Centro de Investigación en Alimentación y Desarrollo, AC.

EVALUACIÓN DE LA BIODISPONIBILIDAD DE PÉPTIDOS INHIBIDORES DE LA ENZIMA CONVERTIDORA DE ANGIOTENSINA DERIVADOS DE LECHE FERMENTADA CON *Lactococcus lactis* NRRL B-50571 Y SU EFECTO PROTECTOR EN UN MODELO MURINO CON HIPERTENSIÓN INDUCIDA

Por

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

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RESUMEN

El efecto antihipertensivo de leche fermentada por *Lactococcus lactis* NRRL B-50571 (FM-571) ha sido reportado en ratas espontáneamente hipertensas y el efecto fue atribuido a péptidos bioactivos. Sin embargo, aún se desconoce la bioaccesibilidad y biodisponibilidad de los péptidos responsables de este efecto, así como el efecto protector de FM-571 como tratamiento preventivo en el desarrollo de la hipertensión. Por lo tanto, el objetivo fue evaluar la bioaccesibilidad y biodisponibilidad de péptidos bioactivos derivados de FM-571 y el efecto protector en un modelo murino con hipertensión inducida con angiotensina II. Se obtuvieron fracciones peptídicas con actividad inhibidora de enzima convertidora de angiotensina (ECA) de FM-571 después de ser sometida a digestión gastrointestinal simulada (DGS) y a un modelo de absorción *ex vivo*. Los péptidos absorbidos en el compartimento serosal fueron identificados mediante HPLC/MS-MS; posteriormente, se determinó cuáles secuencias podrían presentar unión a la ECA mediante un análisis *in silico*. Los resultados mostraron que el IC_{50} para la actividad inhibidora de la ECA disminuyó ($p<0.05$) después de DGIS y de la absorción. Además, la bioaccesibilidad incrementó ($p<0.05$) a 58% después de DGIS mientras que la abundancia de péptidos disminuyó después de la absorción resultando en una biodisponibilidad de 1.36 ± 0.1%. Se identificaron 113 nuevos péptidos de los cuales 13 se reportaron previamente con inhibición de ECA. Adicionalmente, 14 péptidos presentaron alto potencial de unión al sitio activo. Por lo tanto, los resultados sugieren que nuevos péptidos derivados de FM-571 con potencial antihipertensivo fueron biodisponibles para ejercer su efecto. Respecto al efecto protector, los resultados mostraron que la presión sanguínea sistólica y diastólica no fueron significativamente ($p>0.05$) diferente entre los grupos en las primeras dos semanas de administración del tratamiento. Sin embargo, después de la inducción de hipertensión, la presión sanguínea sistólica y diastólica en los grupos FM-571 y Captopril fueron significativamente ($p<0.05$) más bajos que en el grupo control negativo (administrado con Ang II + agua). Las actividades ECA, Glutatión Peroxidasa y Peroxidación Lipídica (malondialdehído) en plasma no fueron significativamente diferentes ($p>0.05$) entre los grupos. Sin embargo, la actividad de Catalasa fue significativamente más alta ($p<0.05$) en el grupo FM-571. Adicionalmente, el índice de estrés oxidativo fue significativamente más bajo ($p<0.05$) en el grupo FM-571. Los resultados sugieren que FM-571 puede ser usada como tratamiento preventivo para

el desarrollo de hipertensión mediante el mejoramiento del sistema de defensa antioxidante primario y disminución del índice de estrés oxidativo.

Palabras clave: digestión gastrointestinal simulada, intestino invertido, leche fermentada, biodisponibilidad, tratamiento preventivo, hipertensión inducida

ABSTRACT

The antihypertensive effect of fermented milk by *Lactococcus lactis* NRRL B-50571 (FM-571) has been previously reported in spontaneously hypertensive rats and the effect was attributed to bioactive peptides. However, the bioaccesibility and bioavailability of the responsible peptides of this effect, as well as the protective effect of FM-571 in an induced hypertensive model has not yet been reported. Therefore, the aim of the present study was to evaluate the bioaccessibility and bioavailability of bioactive peptides derived from FM-571 as well as the protective effect of FM-571 as a preventive treatment in an induced hypertension model with angiotensin II. Peptide fractions with angiotensin-converting enzyme (ACE) inhibitory activity were obtained from FM-571 after being subjected to simulated gastrointestinal digestion (SGD) and an *ex vivo* absorption model. HPLC/MS-MS identified the absorbed peptides into serosal compartment; subsequently, their binding potential to the active sites of ACE was determined by *in silico* analysis. Results showed that the IC_{50} for ACE inhibitory activity decreased ($p<0.05$) after SGD (1259.55 μg/mL) and absorption ($p<0.05$) (10.02 μg/mL). Moreover, the bioaccessibility increased to 58% after SGD, whereas peptide abundance ($p<0.05$) decreased after absorption resulting in a peptide bioavailability of 1.36 ± 0.1%. Moreover, 113 novel peptides were identified from which 13 were previously reported as ACE inhibitors and 100 novel potential ACE inhibitory peptides were identified. Additionally, 14 peptides showed high binding potential to the active sites of ACE. Henceforth, these results suggest that novel peptides derived from FM-571 with antihypertensive potential were bioavailable to exert their effect. Additionally, results showed that systolic and diastolic blood pressure were not significantly ($p>0.05$) different among groups during the first two weeks of run-in periods of treatment administration. However, after hypertension induction with angiotensin II, SBP and DBP in the FM-571 and Captopril groups were significantly ($p<0.05$) lower than in the negative control group (water). ACE activity, glutathione peroxidase and lipid peroxidation (malondialdehyde) in plasma were not significantly different ($p>0.05$) among groups. Nevertheless, catalase activity was significantly higher ($p<0.05$) in the FM-571 group. Moreover, the oxidative stress index was significantly lower ($p<0.05$) in the FM-571 group. Altogether, these results support the fact that FM-571 may be used as a preventive treatment for the development of hypertension by enhancing the primary antioxidant defense system and decreasing the oxidative

stress index.

Key words: Simulated gastrointestinal digestion, everted gut, fermented milk, bioavailability, preventive treatment, induced hypertension

CONTENIDO

1. SINOPSIS

1.1. Justificación

Las enfermedades cardiovasculares son la primera causa de muerte en el mundo y la principal preocupación de las autoridades de salud en los países desarrollados, siendo la hipertensión el principal factor de riesgo (Zhang et al., 2021). Las primeras recomendaciones para disminuir estas enfermedades son cambios de estilo de vida, en específico, los factores modificables tales como los patrones dietarios y el sedentarismo (Gallego, Mora y Toldrá, 2018). Adicionalmente, diferentes fármacos para el tratamiento de la hipertensión arterial son recomendados; no obstante, estos tienen algunos efectos secundarios. En este sentido, mediante la búsqueda de nuevas alternativas más saludables para el manejo y control de la hipertensión, se ha evidenciado que los alimentos funcionales no solo proveen nutrientes al organismo, sino también tienen el potencial de coadyuvar como tratamiento no farmacológico en el control de factores de riesgo de enfermedades cardiovasculares (Brown et al., 2018; Karami y Adergani, 2019), como tal es el caso de las leches fermentadas.

La evidencia científica sugiere que los péptidos con efecto antihipertensivo derivados de leche fermentada son secuencias de 3 a 12 residuos, poseen aminoácidos hidrofóbicos en la posición terminal C y prolina que confieren resistencia a la hidrólisis. Por otro lado, durante la digestión gastrointestinal tiene lugar el mayor grado de hidrólisis de las proteínas por acción de las enzimas del tracto digestivo aumentando la bioaccesibilidad de la fracción proteica del alimento funcional (Kopf-Bolanz et al., 2012). En específico, estudios han reportado que en este proceso ocurre hidrólisis del 50% de las proteínas lácteas, y adicionalmente, el 10% de los péptidos liberados son hidrolizados a aminoácidos libres.

Posteriormente, los péptidos bioactivos pueden ser absorbidos intactos a través de las células epiteliales, estar biodisponibles y exhibir el efecto biológico; mientras que algunos oligopéptidos son hidrolizados por peptidasas de la membrana cepillo-frontera y por lo tanto, estas secuencias más cortas pueden ser absorbidas (Wang et al., 2019). Lo anterior, está en función de las características fisicoquímicas del péptido conferidas por los aminoácidos constituyentes. En

conjunto, el proceso tecnológico de fermentación, así como la digestión y absorción proveen al organismo de péptidos bioactivos con potencial para coadyuvar en el control y manejo de la hipertensión.

En nuestro grupo de investigación, estudios *in vitro* demostraron que leche fermentada por *Lactococcus lactis* NRRL B-50571 (FM-571) presentó actividad inhibidora de la enzima convertidora de angiotensina (ECA) (Rodríguez-Figueroa et al., 2010) y se identificaron las secuencias peptídicas inhibidoras de ECA después del proceso de fermentación (Rodríguez-Figueroa et al., 2012). Posteriormente, en un estudio clínico aleatorizado controlado doble ciego se demostró el efecto antihipertensivo en personas prehipertensas (Beltrán-Barrientos et al., 2018). Además, se estudió el efecto antihipertensivo en un modelo murino con ratas espontáneamente hipertensas (REH) (Rodríguez-Figueroa et al., 2013) y se elucidaron los mecanismos responsables del efecto, los cuales estuvieron asociados a la inhibición de la ECA, el efecto antioxidante y la producción de óxido nítrico (Beltrán-Barrientos et al., 2018b).

No obstante, es imprescindible evaluar la bioaccesibilidad y biodisdisponibilidad de los péptidos de FM-571 liberados después el proceso de digestión gastrointestinal y los que fueron absorbidos en el lumen intestinal que presentan potencial efecto antihipertensivo. Adicionalmente, es necesario determinar el potencial efecto protector de FM-571 ante el desarrollo de hipertensión arterial en un modelo murino con hipertensión inducida.

1.2. Antecedentes

1.2.1. Obtención de Péptidos Bioactivos

El constituyente proteico de los alimentos funcionales provee al organismo aminoácidos y secuencias de péptidos cortos, cuyas funciones como péptidos bioactivos han sido objeto de estudio en los últimos años (Korhonen, 2009). Los péptidos se encuentran inactivos en las proteínas; éstos se han definido como fragmentos específicos (2-12 aminoácidos) que tienen un impacto positivo en las funciones del cuerpo y pueden influir en la salud (Kitts y Weiler, 2003). Entre los efectos

benéficos a la salud que se atribuyen a péptidos bioactivos están las bioactividades antihipertensiva, antioxidante, antitrombótica, antimicrobiana, opioide e inmunomoduladora (Korhonen, 2009).

En el caso de la bioactividad antihipertensiva, la inhibición de ECA juega un papel fundamental, ya que cataliza la conversión de angiotensina I en angiotensina II en el sistema renina-angiotensina; en el cual la angiotensina II es un vasoconstrictor endógeno que incrementa la presión arterial. Por lo tanto, la inhibición de ECA por péptidos bioactivos puede ser un coadyuvante en la prevención, tratamiento y control de la hipertensión. Los compuestos químicos inhibidores de ECA pueden ser sintéticos (como el fármaco Captopril®) o péptidos bioactivos derivados de alimentos que se pueden obtener de alimentos proteicos mediante algunos procesos tecnológicos. A continuación, se describen los principales procesos utilizados para la obtención de péptidos bioactivos.

1.2.1.1. Hidrólisis enzimática de proteínas. Para llevar a cabo la hidrólisis enzimática es importante determinar algunos parámetros (e.g. temperatura, pH y tiempo de hidrólisis) para optimizar la liberación de péptidos a partir de la proteína nativa. Lo anterior, debido a que se relaciona con el grado de hidrólisis, lo cual influye en el tamaño y composición de aminoácidos, y por lo tanto, la bioactividad de las secuencias liberadas (Marcianik et al., 2018). Posterior a la hidrólisis, con la aplicación de técnicas de separación, analíticas e instrumentales se realiza la identificación de las secuencias peptídicas para llevar a cabo análisis más específicos.

Las enzimas más empleadas en la hidrólisis de proteínas alimentarias son la papaína, pepsina de mucosa gástrica porcina (Mohan et al., 2015), tripsina, quimotripsina y proteasas comerciales (Ngo et al., 2015). Abdel-Hamid et al. (2017) hidrolizaron leche descremada de búfala utilizando papaína, pepsina y tripsina para obtener péptidos inhibidores de ECA. El hidrolizado con papaína y tripsina, presentó la actividad inhibidora de ECA más alta ($p<0.05$; $IC_{50}<50$ μg/mL) con respecto al hidrolizado con pepsina ($IC_{50} \approx 100$μg/mL). En otro estudio, Ibrahim et al. (2017) hidrolizaron caseína y proteínas de suero de leche de cabra con pepsina para obtener péptidos inhibidores de ECA. Los autores reportaron que el péptido PEQSLACQCL derivado de β-lactoglobulina f(113-122), ARHPHPHLSFM derivado de κ-caseína f(96-106) y QSLVYPFTGPI derivado de β-caseína f(56-66) mostraron actividad inhibidora de ECA comparable al Captopril.

Corolasa PP es una proteasa comercial que posee las enzimas quimotripsina, elastasa, dipeptidasa, tripsina y aminopeptidasas, por ser un preparado enzimático de amplio espectro que hidroliza

productos y subproductos alimentarios a pH alcalino, se ha empleado para la obtención de péptidos con actividad antioxidante y péptidos inhibidores de ECA (Guan et al., 2018). Quirós et al. (2012) utilizaron caseinato como sustrato con diferentes tratamientos para optimizar la hidrólisis empleando la metodología de superficie de respuesta. La hidrólisis con Corolasa PP a una concentración de 60 mg/g de proteína durante 24 h a 37 ºC permitió obtener la concentración más alta del péptido HLPLP. Por otro lado, Guan et al. (2018) utilizaron tratamiento a altas presiones hidrostáticas combinado con hidrólisis con Corolasa PP para obtener péptidos antioxidantes e inhibidores de ECA; determinaron que la hidrólisis con Corolasa PP a 200 MPa fue más eficiente para la obtención de péptidos, además, disminuyó la hidrofobicidad superficial de los hidrolizados. Fujita et al. (2010) hidrolizaron diferentes alimentos con enzimas; la hidrólisis de músculo de pollo la realizaron con termolisina, mientras que ovoalbúmina fue hidrolizada con pepsina, tripsina, quimotripsina y termolisina. De los péptidos obtenidos con actividad inhibidora de ECA, el que presentó la mayor actividad de inhibición fue IKW con un valor IC_{50} de 0.21μM. También observaron que los péptidos IY, LW, IKW, LAP, LKP, LKPNM, y IWHHT presentaron actividad antihipertensiva similar después de ser administrados vía intravenosa (10mg/kg) a ratas espontáneamente hipertensas. Así mismo, los péptidos IY, LW, KW, LKP, LKPNM, IWHHT, IVGRPR, y IVGRPRHQG también presentaron actividad antihipertensiva después de ser administrados vía oral (60 mg/kg).

1.2.1.2. Procesado de alimentos. Las proteínas poseen propiedades nutricionales, biológicas y funcionales que se ven modificadas durante el procesamiento de los alimentos (Toldrá et al., 2018; Kopf-Bolanz et al., 2014); entre los principales procesos se encuentran la fermentación y el curado. En carnes fermentadas y proteínas de pescado, la fracción proteica se hidroliza por peptidasas microbianas y enzimas endógenas musculares (Mora et al., 2016). En el caso de la leche, el tratamiento térmico influye directamente en la liberación de péptidos de β-lactoglobulina durante la digestión gástrica (Kopf-Bolanz et al., 2014; Sánchez-Rivera et al., 2015). Además, la actividad proteolítica de microorganismos iniciadores de la fermentación lleva a la liberación de péptidos bioactivos y aminoácidos libres por acción de proteasas extra e intracelulares (Hernández-Ledesma et al., 2007; Litopulou-Zanetaki y Tzanetaki 2014).

1.2.1.3. Digestión gastrointestinal. La digestión gastrointestinal in vivo es un proceso dinámico en donde tiene lugar la liberación secuencial de enzimas gástricas (pepsina) e intestinales (tripsina, quimotrisina, elastasas, carboxipeptidasas, etc.) junto con cambios en el pH durante el tiempo de residencia de la matriz alimentaria en el tracto gastrointestinal. Adicionalmente, los movimientos peristálticos en la fase gástrica e intestinal tienen un efecto en la tasa de liberación y desintegración de la matriz alimentaria. Durante el proceso tiene lugar la hidrólisis secuencial de las proteínas, lo cual provee péptidos y aminoácidos al organismo donde la absorción tiene lugar en el duodeno y en el jejuno superior (Sánchez-Rivera et al., 2015).

Con el fin de simular la digestión gastrointestinal *in vivo*, se han desarrollado modelos de digestión gastrointestinal estática y dinámica para diferentes matrices alimentarias (Kopf-Bolanz et al., 2012; Kopf-Bolanz et al., 2014, Egger et al., 2019). Uno de los modelos más empleados para la digestión gastrointestinal simulada de leche, es el método estático desarrollado por Kopf-Bolanz et al. (2012), el cual se realiza en tres fases: fase oral, gástrica e intestinal. El porcentaje de hidrólisis de proteínas reportado es del 53.9%, siendo di y tripéptidos las secuencias mayoritarias, de estos, el 10% es hidrolizado a aminoácidos libres. Este método fue validado comparando los resultados con los valores reportados de estudios de digestión humana. Por otro lado, aunque los métodos de digestión dinámica simulan de manera más cercana el proceso digestivo que ocurre en el organismo, los resultados no presentaron diferencias en la hidrólisis de proteínas de la leche al comparar un método estático y uno dinámico; adicionalmente, estos no fueron diferentes al compararlos con la digestión *in vivo* (Egger et al., 2019).

1.2.2. Bioaccesibilidad y Biodisponibilidad de Péptidos Bioactivos

1.2.2.1. Bioaccesibilidad y estabilidad de péptidos bioactivos. Bioaccesibilidad se define como la cantidad o fracción que se libera de la matriz alimentaria en el tracto gastrointestinal y está disponible para su absorción (Carbonell-Capella et al., 2014). Esto incluye la transformación que tiene lugar en la digestión y está listo para la absorción en el epitelio intestinal y finalmente, el metabolismo pre-sistémico. Por otro lado, Carbonell-Capella et al. (2014) reportaron que la biodisponibilidad se define como la porción y el grado en el cual un compuesto del alimento es

absorbido y es disponible en circulación sistémica para el metabolismo celular del hospedero. En este sentido, en estudios de digestión gastrointestinal simulada de leche se reportó que el 53.9% de las proteínas fueron hidrolizadas a di y tripéptidos; y al comparar estos resultados con estudios *in vivo* no se observaron diferencias en el porcentaje de bioaccesibilidad (Kopf-Bolanz et al., 2012; Egger et al., 2019). Por lo tanto, los di y tripéptidos así como otras secuencias de oligopéptidos bioaccesibles durante la digestión gastrointestinal pueden ser absorbidas en el intestino.

Para el caso de otras matrices alimentarias como semilla de lenteja, soya, frijol pinto y semilla de quinoa, la bioaccesibilidad que se reporta está en rangos que van de 21.92 a 50% expresada como grado de hidrólisis. Adicionalmente, después de la digestión gastrointestinal simulada, las secuencias peptídicas liberadas presentaron actividad inhibidora de ECA, antioxidante y antimicrobiana en ensayos *in vitro* (Guo et al., 2020; Jakubczyk y Baraniak 2013; Capriotti et al., 2015; Tagliazucchi et al., 2015). No obstante, después del proceso de digestión, el bolo alimenticio está en contacto con las peptidasas de membrana cepillo-frontera previo a su absorción, por lo cual, podrían ser posteriormente hidrolizado. Por lo tanto, las secuencias peptídicas y la bioactividad observada pueden ser modificadas después de la absorción.

