regelson, 1996). Moreover, casein-derived peptides inhibit enzymes related to the inflammatory process, e.g. the peptide derived from α_{S1}-casein NENLLRFFVAPFPEVFG (17-33) inhibited matrix metalloproteinase 9 (MMP-9) activity in HT-29 and SW480 cells (Juillerat-Jeanneret et al. 2011; Chatterton et al. 2013). Another peptide derived from α_{s1}-casein corresponding to residues 142–149 (LAYFYPEL) was proven to be an effective inducer of CD8-positive T-cells. Variants of this peptide induced more IFN-γ secretion than residues 142-149 from specific CD8 (+) T cells (Totsuka et al. 1998).

Among the biologically active peptides derived from milk proteins, some of the most studied are casein phosphopeptides (CPPs). These phosphopeptides may form complexes with calcium or other minerals and have several interesting applications (Otani et al. 2000). The sequence 59–79 of α_{S1}-casein (peptide isolated from the phosphoserine-rich region) has showed a humoral immunostimulatory activity in cell cultures, significantly enhancing immunoglobulin production (IgG, IgM and IgA). Also, this sequence inhibited the concanavalin A-induced proliferation of mouse spleen cells and rabbit Peyer's

patch cells, while enhancing the lipopolysaccharide- and phytohaemagglutinin-induced proliferation of both cells (Hata et al., 1998). On the other hand, Kitamura and Otani (2002) demonstrated that ingestion by healthy humans of CPP-enriched cakes, consisting of α_{s2}-casein fragment 1-32 and β-casein fragment 1-28, induced an increase in the faecal IgA content, suggesting a positive effect on mucosal immunity. Bovine β-casein (1-28) purified from a commercially available preparation of casein phosphopeptides also enhanced immunoglobulin activity and cytokine production in human T, B and monocytic cell lines (Kawahara and Otani 2004). Moreover, residues 1-25 from the phosphoserine-rich region of bovine β-casein had a similar effect on the proliferation of spleen cells and rabbit Peyer's patch cells of mice, whether or not these were stimulated by the commercial mitogen (Hata et al. 1998).

The immunomodulatory effect of other peptides derived from β-casein has also been demonstrated. An hexapeptide (54-59) derived from human β-casein by enzymatic hydrolysis with non-pretreated trypsin was purified, sequenced and synthesized, demonstrating the capacity to stimulate phagocytosis by murine peritoneal macrophages of opsonized sheep red blood cells. This hexapeptide administered in a murine model enhanced resistance to infection by *Klebsiella pneumonia* (Parker, 1984). β-Casomorphin-7, another peptide derived from β-casein, was evaluated for its effect on human lymphocyte proliferation; at low concentrations, its effect was suppressed, although it increased at higher concentrations. Coste et al. (1992) observed that the C-terminal sequence 192–209 of bovine β-casein, isolated from a pepsin-chymosin hydrolysate, induced a significant proliferative response on the primed lymph node cells and unprimed spleen cells of rats, at high concentrations.

Kayser and Meisel (1996) found that Tyr-Gly (38-39) and Tyr-Gly-Gly (18-20) sequences in the primary structure of bovine κ-casein and α-lactalbumin, respectively, significantly enhanced the proliferation of peripheral blood

lymphocytes (SRBC). In contrast, β-casomorphin-7 (residues 60–66) and β-casomorphin-10 (residues 193–202) derived from bovine β–casein suppressed proliferation at low concentrations yet stimulated proliferation at high concentrations.

GMP, as previously mentioned, is probably the most studied immunomodulatory peptide. For example, Otani et al. (1995) confirmed that GMP inhibited effects to the immune system in spleen cell cultures of mice in presence of SRBC, and also confirmed that both the lipopolysaccharide (LPS)- and phytohaemaglutinin (PHA)-induced proliferative responses in the spleen and rabbit Peyer's patch cells. Moreover, GMP enhanced the proliferation and phagocytic activity of human macrophage-like cells (Li and Mine, 2004). Requena et al. (2009) demonstrated that GMP enhanced the expression of TNF, IL-1β and IL-8 in monocytes, depending on its concentration.

There is evidence that some peptides derived from milk whey proteins exhibit an immunomodulatory effect. One of them is lactoferricin B, a peptide derived from the N-terminal region of bovine lactoferrin, obtained by hydrolysis with pepsin. It was found that this peptide promotes the phagocytic activity of human neutrophils via dual mechanisms that may involve direct binding to the neutrophil and opsonin-like activity (Miyauchi et al. 1997).

Moreover, it was found that the synthetic peptide Gly-Leu-Phe, corresponding to the sequence f51-53 from α-LA, significantly increased phagocytosis of SRBC by murine peritoneal macrophages and protected mice from lethal *Klebsiella pneumoniae* infections (Berthou et al. 1987). This peptide also stimulated, in a dose-dependent manner, the binding of human senescent RBC to human monocytic-macrophage cells, as well as phagocytosis by these cells (Gattegno et al. 1988). This activity is correlated with the presence of specific binding sites on phagocytic cells in human blood (Jaziri et al. 1992).

The immunomodulatory peptides considered in this review have mainly been derived from casein. This may be attributed to the abundance of casein in the composition of milk, versus that of whey proteins. Nevertheless, it has been reported that some regions of the primary structure of caseins contain overlapping peptide sequences that exert different biological effects. These regions have been referred to as “strategic zones,” which are partially protected from proteolytic breakdown (Meisel, 2004). This behavior was confirmed by Juillerat-Jeanneret et al. (2011), whom reported that the hydrolysates of milk proteins (β-casein in particular) obtained by the addition of LAB produce peptide resistance to the proteolytic enzymes that are involved in several human diseases. Thus, the abundance of immunomodulatory peptides derived from casein and their functioning may be due to the resistance of specific sequences inside this protein to proteolysis.

Structural characteristics and functionality of immunomodulatory peptides

Bioactive peptides derived from milk proteins differ in amino acid sequence and length, including the peptides described in this review. The structural characteristics of proteins might influence the degree of proteolysis and type of peptides released, as well as their localization in the human body, which in turn is dependent on their absorption and bioavailability (Fiat and Jollès, 1989). For example, although many peptides derived from α_{S1}-, β- or κ-caseins have been detected in the stomachs of adult humans after ingestion of milk proteins, small peptides derived from casein and lactoferrin have also been recovered from duodenum. Furthermore, two long peptides, the κ-caseinoglycopeptide and the N-terminal peptide of α_{S1}-casein, have been absorbed and detected in plasma (Chabance et al. 1998). Thus, peptides with biological functions have specific structures, although these may be susceptible to the action of peptidases in the medium to which they were administered or within the *in vivo* system. The survivability of bioactive peptide fractions with different molecular weights has

been evaluated *in vitro* considering a gastrointestinal digestion model; the distribution showed that larger peptides (> 3 KDa) were more easily digested by gastric digestion than smaller peptides (< 3 KDa; Chen and Li, 2012). Therefore, the results of *in vitro* assays should be validated with *in vivo* trials, in considering that the activity of these sequences depends on their absorption and bioavailability, which are strongly related to their structure (Hernández-Ledesma et al. 2014).

In further addressing the importance of the structure of bioactive peptides, the peptides identified in this review to have immunomodulatory activity ranged from 2 to 64 amino acids, which are shown in Table 2. The molecular weights of the sequences reported in this review are around or below 3 KDa, which are proportional to length, except for the sequence corresponding to GMP. However, the majority of these studies have been performed *in vitro*; therefore, the immunomodulatory effects of these sequences exposed to peptidases in living systems are unclear. The generation of smaller peptides could result in similar physiological effects as the parent sequence, or potentially disable them.

Furthermore, the varied length and molecular weights of these sequences suggest that they may also take different transport routes in the intestinal epithelium. This may influence their bioavailability and mode of action, or they may exert a specific bioactivity on the immune system. In some studies on bioactive peptides, di- and tri peptides have been shown to be actively transported via a specific transporter (PepT1), while oligopeptides may be passively transported via the paracellular route across the cell monolayer (Fei et al. 1994; Satake et al. 2002). In addition, oligopeptides can be transported by transcytosis (vesicle-mediated transcellular transport; Shen et al. 1992). The presence of key amino acids also imparts different, specific functionalities. For example, the presence of arginine in the N-or C-terminal region of peptides represents an important structural component that is recognized by specific

membrane bound receptors (Pagelow and Werner, 1986). Likewise, arginine was positioned at the N- or C-terminal in some of the sequences considered in this review.

In general, the structural characteristics, hydrophobicity and basicity, as well as the composition and sequence of amino acids, play a crucial role in determining the biological activities triggered by bioactive peptides (Hancock and Sahl, 2006; Korhonen and Pihlanto, 2006). Hence, in addition to the identification of immunomodulatory sequences, the characterization of these sequences should form a key step in future investigations, followed by an exploration of how immunomodulatory peptides perform biological functions.

Conclusions

Immunomodulatory activity is one of the benefits exhibited by milk protein-derived peptides. Thus far, the evidence indicates that hydrolysates and peptide fractions derived from both casein and whey proteins have an important immunomodulatory effect. Casein is assumed to be the most studied protein in regards to the production of immunomodulatory peptides, although several reports have also focused on whey protein-derived peptides. However, upon evaluating specific single sequences, the results have shown that the vast majority of immunomodulatory peptides are derived from caseins. Accordingly, β-casein was the major source of the peptides identified in this review.

Several studies have shown the immunomodulatory capacity of hydrolysates derived from milk proteins in the presence of bacteria, for example LAB, and the corresponding effects on the modulation of the immune system. However, hydrolysates have shown this effect independently of the presence of live bacteria. In these cases, the resulting effects may be linked to the components of bacterial cells and not solely to immunomodulatory peptides. On the other

391 hand, since natural fermentation processes occur in the gut, bioactive
392 components such as immunomodulatory peptides might be produced de novo.
393 In both cases, the identification of sequences related with immunomodulatory
394 activities is necessary.

395 The molecular mechanisms by which milk protein-derived peptides exert their
396 immunomodulatory effects are not yet defined. In this review, the existence of
397 immunomodulatory sequences of variable length is evident, which in addition to
398 other structural characteristics, leads to a nonspecific action for their targets.
399 Therefore, the identification of new sequences of immunomodulatory peptides
400 and their characterization are necessary in order to support existing information,
401 to discover the mechanisms behind their functioning and finally, to understand
402 their potential biological effects.

403 Finally, although studies covered in this review only addressed the biological
404 activities of milk hydrolysates or peptides, other important issues such as their
405 organoleptic properties should be considered since bioactive peptides produced
406 during milk protein hydrolysis may possess bitter taste. Thus, before these
407 hydrolysates or peptides may be utilized as food products, appropriate
408 processing technology should be used for reducing bitterness without impairing
409 bioactivity.

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660

Table 1. Immunomodulatory effect of milk protein-derived hydrolysates and peptide fractions.

Protein	Enzyme or LAB	Immune effect	Reference
α_{S1}-CN	Pancreatin/trypsin	↓ proliferation of lymphocytes	Otani and Hata, 1995
β-CN	Pancreatin/trypsin	↓ proliferation of lymphocytes	Otani and Hata, 1995
k-CN	Pancreatin	↓ proliferation of lymphocytes	Otani et al. 1995
	Trypsin	↓ proliferation of lymphocytes	Otani et al. 1995
	Pepsin/trypsin	↑ proliferation of lymphocytes	Sutas et al. 1996
	Lactobacillus GG*	↓ proliferation of lymphocytes	
GMP (κ-CN)	Pepsin	↑ proliferation of monocytes ↑ phagocytic activity	Li and Mine, 2004
α-LA	NR	↑ B lymphocyte and T helper cell activities	Bounous 1981 Bounous and Kongshavn, 1985
LF	Pepsin	↑ proliferation of splenocytes ↑ Ig A from Peyer's Patch cells	Miyauchi et al. 1997
Whey proteins	Trypsin/chymotrypsin	↑ proliferation lymphocytes ↑ proliferation of splenocytes ↑ total Ig A ↑serum IFN-γ ↑TGF-β1	Mercier et al. 2004 Saint-Sauveur et al. 2009
β-LG	Trypsin	Carries retinoic acid ↓ lymphocyte blastogenesis	Guimont et al. 1997 Elitsur et al. 1997
	Lactobacillus paracasei NCC2461*	↑ IL-10 production ↓ proliferation of lymphocytes ↓ IFN-γ and IL-4 secretion	Prioult et al. 2004
Milk proteins	*Lactobacillus. delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus* and *Lactobacillus casei* DN-114-001* *Lactobacillus helveticus* R389*	↑IgA, macrophages and dendritic cells ↑ IFN-g, TNF-a, IL-12 production ↑ phagocytic activity ↑IgA-producing cells ↑IL-10, IL-2 and IL-6 cells	Galdeano et al. 2011 LeBlanc et al. 2002 Vinderola et al. 2007

CN: casein, LA: lactoalbumin, LF: lactoferrin, LG: lactoglobulin, NR: not reported, *enzymes derived from acid lactic bacteria.

Table 2. Immunomodulatory effect of the specific sequences of milk protein-derived peptides.

Protein	Peptide	Enzyme	Immune effect	Reference
α_{s1}-CN	RPKHPIKHQGLPQ EVLNENLLRF Isracidin (1-23)	Chymosin	↑Phagocytic activity	Lahov and Regelson, 1996
	NENLLRFFVAPFP EVFG (17-33)	LAB*	↓ activity of MMP-9 (matrix metalloprotease), related to inflammatory process	Juillerat-Jeanneret et al. 2011, Chatterton et al. 2013
	QMEAESISSSEEIV PNSVEQK Casein phosphopeptide (59–79)	trypsin	↑ immunoglobulins production (IgG-IgM-IgA) ↑ or ↓ mitogenic effect type mitogen-dependent on spleen or Peyer´s patch cells	Hata et al. 1998
	LAYFYPEL (142–149)	NR	↑ IFN-γ production ↑ IgE production	Totsuka et al. 1998
α_{s2}-CN	KNTMEHVSSSEE SIISQETYKQEKN MAINPSK Casein phosphopeptide (1-32)	NR	↑ fecal Ig A levels	Kitamura and Otani, 2002
β-CN	RELEELNVPGEIV ESLSSSEESITR Casein phosphopeptide (1-25)	trypsin	↑ or ↓ mitogenic effect mitogen-dependent or independent on spleen or Peyer´s patch cells	Hata et al. 1998
	RELEELNVPGEIV ESLSSSEESITRIN K Casein phosphopeptide (1-28)	NR	↑ Proliferation of T, B and monocyte cells ↑ Ig A production ↑ mRNA expression of IL-6	Kawahara y Otani 2004; Kitamura and Otani, 2002
	VEPIPY (54-59)	trypsin	↑ phagocytosis	Parker et al. 1984
	YPFPGPI β-casomorphine-7 (60-66)	NR	↓ lymphocyte proliferation at lower concentrations ↑ lymphocyte proliferation at higher concentrations	Kayser and Meisel, 1996
	LYQEPVLGPVRGP FPIIV (192–209)	pepsin-chymosin	↑proliferation of lymph node and spleen cells	Coste et al. 1992
	YQEPVLGPVR β–casomorphin-10 (193–202)	NR	↓ lymphocyte proliferation at lower concentrations ↑ lymphocyte proliferation at higher concentrations	Kayser and Meisel, 1996
κ-CN	YG (38–39)	NR	↑ lymphocyte proliferation	Kayser and Meisel, 1996
	MAIPPKKNQDKTE IPTINTIASGEPTST PTTEAVESTVATL EDSPEVIESPPEIN	Chymosin	↓ Spleen or Peyer´s patch cells PHA and LPS-induced ↓ antibody producción	Otani et al. 1995 Li and Mine,

	TVQVTSTAV Glicomacropeptide (106-169)		↑ phagocytic activity ↑ expression of TNF, IL-1β and IL-8	2004 Requena et al. 2009
LF	FKCRRWQWRNK KLGAPSITCVRRA F Lactoferricin B (17-41)	Pepsin	↑ phagocytic activity	Miyauchi et al. 1997
α-LA	LLY (18–20)	NR	↑ lymphocyte proliferation	Kayser and Meisel, 1996
	GLF (51-53)	Trypsin	↑ phagocytic activity	Berthou et al. 1987, Gattegno, et al. 1988, Jaziri et al. 1992

CN: casein, LF: lactoferrin, LA: lactoalbumin, NR: Not reported, *enzymes derived from lactic acid bacteria.

CAPÍTULO III

Milk fermented by specific *Lactobacillus* strains regulate the serum levels of inflammatory cytokines on an LPS-stimulated murine model

Milk fermented by specific *Lactobacillus* strains regulate the serum levels of inflammatory cytokines on an LPS-stimulated murine model

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Abstract

The potential immunomodulatory effect of milk fermented by specific strains of *Lactobacillus* spp*.* on serum cytokines levels was evaluated in a lipopolysaccharide (LPS)-induced murine model. Firstly, 13 strains of *Lactobacillus* were evaluated for their technological properties such as proteolytic and acidifying activities. The selected strains (J20, J23, J25 or J28) were used for milk fermentation administrated in the *in vivo* study. Then, animals were daily gavaged with different fermented milk daily for 4 weeks: fermented milk (FM), pasteurized fermented milk (PFM) and <10 KDa fraction of PFM (PFM10).

Results showed that milk fermented by the selected strains of *Lactobacillus* reduced pro-inflammatory (IL-6 and TNF-α) and increased anti-inflammatory (IL-10) serum cytokines concentration. Furthermore, these effects were enhanced with pasteurization. On the other hand, pro-inflammatory cytokines were reduced for animals treated with PFM10 from milk fermented with J20 or J28. Therefore, the anti-inflammatory effect was related to components present in fermented milk, not necessarily associated to viable cells. Thus, peptides that may be involved in cytokine regulation were identified, which were mainly derived from whey proteins and presented molecular weight < 3 KDa. Several of these peptides presented the amino acid Arg at the extreme of the sequence, hence they have the potential to be recognized by the receptors on the immune cells and in consequence modulate the immune system. Also, several peptides presented a net positive charge, which could bind to the negatively charge LPS molecule, thus diminishing its effect. In conclusion, fermented milk with these specific strains of *Lactobacillus* show potential as novel functional foods for the prevention of systemic inflammatory disorders.

In conclusion, fermented milk with these specific strains of *Lactobacillus* are proposed as novel functional food for the prevention of systemic inflammatory disorders.

***Keywords*:** immunomodulatory effect, fermented milk, cytokines, *Lactobacillus*, lipopolysaccharide.

Introduction

Lactic acid bacteria (LAB) are a large heterogeneous group of Gram-positive, low G+C content, acid-tolerant and nonspore-forming rods or coccobacilli microorganisms. The common agreement is that there is a core group consisting of four genera; Lactobacillus, Leuconostoc, Pediococcus and Streptococcus. Recent taxonomic revisions have proposed several new genera and the remaining group now comprises the following: Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactococcus, Oenococcus, Tetragenococcus, Vagococcus, and Weissella. (Khalid, 2011). Among the genera comprising the LAB, *Lactobacillus* is the largest genus (Claesson et al., 2007).

Taxonomically, *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Lactobacillaceae*. They have limited biosynthetic abilities, and require preformed amino acids, B vitamins, purines, pyrimidines and (usually) a sugar as a carbon and energy source, which is fermented to produce lactic acid as a common end product. These nutritional requirements restrict their habitats to those in which the required compounds are abundant. Nevertheless, *Lactobacillus* occupy a variety of niches including milk and the gastrointestinal tract of humans and other animals (Wells, 2011). Several *Lactobacillus* have an excellent safety profile and they are considered in a “generally-as-safe” status. Besides, they have a preservative action due to their high acidifying activity, contributing to flavor, texture and nutrition enhancement (Iraporda et al., 2014). Thus, a widespread number of *Lactobacillus* species are involved in food production. Hence, these microorganisms play a key role in industrial and artisan food fermentation, including a large variety of fermented dairy products (Kleerebezem et al., 2010).

Lactobacillus of human gut flora are believed to be beneficial to health. In consequence, fermented dairy products have been associated with the ability to

confer and improve health benefits when they are consumed (Bourrie et al., 2016). Moreover, these microorganisms have cell-envelope-associated proteases; thus, fermentation of milk can also serve to create, enrich, or release new milk-associated functional components e.g. peptides, capable of providing health benefits (Hayes et al., 2007; Hebert et al. 2008; Kamau et al. 2010; Espeche Turbay et al. 2012).

Beneficial effects of LAB include the modulation of the immune system (von der Weid et al., 2001; Morita et al., 2002; Prioult et al., 2004; Galdeano et al., 2007; Neumann et al., 2009; Juarez et al, 2013). *Lactobacillus* can elicit innate and adaptive immune responses in the host via binding to pattern recognition receptors (PRR) expressed on immune cells and many other tissues including the intestinal epithelium. PRR recognize conserved molecular structures known as microbe-associated molecular patterns (MAMPs). The PRR-MAMP complex induces the signal for the production of cytokines, chemokines and other innate effectors (Wells, 2011).

Cytokines are mediator molecules distinguished for their activities associated with immune response. The concentrations and variety of cytokines released are considerably increased following stimulation by a diversity of inducers (Fernández-Martínez et al., 2004). Proinflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-a (TNF-a), are produced in response to lipopolysaccharide (LPS) and high levels of these cytokines could trigger serious complications to health with alteration to the immune response (Bastos-Pereira et al, 2014). It has been shown that specific LAB orally administered to animal models can regulate the systemic production of cytokines (Haller et al., 2000; Chiu et al., 2013; Juarez et al, 2013).

Lactobacillus have been investigated for their immunomodulatory effects and these effects have shown to be strain dependent. The health promoting effects ascribed to bacteria and fermented dairy products arise not only from bacteria themselves but also from molecules derived from milk fermentation (Macho et al., 2011; Granier et al., 2013; Mortaz et al., 2015; Agyei et al. 2016). Particularly, milk peptides liberated during fermentation by LAB might have a

crucial role in immunomodulatory activity (Hsieh et al 2015). Studies confirm that hydrolysis of milk proteins by specific strains of *Lactobacillus* have the potential to influence the specific immune response through the modulation of inflammatory cytokine secretion (Prioult et al. 2004; Vinderola et al. 2007; Maldonado Galdeano et al. 2011). Therefore, the aim of the present study was to evaluate between 13 strains of *Lactobacillus* those with desirable technological properties, including proteolytic and acidifying capacities. Then, capacity of milk fermented by the selected strains of *Lactobacillus* on the inflammatory cytokine production was evaluated in an LPS-stimulated murine model. Since fermented milk contain a wide range of potentially active components, including bioactive peptides, the effect of peptide fractions (< 10 KDa) derived from milk fermented by *Lactobacillus* were also tested.

Materials and Methods

Substrates and chemicals

Lactobacilli MRS Broth was purchased from Difco (Sparks, MD, USA). Nonfat dry milk was obtained from Dairy America (Fresno, Cal, USA). O-Phthaldialdehyde (OPA) was purchased from Fluka (Linz, Austria), and TCA, sodium borate, SDS, 2-mercaptoethanol and *E. coli* O11: B4-LPS from Sigma Chemical Co. (St. Louis, MO).

Strains and growth conditions

All the strains of *Lactobacillus* (*Lb. fermentum, Lb. pentosus and Lb. plantarum* species) were obtained from the culture collection from 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 (Heredia, 2011) and previously identified, based on 16S rRNA gene sequencing (Table 1) (Heredia et al., 2015). Prior to all analyses, the strains of *Lactobacillus* were cultured from glycerol at -80°C in 10 mL of sterile MRS Broth. Three consecutive subcultures were inoculated at

1% and incubated for 24, 18 and 12 h respectively at 37 °C in order to obtain fresh cultures. The initial average population of inoculum was obtained (plate counting method) from the 12 h fresh culture.

Preparation of fermented milk

Reconstituted nonfat dry milk (0.1 g/mL) was pasteurized at 110 °C for 10 min, immediately cooled at 4 °C and stored overnight at the same temperature. Fresh cultures of each strain were used to inoculate the reconstituted milk at 1% level and at 37 °C, which were incubated for 12 h at the same temperature and later they were used as starter culture. For the production of fermented milk (FM treatments), an aliquot (3%) of the starter cultures were used to inoculate the reconstituted milk. Then, inoculated milk was incubated for 48 h at 37 °C. pH measurements were directly taken in samples of fermented milk using a pH meter (Orion 4 Star, Singapore). Additionally, titratable acidity was monitored during fermentation. Pasteurized fermented milk (PFM treatments) were prepared by heating fermented milk at 75 °C for 20 min and immediately placed them into the ice bath. Finally, in order to obtain <10 KDa fractions of PFM (PFM10 treatments), PFM was centrifuged at 4500 rpm (J2-21 rotor, Beckman, USA) for 40 min at 4 °C, supernatants were collected and ultra-filtered through 10 KDa cut-off membranes (Pall life Sciences, USA) in an ultrafiltration unit (Millipore Amicon 8050, USA). Permeates were collected, frozen at -80°C and lyophilized with a freeze dryer (Labconco, USA). Samples were storage until peptide sequence was identified by HPLC mass spectrometry analysis.

Proteolytic Activity

Samples of FM were taken at different times (18, 24 and 48h) of fermentation for the determination of proteolytic activity. Proteolysis in fermented milk was quantified by using the OPA method (Donkor et al., 2007). For this purpose, 5 mL of 0.75 N trichloroacetic acid were added to 2.5 mL of fermented milk and vortexed for 1 min. Samples were kept at 4 °C for 30 min, then, they were centrifuged (4500 rpm, 40 min, 4 °C). The supernatants were filtered using

Whatman no. 2 paper. All filtrates were frozen at −20°C until analysis. The OPA reagent was freshly prepared. A 30-µL sample aliquot containing TCA-soluble peptides were added to 600 µL of the OPA reagent. After 2 min at 20°C, absorbance was immediately measured at 340 nm using a spectrophotometer Nanodrop 2000 (Thermo Scientific, USA). Unfermented milk was used as a control, and measurements were taken in duplicate.

Assay in a murine model

This study was approved by the ethical committee of the Food Research and Development Center, A.C. (protocol number CE/007/2015) and was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Mexican Official Regulations (Aluja, 2000) and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources (NRC, 2011). All sacrifices were performed under chloroform anesthesia, and efforts to minimize animal suffering were made during the study.

Male Wistar rats (5-6 weeks old, weighing 110-160 g) used in this study were obtained from Bioinvert & Aprexbio SA de CV, (CDMEX, México). Rats were given rat chow (Standard Rodent Laboratory-Chow 5001 Diet, Purina Feeds, Inc., St. Louis, MO, USA) and purified water *ad libitum*. Animals were kept in a sanitized bioterium, maintained with a relative humidity between 40 to 60%. The temperature was kept at 22 ± 2 °C, with 12 h light/dark cycles. Rats were housed in sanitized polycarbonate cages (60 x 40 x 30 cm) with sterile sawdust bedding that was replaced daily. Cages were arranged on racks and hygiene was continuously monitored.