1.2.3. Absorción de Péptidos Bioactivos

La absorción de péptidos bioactivos en epitelio intestinal está en función de diferentes factores, entre los principales se encuentran: a) el estado de ayuno o condiciones postprandiales al momento de la administración; b) las interacciones entre los componentes del bolo alimenticio en el tracto gastrointestinal; c) la competencia con otros nutrientes que comparten la misma ruta de absorción; d) el estado fisiológico (salud o enfermedad) del organismo, y la edad (Shen y Matsui, 2017; Ozorio et al., 2020). Por lo anterior, es importante determinar la absorción de péptidos potencialmente bioactivos.

Los estudios de absorción se realizan mediante ensayos *in vitro* con el uso de líneas celulares, o mediante estudios *ex vivo* empleando modelos basados en tejido intestinal bajo condiciones fisiológicas. El cultivo de la línea celular Caco-2 en monocapa es utilizado debido a que después de su diferenciación presenta características funcionales y estructurales similares a enterocitos maduros, con microvellosidades de cepillo-frontera, uniones estrechas entre células y expresión de

enzimas y transportadores de membrana (Shen y Matsui 2017), esto lo hace un buen modelo para elucidar los mecanismos de absorción de péptidos específicos así como su resistencia o hidrólisis por las peptidasas de membrana cepillo frontera (Vij et al., 2016). Por otro lado, los modelos *ex vivo* han sido utilizados para determinar la absorción de fármacos con resultados satisfactorios al compararlos con estudios de absorción humana, ya que presentan permeabilidad celular comparable al epitelio del intestino delgado, tienen presencia de mucosa y expresan enzimas de membrana cepillo frontera (Álvarez-Olguín et al., 2022). A continuación se describen los mecanismos de absorción de péptidos bioactivos en el epitelio intestinal.

1.2.3.1. Transportador de Péptidos Pept1. PepT1 es un transportador de péptidos miembro de la familia de transportadores dependiente de H+, transporta di y tripéptidos del lumen intestinal a través de los enterocitos mediante el gradiente de protones entre el lumen intestinal (pH 5.5-6.0) y las células epiteliales (pH 7.0). El gradiente electroquímico se mantiene a través del intercambiador H+/Na+ el cual es balanceado por la ATPasa basolateral de Na+/K+ (Xu et al., 2019). Entre las secuencias peptídicas que transporta PepT1 se encuentran los péptidos antihipertensivos IPP, LKP, IQW (Glesson et al., 2017), el péptido antihipertensivo y antioxidante IRW (Bejjani y Wu 2013) y el péptido YPI (Miguel et al., 2008). PepT1 realiza el transporte de péptidos con carga neutra, hidrofóbicos, y se une a residuos ricos en aminoácidos no polares (Xu et al., 2019).

1.2.3.2. Ruta paracelular pasiva. La ruta paracelular pasiva es mediada a través de las uniones estrechas entre enterocitos, las cuales consisten de la zónula de ocludinas-1, ocludinas y claudinas que forman una barrera biológica estrecha con penetración selectiva. Esta vía de transporte independiente de energía permite la absorción de oligopéptidos por difusión pasiva. Entre los péptidos bioactivos transportados por esta vía de absorción están los previamente reportados con actividad antihipertensiva KVLPVP (Sun et al., 2009), HLPLP (Quiros et al., 2008), RLSFNP (Guo et al., 2018) y con actividad antioxidantes DHHAPQLR, WDHHAP (Xu et al., 2018), RWQ y WQ (Fernández-Musoles et al., 2013).

1.2.3.3. Transcitosis. Este mecanismo de transporte transcelular de péptidos es dependiente de

energía, se realiza a través de interacciones hidrofóbicas entre péptidos y la superficie apical de los enterocitos, lo cual lleva a la formación de vesículas donde los péptidos son embebidos; posteriormente las vesículas son internalizadas en los enterocitos. La absorción mediante este mecanismo se realiza en péptidos con alta hidrofobicidad con más de cuatro aminoácidos en su estructura. Entre las secuencias peptídicas que se absorben por esta ruta está el péptido antioxidante YWDHNNPQIR compuesto por aminoácidos hidrofóbicos (Xu et al., 2017), y los péptidos VLPVPQK y RPPGFSPFR (Vij et al., 2016).

1.2.4. Péptidos Bioactivos en el Lumen Intestinal

Diversos estudios en modelos *in vivo* han evidenciado que solo un 0.059 al 5.18% de péptidos bioactivos bioaccesibles son absorbidos en el lumen intestinal (Sánchez-Rivera et al., 2014; Van der Pijl et al., 2008; Yamada et al., 2015). No obstante, los péptidos y aminoácidos que no se absorben en el intestino delgado, pudieran ejercer funciones específicas a nivel del epitelio intestinal.

Estudios recientes sugieren que los péptidos juegan un papel importante en la regulación de la microbiota intestinal (Asaholu, 2020). Adicionalmente, pueden tener interacciones moleculares con la microbiota y ser metabolizados por ésta, generan nuevas secuencias de péptidos bioactivos, aminoácidos y ácidos grasos de cadena corta con funciones específicas a través de su interacción con receptores intestinales (Wu et al., 2017). En específico, ha sido previamente reportado que péptidos pueden estimular o inhibir el crecimiento de microorganismos específicos (Liu y Ho, 2018), modular la producción de la mucina (Shimizu, 2004; Martínez-Augustin et al., 2014), ser catabolizados como fuente de nitrógeno y carbono, ser metabolizados a ácidos grasos de cadena corta (Gentile y Weir, 2018) y así mejorar la disbiosis intestinal (Beltrán-Barrientos et al., 2021).

1.2.5. Biodisponibilidad *in vivo*

Una vez que los péptidos son absorbidos, estos están en contacto con peptidasas del torrente

sanguíneo donde pueden ser objeto de una hidrólisis final antes de ejercer el efecto bioactivo (Sánchez-Rivera et al., 2014). En este sentido, los estudios de farmacocinética después de la administración oral o intravenosa de péptidos específicos permiten dar seguimiento al metabolismo, el efecto bioactivo y conocer los péptidos resultantes. En el caso específico del péptido LHLPLP, este fue hidrolizado por las peptidasas de membrana cepillo frontera en estudios *in vitro* (Quirós et al., 2008) con hidrólisis entre los enlaces leucina-histidina liberando el pentapéptido HLPLP. Posteriormente, Sánchez-Rivera et al. (2014) observaron que el pentapéptido ejerce efecto antihipertensivo en REH; adicionalmente, el péptido fue hidrolizado a las secuencias LPLP y HLPL por peptidasas del torrente sanguíneo. No obstante, se desconoce si los tetra-péptidos resultantes tienen efecto antihipertensivo.

Por otro lado, la biodisponibilidad que se reporta para las secuencias peptídicas antihipertensivas es relativamente baja, siendo de 5.18% para el péptido LHLPLP que fue administrado por la vía oral (Sánchez-Rivera et al., 2014); mientras que para secuencias más cortas como los tripéptidos IPP, LPP y VPP a pesar de no ser hidrolizados por peptidasas de torrente sanguíneo, la biodisponibilidad reportada en cerdos fue de 0.059 a 0.077% (Van der Pijl et al., 2008). No obstante, aún con la baja biodisponibilidad *in vivo,* los péptidos presentaron efecto antihipertensivo. Yamada et al. (2015) observaron efecto antihipertensivo dependiente de la dosis del péptido ^{14}C-MKP después de una administración oral intragástrica en REH, el porcentaje del péptido intacto después de 4 h fue de 3.6% con la concentración más alta de 0.9 μM a los 8 min después de la administración.

Por otro lado, en los estudios donde no se evalúa la farmacocinética de péptidos, estos reportan el efecto bioactivo *in vivo* mediante la medición de una variable de respuesta primaria. En nuestro grupo de investigación, se reportó el efecto antihipertensivo FM-571 en personas prehipertensas (Beltrán-Barrientos *et al.,* 2018a) y un modelo murino REH (Rodríguez-Figueroa et al., 2013; Beltrán-Barrientos *et al.,* 2018b). De manera similar, se demostró que los mecanismos del efecto hipotensor debido al consumo de esta leche fermentada fueron: la inhibición de la actividad ECA *in vivo*, la producción de óxido nítrico y efecto antioxidante (Beltrán-Barrientos *et al.,* 2018b). El efecto antihipertensivo FM-571 fue atribuido a péptidos bioactivos liberados durante la fermentación de la leche y la digestión gastrointestinal. No obstante, para validar las propiedades funcionales de FM-571 además de la actividad inhibidora de ECA *in vitro* y la bioactividad *in vivo,* es necesario identificar los péptidos liberados después del proceso de digestión gastrointestinal y que se absorben en el lumen intestinal y tienen efecto antihipertensivo, así como las secuencias

peptídicas que no fueron absorbidas en el lumen intestinal. Adicionalmente, aun es necesario determinar la prevención del desarrollo de la hipertensión arterial por FM-571 en un modelo murino.

1.2.6. Estudios de Prevención de Hipertensión

La hipertensión es el principal factor de riesgo para el desarrollo de enfermedades cardiovasculares (Zhang et al., 2021). Las primeras recomendaciones para disminuir estas enfermedades son cambios de estilo de vida, en específico, los factores modificables tales como los patrones dietarios y el sedentarismo (Gallego, Mora y Toldrá, 2018). En este sentido, los alimentos funcionales pueden ser empleados como un coadyuvante en el manejo de la hipertensión arterial. En particular, algunos estudios han probado el efecto antihipertensivo de leche fermentada en modelos murinos de hipertensión espontánea y en personas hipertensas; sin embargo, pocos estudios han probado el efecto protector de estos alimentos al desarrollo de hipertensión.

Sipola et al. (2001) reportaron que la administración *ad libitum* de leche fermentada por *Lactobacillus hevelticus* LBKH16 adicionada con los péptidos IPP y VPP a concentración entre 2.5 – 3.5 mg/kg/día, así como los péptidos disueltos en agua a la misma concentración, atenuaron el desarrollo de hipertensión en ratas SHR al administrar los tratamientos durante 12 semanas; adicionalmente, sugirieron que el posible mecanismo del efecto preventivo fue la inhibición de ECA *in vivo*. En un estudio similar realizado por Jauhiainen et al. (2005) observaron que leche fermentada por *Lactobacillus hevelticus* LBKH16 adicionada con los tripéptidos VPP e IPP a concentración de (1.8 – 2.0 mg/100g) atenuaron de manera más efectiva el desarrollo de hipertensión en ratas SHR al comparar con los péptidos puros y la mezcla de péptidos y minerales (Potasio, Calcio y Magnesio).

Por otro lado, en un modelo murino de hipertensión inducida con Angiotensina II, se observó efecto protector al desarrollo de hipertensión al administrar el péptido DPYKLRP por intubación gástrica a concentración de 10 mg/kg (García-Tejedor et al., 2015). En un estudio similar, El-Fattah et al. (2017) observó efecto antihipertensivo durante cuatro semanas cuando se administró leche fermentada con *Lactibacillus rhamnosus* B-1445, O-114, YC-X11 y *Lactibacillus helveticus* Lh-B 02 en un modelo de hipertensión inducida con Dexametasona vía subcutánea.

De manera similar, Kamkar-Del et al. (2020) demostraron efecto protector al desarrollo de hipertensión al administrar dos extractos de *Ziziphus jujuba* durante cuatro semanas previo a la inducción de hipertensión con Ang vía intravenosa. Los autores sugieren que el efecto protector fue debido a los fenoles y flavonoides presentes en los extractos. Angiotensina II vía su receptor AT_1 es un fuerte activador del estrés oxidativo vascular mediante el estímulo de la producción de NADPH oxidasa, enzima que actúa como una de las más importantes fuentes del ion peróxido y anión superóxido.

1.3. Hipótesis

Hipótesis Primaria

Péptidos derivados de leche fermentada con *Lactococcus lactis* NRRL B-50571 están biodisponibles ya que presentan inhibición de la enzima convertidora de angiotensina después de haber sido sometidos a un modelo de absorción *ex vivo*.

Hipotesis Secundaria

Leche fermentada por *Lactococcus lactis* NRRL B-50571 previene el desarrollo de hipertensión en un modelo murino.

1.4. Objetivo General

- Evaluar la biodisponibilidad de péptidos bioactivos derivados de leche fermentada con *Lactococcus lactis* NRRL B-50571 con actividad inhibidora de la enzima convertidora de angiotensina.
- Evaluar la prevención del desarrollo de hipertensión por leche fermentada con *Lactococcus lactis* NRRL B-50571 en un modelo murino.

1.5. Objetivos Específicos

- Evaluar la bioaccesibilidad y biodisponibilidad de péptidos inhibidores de ECA derivados de leche fermentada con *Lactococcus lactis* NRRL B-50571 después de ser sometida a un modelos de digestión gastrointestinal simulada y absorción *ex vivo*.
- Identificar por LC-MS/MS los péptidos inhibidores de ECA derivados de leche fermentada con *Lactococcus lactis* NRRL B-50571.
- Determinar las propiedades fisicoquímicas, bioactividad y afinidad de unión de los péptidos al sitio activo de ECA por análisis *in silico*.
- Evaluar el efecto de leche fermentada por *Lactococcus lactis* NRRL B-50571 como tratamiento preventivo en el desarrollo de hipertensión arterial inducida con angiotensina II en un modelo murino.
- Evaluar si el efecto de prevención de desarrollo de hipertensión por *Lactococcus lactis* NRRL B-50571 es por inhibición de la ECA y/o actividad antioxidante.

1.6. Sección Integradora del Trabajo

Los artículos que integran esta tesis son tres, a continuación se describen brevemente.

Artículo 1. Current trends and perspectives on bioaccesibility and bioavailability of food bioactive peptides: in vitro and ex vivo studies.

Este artículo presenta una revisión actualizada relacionada a los ensayos *in vitro* y *ex vivo* que se han realizado para evaluar la bioacesibilidad y biodisponibilidad de péptidos bioactivos derivados de alimentos. En este sentido, se hace énfasis en la hidrólisis de la matriz alimentaria durante la digestión gastrointestinal, así como en la estabilidad y propiedades bioactivas de los péptidos liberados bajo los diferentes microambientes en cada una de las etapas de la digestión. La revisión permitió realizar un análisis crítico de los protocolos de digestión gastrointestinal simulada para diferentes matrices, así como las ventajas y desventajas que presenta cada uno. Se evidenció que

los métodos estáticos, a pesar de no simular exactamente la digestión gastrointestinal *in vivo* como lo hacen los protocolos de digestión dinámica, no presentan diferencias significativas con la digestión *in vivo.*

Por otro lado, se reportó que péptidos específicos bioaccesibles pueden ser hidrolizados por las peptidasas de membrana cepillo frontera antes de la absorción, y que las secuencias más cortas pueden tener el efecto bioactivo. Adicionalmente, entre los métodos más empleados para los ensayos de absorción *in vitro* son las líneas celulares Caco-2, mientras que en ensayos *ex vivo*, el protocolo Ussing chamber e intestino invertido son utilizados para evaluar la absorción global de péptidos y la permeabilidad intestinal. En específico, las líneas celulares Caco-2 son utilizados para elucidar los mecanismos de absorción de diferentes compuestos; no obstante, este modelo no logra simular la permeación intestinal humana. En este sentido, el epitelio intestinal incluye mucosa intestinal, células M, células endócrinas y células caliciformes, las cuales no están consideradas en la línea celular Caco-2; por lo que este modelo presenta menor transporte de absorción que el intestino delgado. Por todo lo anterior, estudios recientes han utilizado los modelos *ex vivo* (utilizando tejidos animales) debido a que consideran estos componentes. Así mismo, el protocolo *ex vivo* de intestino invertido destaca por presentar una adecuada permeabilidad paracelular, estructura intestinal y capa de mucosa similar a las condiciones *in vivo* durante el tiempo de ensayo. Este artículo fue publicado en la revista J Sci Food Agric.

Artículo 2. Bioaccesibility and bioavailability of peptides from fermented milk after gastrointestinal digestion and *ex vivo* absorption.

En este artículo se evaluó la bioaccesibilidad y biodisponibilidad de péptidos derivados de leche fermentada con *Lactococcus lactis* NRRL B-50571 (FM-571) después de haber sido sometidos a un modelo de absorción *ex vivo*. Primeramente, la leche fermentada fue sometida a digestión gastrointestinal simulada (DGS) con un protocolo de digestión estática validado para leche. Después, se determinó la actividad inhibidora de ECA en FM-571, FM-571 después de haber sido sometida a la DGS y después de haber sido sometida a DGS y absorción en el modelo *ex vivo*. El ensayo de absorción *ex vivo* se realizó empleando el modelo del intestino invertido utilizando un segmento de jejuno de rata macho Wistar, montado en un dispositivo diseñado para tal efecto. El contenido absorbido en el compartimento serosal fue fraccionado mediante RP-HPLC y se

determinó la inhibición de la ECA, para posteriormente identificar por LC-MS/MS, las fracciones cromatográficas con mejor IC_{50} de actividad inhibidora de ECA. Finalmente, con los péptidos identificados se realizaron ensayos *in silico* para determinar sus propiedades fisicoquímicas, la bioactividad previamente reportada, la probabilidad de poseer una bioactividad de acuerdo a su composición de aminoácidos y secuencia, así como la afinidad de unión de éstos al sitio activo de ECA.

En general, se observó la absorción de péptidos bioactivos intactos derivados de la fermentación que fueron estables durante los procesos de digestión y absorción *ex vivo.* Adicionalmente, se observó que algunos péptidos fueron hidrolizados por peptidasas de membrana cepillo frontera del epitelio intestinal del tejido *ex vivo.* Entre los péptidos bioactivos identificados, en su mayoría fueron hidrofóbicos, e incluso se identificaron trece péptidos previamente descritos como inhibidores de ECA y otros con propiedades antihipertensivas y antioxidantes. Se identificaron 100 nuevas secuencias de péptidos con potencial actividad inhibitoria de ECA. Finalmente, mediante el análisis *in silico* se identificaron 14 secuencias con alto potencial de unión al sitio activo de la ECA *in vivo*. Este artículo se encuentra en revisión en la revista ACS Food Science & Technology.

Artículo 3. Fermented milk as preventive treatment in an induced hypertension murine model with Ang II.

En este artículo se evaluó el efecto de leche fermentada con *Lactococcus lactis* NRRL B-50571 para prevenir el desarrollo de hipertensión en un estudio *in vivo* con ratas macho Wistar sanas. En este estudio, las ratas fueron aleatorizadas y asignadas a cuatro tratamientos: 1) Control sano (agua); 2) Control negativo (administradas con Ang II para inducción de hipertensión + agua); 3) Control positivo (administradas con Ang II para inducción de hipertensión + Captopril®); 4) FM-571 (administradas con Ang II para inducción de hipertensión + FM-571). Los tratamientos se administraron durante dos semanas, y en la tercer semana, los días 15 y 21 se administró por vía intravenosa (i.v.) una sola dosis de Ang II. La presión arterial se determinó cada semana y después de 7 semanas de tratamiento, se les dio muerte a las ratas y se colectó la sangre para evaluar en plasma las actividades enzimática de ECA, catalasa y glutatión peroxidasa, así como la peroxidación lipídica e índice de estrés oxidativo. Los resultados mostraron que se observó efecto protector de FM-571 como tratamiento preventivo en el desarrollo de hipertensión; adicionalmente,

se observó efecto antihipertensivo de FM-571 durante 7 semanas. Respecto a la actividad enzimática en plasma, se observó que la inhibición de ECA *in vivo* no es un mecanismo por la cual se regula la presión sanguínea en este modelo de hipertensión. Por otro lado, la actividad de catalasa en el grupo FM-571 incrementó significativamente respecto a los grupos control, y la actividad de glutatión peroxidasa no fue estadísticamente diferente entre los grupos. De manera similar, los productos de peroxidación lipídica expresados como malondialdehído no presentaron diferencias significativas entre los grupos, mientras que el índice de estrés oxidativo fue estadísticamente menor en el grupo FM-571. Los resultados sugieren que el efecto protector al desarrollo de hipertensión en el modelo murino de hipertensión inducida puede deberse al mejoramiento del sistema de defensa antioxidante primario y a la disminución del índice de estrés oxidativo durante la administración de FM-571. Por lo tanto, FM-571 podría ser administrada como un coadyuvante en la prevención de hipertensión.

2. CURRENT TRENDS AND PERSPECTIVES ON BIOACCESIBILITY AND BIOAVAILABILITY OF FOOD BIOACTIVE PEPTIDES: *in vitro* AND *ex vivo* STUDIES.

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Current trends and perspectives on bioaccessibility and bioavailability of food bioactive peptides: *in vitro* and *ex vivo* studies

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Abstract

The bioaccessibility and bioavailability of food-derived bioactive compounds are important issues when assessing their *in vivo* physiological health-promoting effects. Food components such as proteins and peptides are exposed to different proteases and peptidases during gastrointestinal digestion and absorption. Different *in vitro* approaches have therefore been developed to evaluate the bioaccessibility and stability of bioactive peptides. The static simulated gastrointestinal digestion model (SGD) was widely reported to assess the bioaccessibility of bioactive peptides. On the other hand, although the dynamic SGD model may better simulate human digestion, it has rarely been explored in bioaccessibility studies of food bioactive peptides due to its high cost and lack of standardization. For bioavailability studies, the Caco-2 cell monolayer model has been used extensively for the assessment of food bioactive peptides. In fact, very few reports using alternative methods for determining transepithelial transport of bioactive peptides have been employed. In this sense, *ex vivo* tissue-based models such as the Ussing chamber and the everted sac gut have been used. Current evidence supports the fact that using SGD with cell-based models for evaluating the bioaccessibility, absorption, and bioavailability of food-derived bioactive peptides, is the most commonly used approach. Nevertheless, SGD with *ex vivo* tissue-based models such as the everted sac, remains to be further explored because it seems to be the model that better mimics the physiological process – it is also fast and inexpensive, and several compounds may be tested simultaneously. In the present review, we discuss information available on the different *in vitro* approaches for the determination of bioaccessibility and bioavailability of food-derived bioactive peptides with special emphasis on *ex vivo* tissue-based models such as the everted sac and the Ussing chamber models.

Keywords: simulated gastrointestinal digestion; bioaccessibility; bioactive peptides; bioactivity

INTRODUCTION

In recent decades there has been a strong and growing interest in studying and developing foods with health-promoting effects, also known as functional foods. These health effects are in addition to their natural nutritional contribution,[1] and are attributed to bioactive compounds found in the food matrix.[2] Among them, proteins are fundamental macromolecules playing an important role, not only from the point of view of nutrition and tissue formation but also because they are a rich source of potential bioactive peptides. These peptides must be released from the native proteins in order to exert a beneficial effect. In general, peptides are released during gastrointestinal digestion;[3] however, they may also be produced through different technological processes during food processing,[2] such as by fermentation, the addition of commercial enzymes, food curing, and ripening.[4–7] The resulting peptides may possess different bioactive properties, which depend on their amino acid composition and sequence.[8]

The bioactivity of different food-derived peptides have been studied by *in vitro* and *in vivo* studies including antioxidant, anticoagulant, antimicrobial, chelating, dipeptidyl peptidase IV (DPP-IV) inhibitory, and – the most studied – angiotensin converting enzyme inhibitory (ACEI) (antihypertensive) activity.[3] However, to conclude that peptides have a potential beneficial effect, it is essential to consider how these compounds reach their target tissues, how they interact with biomolecules, and how they are transformed to induce a physiological response.[9] Thus, it is important to determine the stability of bioactive peptides because they may interact with other food components or may be further hydrolyzed during gastrointestinal digestion.[10] Furthermore, when peptides are absorbed, the action of peptidases in the enterocytes and proteases from the blood stream may also hydrolyze and affect their bioactivity.[11] The quantification of bioavailable bioactive peptides is therefore more important than

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determining the amount of peptides present in the food matrix.[12] Hence, studies assessing the bioaccessibility, bioavailability, and bioactivity of bioactive compounds are necessary.[13]

Clinical trials are the gold standard in food and nutrition research; nevertheless different *in vitro* models have been developed to elucidate the bioaccessibility and stability of bioactive peptides, and thus, reduce *in vivo* trials.[9] In this regard, static and dynamic simulated gastrointestinal digestion (SGD) models have been employed.[14] Cell-based models are also used commonly as predictive tools to determine the bioavailability of food bioactives, such as dairy peptides.[9] Current knowledge on the mechanistic transport pathways across the intestinal epithelial barrier of bioactive peptides[15] and peptide absorption through the Caco-2 cell monolayer model have been reviewed.[16] Nevertheless, *ex vivo* tissue-based models that have been extensively used for studying pharmaceutical drugs bioavailability have rarely been applied to study food bioactive peptides and are yet to be reviewed.

The aim of the present review is therefore to present available information on the different *in vitro* approaches for the determination of the bioaccessibility and bioavailability of food-derived bioactive peptides with special emphasis on *ex vivo* tissue-based models.

BIOACCESSIBILITY AND STABILITY OF FOOD DERIVED-PEPTIDES

Bioaccessibility is defined as the portion of a food matrix that is released in the gastrointestinal tract, with the potential to be absorbed into the intestinal lumen and reach the blood stream.[13,17] However, to understand how enzyme activity during gastrointestinal digestion may affect bioactive peptides, it is important to know this process specifically for proteins.[18] In this regard, protein digestion begins in the stomach at an acid pH with the activity of pepsin, which exhibits preferential cleavage of peptides having aromatic or dicarboxylic L-amino acids residues;[19] nonetheless, major hydrolysis occurs in the intestinal lumen and the brush border membrane of the epithelial cells.[18]

In the intestinal lumen with an alkaline environment, pancreatic proteases such as trypsin, α-chymotrypsin, elastase, and carboxypeptidases hydrolyze the polypeptides, release oligopeptides, and free amino acids. Then, oligopeptides undergo a second hydrolysis with peptidases (aminopeptidases, endopeptidases, dipeptidases, and carboxypeptidases) present in the intestinal villi and the pancreatic fluid, releasing tripeptides, dipeptides, and other amino acids.[18,20] Once peptides are absorbed, other proteases in the bloodstream may also further hydrolyze these peptides. On the other hand, unabsorbed peptides from the small intestine enter the large intestine and may be hydrolyzed by gut microbiota or may be absorbed intact.[18,21] Food proteins and peptides undergo a complex process of hydrolysis. It is therefore important to determine the stability of bioactive peptides or the bioactive peptides in a food matrix, preferably with clinical trials; however, this is not always ethical or financially possible.[15] For this purpose, simulated gastrointestinal digestion models have been validated to mimic the *in vivo* process.[22-25] Bioinformatic analysis and *ex vivo* studies have also been developed for different food matrices as alternative protocols.[26]

SIMULATED GASTROINTESTINAL DIGESTION MODELS

Among the different protocols of simulated gastrointestinal digestion (SGD), the static and dynamic models are the most commonly used. A static model is a fast method to evaluate gastrointestinal digestion and several samples may be tested simultaneously; however, as it is oversimplified, it does not include dynamic aspects of gastrointestinal digestion.[15] On the other hand, dynamic digestion models were developed taking into account several digestion conditions, such as fasting, peristaltic movements, rate of gastric and pancreatic enzymes release and time elapsed between gastric and intestinal phases.[23,27,28] This method closely simulates the digestive process that takes place *in vivo*; nonetheless, the main disadvantage is that sophisticated equipment is required to assemble the method and only a few samples may be tested.[29]

Once the food matrix has been subjected to simulated gastrointestinal digestion, the next step is to determine the bioaccessible fraction that may potentially be absorbed into the intestinal lumen. It has been reported that, in a food matrix, 50% of the proteins are degraded into dipeptides and tripeptides, and 10% of those peptides are further hydrolyzed into free amino acids.[24] Moreover, several key parameters to determine the degree of hydrolysis have been employed. In this regard, trinitrobenzenesulfonic acid (TNBS), pH-stat, formol titration and *o*-phthaldialdehyde (OPA) are the methods most frequently used.[30,31]

Static simulated gastrointestinal digestion studies

The static SGD model is a fast method that only comprises two or three steps simulating the mouth (oral), the stomach (gastric), the small intestine (intestinal), and occasionally the fermentation of the large intestine. It includes a single or a combination of enzymes for each step, with a fixed pH and temperature (37 °C)[9] (Fig. 1(a)). In fact, several static SGD models have been proposed to predict the digestibility and bioaccessibility of specific compounds from a food matrix that may be available for absorption through the small intestine.[27] Nevertheless, several differences have been observed between models, such as the variety of enzymes employed from different sources (e.g., porcine, rabbit, or human), which may vary their activity. Other factors, such as pH, mineral type, ionic strength and digestion time, may also affect the outcomes between studies and cause them to differ.[25] Interestingly, only few studies consider a control sample of gastrointestinal juices and enzyme with water instead of the food sample.[32,33] It has also been reported that the food matrix structure may influence nutrient release because the organization of nutrients may directly affect the digestion process.[34]

In this regard, Table 1 summarizes some examples of studies that determine the bioaccessibility and stability of peptides from different food sources after static simulated gastrointestinal digestion. In general, studies reported in-house methods for SGD focusing on determining the bioaccessibility of the sample of interest; however, they were approached differently depending on the food matrix. In this sense, peptide studies in food samples varied, including the whole food matrix, protein isolates and synthetic peptides. Only a few studies of static SGD models included the oral phase and most studies focused on gastric and intestinal digestion with proteases. Not all studies considered the inclusion of digested water as the control; and it is essential to include it because it has been predicted by *in silico* studies that endogenous gastrointestinal proteins may be a source of bioactive peptides.[44] Finally, it is important to highlight that most of these in-house static SGD models did not present validation studies. Nevertheless, it is essential to validate the SGD model for each type of food sample under investigation. In this regard, validation studies involved following the evolution and decomposition of

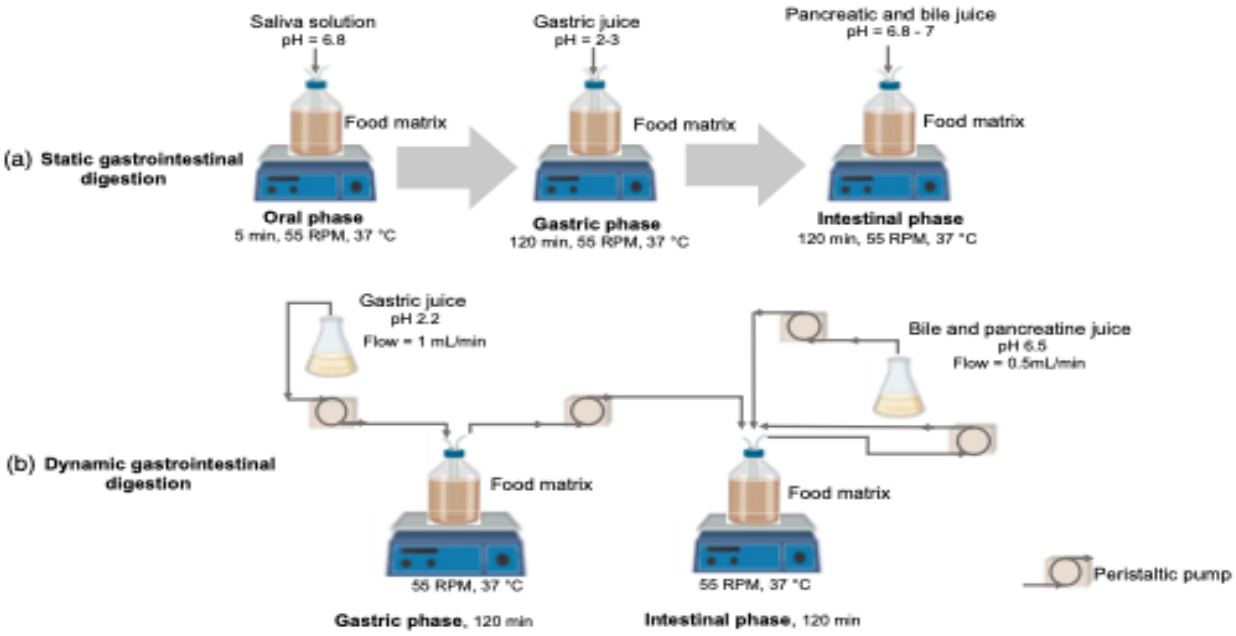

Figure 1. Gastrointestinal digestion conditions for static (a) and dynamic (b) *in vitro* models according to Kopf-Bolanz *et al.*[23] and Egger *et al.*[27]

macronutrients in a particular food, as was the case for homogenized and pasteurized whole milk over the entire gastrointestinal digestive process. Results for all macronutrients in milk were consistent with human physiological values reported in the literature.[23]

Most SGD models originated from previously reported methods, although there is substantial variation between studies, making it difficult to compare and contrast the outcomes among or reach conclusions.[25] An international network from 32 countries therefore consolidated a simulated gastrointestinal digestion protocol (COST Action INFOGEST), which includes oral, gastric, and intestinal phases to improve standardization and provide reproducibility of results among researchers. This protocol was validated with milk proteins using pigs as an animal model.[22] The gastrointestinal digestion of milk proteins by the INFOGEST protocol was compared with *in vivo* digests collected from human jejunum and it was concluded that, although some differences were found, the *in vitro* protocol was a good approximation to what occurs in the *in vivo* intestinal digestion in relation to protein degradation and peptide release.[29]

An *in vitro* batch fermentation protocol was recently reported for studying the contribution of food to gut microbiota composition and functionality following an *in vitro* gastrointestinal digestion model.[45] These consecutive *in vitro* protocols were useful tools for screening work when many food samples were studied. Although the *in vitro* batch fermentation protocol is beyond the scope of this review, it was proposed to be used in conjunction with the INFOGEST protocol.[45]

Interestingly, specific additional factors such as the simulation of gastrointestinal conditions of an infant or an elderly person should also be taken into account.[25] Furthermore, this digestion protocol is constantly improved by the international network of researchers mentioned above.[46] The information presented here highlights the usefulness of the SGD model for studying the bioaccessibility and stability of peptides derived from different food sources. Some studies evaluate *in vitro* bioactivity after subjecting foods to SGD, but others only perform bioinformatic analysis to predict potential bioactivity.[49]

Dynamic simulated gastrointestinal digestion studies

As mentioned previously, the static SGD model has its limitations because it does not mimic real physiological conditions (transit time, pH, and enzymatic conditions) (Fig. 1(a)). Dynamic SGD is therefore a more accurate model that determines the bioaccessibility and stability of food compounds.[47] In this regard, the dynamic SGD model consists of various steps where food is transferred through different compartments with specific physiological conditions (pH, digestive enzymes, and ionic strength) (Fig. 1(b)), depending on the physicochemical properties of food.[48] Nevertheless, to the best of our knowledge, only two studies have employed the dynamic gastrointestinal digestion model to determine the stability of food-derived bioactive peptides with ACEI,[49,50] antioxidant, opioid and antibacterial[49] activity (Table 2).

Sánchez-Rivera *et al.*[49] investigated the impact of heat treatment on hydrolysis with dynamic SGD of bovine milk proteins and the release of bioactive peptides. To achieve this, two reconstituted skim milk powders (unheated and heated) were used. Afterwards, milk samples were subjected to dynamic SGD. After enzymatic digestive hydrolysis, caseins from heated milk presented increased resistance to pepsin. Contrary to this, lactoglobulin was more vulnerable to proteolysis. A total of 12 peptides were identified in both (heated and unheated) skim milks. Six

Table 1. Some examples of bioaccessibility and stability of food-derived peptides after static simulated gastrointestinal digestion

Food source	Digested sample	Static simulated gastrointestinal digestion model	Hydrolysis degree after SGD	Bioactivity of resulting peptides	Reference
Buffalo milk dairy samples	Grana, Ice Cream, Mozzarella, Ricotta, Scamorza and Yoghurt from buffalo milk	Static method: gastric and intestinal phase	ND	Database search: antihypertensive, immunomodulatory, antimicrobial, antidiabetic, anticancer and antioxidant activity	35
Whey protein concentrate	Synthetic peptides	Static method: gastric and intestinal phase	ND	*In vitro* assay: ACE inhibition	36
Lentil seeds	Isolated lentil globulins	Static method: oral, gastric and intestinal phase	31.55%	*In vitro* assay: ACE inhibition	37
Pea seeds	Isolated pea globulins	Static method: oral, gastric and intestinal phase	30.65%	*In vitro* assay: ACE inhibition	38
Soybean seed and soybean milk	Extracted proteins from soybean seed and soybean milk	Static method: gastric phase and bioinformatic analysis	ND	Database search: antimicrobial, ACE inhibitor, antioxidant,	39
Pinto bean (*Phaseolus vulgaris*)	Pinto beans	Static method COST Action FA1005: oral, gastric and intestinal phase	50%	*In vitro* assay: ACE inhibition	40
Whole, semi-skimmed and skimmed bovine milk	Whole, semi-skimmed and skimmed bovine milk	Static method: gastric and intestinal phase	30.70%	*In vitro* assay: antioxidant Database search: DPP-IV inhibitor, ACE inhibition, antioxidant	41
Oil palm kernel expeller	Isolated oil palm kernel expeller glutelin-2	Static method: gastric and intestinal phase	28.44–36.17%	*In vitro* assays: ACE inhibition, antioxidant *In vivo* assay: Antihypertensive effect	42
Fermented donkey milk	Water soluble extracts of fermented milks	Static method COST Action FA1005: oral, gastric and intestinal phase	ND	*In vitro* assays: Antioxidant, antimicrobial and ACE inhibition	32
Fermented goat milk	Fermented goat milk	Static method: gastric and intestinal phase and bioinformatic analysis	47.70%	*In silico* assay: ACE inhibitor, antihypertensive, antioxidant, antibacterial, DPP-IV inhibitor	43
Quinoa seed protein	Extracted quinoa protein	Static method: gastric and intestinal phase	21.92%	*In vitro* assay: ACE inhibition *In vivo* assay: antihypertensive effect	33

Abbreviations: ACE, Angiotensin converting enzyme; DPP-IV: Dipeptidyl peptidase IV; SGD: Simulated gastrointestinal digestion; ND, not described.

Table 2. Bioaccessibility and stability of food-derived peptides after dynamic simulated gastrointestinal digestion

Sequence	Parental protein	Source of protein	Bioactivity	Analysis	Hydrolysis degree (%)	Reference
AYFYPEL, RYLGY	αs1-casein	Skimmed milk powder	ACEI	Dynamic digestion DIDGI®	80	49
SRYPSY	k-casein		Opioid		80	
VYQHQKAMKPWIQPKTKVIPYVRYL	alfas2-casein		Antibacterial		80	
NI/LDTDI/L, WNI/LNA	ND	Flaxseed protein	ACEI	Dynamic model	46.78	50

Abbreviation: ACEI, Angiotensin converting enzyme inhibition; ND: not described.