The animals were adapted (first week) and randomly assigned in pairs to experimental groups (n = 6). Bioassay included the following groups: AM group (acidified milk, positive control), FM, PFM and PFM10 groups administered with milk fermented by each selected strain (treatments), all of them stimulated with LPS. Also, a PBS (phosphate-buffered saline) group was included as a negative control, with no LPS stimulation.

Treatments lasted 4 weeks during which animals were daily gavaged with 1 mL samples. After 28 days of treatment, animals were subcutaneously injected with LPS (7.5 mg/kg diluted in milliQ water) to induce a systemic inflammatory process. Finally, rats were sacrificed at 6 h post-stimulation and blood samples were taken. Blood samples were centrifuged at 2500 rpm for 8 min. Serum was collected, and kept at -20 °C until cytokine analyses were performed (Figure 1).

Cytokine Determinations

Serum cytokine concentrations were determined by the ELISA method (Enzyme-Linked Immunosorbent Assay) using commercially available kits (Thermo Scientific, Rockford, IL). These tests comprised recombinant cytokines from *E. coli* and antibodies against IL-10, IL-6 and TNF-α (3, 5 and 15 pg/mL detection limit respectively) rat cytokines. The results were calculated based on the absorbance of complex cytokines-antibodies. Cytokine concentrations were obtained from calibration curves.

Analysis of peptides by Tandem Mass Spectrometry

Mass spectrometry (MS) 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 nanocolumn was a C18-300 (150 mm × 0.75 µm, 3.5 µm; Agilent Technologies Inc.) The sample injection volume was 1 µL. Solvent A was a mixture of water-acetonitrile-formic acid (10:90:0.1, vol/vol/vol) and solvent B contained water-acetonitrile-formic acid (97:3:0.1, vol/vol/vol). The gradient was based on the increment of solvent B, which was initially set at 3% for 10 min and it took 23 more min to reach 65%. The 0.7 µL/min flow rate was directed into the mass spectrometer via an electrospray interface. Nitrogen (99.99%) was used as the nebulizing and drying gas and operated with an estimated helium pressure of 5×10^2 Pa. The needle voltage was set at 4 kV. Mass spectra were acquired over a range of 300 to 2,500 mass/charge (m/z). The signal threshold to perform auto MS analyses

was 30,000. The precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage ramp from 0.35 to 1.1 V. Peptide sequences were obtained from mass spectrometry data using the Mascot server (Perkins et al., 1999) through the UniProtKB/Swiss-Prot database (http://www.matrixscience.com/help/seq_db_setup_Sprot.html) sequences. Net charges of peptides in this study were also calculated using the informatic tools ProtParam and SAPS (Gasteiger et al., 2005) in the server Expasy (http://www.expasy.org).

Statistical analysis

For statistical analysis, data normality was tested as a prerequisite before one way analysis of variance (ANOVA) was carried out in order to compare groups. Differences among means were assessed by Fisher's least significant difference multiple comparison test and considered significant when $P \leq 0.05$. Data analyses were performed by using the NCSS 2007 statistical program (Hintze, 2007). Resulting data were expressed as means ± S.E.M. of six rats in each group.

Results and discussion

Strain selection

Proteolysis is an important process for the liberation of potent bioactive peptides (Nagpal et al, 2011; Li-Chan, 2015). Production of bioactive peptides could be achieved during the fermentation process by the action of several LAB. These microorganisms have cell-envelope-associated proteases that are responsible of the breakdown of proteins and consequently the release of peptides (Hebert et al. 2008; Kamau et al. 2010; Espeche Turbay et al. 2012). In this study, proteolytic activity was used for the evaluation of *Lactobacillus* species, in order to select those strains with the highest proteolytic activity and thus the greatest potential for bioactive peptide production. A total of 13 *Lactobacillus* strains from three species (*L. fermentum, L. pentosus and L. plantarum*) (Table 1) were evaluated and compared.

Proteolytic activity assessed at 18, 24 and 48 h of fermentation showed differences between the strains of *Lactobacillus* and it was time- and strain-dependent (Figure 2). The strains of *Lactobacillus* J20, J23, J25 and J28 had by far the highest proteolytic activity of all, which increased significantly ($P < 0.05$) over time. Although J31 also increased significantly over time, it was less proteolytic than the strains of *Lactobacillus* J20, J23, J25 or J28 at all fermentation times. On the other hand, the strains J10, J24, J26, J27, J32, J34, J37 and J38 could be considered as weakly-proteolytic. These results were similar as those reported by Leclerc et al. (2002), who demonstrated a linear increase in the extent of proteolysis over fermentation time for *L. helveticus*. Results showed a high proteolytic activity for species *L. fermentum* and *L. plantarum* (strains J20, J23, J25 and J28) at 48 h of fermentation and it was related to a powerful proteolytic system for these strains. These differences in the amounts of amino groups released during milk fermentation could probably be associated to the different proteinases and peptidases produced by these strains (Shihata and Shah, 2000).

In addition, acidifying activity of *Lactobacillus* strains was evaluated by monitoring pH and titratable acidity at different times of fermentation (0, 12, 18, 24 and 48 h) (Figure 3 and 4). pH significantly decreased over time for the strains of *Lactobacillus* J20, J23, J25 and J28. On the other hand, pH did not change for the rest of these strains (Figure 3).

Similarly, lactic acid concentration evaluated by titratable acidity was significantly ($P < 0.05$) different over time for the strains of *Lactobacillus* J20, J23, J25 and J28 (Figure 4). On the other hand, titratable acidity did not change over time for the rest of the strains (Figure 4).

As it appears, the rate of lactic acid production and pH decrease by *Lactobacillus* was strain dependent and could be explained in terms of differences in metabolic ability and growth requirements. This fact was previously reported by Widyastuti et al. (2014). Certain strains of lactic acid bacteria can utilize lactose fully as opposed to some others than can mainly convert a part of lactose, namely glucose, into lactic acid (Donkor et al., 2007).

While pH decrease over time depends on the amount of lactic acid and other organic acids released, which is directly linked to the culture metabolic capacity, titratable acidity depends only on the lactic acid produced (Widyastuti et al., 2014). Organic acids, included lactic acid, is the end product of carbohydrate metabolism by LAB, as a hallmark among other metabolites that may contribute to product characteristics (Garrote et al., 2015).

Since the strains of *Lactobacillus* J20, J23, J25 and J28 showed the highest proteolytic and acidifying activities, they were selected for further studies.

Previous to bioassays, the initial average population of inoculum for milk fermentation with the selected strains was established. Growth during 24 h was monitored for the strains of *Lactobacillus* J20, J23, J25 and J28 and the point corresponding to the end of the exponential phase, that reached 10^9 CFU/mL at 12 h, was chosen as the initial average population for milk inoculum (Figure 5).

Cytokine analysis in an LPS-stimulated murine model

Milk fermented by the four different strains of *Lactobacillus* previously selected were evaluated according to their capacities to modulate the production of IL-6, TNF-α and IL-10 in a LPS-stimulated murine model. Results showed an increase in the production of each serum cytokine evaluated in groups stimulated with LPS. PBS group (with no LPS stimulation), showed concentrations near to zero for all cytokine determinations.

FM treatments with the strains of *Lactobacillus* J20, J23, J25 or J28 did not modified the values of IL-6, TNF-α and IL-10 compared to AM-LPS control group, except for milk fermented by the strains of *Lactobacillus* J25 or J28, which significantly reduced TNF-α serum levels (figure 6).

PFM treatments with the strains of *Lactobacillus* J20, J23, J25 or J28 decreased the pro-inflammatory cytokines values compared to AM-LPS control group. PFM decreased IL-6 levels and this decrease was significant for PFM with strains *Lactobacillus* J23, J25 or J28. On the other hand, decrements for TNF-α, were significant for PFM with each one of these four strains. Moreover,

serum levels of IL-10 were significantly increased for all treatments except for treatment with *Lactobacillus* J28 (figure 7).

Additionally, in this study the administration of PFM10 treatments were assessed on cytokine production. These results showed that treatments with the strain *Lactobacillus* J28 significantly decreased levels of IL-6 and treatments with the strains *Lactobacillus* J20 or J28 significantly decreased the levels of TNF-α. However, IL-10 did not show differences compared to the control AM-LPS (Figure 8).

TNF-α is the most important pro-inflammatory cytokine and it is considered to play a key role in the acute inflammatory response. It is released by monocytes and macrophages in response to various stimuli, including bacterial LPS and their overproduction is associated with a wide range of pathologic conditions. (Chamulitrat et al., 1995; Marriot et al., 1998). Therefore it is necessary to find ways to down-regulate its production or inhibit its effects *in vivo*. In this study, the milk fermented by the strains J25 or J28 showed a regulatory effect over this cytokine since TNF-α production was decreased.

On the other hand IL-6, another important pro-inflammatory cytokine produced by monocytes/macrophages, showed high levels of production at 6 h after stimulation with LPS compared to the others cytokines in this study.

IL-10 is a potent and pleiotropic anti-inflammatory cytokine produced by lymphocytes and macrophages (Thompson et al., 1998). It inhibits the synthesis of pro-inflammatory cytokines, such as IL-6 and TNF-α, by T helper type 1 cells, mono/macrophages, and polymorphonuclear cells, and reduces T-cell activation *in vitro* and *in vivo* (Louis et al., 1997; Sang et al., 1999). This cytokine was regulated in the present study, since PFM treatments enhanced the cytokine IL-10 production.

The regulatory effect on pro and anti-inflammatory cytokines production after the administration of fermented milk (with or without pasteurization) on a LPS-stimulated murine model was demonstrated. Nevertheless, this effect was more pronounced after the administration of pasteurized fermented milk.

Some reports have demonstrated that molecules, possibly bioactive peptides, released by LAB during fermentation are able to modulate the production of cytokines and therefore, to modulate the immune system (Abdou et al., 2006; Granier et al., 2013; Agyei et al. 2016). Additionally, it has been reported that heat treatment changes the structural conformation of proteins and peptides in milk. The formation of bioactive peptides through digestion could also be affected by heat treatment, as different protein bonds will be available for enzymes in the gastrointestinal tract (Sánchez-Rivera et al., 2015).

Identification of peptides in milk fermented by *L. fermentum* with regulatory effect on cytokine production

The decreased on IL-6 or TNF-α serum levels at 6 h after LPS-induction with PFM10 treatments obtained from milk fermented by *L. fermentum* J20 and J28 could be associated to the presence of peptides present in these fermented milk. Therefore, peptides in fractions (< 10 KDa) of milk fermented by these strains were identified by tandem mass spectrometry (Table 1).

The proteolytic activity of *L. fermentum* J20 or J28 targeted mainly whey proteins, especially lactotransferrin. Twenty six and fifteen peptide sequences derived from theses proteins were identified in milk fermented by *L. fermentum* J20 and J28, respectively. The proteolysis process gave rise to medium-sized peptides, in a length ranging from 7 to 34 amino acid and a molecular weight mostly < 3 KDa. These sequences were variable and different in the type and order of amino acids, which was dependent on the strain used for milk fermentation. This variability suggests that *L. fermentum* J20 and J28 may present different proteolytic systems and consequently release different peptides. Peptides listed in Table 1 showed the action of mainly serine proteases, specifically trypsin, which cleaves the peptide chains mainly at the carboxyl side of the amino acids lysine or arginine (Rodriguez et al., 2008).

A key point in identified sequences is the type of amino acid in the N-terminal and C-terminal positions. The evidence suggests that arginine (Arg) in

the extreme of bioactive peptides is the dominant entity recognized by receptors on macrophages and lymphocytes, which enhances their maturation and proliferation (Meisel and FitzGerald, 2003; Haque and Chand, 2008). In this study, six sequences had the amino acid Arg in the N-terminal and C-terminal positions of peptides identified in milk fermented with *Lactobacillus* J20. These peptides were NLNREDFR, GCAPGSPR, WDQVKR, DSALGFLR, RPKHPIK and KVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVR. On the other hand, only one sequence with Arg (RYPSYGLN) was present in milk fermented with *Lactobacillus* J28. These sequences could be recognized by receptors in immune cells and in consequence, they could show an immunomodulatory effect.

Based on the presence of arginine and lysine (positively charged residues), as well as aspartic acid and glutamic acid (negatively charged residues) in the sequence, peptide net charge was calculated. Results showed that these sequences had a net charge ranging from -4 to +3 at physiological pH. These peptides were identified as anionic (35 and 40 %), neutral (23 and 7 %) and cationic (42 and 53 %), when derived from milk fermented by *L. fermentum* J20 and J28, respectively.

Lipopolysaccharide (LPS) is the major constituent of the outer membrane of Gram-negative bacteria that when released into the bloodstream causes inflammation via activation of monocytes and endothelial cells. It can lead to septic shock and even death. One strategy to opposing endotoxic shock is to neutralize the most conserved part and major mediator of LPS activity (lipid A), which present negatively charged phosphoryl groups by positively charged LPS-binding molecules, such as proteins or peptides (Van Amersfoort et al., 2003; Martínez-Sernández et al., 2016).