Table 3. Absorption of bioactive peptides from different food sources with Caco-2 cell monolayer

Peptide Source	Biological effect	Time of incubation (h)	Original peptides	Outcomes after exposure		Reference
				Apical	Basolateral	
Caseins from Parmigiano Reggiano cheese	ACEI	1	VPP, IPP, RYLGY, RYLG, AYFYPEL, AYFYPE, LHLPLP, HLPLP	VPP, IPP, RYLGY, AYFYPEL, AYFYPE, LHLPLP, HLPLP,	LHLPLP, HLPLP	62
Milk proteins	DPP-IV inhibitory	2	LKPTPEGDL, LPYPY, IPIQY, IPI, WR	LKPTPEGDL, LPYPY, IPIQY, IPI, WR	LPYPY WR	57
Soybean hydrolysate	Antioxidant	2	ND	GNPDIEHPE, TNDRPSKG, SVIKPPTDE, VIKPPTDE, GNPDIEHPET, LVPPQESQ, EITPEKNPQ, NSQHPEL, FEEPQQPQ	GNPDIEHPE, SVIKPPTDE, VIKPPTDE, GNPDIEHPET, FEEPQQPQ	60
Egg white (Ovokinin)	ACEI	1	FRADHPFL, RADHP, YAEERYPIL YAEER, YPI	FRADHPFL, RADHPFL, ADHPFL, DHPFL, RADHP, YAEERYPIL, YAEER, YPI	FRADHPFL, ADHPFL, DHPFL, YAE, YPI, YAEER, YPI	63
Lactoferrin derived peptides	ACEI	2	RRWQWR RRWQ, WQ	RRWQ, RRW, QWR, WR, RWQ, WQ	RRWQ, WR, RWQ, WQ, W	64
Ovotransferrin	Antihypertensive	1	IRW	IRW	IRW	65
Ovotransferrin	Antihypertensive	2	RVPSL	RVPSL		66
Egg white	ACEI	2	TNGIIR	TNGIIR	TNGIIR	67
Casein hydrolysate	ACEI, Antioxidant	1	VLPVPQK	VLPVPQK	VLPVPQK, VLPVPQ	68
Casein	Antioxidant	2	Fractions 1, 2 and 3	Fractions 1, 2 and 3	Fractions 1, 2 and 3	69
Spent hen	ACEI, antioxidant and anti-inflammatory	2	IWHHT	IWHHT IWH IW	IWHHT IHW IW	58
Egg white	Antihypertensive	1	LKP IQW	LKP IQW	LKP IQW	61
Rapeseed protein	Antioxidant	2	YWDHNNPQIR	WDHNNPQIR DHNNPQIR YWDHNNPQ	WDHNNPQIR DHNNPQIR YWDHNNPQ	70
Tilapia skin gelatin	ACEI	2	QAGLSPVR	QAGLSPVR	QAGLSPVR	59
Dry edible beans	Anti-inflammatory	2 and 6	γ-glutamyl valine	γ-glutamyl valine	γ-glutamyl valine	71

Abbreviation: ACEI, Angiotensin converting enzyme inhibition; DPP-IV: Dipeptidyl peptidase IV; ND: Not described.

peptides were identified only in the heated milks. In fact, after bioinformatic analysis, angiotensin converting enzyme (ACE) inhibition, opioid, antimicrobial and antioxidant activity was reported on the peptides that were identified.[49]

Marambe *et al.*[50] investigated whether flaxseed proteins may be a source of ACEI peptides, with potential cardiovascular beneficial effects. First, proteins were extracted from flaxseed, and then they were subjected to static and dynamic SGD. In fact, after digestion exposure, results showed that the dynamic SGD model presented a significantly higher hydrolysis degree (46.78%) than the static SGD model (43.95%). The dynamic SGD model presented significantly higher ACEI activity than the static SGD model. Furthermore, two peptides were identified NI/LDTDI/L and WNI/LNA, which may be potentially absorbed through the intestinal epithelium; however, further *in vivo* studies are necessary to confirm this fact.[50]

Bioavailability of bioactive compounds includes the full process of ingestion, digestion, absorption, and metabolism, reaching blood circulation where they may exert their physiological effect. Bioavailability is therefore frequently measured by *in vivo* studies using animal models or clinical trials.[13] However, Caco-2 cell monolayers and *ex vivo* tissue-based models have been also used to study the bioavailability of bioactive compounds or drugs.[16] After evaluating the stability of bioactive peptides exposed to SGD, further experiments determining bioavailability are therefore usually carried out.

ABSORPTION AND BIOAVAILABILITY OF FOOD DERIVED-PEPTIDES

Bioavailability is defined as the portion and degree to which any food compound is absorbed and becomes available in systemic circulation for the cellular metabolism of the host.[12,27] In general, bioavailability is determined with *in vivo* (animal) and clinical studies by quantifying a determined compound in plasma circulation after the administration of a specific dose.[12] In fact, bioavailability depends strictly on stability after gastrointestinal digestion, the release from the food matrix, and the efficiency of absorption through the transepithelial cells.[9]

This barrier contains numerous peptidases, which further hydrolyze peptides and thus affect their bioactivity.[51] Thus, it is necessary to evaluate the absorption process by *in vitro* or *ex vivo* tests that closely mimic the *in vivo* absorption in order to further determine the potential bioactivity of the absorbed peptides. In this sense, the most widely used model is the Caco-2 cell lines monolayer.[26] Other studies have explored *in vitro* tissue-based models, also called *ex vivo* models to determine bioactive peptides absorption, such as the Ussing chamber and the everted sac gut.[15,52-54] Tissue-based models use living functional tissues or organs isolated from an organism that are cultivated outside the organism under controlled conditions, and they present several advantages over cell-based models such as adequate paracellular permeability provided by the small intestinal epithelium and the presence of a mucus layer.[55]

Caco-2 cell line monolayer

Caco-2 cells culture models are originally obtained from colon carcinomas. Despite their origin, these differentiated cells may maintain some morphological and functional traits from mature enterocytes.[56] Interestingly, this model has been widely used to complement the *in vitro* gastrointestinal models and help predict the bioavailability of bioactive food compounds and different drugs[12] as they comprise numerous peptidases and possess carrier-mediated transport systems and functional tight junctions.[26,51,56]

Several studies have evaluated the absorption of bioactive peptides from different food sources with the Caco-2 cell monolayer models (Table 3). In this regard, studies have focused on determining and identifying which bioactive peptides are resistant to brush border peptidases and are absorbed from the apical to the basolateral chambers (Fig. 2). Interestingly, results have shown that DPP-IV inhibitory peptides from milk,[57] ACEI peptides from spent hen[58] and Tilapia skin gelatin,[59] and antioxidant peptides from soybean[60] and rapeseed[61] may be absorbed intact through the transepithelial cells and exert their biological effect. Other studies with the Caco-2 cell monolayer models have shown that peptides may still be susceptible to brush border peptidases and new sequences are further absorbed.[63,64,66,68]

Peptide absorption has been suggested to be through three different mechanisms (Fig. 3). In this sense, paracellular transport via tight junctions is an energy-independent passive pathway for water soluble low molecular compounds such as oligopeptides. Absorption by specific carrier proteins such as PepT1 (H^+-coupled peptide bidirectional transporter) may also transport di- and tripeptides. Finally, transcytosis via endocytosis by cells has also been observed. Mechanisms of absorption may occur in a single mode or in combination.[69,72,73]

Interestingly, the Caco-2 cell models have also been employed to explore and identify the mechanistic transport of specific peptides.[71] In this regard, inhibitors for the specific transport mechanisms mentioned above have been used, such as Gly-Ser for PepT1, wortamanin for transcytosis inhibition and cytochalasin D for gap junction disruption.[65,71] In fact, small peptides that were resistant to further brush border peptidases, such as YPI,[63] IRW[65] and other larger peptides such as VLPVPQK, were transported intact by the mechanism of absorption of PepT1.[63] Also, paracellular pathways have been observed in RWQ and WQ,[64] RVPSL,[66] TNGIIR[67] and QAGLSPVR[59] bioactive peptides. Finally, the transcytosis route has been reported in YWDHNNPQIR.[70] Interestingly, as previously mentioned, some peptides may be absorbed with different mechanisms, as observed with ACEI peptides IWH,[58] LKP and IQW,[61] and the anti-inflammatory peptide γ-glutamyl valine, which were transported by PepT1 and paracellular pathways.[58]

In general, although several studies have employed the Caco-2 cell monolayer models to elucidate mechanisms of absorption, to date it is still uncertain if it may mimic human intestinal permeation. In this last aspect, the intestinal epithelium also involves goblet cells, endocrine cells, M cells and mucus, which are not considered in the Caco-2 cell monolayer model.[26,72] It has been suggested that the Caco-2 cell model presents lower transport activity than the human small intestine.[74] Thus, *ex vivo* models have been proposed to cover these aspects using animal tissues.[26]

Ex vivo tissue-based models: Ussing chamber, jejunal segment and everted gut

The Ussing chamber (UC) is an *in vitro* system applied for evaluating the absorption and the permeability of peptides and drugs over an excised intestinal animal tissue segment (Fig. 4).[75,76] In the UC, a section of the intestinal tissue is excised, cut in segments of appropriate size, and opened to form a flat sheet, which is placed between the two halves of the chamber filled with a physiological buffer continuously gassed with carbogen (95% O_2, 5% CO_2) and maintained at 37 °C. The compound to be tested is

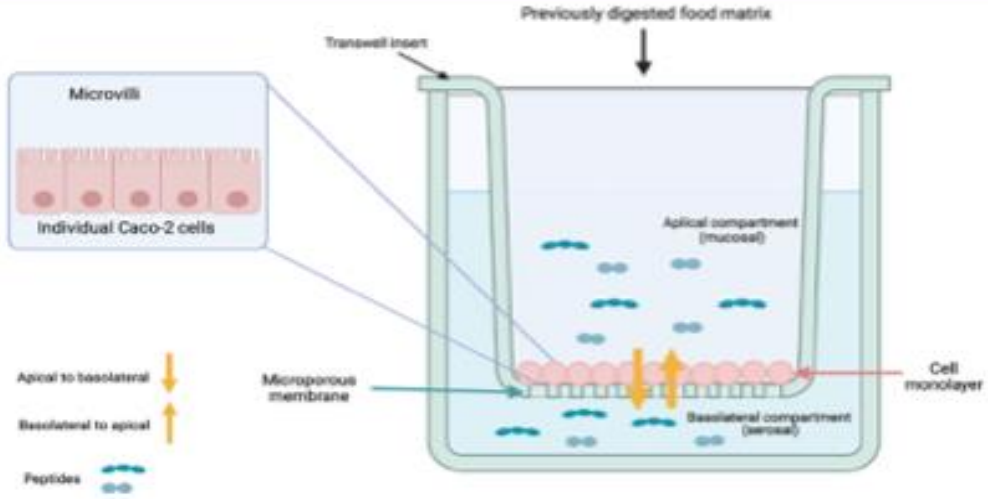

Figure 2. Schematic drawing of Caco-2 cells monolayer as a model to predict intestinal absorption of food peptides. Peptides are absorbed from the apical (mucosal) compartment to basolateral (serosal) compartments.

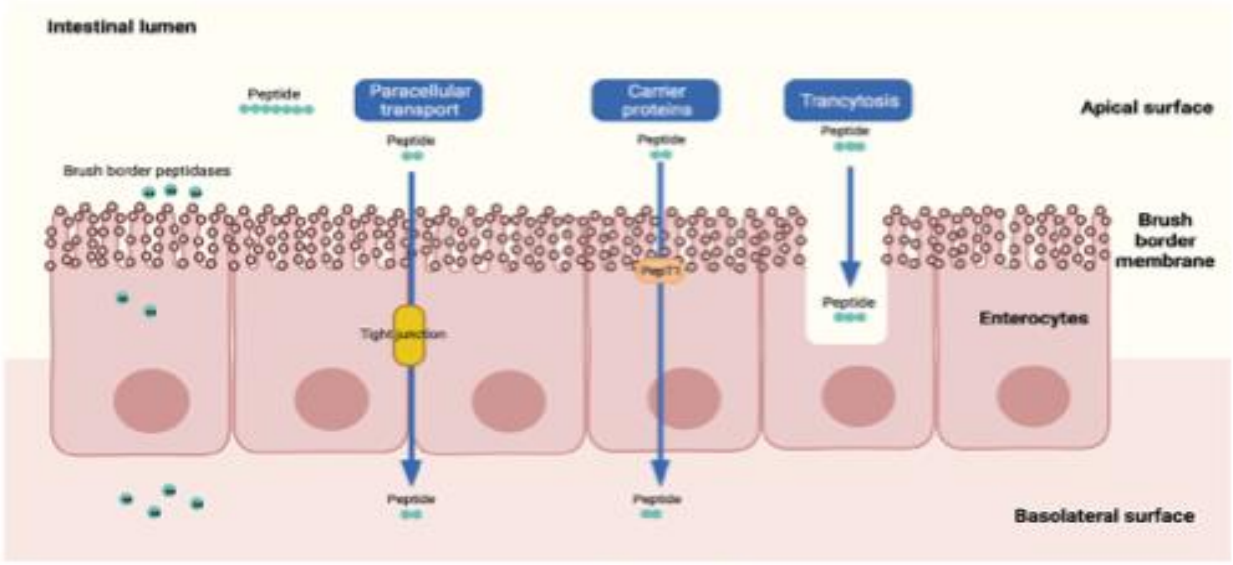

Figure 3. Mechanisms for peptide absorption (adapted from Gleeson *et al.*[76] and Xu *et al.*[15]).

placed in the buffer at the apical chamber side and samples are removed for evaluation from the basolateral chamber. The volume of sample removed in the basolateral chamber is then replaced by the same volume of prewarmed buffer to maintain a constant volume.[55]

Interestingly, this model has been used widely to determine drug absorption, as a satisfactory association has been observed with human absorption studies.[26] In fact, compared to the Caco-2 cell model, the UC may be a more adequate model, because it offers a paracellular permeability more comparable to the small intestine epithelium, the presence of a mucus layer, and more expression of metabolic enzymes and transport proteins.[74] Studies with food compounds such as peptides may therefore also be suitable.[26] So far, only few transepithelial studies with the UC model have been explored.

In this regard, Foltz *et al.*[74] reported three different absorption models to study the bioavailability of ACEI peptides (IPP and VPP). The authors explored the Caco-2 cell model, the UC model,

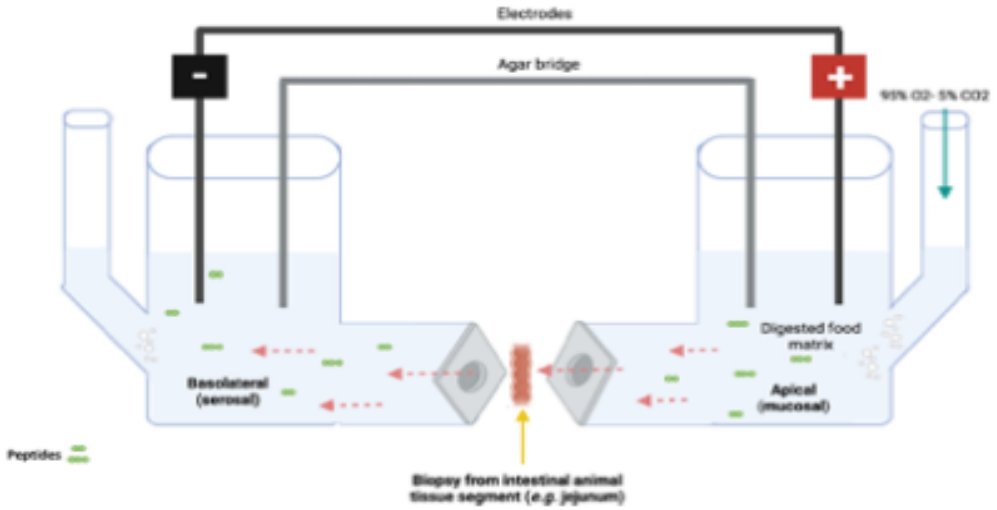

Figure 4. Schematic drawing of Ussing chamber as *in vitro* system applied for evaluating the absorption and permeability of peptides. Intestinal tissue must be mounted flat between two films, that are placed between two half chambers (apical and basolateral). The epithelial side is exposed to the apical (mucosal) compartment and the lamina propria is exposed in the basolateral (serosal) compartment. Chambers are kept at 37 °C and continually oxygenated with gas flow (95% O_2, 5% CO_2).

and *in situ* intestinal perfusion with rats. Interestingly, results with the UC model showed that the absorption was 5-fold and 10-fold higher for IPP and VPP, respectively, in comparison with the Caco-2 cell model. Thus, the authors concluded that the Caco-2 cell model may underestimate permeability. Moreover, although the *in situ* intestinal perfusion model may represent a more appropriate model, as blood flow is preserved, results showed that it was not a suitable technique because the experiment was not reproducible. However, the authors suggested that the UC model was more vigorous in terms of reproducibility and was more suitable for exploring peptide absorption.[74]

Similarly, the stability and intestinal permeation of two antihypertensive peptides (IPP and LKP) were determined with the UC model using rat intestinal tissue.[77] They were stable against brush border, intestinal, and liver proteolytic enzymes, which may indicate that these peptides may be transported intact into the circulation system. Nonetheless, after exposure in the UC model, the results showed low permeability even with permeation enhancers. The authors therefore concluded that further *in vivo* studies with permeation enhancers are needed to determine an *in vivo* hypotensive effect.[77] Later on, the authors determined the mechanism of absorption and transport studies with the Caco-2 cell monolayer and the UC model of IPP and LKP peptides.[78] After several experiments, the results elucidated that the PepT1 and paracellular routes were the main mechanisms of absorption.[78]

Ozorio *et al.*[26] explored the stability and the bioavailability of a digested and a nondigested vasodilatory whey protein hydrolysate with the UC model employing a piglet proximal jejunum. The results evidenced intense hydrolysis activity from brush border cells in the UC model. Also, 360 and 286 new peptides from undigested and digested whey hydrolysates were detected in the basolateral compartment in the UC with a piglet proximal jejunum, suggesting that peptides with different molecular weights are able to be absorbed in the small intestine. Finally, the authors suggested that SGD coupled to the UC model may represent a new approach to study bioavailability of food components. This is still under review by the INFOGEST international network on food digestion.[26]

The everted sac model is another *ex vivo* assay employed to study intestinal transport, specifically of drugs and nowadays of food components (Fig. 5). In the everted sac model, a small piece of intestine is removed from anesthetized animals; it is flushed with buffer and everted over a tube with the serose part inside the sac and the mucose side facing the buffer solution. Both ends of the sac are tied and filled with oxygenated buffer and placed in the beaker with the compound to be evaluated. Accumulation of the sample in the inner compartment is measured after a specified time period.[55]

Major disadvantages of the everted sac model are the viability of the tissue, the morphological damage while everting,[52] and the potential for severe damage, which would reduce the metabolizing capacity of the everted sac during the freezing and thawing process. Furthermore, a potential disadvantage of this approach is the presence of the muscularis mucosa, which is usually not removed from the everted sac preparations. Nevertheless, it is a fast and an inexpensive model. Several compounds may be tested simultaneously. A mucus layer is present and presents a large surface area available for absorption.[55]

Moreover, it is important to take notice that the viability of this model is approximately 2 h; nonetheless, it is considered a useful tool to screen intestinal absorption.[79] To the best of our knowledge, only one study has explored the stability and transport of DPP-IV egg white peptides with the Caco-2 cells monolayer model and the everted rat sac model.[54] Interestingly, results evidenced that LGAKDSTRT, DGSRQPVDN, VNDLQGKTS, and GKKDPVLKD peptides were identified in the basolateral compartment of the Caco-2 cell monolayers and the serous side of the everted rat

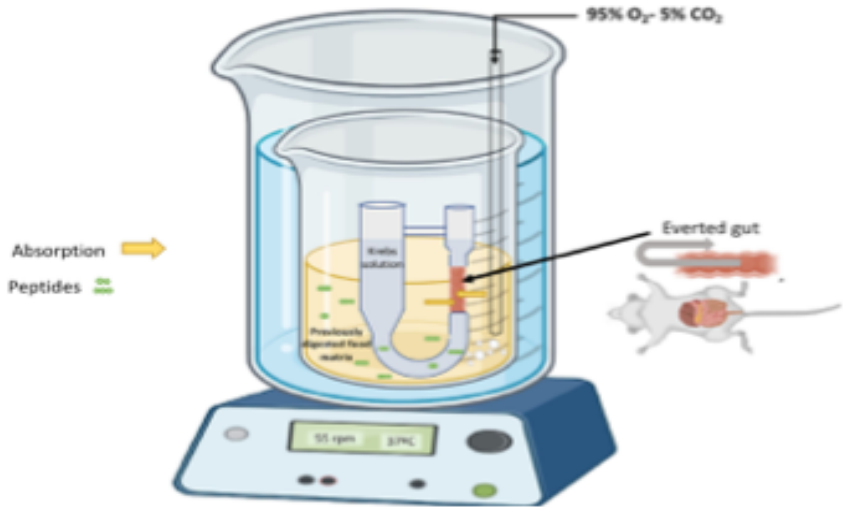

Figure 5. Schematic drawing of everted gut as an *in vitro* system used for evaluating the absorption and permeability of peptides. Intestinal tissue must be everted, placing the epithelial side exposed and mounted on the glass tubes. Krebs solution is added inside the glass compartment. The digested food matrix is placed in the beaker provided with a water bath (37 °C) and continually oxygenated with gas flow (95% O_2, 5% CO_2) (adapted from Dixit *et al.*[52]).

sac, indicating resistance to border brush peptidases. Although there were more peptides in the everted rat sacs than in the Caco-2 cell monolayer, it presented less permeability; thus, the everted rat sac may be characterized by higher brush border membrane peptidase-induced hydrolysis.[54]

A three-dimensional intestinal model, using organoids, is a novel promising *in vitro* model for studying nutrient transport and drug absorption. Interestingly, organoids comprise all kinds of intestinal epithelium cells, thus, presenting all of the epithelium intestinal functions. However, this model has been used for studying the bioavailability of some food compounds such as phytochemicals[80–82] and has not been used for studying food bioactive peptides.

Although tissue-based models, such as the UC, have been used for the evaluation of food peptide absorption, high throughput systems based on the UC, such as the InTESTine™ have been proposed for studying human intestinal absorption of digested foods.[82] This system studies multiple segments (duodenum, jejunum, ileum, and colon) in parallel, using a disposable multi-well setting and standardized culture conditions in a humidified high O_2/CO_2 incubator on a rocker platform. The presence of the mucus layer allows intestinal absorption to be studied in the absence or presence of microbiota, which is an advantage over Caco-2 cells.[83] Thus, this high throughput system offers potential for studying food bioactive peptides intestinal absorption.

FUTURE PERSPECTIVES AND CONCLUSIONS

Among the different *in vitro* models used to evaluate the stability and bioavailability of bioactive peptides (simulated gastrointestinal digestion, Caco-2 cell line monolayer, Ussing chambers, and everted rat sacs), further hydrolysis may be observed either by the action of the digestive enzymes, by the brush border membrane peptidases, or by intracellular enzymes during the transport process. Nevertheless, only a few studies determined peptide bioactivity after absorption evaluation. Comparison between different absorption models (cell-based versus tissue-based) is also necessary for bioavailability evaluation of food bioactive peptides because the Caco-2 cell monolayer model was the only model extensively studied. In fact, organoids constitute a novel promising *in vitro* model for bioavailability evaluation. Nevertheless, this model has not yet been explored with bioactive food peptides. Knowledge of the effect of food components on gut microbiota composition and functionality by an *in vitro* batch fermentation procedure carried out after *in vitro* gastrointestinal digestion may also be useful in order to study the relationship between food peptides, gut microbiota, and health.

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3. BIOACCESIBILITY AND BIOAVAILABILITY OF ACE INHIBITORY PEPTIDES FROM FERMENTED MILK WITH *Lactococcus lactis* NRRL B-50571.

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Bioaccessibility and bioavailability of ACE inhibitory peptides from fermented milk with *Lactococcus lactis* NRRL B-50571

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Abstract

The present study aimed to determine the bioaccessibility and bioavailability of peptide fractions with angiotensin-converting enzyme (ACE) inhibition obtained from fermented milk with *L. lactis* NRRL B-50571 (FM-571) after being subjected to simulated gastrointestinal digestion (SGD) and an *ex vivo* absorption model. First, FM-571 was subjected to SGD, and then, the absorption process was carried out with an *ex vivo* model using the everted sac. Afterward, peptide identification with HPLC/MS-MS was carried out, and an *in silico* analysis was performed to identify which peptide sequences may be present with potential ACE binding. Results showed that ACE inhibition increased ($p<0.05$) (93.66 ± 7.33%) after SGD and decreased after absorption ($p<0.05$) (32.88 ± 13.20%). Conversely, the IC_{50} decreased ($p<0.05$) after SGD (1259.55 µg/mL) and after absorption ($p<0.05$) (10.02 µg/mL). Moreover, as expected, peptide abundance increased ($p<0.05$) by 58% after SGD indicating augmented bioaccessibility. On the other hand, peptide abundance ($p<0.05$) decreased after absorption, resulting in a calculated peptide bioavailability of 1.36 ± 0.1%. Nevertheless, RP-HPLC collected fractions from the serosal (absorbed) compartment exhibited the lowest IC_{50} ranging from 17.8 to 62.69 µg/mL. One hundred thirteen peptides were identified in the serosal compartment, from which 13 were previously reported with ACE inhibition. Thus, 100 novel potential ACE inhibitory peptides were identified. Additionally, 14 peptides showed high binding potential to the active sites of ACE. Henceforth, these results suggest that novel peptides derived from FM-571 with antihypertensive potential were bioavailable to exert their effect.

Keywords: ACE inhibitory peptides, simulated gastrointestinal digestion, fermented milk, everted gut, bioavailability.

INTRODUCTION

Recently, fermented milk (FM) with specific *Lactococcus lactis* was reported to present an antihypertensive effect[1-5]. In particular, FM with *L. lactis* NRRL B-50571 (FM-571) presented antihypertensive and hypocholesterolemic effects in spontaneously hypertensive rats[3]. Additionally, the antihypertensive effect in prehypertensive subjects treated with FM-571 was observed in a double-blind, randomized, controlled clinical study[4]. Furthermore, angiotensin-I converting enzyme (ACE) inhibition, nitric oxide production enhancement, and antioxidant effect were the mechanistic pathways established for the antihypertensive effect of FM-571[5]. However, which peptides may be responsible for this effect is still unknown since they need to show stability and reach target organs.

In this sense, bioaccessibility is the fraction of available peptides for absorption released from the food matrix during gastrointestinal digestion. Additionally, these peptides become bioavailable in the bloodstream after absorption and reach the target organ to exert a specific effect[6]. Over the last decades, different *in vitro* models have been developed to determine the bioaccessibility and bioavailability of other peptides. In this regard, simulated gastrointestinal digestion (SGD) models have been used to assess the bioaccessibility and stability of bioactive peptides after exposure to gastrointestinal proteases and peptidases[7], where the hydrolysis degree of proteins is reported around 53% corresponding to peptides with 6 – 10 amino acids[8-10]. These outcomes are comparable to digests obtained from *in vivo* tests[7, 11-13]. Moreover, to establish the bioavailability of peptides, the Caco-2 cell monolayer model is the most widely used; however, *ex vivo* tissue-based models (the Ussing chamber and the everted sac gut) have recently been employed[13], since they may better mimic the physiological process. Additionally, several studies have simultaneously determined bioaccessibility and bioavailability by using SGD followed by cell-based models[14]; however, it is still uncertain if cell-based models may mimic intestinal permeation[6]. Thus, SGD with an *ex vivo* tissue-based model may be a promising tool for assessing bioaccessibility and bioavailability.

On the other hand, integrating into *in vitro* studies with the 'omics' approach has recently gained interest. Several universal protein and peptide databases, such as UniProtKB, SwissProt, PepBank, and BIOPEP, predict peptides with potential bioactivity[15]. Additionally, knowledge about the possible binding between peptides and ACE is noteworthy to help elucidate which peptides are potentially responsible for the antihypertensive effect. Therefore, the present study aimed to determine the bioaccessibility and bioavailability of peptides with ACE inhibition obtained from FM with *L. lactis* NRRL B-50571 after being subjected to SGD and an *ex vivo* model. Additionally, peptide identification with HPLC/MS-MS was carried out, and an *in silico* analysis was performed to identify which peptide sequences may present ACE binding potential.

MATERIALS AND METHODS

Materials and chemicals

Pepsin (E. C. 3.4.23.1), pancreatin (EC: 232-468-9), bile salts, α-amylase (EC: 3.2.1.1), lysozyme (EC: 3.2.1.17), galactose, glucosamine and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO USA). M17 broth and lactose were purchased from DIFCO (Sparks, MD USA). The DC Lowry protein assay was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Acetonitrile was purchased from Honeywell (Charlotte, NC, USA) grade HPLC. All reagents used for the simulated fluids were analytical grade.

Strain and growth conditions

The growth and propagation of *Lactococcus lactis* NRRL B-50571 were carried out as previously reported by Rodríguez-Figueroa et al.[2]. *Lactococcus lactis* NRRL B-50571 was obtained from the Dairy Laboratory Collection at Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD, A.C., Hermosillo Sonora, México). First, sterilized M17 broth was prepared with lactose (10% w/v) and

inoculated with *Lactococcus lactis* NRRL B-50571 at (1% v/v). Then, tubes were incubated at 30 ºC for 24 h. This procedure was repeated twice to obtain a fresh pre-culture and reach a concentration of viable colonies between 10^6–10^7 UFC/mL. Finally, a fresh preculture was then inoculated (3% v/v) in reconstituted sterilized (110 ºC/10 min) skim milk (10% w/v) and incubated at 30 ºC for 12 h to obtain the working culture.

Fermented milk with *Lactococcus lactis* NRRL B-50571 (FM-571)

Skim commercial milk was reconstituted (10% w/v) and pasteurized at 80 ºC for 30 min, and after cooling, it was inoculated with the working culture (3% v/v). Afterward, it was incubated at 30 ºC for 48 h to obtain the FM. Then, fermentation was stopped by heat treatment at 75 ºC for 15 min and cooled in an ice bath[4]. Finally, samples were frozen at –20 ºC for further analysis.

Simulated gastrointestinal digestion model (SGD)

FM-571 or water (blank) was subjected to SGD, as previously reported by the validated method of Kopf-Bolanz et al.[8]. The process consisted of three phases: oral, gastric, and intestinal. First, samples (9.5 mL) were mixed with saliva solution (13 mL) and incubated for 5 min at pH 6.8, with orbital agitation (55 rpm) at 37 ºC. Afterward, gastric solution (25 mL) was added, pH was adjusted to 2.2, and incubated for 120 min at 37 ºC. After that, pancreatic (25 mL) and bile juices (12.5 mL) were added, pH was increased to 6.8, and incubated for 120 min at 37 ºC. After digestion, samples were cooled in an ice bath. Digestion enzymes were inactivated at 80 ºC for 10 min and then cooled. Finally, digested samples were frozen at -20 ºC until further analysis.

***Ex vivo* experimental protocol**

A total of twelve male Wistar rats (9 weeks old; 275 ± 10.5 g body weight (BW)) were randomly placed in individual stainless-steel cages at 22 ± 2 ºC, 12 h light-dark cycles, and 55 ± 3% relative humidity, with an *ad libitum* standard rodent diet (Labdiet 5008, México City, México) and purified water. After one week of adaptation, rats were randomized (p=0.98) into two groups of six rats (n = 6): 1) Water (negative control) and 2) FM-571. Then, with some modifications, the ex vivo assay was performed, as previously reported by Dixit et al.[16]. Rats were fasted for 24 h, anesthetized (sodic pentobarbital 40 mg/kg BW), and euthanized. A segment of 3 cm from the small intestine (jejune) was obtained, submerged in Krebs solution at 4 ºC, and carefully washed to remove the remaining intestinal content. Afterward, the intestinal segment was gently everted using a glass rod to avoid damage to the intestinal epithelium (**Figure 1**) and placed in a beaker of 100 mL containing 85 mL of each digested treatment. The basolateral (serosal compartment) was filled with 15 mL of Krebs solution. The beaker was placed in a water bath at 37 ºC and homogenized with a magnetic stirrer at 55 rpm. It was also aerated with 95% O_2 and 5% CO_2 (40 bubbles/min). After 45 min, the absorbed content on the serosal compartment was recovered and ultra-filtered (stirred ultrafiltration cell model 8050, Amicon, Bedford, MA USA) through 3 kDa cut-off membranes (Ultracell 3 kDa, Millipore, Billerica, MA, USA). The recovered fraction was filtered in a pore diameter of 0.20 µm (Millex Millipore, Billerica, MA, USA) and frozen to -20 ºC, for further analysis. The experimental protocol was approved by the Bioethics Committee of the Research Center for Food and Development (CIAD, A.C., Hermosillo Sonora, México) (CE/017/2019).

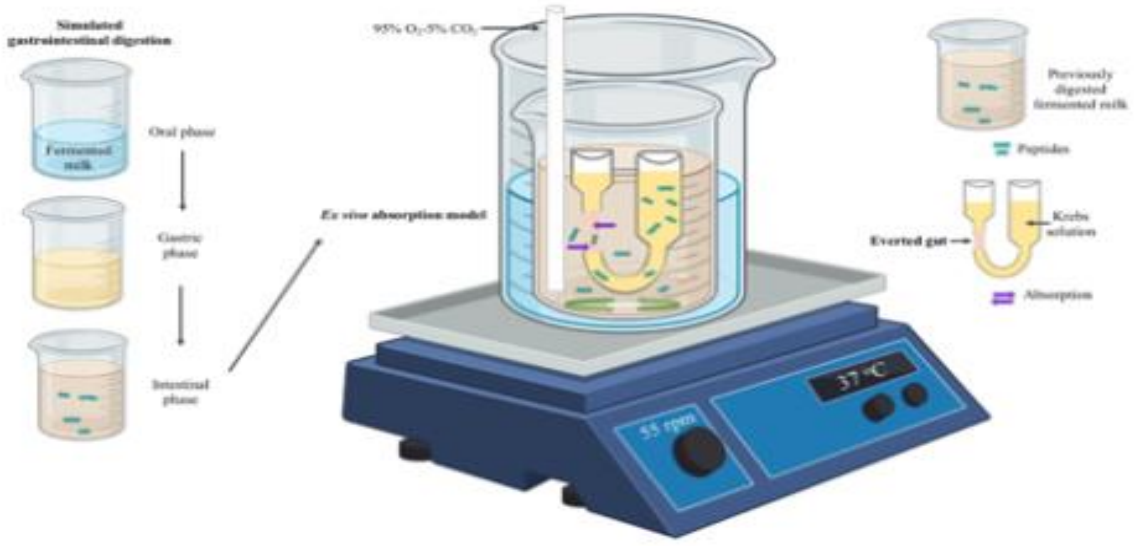

Figure 1. General scheme of the simulated gastrointestinal digestion and *ex vivo* everted gut model. Adapted from Álvarez-Olguín et al.[6].

Angiotensin I-converting enzyme inhibitory activity

ACE inhibitory activity was determined in FM-571, FM-571 subjected to SGD and FM-571 after SGD and *ex vivo* absorption model, as previously reported by Wu et al.[17] with modifications by Rendón-Rosales et al.[18]. Briefly, 2.17 mM hippuryl-histidyl-leucine (HHL) was used as substrate and prepared in a 100 mM sodium metaborate solution (containing 300 mM NaCl, pH 8.3). And the enzyme was prepared at 0.2 U/mL. All solutions and samples were tempered at 37 °C for 10 min before mixing, and 50 µL of HHL were added to 10 µL of the peptide absorbed sample and 10 µL of ACE solution and incubated for 40 min (37 °C, 450 rpm; Eppendorf thermomixer, Brinkmann instruments, NY, USA). The reaction was stopped with the addition of 85 µL of HCl 1 M, vortexed, and the released hippuric acid (HA) was analyzed by HPLC using a ZORBAX 300Extend-C18 (4.6 × 250 mm, 5 µm) column in an Agilent 1260 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with OpenLAB Chromatography

Data System version A.02.02 (Agilent Technologies, Germany). Solvent A was water, and solvent B was acetonitrile, containing 0.05% TFA. A gradient elution from 5% to 60% (solvent B in A) for 10 min was used. After 2 min, 60% solvent B in A was used, then decreased from 60% to 5% between 12-13 min, and was maintained for 5 min at 5%. The flow rate was adjusted to 0.5 mL/min for a sample injection of 20 µL and detection at 228 nm. ACE inhibitory activity was calculated using the equation: ACE inhibitory activity (%) = $(\frac{A-B}{A})$x100.

Where A was the peak area of HA because of the ACE reaction with the substrate HHL and B was peak area of HA after the ACE reaction with the substrate in the presence of the digested sample. The IC_{50} (peptide content necessary to inhibit ACE by 50%) was calculated. Based on the Lowry method, the peptide content was determined with the DC protein assay, and bovine serum albumin was used as the standard (7.25–500 µg/mL).

Peptide profiles by reverse-phase HPLC

Peptide profiles from the absorbed contents were analyzed by reverse-phase HPLC (1100 series; Agilent Technologies Japan Ltd., Tokyo, Japan) following the methodology by Torres-Llanez et al.[19] with the modifications of Reyes-Díaz et al.[20]. Injected sample (20 μL) was separated with an Eclipse AAA C-18 column (4.6 mm x 150 mm, 3.5 µm particle size, Agilent Technologies, Santa Clara, CA, USA) at a temperature of 30 ºC with a solvent flow of 0.25 mL/min. Solutions were eluted with solvent A (0.01% TFA in water) and solvent B (0.01% TFA in acetonitrile). Once equilibrated the column with solvent A, 20 µL of the sample was injected, and samples were eluted in a linear gradient of solvent B in solvent A from 0.1% to 60% for 30 min and of 60% to 99.9% between 30 and 35 min, finally from 99.9% to 0.1% between 35- and 40-min. Profile peptides were monitored at 214 nm of absorbance. Afterward, eight peptide fractions were collected and lyophilized for ACE inhibition determination and peptide identification analysis.

Additionally, bioavailability (%) was calculated as previously reported by Xie et al.[21] with modifications. In this regard, bioavailability was based on the area under the curve (AUC_1) of the peptide profiles from digested FM-571 and the AUC_2 from the absorbed content in the serosal compartment. Bioavailability (%) = (AUC_2/ AUC_1) x 100.

Analysis of peptides by Tandem Mass Spectrometry

Mass spectrometry 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 Zorbax 300SB-C18 (0.075 x 150 mm, 3.5 µm; Agilent Technologies Inc.). The sample injection volume was 1 µL, where Solvent A was a mixture of water-acetonitrile-formic acid (10:90:0.1, v/v/v) and solvent B contained water-acetonitrile-formic acid (97:3:0.1, v/v/v). The gradient was based on the increment of solvent A in B that started at 3% for 10 min; at 20 min, solvent A increased to 45%, 97% was reached at 35 min, finally, it decreased to 3% at 37 min. The sample injection volume was 1 µL with a flow rate of 0.5 µL/min directed into the mass spectrometer via an electrospray interface.

The flow was directed into the Mass Spectrometer via an electrospray ionization source. Nitrogen (99%) was used as nebulizing and drying gas and operated with an estimated helium pressure of $5x10^2$ Pa. The needle voltage was set to 4 kV, and the mass-charge ratio was 50-2200 (m/z). The signal threshold to perform auto MS analysis 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. Peptides sequences were obtained from mass spectrometry data using the Mascot server (UnitProtKB/Swiss-Prot database sequences).

Prediction of peptide sequences using *in silico* analysis

The database Peptide Ranker tool (http://distilldeep.ucd.ie/PeptideRanker/) was used to predict the probability of bioactivity on peptide sequences that were not previously reported as bioactive in the

BIOPEP database. Such prediction is based on the physicochemical characteristics of peptides where a threshold of 0.5 or higher was labeled as bioactive. Additionally, the chemical properties of these peptides, such as isoelectric point, charge, and hydrophobicity, were predicted using PepDraw (Tulane University, 2011). Furthermore, fractions with the lowest IC_{50} value were subjected to an *in silico* analysis with Pepsite 2 database (Pepsite 2) (http://pepsite2.russelllab.org) to predict specific peptide binding to ACE. Finally, the ACE (1O86) PDB code number from *homo sapiens* was used as an enzyme model; peptides with high binding potential were considered statistically significant when $p<0.01$.