In this study, nineteen sequences with a net positive charge were identified that may bind to LPS thus reducing its toxicity. The identified sequences were: VPVLAENRK, GCAPGSPR, WDQVKR, GECAQKKI, KHSSLDCVLRPT, EKNRLNF, IIAEKTK, MSFVSLLLVGILFHATQAE, AVAKFFSASCV,

GPVRGPFPIIV, RPKHPIK, KTKIPAVFK, AIAEKKA, AKKTYDS, AMTNLRQ, RYPSYGLN, VLPVPQKAV, QVLLHQQALFGKNGKNCPDK and KYLLFCM.

The literature has reported the immunomodulatory effect of peptides mainly derived from caseins (Reyes-Díaz et al., 2016). In this study, a large number of the identified peptides in milk fermented by *L. fermentum* J20 or J28 were derived from whey proteins. Thus these results open the possibility for finding *de novo* sequences with immunomodulatory activity. However, more studies are needed in order to identify the role of these peptides on the observed effect.

Conclusions

It was concluded that milk fermented by specific strains of *Lactobacillus* are able to modulate the balance of LPS-stimulated pro- and anti-inflammatory serum cytokines. The potential immunomodulatory effect was possibly due to components present in fermented milk, not necessarily associated to viable cells, since pasteurized fermented milk and fractions < 10 KDa also showed an effect. Thus, cytokine modulation could be associated to peptides present in fractions (<10 KDa) obtained from fermented milk. Mass spectrometry analyses showed that these peptides were mainly derived from whey proteins and had a molecular weight < 3 KDa. Some of these structures presented the amino acid Arg at the extreme of the sequence, hence they have the potential to be recognized by the receptors on the immune cells and in consequence modulate the immune system. Also, several peptides in fermented milk presented a positive charge, which could bind to the negatively charge LPS molecule. However, more studies are needed in order to identify the components responsible for the observed effect. In conclusion, fermented milk with these specific strains of *Lactobacillus* show potential as novel functional foods for the prevention of systemic inflammatory disorders.

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Table 1. *Lactobacillus* strains under study (Heredia-Castro et al., 2015).

Strain code	LAB
J10	*Lactobacillus fermentum*
J20	*Lactobacillus fermentum*
J23	*Lactobacillus fermentum*
J24	*Lactobacillus pentosus*
J25	*Lactobacillus plantarum*
J26	*Lactobacillus pentosus*
J27	*Lactobacillus pentosus*
J28	*Lactobacillus fermentum*
J31	*Lactobacillus pentosus*
J32	*Lactobacillus fermentum*
J34	*Lactobacillus pentosus*
J37	*Lactobacillus pentosus*
J38	*Lactobacillus fermentum*

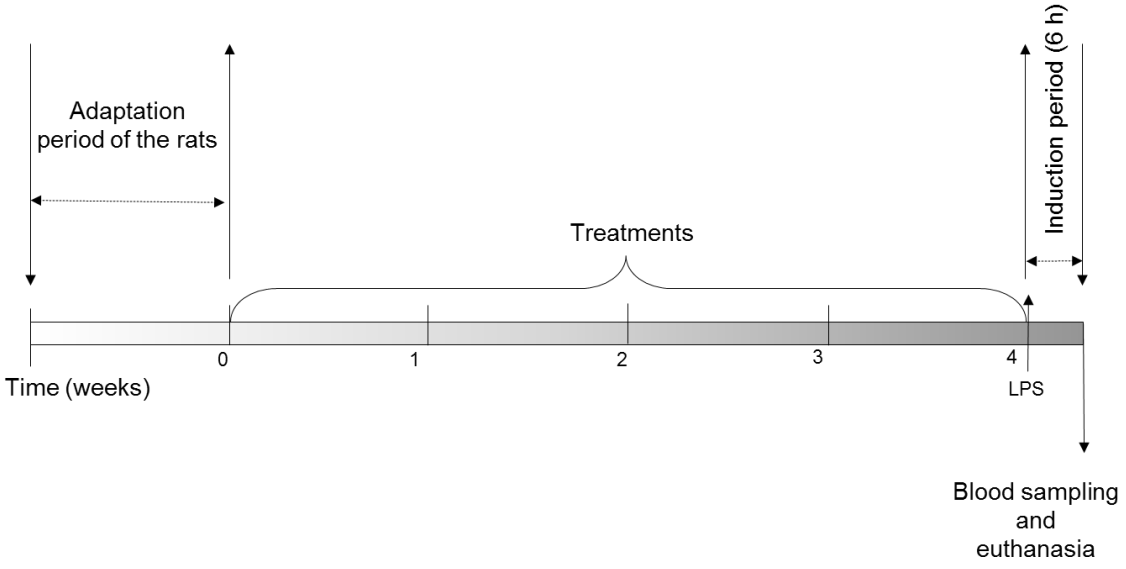

Figure 1. Experimental design for bioassay in a LPS-stimulated murine model.

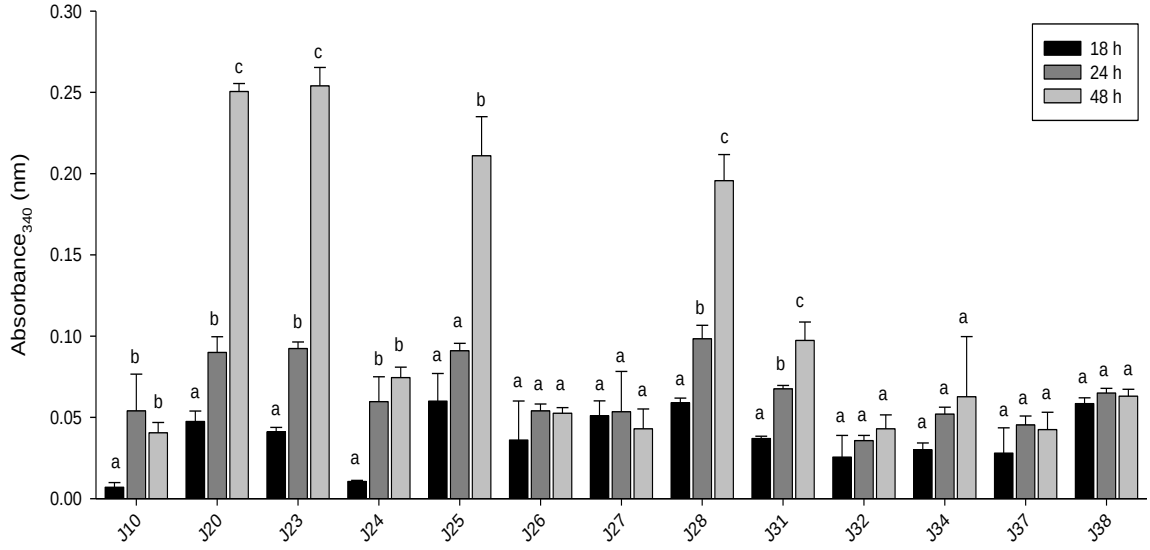

Figure 2. Extent of proteolytic activity (o-phtaldialdehyde method) in samples of milk fermented by strains of *Lactobacillus* spp. at 18, 24 and 48 h of fermentation (37 °C). Mean ± SD (n=3). Different letters indicate significant differences ($p < 0.05$) among fermentation times for each strain.

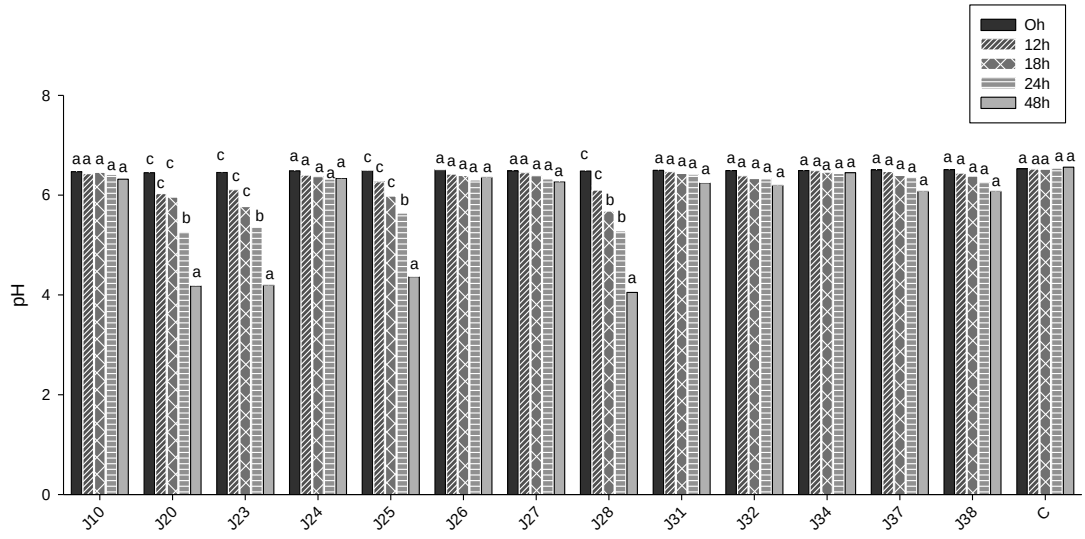

Figure 3. pH of milk fermented by strains of *Lactobacillus* spp. at 0, 12, 18, 24 and 48 h of fermentation. C=control (unfermented milk). Mean ± SD (n=3). Different letters indicate significant differences ($p < 0.05$) among fermentation times for each strain.

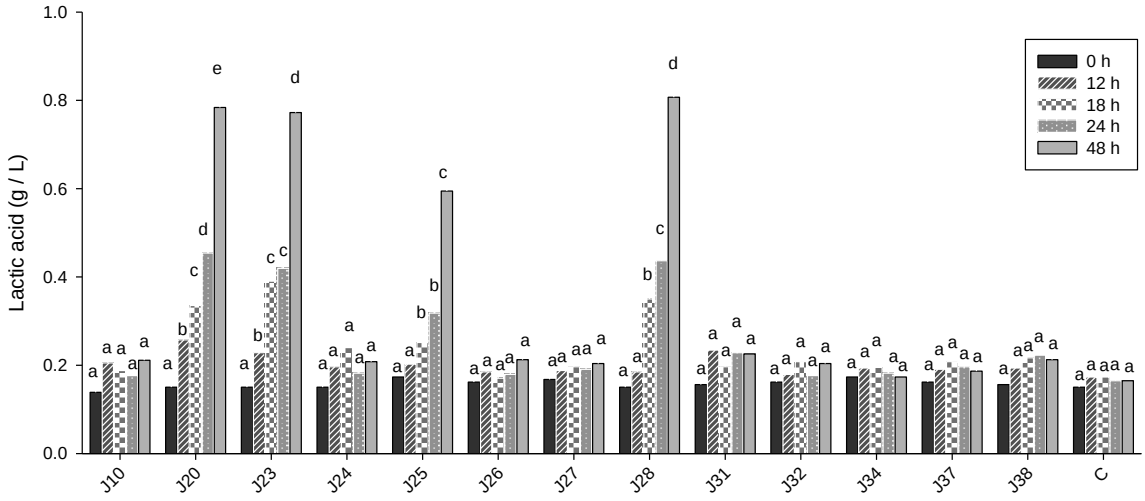

Figure 4. Lactic acid concentration in milk fermented by strains of *Lactobacillus* spp. at 0, 12, 18, 24 and 48 h of fermentation. C=control (unfermented milk). Mean ± SD (n=3). Different letters indicate significant differences ($p < 0.05$) among fermentation times for each strain.

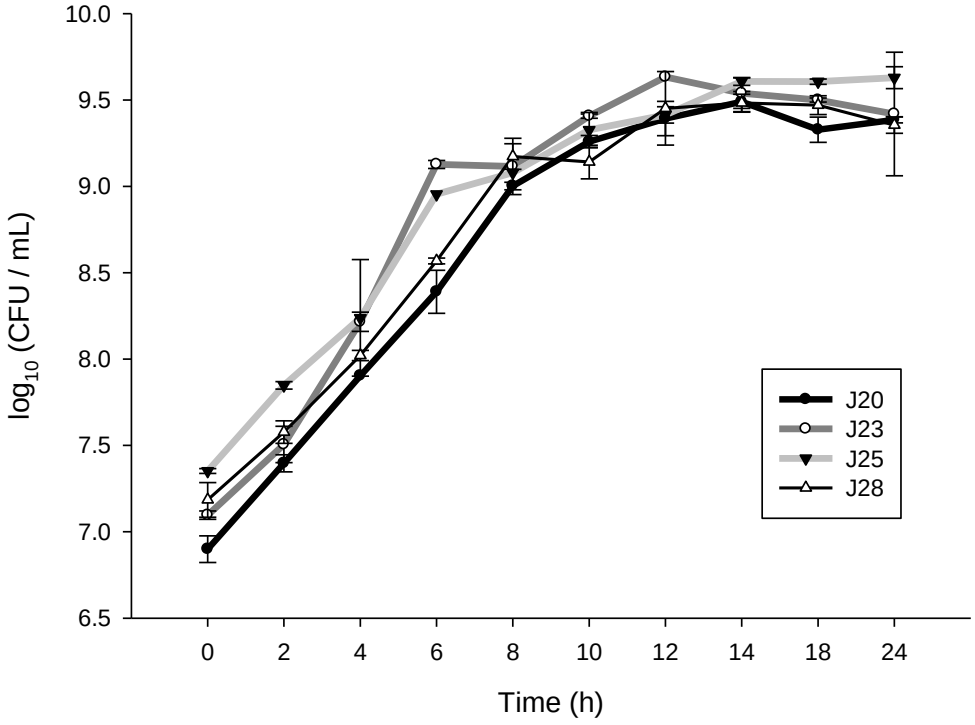

Figure 5. Growth curves of the different strains of *Lactobacillus* spp. in MRS both at 37 °C. Mean ± SD (duplicates).