Statistical analysis

Data are presented as means ± SD. Data normality was evaluated as a prerequisite before the one-way ANOVA test. Differences among means were assessed by Scheffe for multiple comparison tests and considered significant when $p<0.05$. Kruskal–Wallis test was performed for data that did not present a normal distribution and were considered significant when $p<0.05$; and are presented as medians. All data analyses were performed using the STATA 11 statistical program (version 11, StataCorp., College Station, TX, USA).

RESULTS AND DISCUSSION

Bioavailability of fermented milk with *L. lactis* NRRL B-50571

The chromatographic peptide profiles of FM-571 before and after SGD and after absorption are depicted in **Figure 2**. The peptide profile of FM-571 before being subjected to simulated gastrointestinal digestion showed a total area of 100,100 mAU. As expected, the total area ($p<0.05$) significantly increased to 158,660 mAU after SGD, indicating an increment in the peptide abundance of 58%, augmenting

bioaccessibility. However, after that, the total area significantly ($p<0.05$) decreased to 2,086.67 mAU after absorption in the serosal compartment. In fact, in the mucosal compartment, results showed a total area of 130,696 mAU for the unabsorbed peptides.

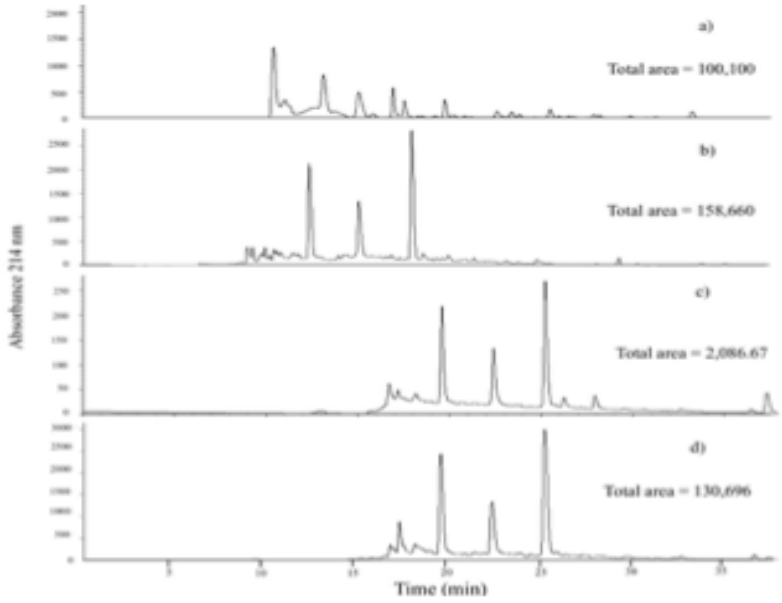

Figure 2. Chromatographic peptide profiles obtained by RP-HPLC from fermented milk with *Lactococcus lactis* NRRL B-50571 a) before and b) after simulated gastrointestinal digestion; and after the absorption process from the c) serosal and d) mucosal compartments.

Moreover, a calculated bioavailability of 1.36 ± 0.1% was observed after the *ex vivo* absorption model. Our results showed that peptide abundance decreased 76 times after absorption, potentially negatively affecting bioavailability. This difference may be due to peptide resistance to intestinal peptidases and, thus, affecting the absorption process.

It has been widely reported that bioactive peptides must be bioaccessible (released from the food matrix in the gastrointestinal tract) and bioavailable (absorbed in the intestinal lumen) to interact with a specific

organ and exert physiological effects[22]. In this regard, the gastrointestinal tract is a significant barrier for bioactive components such as peptides[23]; since it includes several peptidases that may affect bioavailability (Álvarez-Olguín et al., 2022)[6]. Therefore, several studies have been focused on simulating the absorption process with different models, mainly Caco-2 cells[23].

In this sense, Wang et al.[23] studied the bioavailability of antioxidant peptides from casein after SGD and the absorption process with the Caco-2 cell model. They reported a nitrogen peptide reduction after absorption. Nevertheless, Wang et al.[24] showed that the everted sac model presented higher brush border membrane peptidase-induced hydrolysis than the Caco-2 cells. Moreover, the Caco-2 cells model helps to elucidate mechanisms of absorption; however, they may also underestimate permeability, since they may not mimic the intestinal permeation like tissue-based models[6]. Henceforth, the present study determined bioavailability with the everted sac, a tissue-based model (*ex vivo*). Also, it has been reported that the bioavailability of antihypertensive peptides derived from dairy proteins is between 5 to 23% using the Caco-2 cell line monolayer as the absorption model[23]. While bioavailability of 5.1% was reported for specific peptides in *vivo* studies[25], the nanomolar concentration of peptides was also reported in human plasma[26].

ACE inhibitory activity in milk fermented with *L. lactis* NRRL B-50571

The ACE inhibitory activity and IC_{50} values of FM-571 before and after being subjected to SGD and after the absorption model are depicted in **Figure 3**. FM-571 showed 37.8 $\pm$ 14.65% ACE inhibition and significantly ($p<0.05$) increased after SGD, reaching 93.66 $\pm$ 14.65% of inhibition. However, after fraction absorption from the serosal compartment, ACE inhibition significantly ($p<0.05$) decreased to 32.87 $\pm$ 13.20%. Moreover, FM-571 showed the highest ($p<0.05$) IC_{50} value (2520 $\pm$ 979 μg/mL), and after SGD, the IC_{50} decreased to 1903 $\pm$ 148.49 μg/mL. Interestingly, after absorption, the IC_{50} significantly decreased ($p<0.05$) to 9.26 $\pm$ 0.83 μg/mL.

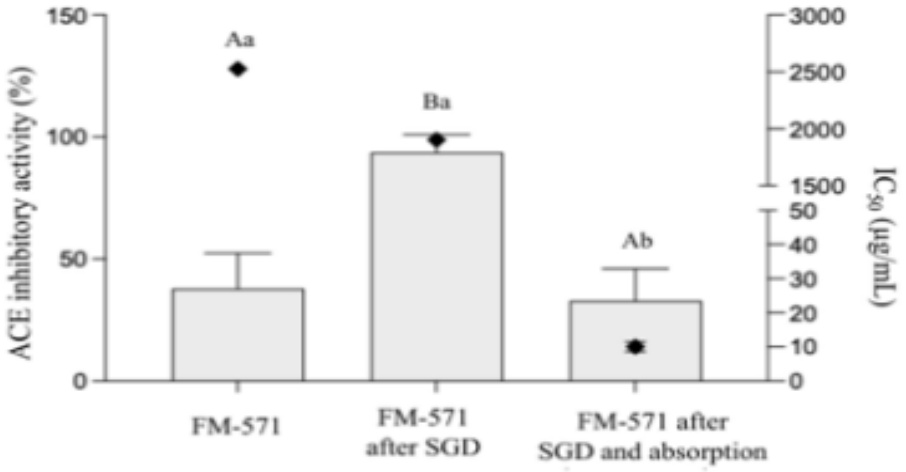

Figure 3. Angiotensin converting enzyme (ACE) inhibitory activity (%, bar) and IC_{50} (□) from fermented milk with *Lactococcus lactis* NRRL B-50571 (FM-571) before and after simulated gastrointestinal digestion (SGD), and after absorption. Data are presented as means ± SD. Different letters indicate significant differences ($p<0.05$) between samples for ACE inhibitory activity (uppercase) and IC_{50} (lowercase).

Similarly, several studies have determined the stability or resistance of ACE inhibitory peptides in fermented milk after SGD[27]. For example, an increased ACE inhibition was observed in fermented whey protein (reaching 100% inhibition)[28], commercial fermented milk (2 to 3-fold)[29], kefir (8-fold)[30] and fermented milk (1.2-fold)[31] after being subjected to SGD. Thus, this may suggest that gastrointestinal digestion may enhance the release of new active ACE inhibitory peptides. On the other hand, peptides may also resist degradation by gastrointestinal enzymes; specifically with proline in their sequence[32].

Additionally, other studies have determined the stability and concentration of ACE inhibitory peptides after the absorption process[33-37]; and to the best of our knowledge, there are no studies that determine

ACE inhibition after this process. Moreover, after absorption, peptides may be further hydrolyzed by peptidases in the enterocytes and serum proteases, affecting their bioactivity[38]. Therefore, after absorption, the chromatographic peptide profile from FM-571 was subjected to further fractionation (F1 to F8; **Figure 4**), and ACE inhibition was determined (**Figure 5**). Results showed that F2, F3, F4, and F7 showed the highest ($p<0.05$) ACE inhibition, followed by F1, F6 and F8, and were significantly ($p<0.05$) different between them. In fact, F1, F7 and F8 showed the lowest ($p<0.05$) IC_{50}; followed by F2 and F4.

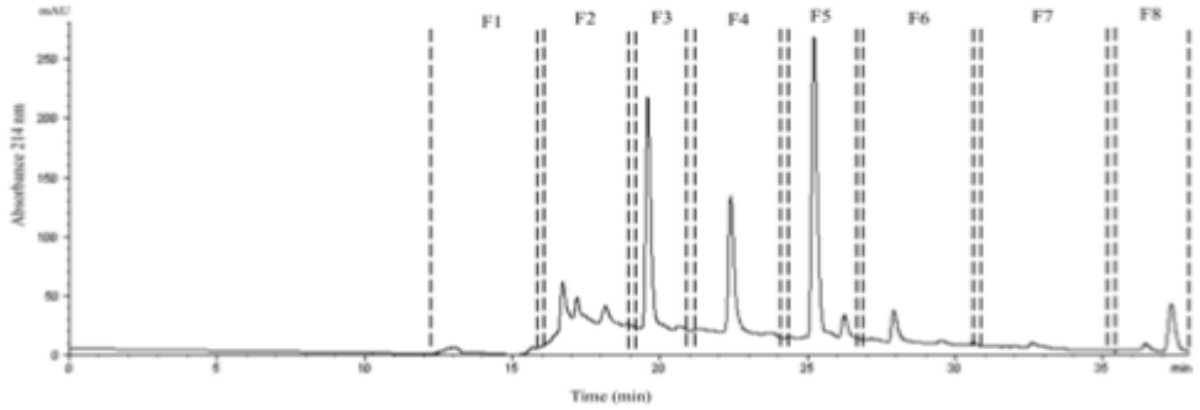

Figure 4. Peptide fraction profile (F1−F8) from water-soluble extracts (< 3 kDa) obtained from fermented milk with *Lactococcus lactis* NRRL B-50571 after the absorption process (basolateral compartment).

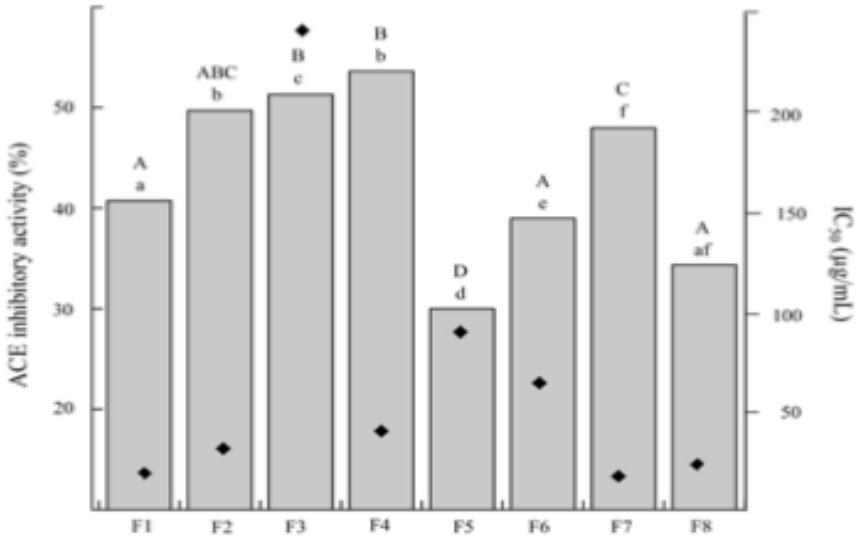

Figure 5. Angiotensin converting enzyme (ACE) inhibitory activity (%, bar) and IC_{50} (□) from collected fractions from fermented milk with *Lactococcus lactis* NRRL B-50571 after *ex vivo* absorption model. Data are presented as median and were analyzed by non-parametric test (Kruskal–Wallis $p<0.05$). Different letters indicate significant differences ($p<0.05$) between fractions for ACE inhibitory activity (uppercase) and IC_{50} (lowercase).

Recently, structure-activity studies have suggested that the ACE inhibitory activity of peptides is strongly related to the number and specific amino acid residues in their sequence, particularly in the hydrophobic amino acids ratio[39]. Also, it has been widely reported that amino acids in the C-terminal position are the most viable to bind ACE, specifically aromatic and hydrophobic residues and proline[32]. Additionally, peptides with 2 to 12 amino acid residues contribute to ACE inhibitory activity since they may easily bond to the active sites of ACE than larger peptides[40]. On the other hand, Gleeson et al.[37] reported that ACE inhibitory peptides containing proline were highly permeable and may be absorbed into the small

intestinal epithelium. Therefore, in the present study, these specific amino acid sequences may be present and absorbed into the basolateral compartment and be responsible for the ACE inhibitory activity. Hence, these fractions' bioavailable potential ACE inhibitory peptides were further identified with LC-ESI-MS.

Identification of peptides by Tandem Mass Spectrometry

Overall, 113 peptides were identified in the serosal compartment after absorption (**Table 1**). These digested and absorbed peptides were from the native milk proteins β-casein (33%), αS1-casein (16%), κ-casein (12%), β-lactoglobulin (10%), lactotransferrin (9%), αS2-casein (8%), α-lactalbumin (7%) and serotransferrin (5%). Additionally, these peptides presented a mass ranging from 207.90 to 1173.80 Da and showed from 2 to 10 amino acid residues in their sequence. **Figure 6** depicts a typical MS/MS spectrum of peptides identified in F4 (SDIPNPI) and F5 (FPIIV). Moreover, the structural characteristics and the physicochemical properties of peptides establish the mechanism of absorption[13], the potential function and the bioactivity they may provide[41-42].

In this regard, it has been widely reported that peptide absorption may be through three different mechanisms: paracellular transport via tight junctions, protein carriers (*e.g.* PepT1), and transcytosis via endocytosis[6]. Specifically, PepT1 shows a preference with neutral di and tri peptides[43]; and in the present study, only 7 peptides were neutral. Furthermore, although differences in the transport of negatively and positively charged peptides have been hypothesized, it is still unclear which peptide charge is more favorable[44]. However, authors have suggested that hydrophilic and negatively charged peptides may be absorbed via paracellular transport, while hydrophobic and positively charged peptides may be absorbed via transcytosis[44-45]. In particular, results showed that 22 peptides were positively charged from the serosal compartment, which may have been absorbed through transcytosis. Meanwhile, 40 negatively charged peptides were also identified and may have been absorbed by paracellular transport.

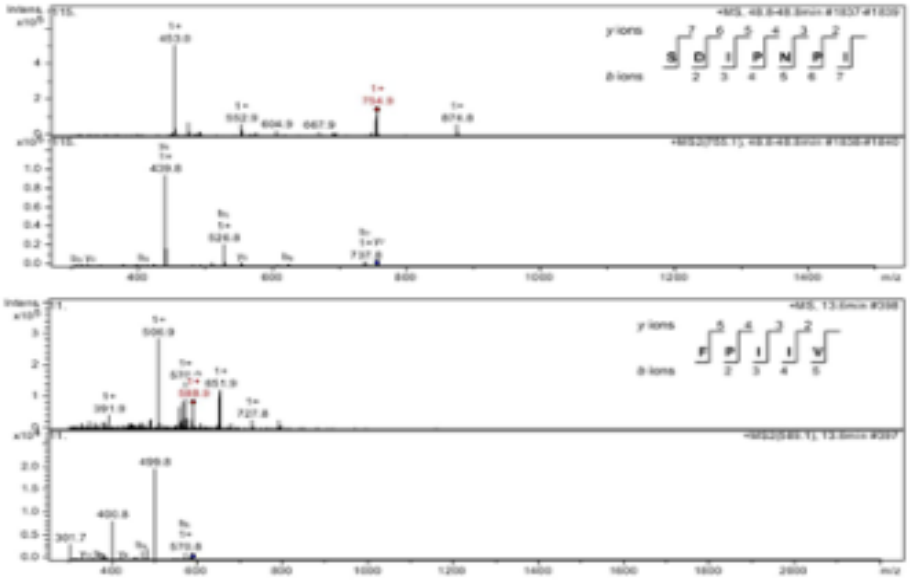

Figure 6. Tandem mass (MS/MS) spectra of ion *m/z* a) 754.9 and b) 588.9 from fractions 4 and 5. Following sequence interpretation and database searching, the spectrum was matched to SDIPNPI and FPIIV, respectively.

In fact, 71 peptides showed proline in their sequence, which may have also favored resistance to degradation and permeability[32,37]. Conversely, nine different peptides from F1, F5, F6, F7 and F8 were further hydrolyzed during absorption; specifically, QLPLPPT, NVPGEIVE, SKVLPVP, NAVPITPT, EIVPNSVEQK, LPLPLLQ, DIPNPI, DIGSESTE and VSLP; and the resulting peptides were also absorbed. Hence, brush border peptidases may have degraded these peptides[46]. Similarly, VLPVPQ from β-CN (f170-1745) and LNVPGE from β-CN (f6-12) were reported after SGD of casein with the INFOGEST Protocol. When brush border membrane peptidases isolated from porcine jejune were added, they were hydrolyzed to minor fragments[68].

Table 1. Identified peptides obtained from the absorbed fractions from fermented milk with *Lactococcus lactis* NRRL-B50571

Fraction	Exp. Mass	Molecular ion (*m/z*) selected for MS/MS (charge)	Suggested fragment of protein	Sequence	Isoelectric point (pI)	Charge net	Hydrophobicity	Peptide ranker	Reference
F1	324.78	325.79 (+1)	κ-CN (f139-141), β-CN (f89-91)	IPP	5.62	0	+7.06 Kcal/mol	0.76	47-48
	324.77	325.78 (1+)	β-CN (f166-168)	LPP	5.63	0	+6.93 Kcal/mol	0.82	49
	452.50	453.51 (+1)	β-CN (f164-167)	QPLP	5.47	0	+7.7 KCal/mol	0.75	
	314.70	315.71 (1+)	β-CN (f118-120)	APK	9.8	+1	+111.34 Kcal/mol	0.31	50
	445.50	446.51 (1+)	β-Lg (f44-47)	DISL	2.95	-1	+9.63 Kcal/mol	0.23	
	385.79	386.80 (1+)	κ-CN (f84-87)	KPAA	10.21	+1	+11.84 Kcal/mol	0.25	
	1391.81	464.95 (3+)	κ-CN (f106-118)	AKSCQAQPTTMAR	9.82	+2	15.96 Kcal/mol	0.40	
	427.64	428.65 (1+)	κ-CN (f176-179)	SPPE	3.13	-1	+12.27 Kcal/mol	0.41	
F2	324.87	325.88 (1+)	β-CN (f155-157)	PLP	5.25	0	+6.93 Kcal/mol	0.86	51
	451.16	452.17 (1+)	α_{s2}-CN (f220-222)	RYL	9.91	+1	+7.75 Kcal/mol	0.56	38

	295.24	148.63 $^{(2+)}$	α_{s1}-CN (f106-107; 109-110), α_{s2}-CN (f110-111; 113-114; 194-195; 221-222), β-Lg (f118-119), Serotransferrin (f265-266; 337-338; 417-418; 449-450; 675-676), Lactotransferrin (f127-128; 311-312; 316-317; 425-426; 652-653)	YL	5.45	0	+5.94 Kcal/mol	0.57	
	295.24	148.63 $^{(2+)}$	α_{s1}-CN (f114-115), β-CN (f207-208), Serotransferrin (f153-154; 336-337; 418-419; 490-491)	LY	5.48	0	+5.94 Kcal/mol	0.51	[52]
	295.28	148.65 $^{(2+)}$	Lactotransferrin (f73-74; 391-392)	IY	5.48	0	+6.07 Kcal/mol	0.31	
	385.60	193.81 $^{(2+)}$	κ-CN (f29-31)	PIR	10.73	+1	+8.73 Kcal/mol	0.60	
	363.76	464.77 $^{(1+)}$	β-CN (f67-69)	FAQ	5.38	0	+7.46 Kcal/mol	0.66	
	385.60	193.81 $^{(2+)}$	β-Lg (f54-56)	PLR	10.73	+1	+8.60 Kcal/mol	0.74	
	314.82	315.83 $^{(1+)}$	β-CN (f97-99)	VVV	5.63	0	+6.52 Kcal/mol	0.02	
	473.96	474.97 $^{(1+)}$	β-Lg (f30-34)	KVAGT	9.82	+1	+12.14 Kcal/mol	0.07	[53]
	698.04	350.03 $^{(2+)}$	β-Lg (f105-110)	ENKVLV	6.81	0	+13.01 Kcal/mol	0.06	
	403.52	202.77 $^{(2+)}$	Lactotransferrin (f153-155)	IDR	6.42	0	+12.23 Kcal/mol	0.23	
	344.12	173.07 $^{(2+)}$	Lactotransferrin (f341-344)	AVGP	5.65	0	+9.23 Kcal/mol	0.34	

	302.76	303.77 $^{(1+)}$	Serotransferrin (f676-678)	LGD	3.12	-1	+11.44 Kcal/mol	0.33	
F4	753.85	754.86 $^{(1+)}$	α_{s1}-CN (f195-201)	SDIPNPI	3.05	-1	+10.89 Kcal/mol	0.57	
	613.92	614.93 $^{(1+)}$	κ-CN (f26-30)	QEQPI	3.12	-1	+12.09 Kcal/mol	0.16	
	754.03	755.04 $^{(1+)}$	κ-CN (f67-73)	KPVALIN	9.63	+1	+ 9.36 Kcal/mol	0.23	
	586.21	587.22 $^{(1+)}$	κ-CN (f101-106)	SNTVPA	5.46	0	+9.64 Kcal/mol	0.18	
	654.85	655.86 $^{(1+)}$	κ-CN (f176-181)	SPPEIN	3.13	-1	+12 Kcal/mol	0.42	[54]
	514.01	515.02 $^{(1+)}$	β-CN (f22-26)	NVPGE	3.11	-1	+13.21 Kcal/mol	0.14	[54]
	726.83	727.98 $^{(1+)}$	β-CN (f22-28)	NVPGEIV	3.11	-1	+11.63 Kcal/mol	0.10	[54]
	469.97	470.98 $^{(1+)}$	β-CN (f87-90)	QNIP	5.47	0	+8.54 Kcal/mol	0.33	[54]
	440.05	441.06 $^{(1+)}$	β-CN (f130-133)	PVEP	3.01	-1	+11.35 Kcal/mol	0.17	[55]
	677.92	678.93 $^{(2+)}$	α_{s1}-CN (f23-28)	HQGLPQ	7.59	0	+11.81 Kcal/mol	0.29	[54]
	673.85	674.86 $^{(1+)}$	α_{s1}-CN (f99-104)	EDVPSE	2.79	-3	+18.94 Kcal/mol	0.06	
	1173.80	587.91 $^{(2+)}$	α_{s1}-CN (f109-117)	YLEQLLRLK	9.47	+1	+11.20 Kcal/mol	0.34	
	651.96	652.97 $^{(1+)}$	α_{s1}-CN (f188-193)	YTDAPS	3.05	-1	+12.18 Kcal/mol	0.12	
	976.78	489.40 $^{(2+)}$	α_{s1}-CN (f45-53)	PSKENLCST	6.14	0	+15.22 Kcal/mol	0.28	
	1042.80	522.41 $^{(2+)}$	β-Lg (f99-107)	KIDALNENK	6.64	0	+20.60 Kcal/mol	0.11	[56]
	802.79	803.80 $^{(1+)}$	β-Lg (f141-147)	TPEVDDE	2.7	-4	+22.77 Kcal/mol	0.04	[57]

F5	573.83	574.84 (3+)	β-CN (f172-176)	FPPQS	5.39	0	+7.70 Kcal/mol	0.7	58
	587.82	588.83 (3+)	β-CN (f220-224)	FPIIV	5.46	0	+3.63 Kcal/mol	0.62	
	650.87	651.88 (3+)	β-CN (f164-169)	QPLPPT	5.35	0	+8.09 Kcal/mol	0.67	59
	522.86	523.87 (3+)	β-CN (f165-169)	PLPPT	5.17	0	+7.32 Kcal/mol	0.71	
	563.78	564.79 (3+)	β-Lg (f166-170)	SFNPT	5.36	0	+7.89 Kcal/mol	0.64	
	558.83	559.84 (3+)	κ-CN (f138-142)	TEIPT	3.08	-1	+11.05 Kcal/mol	0.08	
	755.75	756.76 (3+)	κ-CN (f176-182)	SPPEINT	3.13	-1	+12.25 Kcal/mol	0.34	
	626.88	627.89 (3+)	β-CN (f21-26)	LNVPGE	3.2	-1	+11.96 Kcal/mol	0.16	54
	854.83	855.84 (3+)	β-CN (f22-29)	NVPGEIVE	2.94	-2	+15.96 Kcal/mol	0.07	54
	740.96	741.97 (3+)	β-CN (f23-29)	VPGEIVE	3.06	-2	+14.41 Kcal/mol	0.08	54
	512.93	513.94 (3+)	β-CN (f23-27)	VPGEI	3.23	-1	+11.24 Kcal/mol	0.18	54
	583.94	584.95 (3+)	β-CN (f103-107)	LQPEV	3.2	-1	+10.73 Kcal/mol	0.11	60
	602.82	603.83 (3+)	β-CN (f129-133)	YPVEP	3.14	-1	+10.64 Kcal/mol	0.24	61
	578.73	579.74 (3+)	β-CN (f133-137)	PFTES	3.01	-1	+10.67 Kcal/mol	0.20	
	556.04	557.05 (3+)	β-CN (f153-157)	PLLQS	5.18	0	+ 6.77 Kcal/mol	0.21	
	739.82	370.92 (2+)	β-CN (f183-189)	SKVLPVP	10.59	+1	+9.27 Kcal/mol	0.25	

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	650.90	651.91 (1+)	β-CN (f185-190)	VLPVPQ	5.52	0	+6.78 Kcal/mol	0.21	62
	551.88	552.89 (1+)	β-CN (f186-190)	LPVPQ	5.48	0	+7.24 Kcal/mol	0.32	59
	633.77	634.78 (1+)	β-CN (f208-212)	YQEPV	3.14	-1	+11.27 Kcal/mol	0.14	
	810.83	811.84 (1+)	α_{s1}-CN (f130-137)	NAVPITPT	5.32	0	+8.35 Kcal/mol	0.21	
	612.91	613.92 (1+)	α_{s1}-CN (f130-135)	NAVPIT	5.32	0	+8.06 Kcal/mol	0.20	
	499.87	500.88 (1+)	α_{s1}-CN (f131-135)	AVPIT	5.49	0	+7.21 Kcal/mol	0.19	
	1139.74	570.88 (2+)	α_{s1}-CN (f85-94)	EIVPNSVEQK	4.08	-1	+18.14 Kcal/mol	0.07	
	569.88	570.89 (1+)	α_{s1}-CN (f85-89; 125-129)	EIVPN	3.09	-1	+10.94 Kcal/mol	0.11	63
	1039.74	520.88 (2+)	β-Lg (f59-67)	VEELKPTPE	3.8	-2	+20.41 Kcal/mol	0.07	64
	850.05	851.06 (1+)	β-Lg (f170-176)	TQLEEQC	2.92	-2	+15.68 Kcal/mol	0.05	
	600.77	601.78 (1+)	α-La (f74-78)	INNKI	10.15	+1	+10.16 Kcal/mol	0.12	65
	353.63	354.64 (1+)	α-La (f125-127)	AHK	9.8	+1	+13.53 Kcal/mol	0.13	
	903.66	452.84 (2+)	α-La (f131-137)	SEKLDQW	4	-1	+15.86 Kcal/mol	0.27	
F6	967.87	968.88 (1+)	β-CN (f21-29)	LNVPGEIVE	3.03	-2	+14.01 Kcal/mol	0.07	54
	574.90	575.91 (1+)	β-CN (f149-153)	HLPLP	8.32	0	+8.01 Kcal/mol	0.75	25
	437.92	438.93 (1+)	β-CN (f150-153)	LPLP	5.63	0	+5.68 Kcal/mol	0.79	25
	551.86	552.87 (1+)	β-CN (f150-154)	LPLPL	5.58	0	+4.43 Kcal/mol	0.81	25

	224.94	225.95 (3+)	α-La (f 15-16); α_{s1}-CN (f143-144): Serotransferrin (f272-273; 293-294; 611-612); Lactotransferrin (f245-246; 587-588)	HA	7.95	0	+10.53 Kcal/mol	0.18	
	437.95	438.96 (3+)	β-CN (f161-154)	PLPL	5.23	0	+5.68 Kcal/mol	0.84	
	666.80	667.81 (3+)	α_{s1}-CN (f196-201)	DIPNPI	2.95	-1	+10.43 Kcal/mol	0.54	
	551.90	552.91 (3+)	α_{s1}-CN (f197-201)	IPNPI	5.57	0	+6.79 Kcal/mol	0.55	
	207.90	208.91 (+1)	α-La (f95-96); κ-CN (f108-109); Serotransferrin (f56-57; 135-136; 175-176; 440-441; 472-473); Lactotransferrin (f106-107; 148-149; 448-449; 472-473; 482-483; 578-579)	SC	5.13	0	+8.34 Kcal/mol	0.62	
	558.86	559.87 (3+)	κ-CN (f98-102)	QVLSN	5.25	0	+8.27 Kcal/mol	0.07	
	739.80	740.81 (3+)	β-CN (f21-27)	LNVPGEI	3.2	-1	+10.84 Kcal/mol	0.15	
	605.88	606.89 (3+)	β-CN (f96-101)	PVVVPP	5.25	0	+6.94 Kcal/mol	0.26	
	860.82	861.83 (3+)	β-CN (f177-184)	VLSLSQSK	9.8	+1	+9.89 Kcal/mol	0.08	
	522.95	523.96 (3+)	β-CN (f185-189)	VLPVP	5.69	0	+6.01 Kcal/mol	0.32	[66]
	816.85	817.87 (3+)	α_{s1}-CN (f15-21)	ARPKHPI	11.55	+2	+14.50 Kcal/mol	0.46	
	473.86	474.87 (3+)	α_{s1}-CN (f52-55)	VNEL	3.23	-1	+10.67 Kcal/mol	0.06	
	543.84	544.85 (3+)	α_{s1}-CN (f195-199)	SDIPN	3.05	-1	+11.87 Kcal/mol	0.39	
	674.84	675.85 (3+)	α_{s1}-CN (f115-120)	YQGPIV	5.45	0	+7.67 Kcal/mol	0.32	
	511.98	512.99 (3+)	α_{s1}-CN (f130-134)	NAVPI	5.41	0	+7.81 Kcal/mol	0.22	

6

	476.03	474.05 (3+)	β-Lg (f83-86)	AQKK	10.57	+2	+14.77 Kcal/mol	0.09	
	678.03	679.40 (3+)	Lactotransferrin (f195-201)	AGDVAFV	3.13	-1	+11.06 Kcal/mol	0.42	
F7	343.62	172.82 (2+)	κ-CN (f183-185)	VQV	5.63	0	+7.75 Kcal/mol	0.03	
	263.72	132.87 (2+)	α_{s1}-CN (f46-47); α_{s2}-CN (f161-162)	VF	5.56	0	+5.73 Kcal/mol	0.81	
	187.84	188.85 (1+)	α_{s1}-CN (f25-26); β-Lg (f25-26); α-La (f70-71); κ-CN (f60-61); Serotransferrin (f83-84; 89-90; 139-140; 425-426; 488-489); Lactotransferrin (f110-111; 398-399; 437-438; 464-465; 503-504)	GL	5.6	0	+7.80 Kcal/mol	0.8	
	385.09	193.56 (2+)	β-Lg (f138-140)	LVR	10.73	+1	+8.00 Kcal/mol	0.14	
	1151.33	384.79 (3+)	β-CN (f160-169)	HQPHQPLPPT	7.73	0	+13.66 Kcal/mol	0.63	67
	374.91	375.92 (1+)	β-CN (f220-222)	FPI	5.46	0	+5.21 Kcal/mol	0.93	
	493.83	494.84 (1+)	α_{s1}-CN (f173-177)	AYPSG	5.58	0	+9.44 Kcal/mol	0.51	
	706.25	707.26 (1+)	β-CN (f133-138)	PFTESQ	3.01	-1	+11.44 Kcal/mol	0.15	
	721.80	361.91 (2+)	α-La (f21-26)	QLTKCE	6.16	0	+14.08 Kcal/mol	0.09	
	841.86	842.87 (1+)	α-La (f63-69)	NNDSTEY	2.89	-2	+16.87 Kcal/mol	0.09	
	836.00	837.01 (1+)	α_{s1}-CN (f58-65)	DIGSESTE	2.74	-3	+20.00 Kcal/mol	0.06	
	722.10	362.06 (2+)	α_{s1}-CN (f60-66)	GSESTED	2.91	-3	+21.12 Kcal/mol	0.06	

	344.82	345.83 $^{(1+)}$	α_{s1}-CN (f55-57); Lactotransferrin (f263-265)	LSK	9.8	+1	9.91 Kcal/mol	0.12	
	443.97	443.97 $^{(1+)}$	Lactotransferrin (f247-250); Serotransferrin (f274-277)	VVAR	10.73	+1	+9.29 Kcal/mol	0.06	
F8	791.63	396.83 $^{(2+)}$	β-CN (f150-156)	LPLPLLQ	5.48	0	+3.95 Kcal/mol	0.57	
	474.61	159.21 $^{(3+)}$	Lactotransferrin (f161-164)	LCQL	5.21	0	+6.15 Kcal/mol	0.57	
	1049.71	350.91 $^{(3+)}$	κ-CN (f117-125)	ARHPHPHLS	10.85	+1	+16.69 Kcal/mol	0.47	
	388.62	130.55 $^{(3+)}$	β-CN (f38-40)	ITR	10.73	+1	+8.84 Kcal/mol	0.12	
	514.98	172.67 $^{(3+)}$	β-CN (f44-47)	KIEK	9.63	+1	+16.01 Kcal/mol	0.04	
	549.09	184.04 $^{(3+)}$	β-CN (f114-118)	KEAMA	6.86	0	+14.66 Kcal/mol	0.11	
	705.56	383.79 $^{(2+)}$	α_{s1}-CN (f58-64)	DIGSEST	2.82	-2	+16.37 Kcal/mol	0.07	
	636.59	160.16 $^{(4+)}$	α_{s1}-CN (f196-200)	KTVYQ	9.51	+1	+10.55 Kcal/mol	0.04	
	414.90	415.91 $^{(1+)}$	α-La (f40-43)	VSLP	5.69	0	+6.79 Kcal/mol	0.24	
	443.61	444.62 $^{(1+)}$	α-La (f41-44)	SLPE	3.13	-1	+10.88 Kcal/mol	0.21	
	707.24	354.63 $^{(2+)}$	Lactotransferrin (f216-220)	RDQYE	4	-1	+17.04 Kcal/mol	0.10	

Lactoalbulmin: La, lactoglobulin: Lg, casein: CN

Additionally, it has been reported that specific amino acid residues in the N-terminal of tetrapeptides, such as leucine, methionine, valine, isoleucine, and cysteine, may show high permeability[13]. Notably, in this study, four tetrapeptides presented leucine in the N-terminal. Finally, it has been suggested that oligopeptides (tetrapeptides or longer peptides) may not be easily transported into the bloodstream; however, other studies have evidenced their presence in plasma[69]. In this sense, 89 oligopeptides (≥ 4 amino acid residues) were identified in the serosal compartment; nevertheless, these peptides must also show resistance to serum proteases in the bloodstream and exert the physiological effect[38].

On the other hand, 110 unabsorbed peptides were identified in the mucosal compartment (**Table 2).** Interestingly, 21 peptides identified in the serosal compartment were also present in the mucosal compartment. Moreover, 48 identified peptides were further hydrolyzed into smaller fragments, although not absorbed. Recent studies have suggested that peptides may play a role in the modulation of gut microbiota through different mechanisms[70]. In this regard, peptides may enhance the production of mucin[71-72], may affect the biochemical response during microbiome-host or microbiome-microbiome interactions[70], act as prebiotics[73] or provide essential carbon and nitrogen[74]. Overall, this modulation may affect microbial diversity and composition of gut microbiota[75]. Also, gut dysbiosis has been associated with different diseases such as hypertension. Therefore, it has been suggested that fermented dairy products may improve gut microbiota balance[76]. In fact, it has been suggested that kefir's attenuation of elevated blood pressure was associated with restoring intestinal structure and reducing neuroinflammation[77]. Henceforth, it is noteworthy to determine further how fermented milk and its peptidic fractions in this study may affect gut microbiota and diminish hypertension.

Furthermore, each absorbed peptide's probability of possessing bioactivity was analyzed with the Peptide Ranker program as a predictor (**Table 1**). In this regard, 23 peptides showed potential bioactivity presenting score values >0.5 in F1 (IPP and QPLP), F2 (RYL, YL, LY, PIR, FAQ, and PLR), F4

(SDIPNPI), F5 (FPPQS, FPIIV, QPLPPT, PLPPT and SFNPT), F6 (HLPLP, LPLP, DIPNPI, IPNPI, and SC), F7 (HQPHQPLPPT and AYPSG) and F8 (LPLPLLQ and LCQL). Meanwhile, three previously reported peptides exhibited predicted bioactive scores >0.8 in F1 (LPP), F2 (PLP), and F6 (LPLPL). Interestingly, four novel peptides displayed high predicted scores (>0.8) from F6 (PLPL) and F7 (VF, GL and FPI). It has been reported that the closer the predicting score is to 1, the more likely the peptide will be bioactive[78].