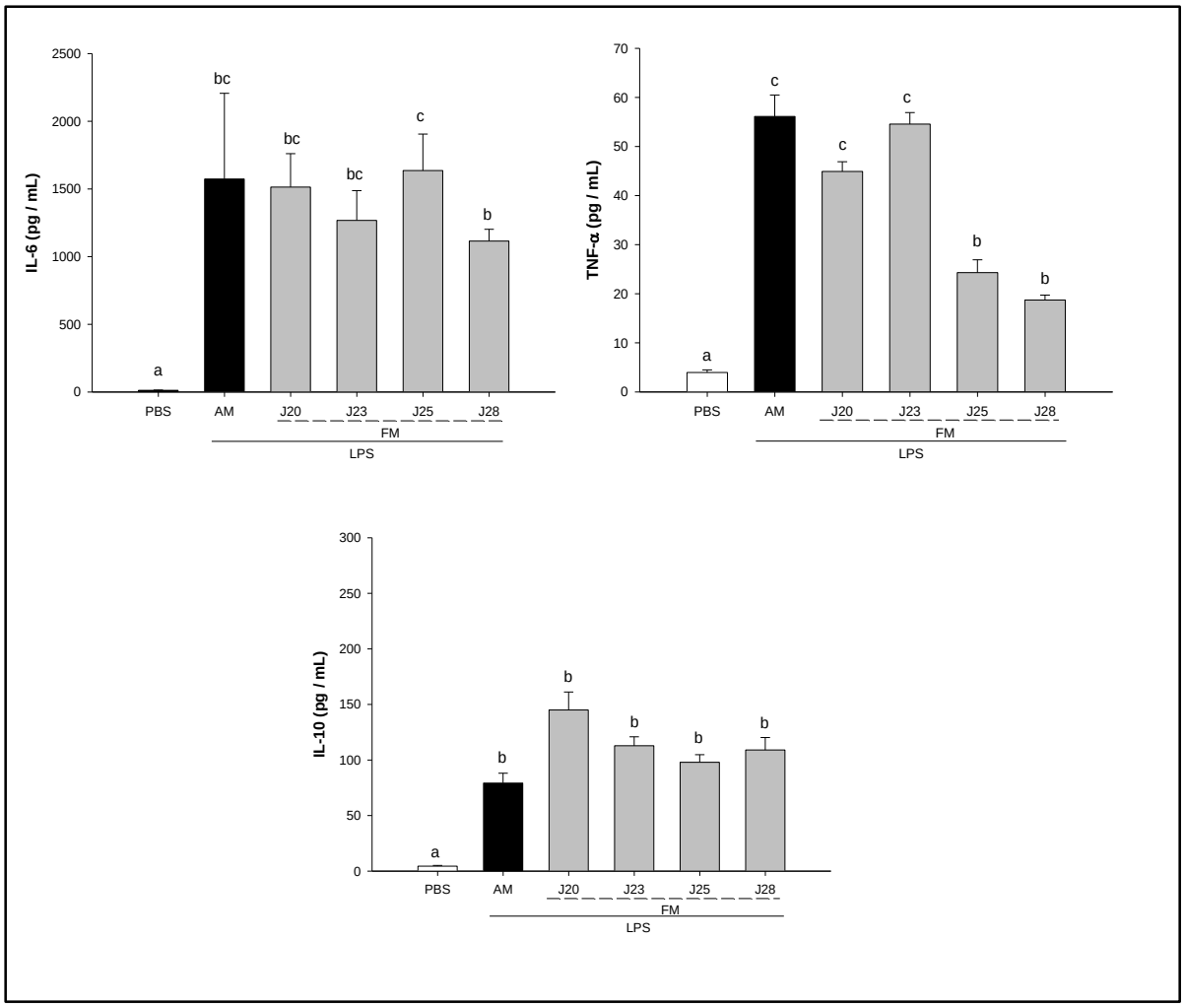

Figure 6. Serum concentration of IL-6, TNF-α and IL-10 determined by ELISA in Wistar rat (n=6) daily administrated for 4 weeks with fermented milk (FM) by strains of *Lactobacillus* J20, J23, J25 or J28. At day 29, rats were injected with LPS (7.5 mg/kg). Six hours after injection, rats were sacrificed and blood samples were taken. In addition, phosphate-buffered saline (PBS) and acidified milk (AM) groups were included. Columns represent means ± SE. Different letters indicate significant differences ($p < 0.05$) between groups.

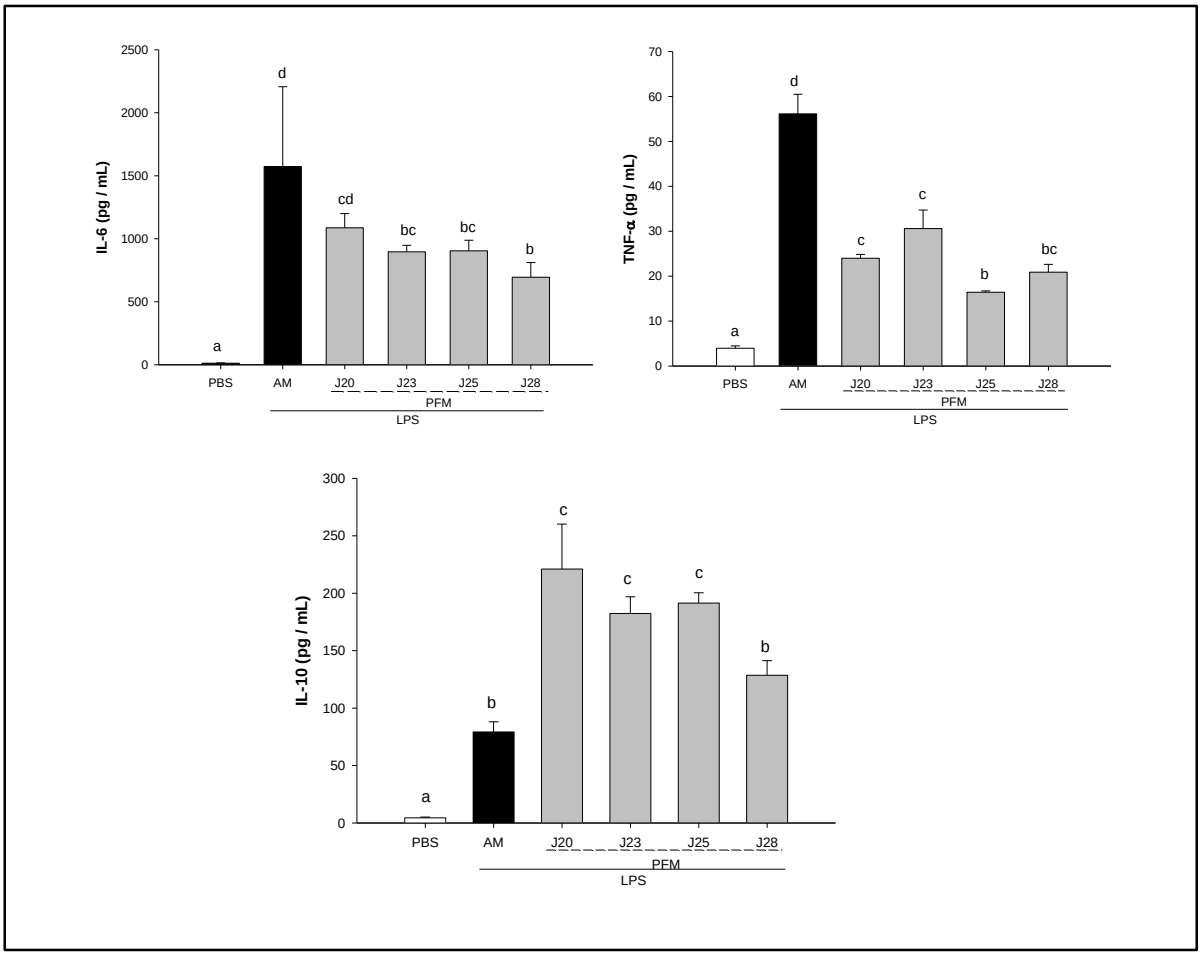

Figure 7. Serum concentration of IL-6, TNF-α and IL-10 determined by ELISA in Wistar rat (n=6) daily administrated for 4 weeks with pasteurized fermented milk (PFM) by strains of *Lactobacillus* J20, J23, J25 or J28. At day 29, rats were injected with LPS (7.5 mg/kg). Six hours after injection, rats were sacrificed and blood samples were taken. In addition, phosphate-buffered saline (PBS) and acidified milk (AM) groups were included. Columns represent means ± SE. Different letters indicate significant differences ($p < 0.05$) between groups.

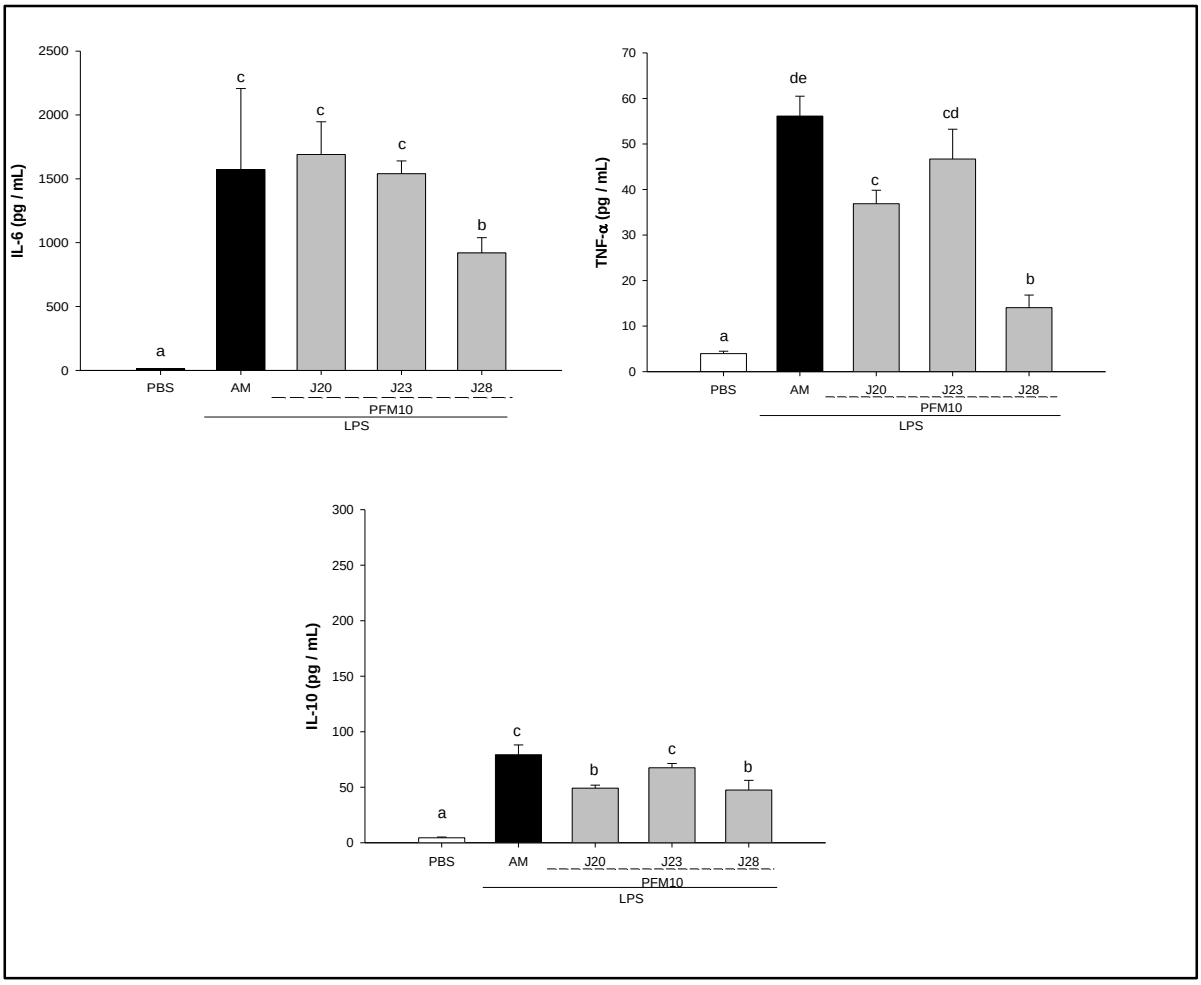

Figure 8. Serum concentration of IL-6, TNF-α and IL-10 determined by ELISA in Wistar rat (n=6) daily administrated for 4 weeks with <10 KDa fraction of pasteurized fermented milk (PFM10) by strains of *Lactobacillus* J20, J23, J25 or J28. At day 29, rats were injected with LPS (7.5 mg/kg). Six hours after injection, rats were sacrificed and blood samples were taken. In addition, phosphate-buffered saline (PBS) and acidified milk (AM) groups were included. Columns represent means ± SE. Different letters indicate significant differences ($p < 0.05$) between groups.

Table 1. Identification of peptides in < 10 KDa fractions of milk fermented by specific *Lactobacillus fermentum* strains.

Sample[1]	Experimental mass	Theorical mass	Molecular Ion (m/z) selected for MS/MS[2] (charge)	Protein fragment	Secuence
J20					
F1	678.2127	677.3385	679.2 (+1)	Lactotransferrin (f209-215)	AGDVAFV
	1025.5507	1024.6029	342.9 (+3)	Lactotransferrin (f427-435)	VPVLAENRK
	1062.4654	1062.5206	532.2 (+2)	Lactotransferrin (f582-589)	NLNREDFR
	743.0782	743.3385	248.7 (+3)	Serotransferrin (f506-513)	GCAPGSPR
F2	2150.9927	2152.1602	2152.0 (+1)	β-CN (f93-112)	TQTPVVVPPFLQPEVMGVSK
	829.7250	830.4398	415.9 (+2)	α_{S2}-CN (f124-129)	WDQVKR
	875.2962	875.4537	438.7 (+2)	β-Lg (f80-87)	GECAQKKI
	978.3448	978.5023	490.0 (+2)	β-Lg (f147-154)	EALEKFDK
	877.2025	877.4657	293.4 (+3)	Lactotransferrin (f321-328)	DSALGFLR
	1343.2166	1343.6544	448.7 (+3)	Lactotransferrin (f364-375)	VVWCAVGPEEQK
	1343.5166	1343.6544	448.8 (+3)	Lactotransferrin (f364-375)	VVWCAVGPEEQK
	1355.0333	1354.7029	1356.0 (+1)	Lactotransferrin (f438-449)	KHSSLDCVLRPT
	920.3655	919.4876	461.2 (+2)	α_{S2}-CN (f172-178)	EKNRLNF
F3	1277.6482	1277.6690	426.9 (+3)	Serotransferrin (f48-59)	ILESGPFVSCVK
	740.7333	740.3857	371.4 (+2)	β-CN (f77-83)	FPGPIPN
	1179.8125	1178.6336	1180.8 (+1)	α_{S1}-CN (43-52)	FPEVFGKEKV
	800.5854	801.4960	401.3 (+2)	β-Lg (f87-93)	IIAEKTK
	2075.2257	2075.1125	2076.2 (+1)	α-La (f2-20)	MSFVSLLLVGILFHATQAE
	2075.6397	2074.8898	2076.6 (+1)	α-La (f95-112)	SCDKFLDDDLTDDIMCVK
	2074.0397	2074.8898	2075.0 (+1)	α-La (f95-112)	SCDKFLDDDLTDDIMCVK
F4	1129.6703	1128.5638	377.6 (+3)	Lactotransferrin (f167-177)	AVAKFFSASCV
	1150.0854	1150.6863	576.1 (+2)	β-CN (f214-224)	GPVRGPFPIIV
	874.2993	874.5501	438.2 (+2)	α_{S1}-CN (16-22)	RPKHPIK
F5	3949.5839	3948.9499	1975.8 (+2)	β-Lg (f107-140)	KVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVR
	2074.8397	2074.8898	2075.8 (+1)	α-La (f95-112)	SCDKFLDDDLTDDIMCVK
	2074.3397	2073.9058	2075.3 (+1)	α-La (f95-112)	SCDKFLDDDLTDDIMCVK
J28					
F1	1591.2874	1590.7810	531,4 (+3)	α-La (f100-113)	LDDDLTDDIMCVKK
	2028.5295	2028.0892	2029.5 (+1)	β-Lg (f48-65)	LDAQSAPLRVYVEELKPT
F2	1877.7400	1878.8694	1878.7 (+1)	α_{S1}-CN (50-66)	EKVNELSKDIGSESTED
	708.2083	708.3443	237.1 (+3)	α-La (f33-39)	DLKGYGG
F3	1029.8579	1030.6539	344.3 (+3)	β-Lg (f91-99)	KTKIPAVFK
	729.2214	729.4385	244.1 (+3)	Lactotransferrin (f67-73)	AIAEKKA
	3409.0424	3409.7340	1705.5 (+2)	Lactotransferrin (f73-104)	ADAVTLDGGMVFEAGRDPYKLRPVAAEIYGTK
	2036.3834	2036.8710	2037.4 (+1)	Lactotransferrin (f564-581)	NDTVWENTNGESTADWAK
	810.6544	811.4075	407.3 (+2)	Serotransferrin (f668-674)	AKKTYDS
	831.4441	832.4226	832.4 (+1)	Serotransferrin (f683-689)	AMTNLRQ
F4	967.9454	968.4716	484.9 (+2)	κ-CN (f55-62)	RYPSYGLN
	949.2333	949.5961	317.4 (+3)	β-CN (185-193)	VLPVPQKAV
	2236.1196	2237.1739	2237.1 (+1)	Lactotransferrin (f628-647)	QVLLHQQALFGKNGKNCPDK
F5	916.0054	916.4550	459.0 (+2)	β-Lg (f117-123)	KYLLFCM
	1133.6193	1133.5023	284.4 (+4)	Lactotransferrin (f523-533)	LCAGDDQGLDK

[1] Fractions collected from milk fermented with *L. fermentum* J20 and J28. [2]MS/MS: tandem mass spectrometry.

CAPÍTULO IV

Regulation of lipopolysaccharide-induced serum cytokines in a murine model by milk fermented with specific *Lactococcus lactis* strains

Regulation of lipopolysaccharide-induced serum cytokines in a murine model by milk fermented with specific *Lactococcus lactis* strains

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ABSTRACT

The potential immunomodulatory effect of milk fermented by two specific strains of *Lactococcus lactis* NRRL B-50 571 or NRRL B-50 572 was evaluated on serum cytokines levels (IL1-β, lL-6, TNF-α, and lL-10) in a LPS-stimulated murine model. Three treatments were evaluated: fermented milk (FM), pasteurized fermented milk (PFM) and <10 KDa fraction of PFM (PFM10). Results showed the regulation of the production of these cytokines. The FM treatment with NRRL B-50 571 decreased TNF-α serum levels, the FM treatment with NRRL B-50 572 increased IL-10 serum levels and the PFM and PFM10 treatments with NRRL B-50 571 or NRRL B-50 572, respectively decreased IL-6 serum levels at 6 h after LPS-induction. Cytokine regulation by the administration of fermented milk could be related to the presence of peptides derived from milk proteins. Mass spectrometric analysis of identified peptides revealed that they had a molecular weight < 4 KDa and were mainly derived from whey proteins. Several of these peptides presented arginine at the extremes that may be recognized by receptors in immune cells. Additionally, several positively charged peptides were identified which could bind to the negatively charged LPS molecule thus reducing its toxic effect. In conclusion, fermented milk by these specific strains of *Lactococcus lactis* present potential as novel functional foods with immunomodulatory effect for the prevention of LPS-induced systemic disorders.

Keywords**:** fermented milk, cytokines, *Lactococcus lactis*, lipopolysaccharide.

INTRODUCTION

Lactic acid bacteria (LAB) from both plant and animal origin, play an important role in food processes. They are gram positive bacteria that include Lactococci, streptococci, and lactobacilli, which have long been used as starters for food fermentation, mainly in dairy and meat products (D'Souza et al., 2012). Therefore, they are among the most studied microorganisms in food research. Fermented dairy products with LAB usually contain bioactive components that have shown several benefits for human health (Griffiths & Tellez, 2013). Most of these benefits have been displayed by different species of LAB, mainly by *Lactobacillus* species that have been widely studied for their probiotic properties and bioactive peptide production (Widyastuti et al., 2014).

It is generally recognized that milk proteins are some of the best sources of biologically active peptides (Agyei et al., 2016). These peptides, encrypted in food proteins show different physiological effects; such as satiety, cardiovascular function, gut-brain axis, microbial inhibition and others (Korhonen, 2009; Pessione & Cirrincione, 2016).

Among the physiological effects, immunomodulation is one of the most interesting effects exhibited by bioactive peptides. Immunomodulation has been reported mainly for strains of *Lactobacillus* and *Bifidobacterium,* which are effective at enhancing innate and adaptive immunity. In addition, specific strains of these genera have shown to prevent gastric mucosal lesion development, alleviate allergies and put up defense against intestinal pathogen infection (Yueh-Ting et al., 2012). Specific strains of LAB may enhance immune responses, such as T-cell proliferation (Marin, 1998), antibody production (Perdigon, 1990) and regulation in pro- and anti-inflammatory cytokines production (Mozzi et al., 2015).

Thus, since an effective way to enhance the immune response is by the action of immunomodulatory peptides, it is feasible to increase their production in dairy products by milk fermentation with strains of highly proteolytic LAB. Peptides produced are dependent on specific proteolytic and peptidolytic

systems present in the bacterial cells (Hugenholtz, 2008). In fact, the immunomodulatory effect is clearly strain-dependent and modulated by growth within a fermented product (Hak-Jong et al., 2015; Foligne et al., 2016). For instance, *L. lactis*, an important component of most starter cultures used in cheese manufacture, has complex amino acid requirements. Thus, for optimal growth in milk, it relies on a sophisticated proteolytic system that includes a cell wall-associated proteinase, several membrane-located peptide transport systems, and an array of intracellular peptidases (Picon, 2010). As result of this complex proteolytic system, *L. lactis* is one of the most well-studied LAB because of its importance as part of commercial starter cultures used in the manufacture of fermented dairy products (Odamaki, 2011). However, in comparison to *Lactobacillus*, few studies have been related to the regulation of immune system including strains of *Lactococcus*.

Studies by Yang et al. (2015), have demonstrated that *L*. *lactis* induced cytokine production in the J.774.1 mouse cell line and ovomucoid-specific responses in mice (Kimoto et al., 2004). In vaccination applications, the strain *L. lactis* D1813 isolated from kuruma shrimps induced IFN-gamma production and helped to resist the infection of *Vibrio penaeicida* (Maeda et al., 2014). *L. lactis* subsp. *cremoris* A17 isolated from Taiwan fermented cabbage, induced IFN-gamma production in human peripheral blood mononuclear cells, suggesting an immunomodulatory activity toward the T-helper cell type 1 response (Hui-Ching et al., 2013).

On the other hand, *L. lactis* IL-27 significantly reduced inflammatory cytokines levels such as IL-17 and increasing the production of IL-10 in a colitis model (Hanson et al., 2014).

Previous studies in our laboratory have shown that milk fermented by specific strains of *L. lactis* isolated from native ecosystems were able to reduce blood-pressure in a murine model (Rodríguez-Figueroa et al., 2013). Since beneficial effects were shown for these fermented milk, it is interesting to explore another important bioactivities, such as the regulation of cytokine

production, directed to an anti-inflammatory response. Therefore, the objective of the present study was to evaluate the anti-inflammatory potential of milk fermented by specific strains of *L. lactis* on the regulation of LPS-induced cytokines in a murine model.

MATERIALS AND METHODS

Substrates and chemicals

M17 Broth was obtained from Difco (Sparks, MD, USA). Nonfat dry milk was obtained from Dairy America (Fresno, CA, USA) and *E. coli* O11: B4-LPS from Sigma Chemical Co. (St. Louis, MO, USA).

Strains and growth conditions

The strains of *L. lactis* (NRRL B-50 571 and NRRL B-50 572) were obtained from the Dairy Laboratory collection at the Food Research and Development Center, A.C. (CIAD, A.C., Hermosillo, Sonora, Mexico). These strains were deposited in the Agricultural Research Service Culture Collection (NRRL, Peoria, IL) of the US Department of Agriculture and were selected for showing a high proteolytic activity in milk (Rodríguez-Figueroa et al, 2010). The strains of *L. lactis* maintained in glycerol at -80°C were reactivated by inoculation into sterile M17 broth supplemented at 10 % (vol/vol) with a sterile solution of lactose (10 % wt/vol). Three consecutive subcultures were prepared using 1% inoculum and incubation conditions of 24, 18 and 10 h, respectively, at 30 °C in order to obtain fresh cultures. The initial average population of inoculum was obtained from the 10 h fresh culture, reaching 10^8 cfu/mL as enumerated on M17 agar.

Preparation of fermented milk

Reconstituted nonfat dry milk (0.1 g/mL) was pasteurized at 110 °C for 10 min, immediately cooled at 4 °C and stored overnight at the same temperature. Fresh cultures of each strain were used to inoculate the reconstituted milk at 1% level and at 30 °C, which were incubated for 10 h at the same temperature and

later they were used as starter culture. For the production of fermented milk (FM treatments), an aliquot (3%) of the starter cultures were used to inoculate the reconstituted milk. Then, inoculated milk was incubated for 48 h at 37 °C. pH measurements were directly taken in samples of fermented milk using a pH meter (Orion 4 Star, Singapore). Additionally, titratable acidity was monitored during fermentation. Pasteurized fermented milk (PFM treatments) were prepared by heating fermented milk at 75 °C for 20 min and immediately placed them into the ice bath. Finally, in order to obtain <10 KDa fractions of PFM (PFM10 treatments), PFM was centrifuged at 4500 rpm (J2-21 rotor, Beckman, USA) for 40 min at 4 °C, supernatants were collected and ultra-filtered through 10 KDa cut-off membranes (Pall life Sciences, USA) in an ultrafiltration unit (Millipore Amicon 8050, USA). Permeates were collected, frozen at -80°C and lyophilized with a freeze dryer (Labconco, USA). Samples were storage until peptide sequence was identified by HPLC mass spectrometry analysis.

Assay in a murine model

This study was approved by the ethical committee of the Food Research and Development Center, A.C. and was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Mexican Official Regulations (Aluja, 2000) and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources (NRC, 2011). All sacrifices were performed under chloroform anesthesia, and efforts to minimize animal suffering were made during the study.

Male Wistar rats (Bioinvert & Aprexbio SA de CV, CDMEX), 5-6 weeks old weighing 110-160 g were used in this study. Rats were given rat chow (Standard Rodent Laboratory-Chow 5001 Diet, Purina Feeds, Inc., St. Louis, MO, USA) and purified water *ad libitum*. Animals were kept in a sanitized bioterium, maintained with a relative humidity between 40 to 60%. The temperature was kept at 22 ± 2°C, with 12 h light/dark cycles starting at 06:30 a.m. Rats were housed in sanitized polycarbonate cages (60 x 40 x 30 cm) with

sterile sawdust bedding that was replaced daily. Cages were arranged on racks and their hygiene was continuously monitored.

The animals were adapted (first week) and randomly assigned in pairs to eight experimental groups (n = 6). The bioassay included the following groups: FM (fermented milk), PFM (pasteurized fermented milk) and PFM10 (fraction < 10 KDa from PFM) all of them obtained with the strains *L. lactis* NRRL B-50 571 or NRRL B-50 572. Moreover, LPS and BASAL control groups (with or without LPS stimulation respectively) were included, both groups were not feeded with any treatment.

Treatments lasted 4 weeks during which animals were daily gavaged with 1 mL of samples. After 28 days, animals were subcutaneously injected with LPS (7.5 mg/kg diluted in milliQ water) to induce a systemic inflammatory process. Finally rats were sacrificed at 6 h post-stimulation. Blood samples were taken before and after LPS stimulation. Blood samples were centrifuged at 2500 rpm for 8 min. Serum was collected, and kept at -20 °C until cytokine analyses were performed (Figure 1).

Cytokine Determinations

Serum cytokine concentrations were determined by the ELISA method (Enzyme-Linked Immunosorbent Assay) using commercially available kits for IL-1β, IL-6, TNF-α and IL-10 Rat (R&D Systems, Inc., USA). These tests comprised recombinant cytokines from *E. coli* and antibodies against rat cytokines. The results were calculated based on the absorbance of complex cytokines-antibodies. Cytokine concentrations were obtained from calibration curves with a 5 pg/mL for TNF-α or IL-1β, 21 pg/mL for IL-6 and 10 pg/mL for IL-10 minimum detection limit.

Analysis of peptides by Tandem Mass Spectrometry

Mass spectrometry (MS) 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 nanocolumn was a C18-300

(150 mm × 0.75 µm, 3.5 µm; Agilent Technologies Inc.) The sample injection volume was 1 µL. Solvent A was a mixture of water-acetonitrile-formic acid (10:90:0.1, vol/vol/vol) and solvent B contained water-acetonitrile-formic acid (97:3:0.1, vol/vol/vol). The gradient was based on the increment of solvent B, which was initially set at 3% for 10 min and it took 23 more min to reach 65%. The 0.7 µL/min flow rate was directed into the mass spectrometer via an electrospray interface. Nitrogen (99.99%) was used as the nebulizing and drying gas and operated with an estimated helium pressure of 5×10^2 Pa. The needle voltage was set at 4 kV. Mass spectra were acquired over a range of 300 to 2,500 mass/charge (m/z). The signal threshold to perform auto MS analyses was 30,000. The precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage ramp from 0.35 to 1.1 V. Peptide sequences were obtained from mass spectrometry data using the Mascot server (Perkins et al., 1999) through the UniProtKB/Swiss-Prot database (http://www.matrixscience.com/help/seq_db_setup_Sprot.html) sequences. Net charges of peptides in this study were calculated using the informatic tools ProtParam and SAPS (Gasteiger et al., 2005) in the server Expasy (http://www.expasy.org)

Statistical analysis

For statistical analysis, data normality was tested as a prerequisite before one way analysis of variance (ANOVA) was carried out to compare groups. Differences among means were assessed by Fisher's least significant difference multiple comparison test and considered significant when P≤0·05. Data analyses were performed by using the NCSS 2007 statistical program (Hintze, 2007). Data are expressed as means ± S.E.M. of six rats in each group.

Results and discussions

Cytokine analysis in an LPS-stimulated murine model

Cytokines showed a similar behavior in the different animal groups previous to induction with LPS. Cytokine levels in basal group were below detection limit and some of the other groups showed a significant slight increase. However, these levels were much lower when compared with the levels of cytokines in the same groups after LPS induction. These increases were attributable to external stressors, such as the effect of oral gavage.

On the other hand; as expected, the levels for all cytokines in all groups were increased after induction with LPS. IL-1β serum levels were tested; however, although PFM treatment obtained from milk fermented by *L. lactis* NRRL B-50 571 was able to reduce IL-1β levels compared with LPS group, differences were not significant (Figure 2).

A marked increase was observed for IL-6 levels in all groups after stimulation with LPS. Both PFM10 treatment obtained from milk fermented by *L. lactis* NRRL B-50 572 and PFM treatment obtained from milk fermented by *L. lactis* NRRL B-50 571 inhibited IL-6 production (Figure 3).

Additionally, FM treatment obtained from milk fermented by *L. lactis* NRRL B-50 571 after LPS-stimulation significantly decreased TNF-α serum levels, compared with LPS group (Figure 4).

Moreover, levels of IL-10 showed a significant increase for FM treatment obtained from milk fermented by *L. lactis* NRRL B-50 572 (Figure 5).

It is known that LPS-induced pro- and anti-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and IL-10, play a key role in the process of inflammatory diseases. The excessive production of pro-inflammatory cytokines may result in a systemic inflammatory response. Hence, inhibition of these inflammatory mediators has become to be an important area of investigation for the regulation of these systemic disorders. In this study the regulation of pro- and anti-inflammatory cytokines was observed with fermented milk by *L. lactis* strains, with or without pasteurization and their fractions (<10 KDa).

Identification of peptides in milk fermented by *L. lactis* with regulatory effect on cytokine production

PFM and PFM10 treatments obtained from milk fermented by *L. lactis* with NRRL B-50 571 or NRRL B-50 572, respectively decreased IL-6 serum levels at 6 h after LPS-induction, this effect could be associated to the presence of peptides present in this fermented milk. These peptides were identified by tandem mass spectrometry (Table 1).

Twenty four and fourteen peptide sequences derived mostly from whey proteins were identified in milk fermented by *L. lactis* NRRL B-50 572 and NRRL B-50 571, respectively. These structures had a length ranging from 7 to 41 amino acid and a molecular weight < 3 KDa. Only the sequences serotransferrin (f172-212) derived from milk fermented by NRRL B-50 572 and β-Lg (f35-72) derived from milk fermented by NRRL B-50 572 had a molecular weight of 4 KDa.

Arginine (Arg) in the extreme of bioactive peptides is a dominant entity recognized by receptors on macrophages and lymphocytes, which enhances their maturation and proliferation (Meisel & FitzGerald, 2003; Haque & Chand, 2008). In this study, four sequences identified in treatments with the strain *L. lactis* NRRL B-50 572: NLNREDFR, LGAPITCVRR, TLDGMVFEAGR and NERYYGYTGAFR, and two sequences identified in treatments with the strain *L. lactis* NRRL B-50 571: ACQCLVR and YLGYLEQLLR had the amino acid Arg in the N-terminal and C-terminal positions. These sequences could be recognized by receptors in immune cells and in consequence they could show an immunomodulatory effect. Furthermore, the type of amino acid in the N-terminal and C-terminal position of the peptides may be related to the action of specific peptidases and proteinases. Data in table 1 showed the action of mainly serine proteases, specifically trypsin, which cleaves the peptide chains mainly at the carboxyl side of the amino acids lysine or arginine (Rodriguez et al., 2008).

Identified peptide sequences presented a net charge ranging from -5 to +2 at physiological pH. These peptides were identified as anionic (29 and 50 %),

neutral (38 and 21 %) and cationic (33 and 29 %), when derived from milk fermented by *L. lactis* NRRL B-50 571 and NRRL B-50 572, respectively.

It has been hypothesized that proteins and peptides with an exposed positively charged domain could interact with the negatively charged phosphoryl groups of LPS, thus promoting effective binding via electrostatic forces. Also, hydrophobic interactions involving the fatty acid residues of lipid A and hydrophobic amino acids have also been postulated to participate in the mechanism of LPS binding (Martínez-Sernández et al., 2016). In this sense, some examples of cationic molecules with proven LPS-binding ability that can reduce LPS activity have been reported. Hydrolysates of casein glycomacropeptide may form a complex with lipid A of LPS and partially inhibit the LPS-induced release of pro-inflammatory mediators by macrophages (Cheng et al., 2015). Likewise, hydrolysates of whey proteins reduced LPS binding to surface TLR4, with a decrease on IL-8 secretion induced by TNF-α or IL-1β on respiratory epithelial cell lines (Iskandar et al., 2013). The peptide cationic lactoferricin derived from lactoferrin neutralized the effect of LPS-induced toxicity by binding to LPS. Synthetic peptides derived from human and bovine lactoferricin have shown interaction with LPS (Sinha et al., 2013).

In the present study, twelve identified sequences could have the potential to bind LPS, since these were positively charged. These sequences were: FALPQYLK, LGAPITCVRR, AQEKFGK, NERYYGYTGAFR, KVLILAC, GPVRGPFPIIV, HLSFMAIPPK, CLLLALALT, ACQCLVR, GALGLCLAAPRK, KIDALNENKVLVLDTDYK and FFSDKIAK.

Although the literature have reported the immunomodulatory effect of peptides mainly derived from caseins (Reyes-Díaz et al., 2016), a large number of peptides derived from whey proteins in milk fermented by *L. lactis* were identified in this study. These results highlight the possibility of finding *de novo* sequences with immunomodulatory activity. However, more studies are needed in order to identify the role of these peptides on the observed effect.

Conclusions

In this study, the immunomodulatory effect of milk fermented by specific strains of *L. lactis* on serum cytokines levels was demonstrated. FM treatment with NRRL B-50 571 decreased TNF-α serum levels and FM treatment with NRRL B-50 572 increased IL-10 serum levels at 6 h after LPS-induction. Moreover, PFM and PFM10 treatments with NRRL B-50 571 or NRRL B-50 572, respectively, decreased IL-6 serum levels at 6 h after LPS-induction. These results suggested an inhibitory effect on pro-inflammatory cytokine IL-6 production, attributable to bioactive components present in the fermented milk. Since the effect was present not only in pasteurized fermented milk but also in fractions <10 KDa, it may be associated to the presence of peptides derived from milk proteins. Several of the identified peptides could possibly bind to the negatively charge LPS molecule, since they presented a positive net charge. However, further studies need to be carried out in order to determine these interactions.

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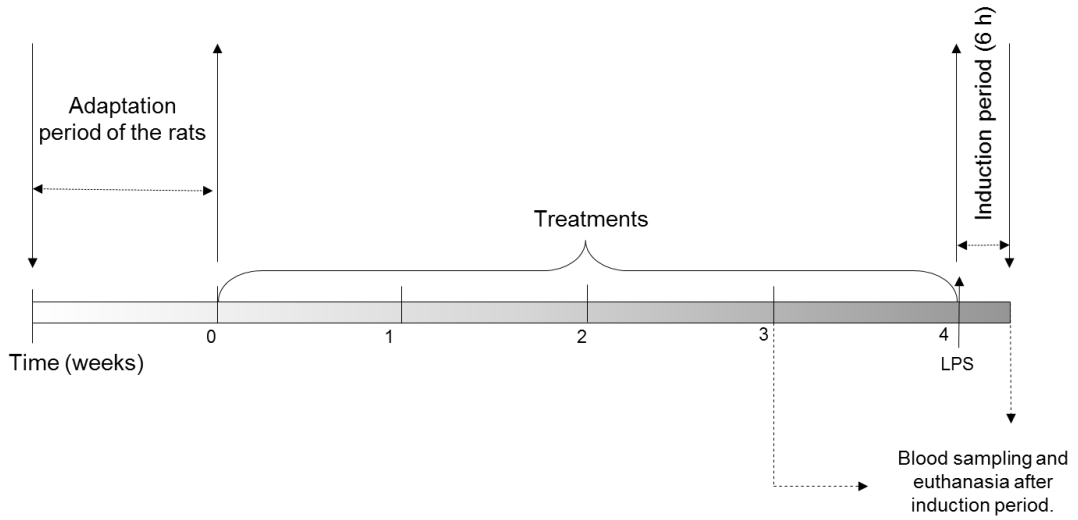

Figure 1. Experimental design for bioassay in a LPS-stimulated murine model.

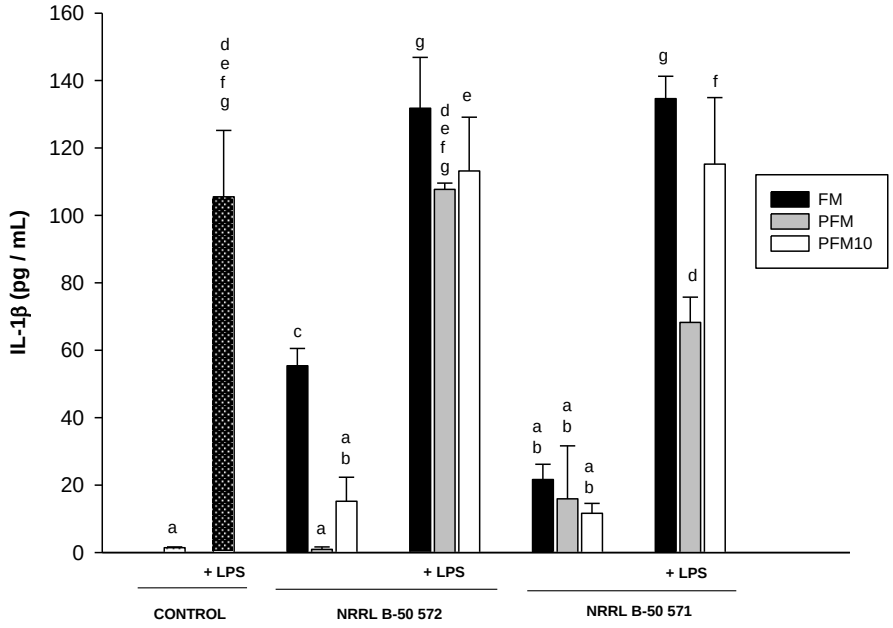

Figure 2. Serum concentration of IL-1β determined by ELISA in Wistar rat (n=6) daily administrated for 4 weeks with fermented milk (FM), pasteurized fermented milk (PFM) or <10 KDa fraction of PFM (PFM10) by *L. lactis* (NRRL B-50 572 and NRRL B-50 571). After 28 days of treatment, rats were injected with LPS (7.5 mg/kg) and sacrificed six hours after injection. Blood samples were taken before and after induction with LPS. BASAL: without treatment, LPS: lipopolysaccharide. Columns represent means ± SE. Different letters indicate significant differences ($p < 0.05$) between groups.

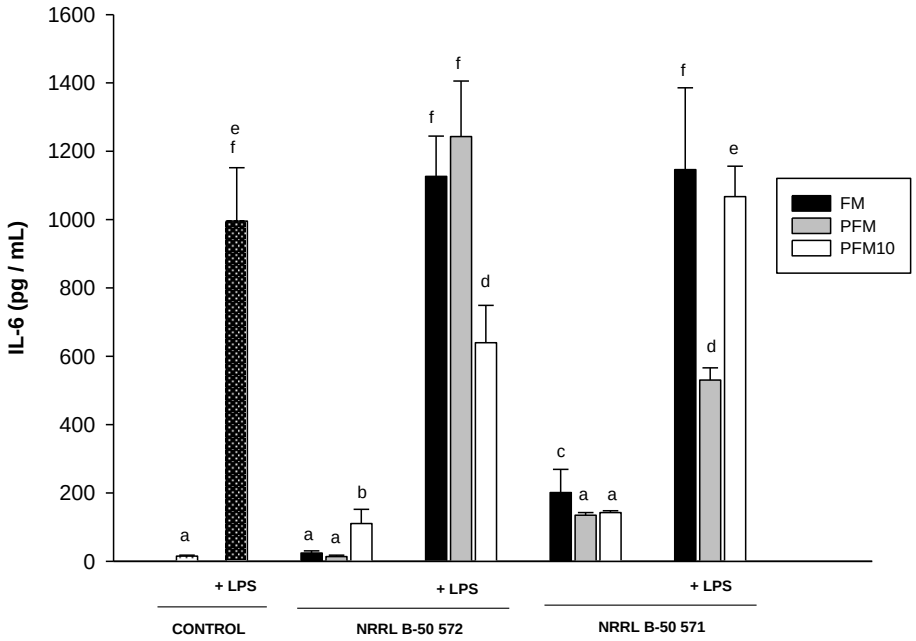

Figure 3. Serum concentration of IL-6 determined by ELISA in Wistar rat (n=6) daily administrated for 4 weeks with fermented milk (FM), pasteurized fermented milk (PFM) or <10 KDa fraction of PFM (PFM10) by *L. lactis* (NRRL B-50 572 and NRRL B-50 571). After 28 days of treatment, rats were injected with LPS (7.5 mg/kg) and sacrificed six hours after injection. Blood samples were taken before and after induction with LPS. BASAL: without treatment, LPS: lipopolysaccharide. Columns represent means ± SE. Different letters indicate significant differences ($p < 0.05$) between groups.

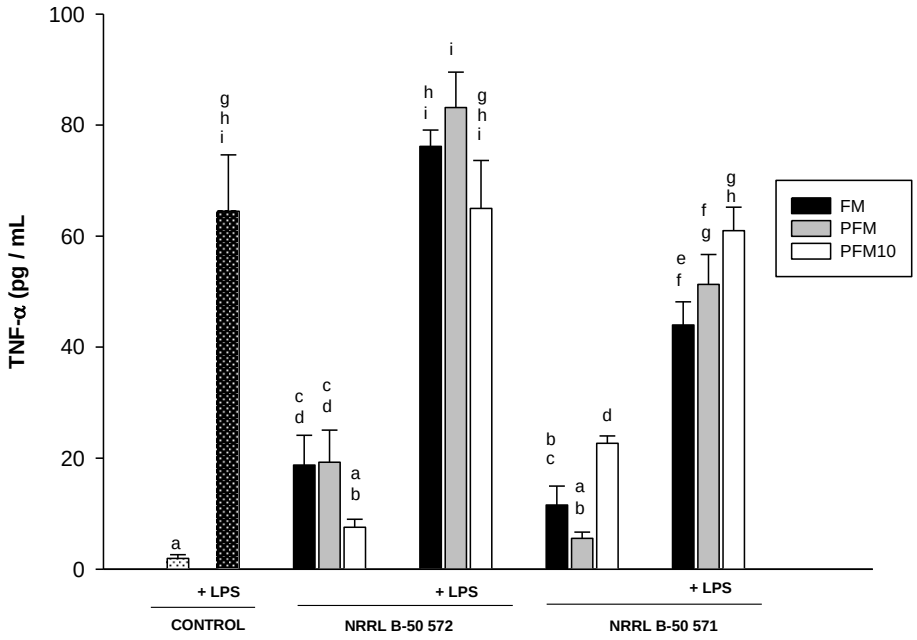

Figure 4. Serum concentration of TNF-α determined by ELISA in Wistar rat (n=6) daily administrated for 4 weeks with fermented milk (FM), pasteurized fermented milk (PFM) or <10 KDa fraction of PFM (PFM10) by *L. lactis* (NRRL B-50 572 and NRRL B-50 571). After 28 days of treatment, rats were injected with LPS (7.5 mg/kg) and sacrificed six hours after injection. Blood samples were taken before and after induction with LPS. BASAL: without treatment, LPS: lipopolysaccharide. Columns represent means ± SE. Different letters indicate significant differences ($p < 0.05$) between groups.

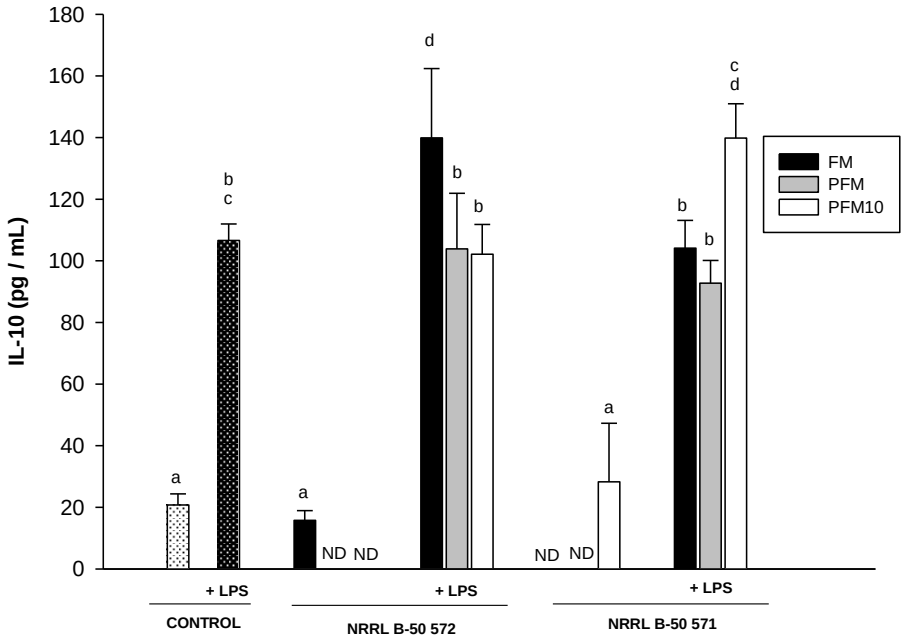

Figure 5. Serum concentration of IL-10 determined by ELISA in Wistar rat (n=6) daily administrated for 4 weeks with fermented milk (FM), pasteurized fermented milk (PFM) or <10 KDa fraction of PFM (PFM10) by *L. lactis* (NRRL B-50 572 and NRRL B-50 571). After 28 days of treatment, rats were injected with LPS (7.5 mg/kg) and sacrificed six hours after injection. Blood samples were taken before and after induction with LPS. BASAL: without treatment, LPS: lipopolysaccharide, ND: not detectable. Columns represent means ± SE. Different letters indicate significant differences ($p < 0.05$) between groups.

Table 1. Peptides identified in fractions (<10 KDa) derived from milk fermented by *Lactococcus lactis* NRRL B-50 572 and NRRL B-50 571.

Sample[1]	Experimental mass	Theorical mass	Molecular Ion (m/z) selected for MS/MS[2] (charge)	Protein fragment	Sequence
NRRL B-50 572	978.4975	978.5539	979.5 (+1)	α_{S2}-CN (f189-196)	FALPQYLK
F1	1852.1958	1851.9511	927.1 (+2)	α_{S1}-CN (f124-139)	LEIVPNSAEERLHSMK
	1006.9452	1007.4788	504.5 (+2)	β-CN (f123-130)	EMPFPKYP
	4385.9473	4384.8971	1461.9 (+3)	Serotransferrin (f172-212)	FSASCVPCADQSSFPKLCQLCAGKGTDKCACSNHEPYFGYS
F2	1063.5454	1062.5206	532.8 (+2)	Lactotransferrin (f582-589)	NLNREDFR
	1062.9254	1062.5206	532.5 (+2)	Lactotransferrin (f582-589)	NLNREDFR
	1062.6254	1062.5206	532.3 (+2)	Lactotransferrin (f582-589)	NLNREDFR
	1171.9770	1171.6495	1173.0 (+1)	Lactotransferrin (f48-58)	LGAPITCVRR
	1250.5927	1251.5918	1251.6 (+1)	Lactotransferrin (f77-88)	TLDGMVFEAGR
	807.6054	806.4286	404.8 (+2)	Lactotransferrin (f293-299)	AQEKFGK
	1888.2537	1889.0080	1889.3 (+1)	Serotransferrin (f43-57)	ENVLRILESGPFVSCVK
	1496.4468	1495.6843	749.2 (+2)	Serotransferrin (f537-548)	NERYYGYTGAFR
F3	759.5497	758.4724	380.8 (+2)	β-CN (2-8)	KVLILAC
F4	904.1254	904.4866	453.1 (+2)	κ-CN (f182-190)	TVQVTSTAV
	918.9854	919.4321	460.5 (+2)	Lactotransferrin (f694-702)	CSTSPLLEA
	980.1445	649.2670	341.1 (+2)	Serotransferrin (f217-223)	CLMEGAG
	975.9682	975.4331	326.3 (+3)	Serotransferrin (f595-603)	KPVTDAENC
F5	773.9101	773.3191	387.0 (+2)	α_{S2}-CN (f20-26)	EHVSSSE
	665.8777	665.2868	333.9 (+2)	α_{S2}-CN (f68-74)	SIGSSSE
	1717.2254	1716.9927	859.6 (+2)	β-CN (f209-224)	QEPVLGPVRGPFPIIV
	1149.8054	1150.6863	575.9 (+2)	β-CN (f214-224)	GPVRGPFPIIV
	925.7854	926.4538	463.9 (+2)	κ-CN (f75-81)	QFLPYPY
	1139.7709	1139.6161	570.9 (+2)	κ-CN (f123-132)	HLSFMAIPPK
	928.9916	929.5619	465.5 (+2)	β-Lg (f3-11)	CLLLALALT
NRRL B-50 571					
F1	739.2054	739.4228	370.7 (+2)	κ-CN (f64-74)	PVALINN
	4188.2692	4189.1294	2095.1 (+2)	β-Lg (f35-72)	WYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEI
	790.9582	791.3782	264.7 (+3)	β-Lg (f134-140)	ACQCLVR
	1181.3352	1180.5468	394.8 (+3)	α-La (f103-112)	DLTDDIMCVL
	974.4382	975.4331	325.8 (+3)	Serotransferrin (f595-603)	KPVTDAENC
F2	2075.5237	2074.8898	2076.5 (+1)	α-La (f95-112)	SCDKFLDDDLTDDIMCVK
F3	2075.7237	2074.8898	2076.7 (+1)	α-La (f95-112)	SCDKFLDDDLTDDIMCVK
	2074.5397	2074.8898	2075.5 (+1)	α-La (f95-112)	SCDKFLDDDLTDDIMCVK
F4	1168.0751	1168.6750	390.4 (+3)	Lactotransferrin (f12-23)	GALGLCLAAPRK
	1266.0382	1266.6972	423.0 (+3)	α_{S1}-CN (106-115)	YLGYLEQLLR
	664.4377	665.2868	333.2 (+2)	α_{S2}-CN (f68-74)	SIGSSSE
	2090.9768	2090.1260	2091.9 (+1)	β-Lg (f99-116)	KIDALNENKVLVLDTDYK
F5	1265.8615	1266.5511	1266.9 (+1)	Lactotransferrin (f383-395)	QSGQNVTCATAST
	954.1854	954.5175	478.1 (+2)	κ-CN (f38-45)	FFSDKIAK

[1] Fractions collected from milk fermented with *L. lactis* NRRL B-50 572 and NRRL B-50 571. [2]MS/MS: tandem mass spectrometry