Table 2. Identified peptides obtained from the not absorbed (mucosal compartment) from fermented milk with *Lactococcus lactis* NRRL-B50571

Exp. Mass	Molecular ion (*m/z*) selected for MS/MS (charge)	Suggested fragment of protein	Sequence
374.99	376.00 $^{(1+)}$	κ-CN (f16-18)	LPF
385.48	193.75 $^{(2+)}$	κ-CN (f18-21)	LGAQ
385.26	193.64 $^{(2+)}$	κ-CN (f29-31)	PIR
504.13	505.14 $^{(1+)}$	κ-CN (f46-49)	YIPI
976.70	489.36 $^{(2+)}$	κ-CN (f48-55)	PIQYVLSR
632.55	211.86 $^{(3+)}$	κ-CN (f53-57)	LSRYP
1057.86	529.94 $^{(2+)}$	κ-CN (f54-62)	SRYPSYGLN
626.95	626.96 $^{(1+)}$	κ-CN (f68-73)	PVALIN
503.00	504.01 $^{(1+)}$	κ-CN (f75-78)	QFLP
634.87	635.88 $^{(1+)}$	κ-CN (f76-80)	FLPYP
1190.74	596.38 $^{(2+)}$	κ-CN (f08-118)	SCQAQPTTMAR
330.13	331.14 $^{(1+)}$	κ-CN (f140-142)	IPT
659.70	330.86 $^{(2+)}$	κ-CN (f141-146)	PTINTI
1057.96	529.99 $^{(2+)}$	κ-CN (f145-155)	TIASGEPTSTP
678.04	679.05 $^{(1+)}$	κ-CN (f1148-154)	SGEPTST

884.80	885.81 $^{(1+)}$	κ-CN (f175-182)	ESPPEINT
428.81	429.82 $^{(1+)}$	κ-CN (f176-179)	SPPE
541.08	542.09 $^{(1+)}$	κ-CN (f176-180)	SPPEI
455.11	456.12 $^{(1+)}$	κ-CN (f177-180)	PPEI
755.94	756.95 $^{(1+)}$	κ-CN (f176-182)	SPPEINT
626.84	627.85 $^{(1+)}$	β-CN (f21-26)	LNVPGE
627.99	628.00 $^{(1+)}$	β-CN (f22-27)	NVPGEI
726.85	727.86 $^{(1+)}$	β-CN (f22-28)	NVPGEIV
854.86	855.87 $^{(1+)}$	β-CN (f22-29)	NVPGEIVE
728.90	729.91 $^{(1+)}$	β-CN (f24-30)	PGEIVES
617.94	618.95 $^{(1+)}$	β-CN (f58-62)	DELQD
620.99	622.00 $^{(1+)}$	β-CN (f74-78)	VYPFP
887.75	888.76 $^{(1+)}$	β-CN (f74-81)	VYPFPGPI
522.08	523.09 $^{(1+)}$	β-CN (f75-78)	YPFP
788.94	789.95 $^{(1+)}$	β-CN (f75-81)	YPFPGPI
625.90	626.91 $^{(1+)}$	β-CN (f76-81)	PFPGPI
529.92	529.92 $^{(1+)}$	β-CN (f77-81)	FPGPI
626.09	627.10 $^{(1+)}$	β-CN (f77-82)	FPGPIP
381.80	382.81 $^{(1+)}$	β-CN (f78-81)	PGPI
381.82	382.83 $^{(1+)}$	β-CN (f79-82)	GPIP
638.93	640.79 $^{(1+)}$	β-CN (f81-86)	IPNSLP
1106.14	554.08 $^{(2+)}$	β-CN (f86-95)	PQNIPPLTQT
551.90	552.91 $^{(1+)}$	β-CN (f88-92)	NIPPL
653.01	654.02 $^{(1+)}$	β-CN (f88-93)	NIPPLT

438.01	439.02 $^{(1+)}$	β-CN (f89-92)	IPPL
325.88	325.89 $^{(1+)}$	β-CN (f90-92)	PPL
757.06	758.07 $^{(1+)}$	β-CN (f92-98)	LTQTPVV
838.73	839.74 $^{(1+)}$	β-CN (f93-100)	TQTPVVVP
854.85	855.86 $^{(1+)}$	β-CN (f95-102)	TPVVVPPF
606.02	607.03 $^{(1+)}$	β-CN (f96-101)	PVVVPP
753.03	754.04 $^{(1+)}$	β-CN (f96-102)	PVVVPPF
602.96	603.97 $^{(1+)}$	β-CN (f129-133)	YPVEP
586.94	587.95 $^{(1+)}$	β-CN (f130-134)	PVEPF
1151.52	576.77 $^{(2+)}$	β-CN (f142-151)	LTDVENLHLP
473.82	747.83 $^{(1+)}$	β-CN (f145-148)	VENL
577.86	193.63 $^{(3+)}$	β-CN (f149-153)	HLPLP
438.06	439.07 $^{(1+)}$	β-CN (f150-153)	LPLP
552.01	553.02 $^{(1+)}$	β-CN (f150-154)	LPLPL
438.08	439.10 $^{(1+)}$	β-CN (f151-154)	PLPL
650.91	651.92 $^{(1+)}$	β-CN (f164-184)	QPLPPT
522.94	523.95 $^{(1+)}$	β-CN (f165-184)	PLPPT
945.68	473.85 $^{(2+)}$	β-CN (f175-183)	QSVLSLSQS
945.74	473.88 $^{(2+)}$	β-CN (f176-184)	SVLSLSQSK
425.99	427.00 $^{(1+)}$	β-CN (f185-188)	VLPV
522.96	523.97 $^{(1+)}$	β-CN (f185-189)	VLPVP
650.99	652.00 $^{(1+)}$	β-CN (f185-190)	VLPVPQ
439.85	440.86 $^{(1+)}$	β-CN (f187-190)	PVPQ
886.94	887.95 $^{(1+)}$	β-CN (f197-203)	QRDMPIQ
631.77	211.60 $^{(3+)}$	β-CN (f198-202)	RDMPI

602.87	603.88 $^{(1+)}$	β-CN (f199-203)	DMPIQ
633.84	634.85 $^{(1+)}$	β-CN (f208-212)	YQEPV
583.95	584.96 $^{(1+)}$	β-CN (f209-213)	QEPVL
375.04	376.05 $^{(1+)}$	β-CN (f220-222)	FPI
440.06	441.07 $^{(1+)}$	β-CN (f221-224)	PIIV
946.71	473.86 $^{(2+)}$	α_{S1}-CN (f15-22)	ARPKHPIK
549.92	550.93 $^{(1+)}$	α_{S1}-CN (f23-27)	HQGLP
677.85	339.90 $^{(1+)}$	α_{S1}-CN (f23-28)	HQGLPQ
657.86	658.87 $^{(1+)}$	α_{S1}-CN (f40-45)	VAPFPE
757.97	757.98 $^{(1+)}$	α_{S1}-CN (f40-46)	VAPFPEV
559.01	560.02 $^{(1+)}$	α_{S1}-CN (f41-45)	APFPE
658.08	659.09 $^{(1+)}$	α_{S1}-CN (f41-46)	APFPEV
490.00	491.01 $^{(1+)}$	α_{S1}-CN (f43-46); Lactotransferrin (f195-198)	FPEV
677.66	339.84 $^{(2+)}$	α_{S1}-CN (f44-49)	PEVFGK
945.92	473.97 $^{(2+)}$	α_{S1}-CN (f50-57)	EKVNELSK
569.89	570.90 $^{(1+)}$	α_{S1}-CN (f85-89; f125-129)	EIVPN
569.05	570.06 $^{(1+)}$	α_{S1}-CN (f124-128)	LEIVP
943.68	472.85 $^{(2+)}$	α_{S1}-CN (f144-151)	AQQKEPMI
886.85	887.86 $^{(1+)}$	α_{S1}-CN (f148-155)	EPMIGVNQ
716.82	717.83 $^{(1+)}$	α_{S1}-CN (f159-163)	YFYPE
329.93	330.94 $^{(1+)}$	α_{S1}-CN (f175-178)	PSGA
421.56	211.79 $^{(2+)}$	α_{S1}-CN (f191-194)	APSF
640.84	641.85 $^{(1+)}$	α_{S1}-CN (f195-200)	SDIPNP

753.82	754.83 $^{(1+)}$	α_{S1}-CN (f195-201)	SDIPNPI
554.01	555.02 $^{(1+)}$	α_{S1}-CN (f196-200)	DIPNP
666.97	667.98 $^{(1+)}$	α_{S1}-CN (f196-201)	DIPNPI
342.88	343.89 $^{(1+)}$	α_{S1}-CN (f197-199); β-CN (f81-83)	IPN
439.06	440.07 $^{(1+)}$	α_{S1}-CN (f197-200)	IPNP
552.08	553.09 $^{(1+)}$	α_{S1}-CN (f197-201)	IPNPI
439.95	440.96 $^{(1+)}$	α_{S1}-CN (f198-201)	PNPI
925.62	463.82 $^{(2+)}$	α_{S2}-CN (f98-104)	NEINQFY
675.04	676.05 $^{(1+)}$	α_{S2}-CN (f115-120)	YQGPIV
512.11	513.12 $^{(1+)}$	α_{S2}-CN (f116-120)	QGPIV
966.96	967.97 $^{(1+)}$	α_{S2}-CN (f129-137)	RNAVPITPT
511.88	512.89 $^{(1+)}$	α_{S2}-CN (f130-134)	NAVPI
810.89	811.90 $^{(1+)}$	α_{S2}-CN (f130-137)	NAVPITPT
945.76	473.89 $^{(2+)}$	α_{S2}-CN (f211-218)	PKTKVIPY
545.01	546.02 $^{(1+)}$	β-Lg (f25-29)	GLDIQ
563.84	564.85 $^{(1+)}$	β-Lg (f166-170)	SFNPT
529.00	530.01 $^{(1+)}$	α-La (f38-43)	GGVSLP
656.91	657.92 $^{(1+)}$	α-La (f38-44)	GGVSLPE
414.91	415.92 $^{(1+)}$	α-La (f40-43)	VSLP
1011.76	506.89 $^{(2+)}$	α-La (f63-71)	NNDSTEYGL
487.98	488.99 $^{(1+)}$	Lactotransferrin (f177-180)	SREP
678.02	679.03 $^{(1+)}$	Lactotransferrin (f195-201); Sero-transferrin (f222-228)	AGDVAFV
490.02	491.03 $^{(1+)}$	Lactotransferrin (f225-228)	NNSR

Lactoalbulmin: La, lactoglobulin: Lg, casein: CN

Moreover, 34 peptides were previously reported with potential bioactivity. Well-recognized ACE inhibitory peptides were identified in F1 such as IPP[47] and LPP[49]; F2 PLP[51], RYL[38] and LY[52]; F4 HQGLPQ[54]; F5 NVPGEIVE[54] and VLPVPQ[62]; and F6 LNVPGEIVE[54], HLPLP[25]; LPLP[25] and LPLPL[25]. Thus, these results suggest that 100 novel peptides with potential ACE inhibitory activity were identified. In this regard, hydrophobic and branched amino acid residues at the C-terminal, such as alanine, valine, leucine, isoleucine, methionine, tyrosine, phenylalanine and tryptophan, may enhance ACE inhibition[79]. The structural characteristics and physicochemical properties of peptides provide the function and bioactivity they exert *in vitro* or *in vivo*[80]; these parameters are fundamentals to establish interactions of enzymatic inhibition[81] where the hydrophobicity of a peptide is closely related to its ACEI activity[82]. The C-terminal domain of ACE is a hydrophobic environment, therefore, there is higher affinity interaction among hydrophobic peptides and the main active site pockets of ACE. Also, other studies reported that glycine, isoleucine, leucine, and valine in the N-terminal may also be adequate for ACE inhibition[82]. Interestingly, in the present study, 35 and 14 peptides presented these amino acid residues in the C-terminal and N-terminal; respectively.

Additionally, an *in silico* analysis with Peptide 2 was performed with the selected novel peptides from the absorbed fractions that showed the lowest IC_{50} value for ACE inhibition and presented a peptide ranker score >0.5 (**Table 3).** Fourteen peptides from F1, F2, F4, F5, F7 and F8 resulted with significantly ($p<0.01$) high binding potential to ACE. In this regard, ACE comprises three active sites, S1, S2 and S1′, whereas their interacting residues Ala354, Glu384, and Tyr523 for S1; Gln281, His353, Lys511, His513 and Tyr520 for S2; and Glu162 for S1′ have been reported[83]. In this study, all 14 peptides showed binding potential to the three active sites but not for all the interacting residues.

Table 3. Potential binding sites between identified bioactive peptides obtained from fermented milk by *Lactococcus lactis* NRRL B-50572 and angiotensin converting enzyme-I using Peptide 2.

Fraction	Sequence	P-value	Reactive residues in peptide	Bound amino acids residues of angiotensin-I converting enzyme
F1	QPLP	<0.01	$Q_1P_2L_3P_4$	Tyr146, His353, His383, Lys511, Phe512, His513, Tyr520, Tyr523
F2	PIR	<0.01	$P_1I_2R_3$	His353, Ala354, His383, Glu384, His387, Glu411, Asp415, Phe457, His513, Tyr520, Tyr523
	FAQ	<0.01	$F_1A_2Q_3$	His353, Ala354, His383, Glu384, Glu411, His513, Tyr520, Tyr523
	PLR	<0.01	$P_1L_2R_3$	His353, Ala354, His383, Glu384, His387, Glu411, Asp415, Phe457, His513, Tyr520, Tyr523
F4	SDIPNPI	<0.01	$I_3P_4N_5P_6I_7$	Trp279, Gln281, His353, Ala354, His383, Glu384, His387, Glu411, Phe457, Phe460, Lys511, His513, Tyr520, Tyr523, Phe527
F5	FPIIV	<0.01	$F_1P_2I_3I_4$	His353, Ala354, His383, Glu384, His387, Glu411, Phe457, His513, Tyr520, Tyr523
	PLPPT	<0.01	$P_1P_3P_4T_5$	Tyr146, His353, His383, His387, Glu411, Asp415, Phe457, Lys511, Phe512, His513, Tyr520, Tyr523
	SFNPT	<0.01	$F_2N3P_4T_5$	His353, Ala354, Ser355, His383, Glu384, His387, Phe457, Lys511, Phe512, His513, Tyr520, Tyr523
F7	VF	<0.01	V_1F_2	His353, Ala354, Ser355, His383, Glu384, His387, Lys511, Phe512, His513, Tyr520, Tyr523
	GL	0.01	G_1L_2	His353, Ala354, Ser355, Ser356, His383, Glu384, His387, His513, Tyr523
	FPI	<0.01	$F_1P_2I_3$	His353, Ala354, His383, Glu384, His387, Glu411, Phe457, His513, Tyr520, Tyr523

	AYPSG	<0.01	$A_1Y_2P_3S_4$	His353, Ala354, His383, Glu384, His387, Glu411, Asp415, Phe457, His513, Tyr520, Tyr523, Ser526
F8	LPLPLLQ	<0.01	$L_1P_2L_3P_4L_6Q_7$	Tyr146, Trp270, Gln281, His353, Ala354, Ser355, Ala356, Glu384, His387, Glu411, Phe457, Phe460, Lys511, Phe512, His513, Tyr520, Tyr523
	LCQL	<0.01	$L_1C_2Q_3L_4$	Trp279, Gln281, His353, Ala354, His383, Glu384, Glu411, Phe457, Phe460, Lys511, Phe512, His513, Tyr520, Tyr523

Additionally, other ACE residues not characterized as active sites may also participate in the inhibition of ACE. Molecular docking studies have indicated that these non-catalytic enzyme-peptide complexes are through hydrogen bond interaction forces[84]. In fact, lisinopril (a widely known ACE inhibitor) has been shown to bind to non-catalytic sites, specifically, His353, Ala354, His383, Glu384, His387, Glu411, Lys511 and Tyr520[85]. Interestingly, all 14 peptides also presented binding to these residues, particularly SDIPNPI and LCQL from fractions F4 and F8; respectively.

Although *in silico* studies may provide insight at a molecular level, some restrictions must be recognized. In this sense, these studies do not consider peptide combinations that may influence the binding activity. Also, the prediction of peptide binding to the active site of a specific enzyme may not be necessary to inhibit enzyme activity[86]. In this sense, it is noteworthy to mention that some peptides may possess specific interactions with an allosteric site (a non-active site); changing the structure of the active site and resulting in no enzyme effect[39]. Therefore, further structure-activity relationship studies with these peptides with potential ACE inhibitory effects are necessary.

The present study determined the bioaccesability and bioavailability of ACE inhibitory peptides from fermented milk with *Lactococcus lactis* NRRL B-50571. A total of one hundred new potential ACE inhibitory peptides were identified in the serosal compartment after simulated gastrointestinal digestion and *ex vivo* absorption model. These peptides also showed high binding potential to active sites of ACE.

Therefore, these peptides may be associated with the *in vivo* antihypertensive effect; nevertheless, further *in vivo* studies using synthesized peptides are necessary. Also, it is noteworthy to determine further how the non-absorbed peptides identified in this study may affect the gut microbiota.

Abbreviations

Angiotensin I-converting enzyme (ACE)

Area under the curve (AUC)

Casein (CN)

Fermented milk (FM)

Fermented milk with Lactococcus lactis NRRL B-50571 (FM-571)

Hippuric acid (HA)

Hippuryl-histidyl-leucine (HHL)

Lactoalbulmin (La)

Lactoglobulin (Lg)

Simulated gastrointestinal digestion (SGD)

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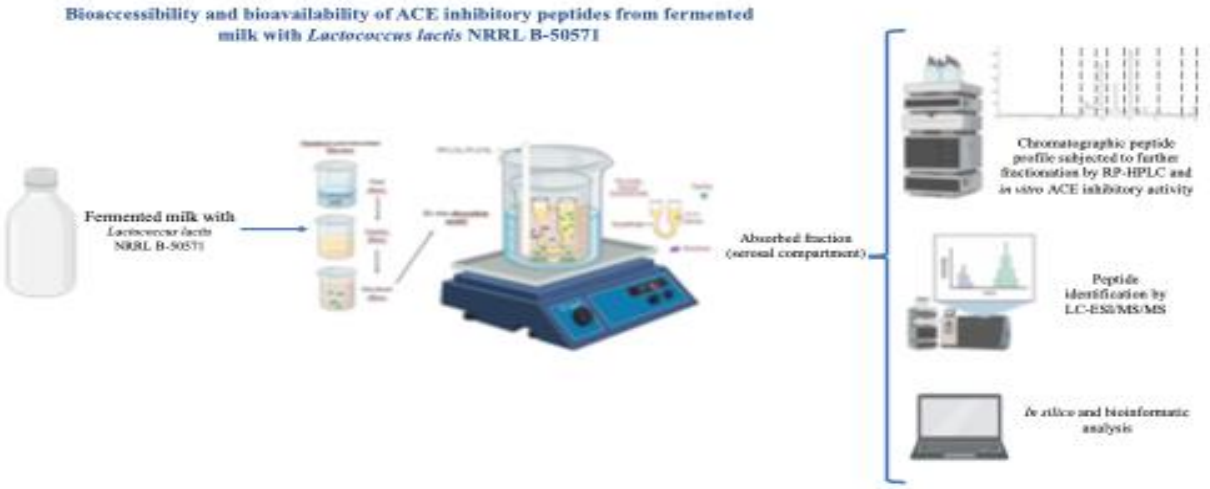
Graphic for table of contents



4. FERMENTED MILK WITH *Lactococcus Lactis* NRRL B-50571 AS A PREVENTIVE TREATMENT OF HYPERTENSION IN AN INDUCED HYPERTENSION MODEL WITH ANGIOTENSIN II.

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Abstract

The mechanistic pathways of the antihypertensive effect of fermented milk by *Lactococcus lactis* NRRL B-50571 (FM-571) have been previously reported in spontaneously hypertensive rats. However, the protective effect of FM-571 in an induced hypertensive model has not been reported. Therefore, the aim of the present study was to evaluate the effect of FM-571 as a preventive treatment in an induced hypertension murine model with angiotensin II (Ang II). Results showed that systolic (SBP) and diastolic (DBP) blood pressure were not significantly ($p>0.05$) different among groups during the first two weeks run-in periods of treatment administration. However, after hypertension induction with Ang II, SBP and DBP in the FM-571 and Captopril groups were significantly ($p<0.05$) lower than in the negative control group (water). Angiotensin converting enzyme activity, glutathione peroxidase and lipid peroxidation (malondialdehyde) in plasma were not significantly different ($p>0.05$) among groups. Nevertheless, catalase activity was significantly higher ($p<0.05$) in the FM-571 group. Moreover, the oxidative stress index was significantly lower ($p<0.05$) in the FM-571 group. Altogether, these results support the fact that FM-571 may be used as a preventive treatment for the development of hypertension by enhancing the primary antioxidant defense system and decreasing the oxidative stress index.

Keywords: preventive treatment, induced hypertension, fermented milk, enzymatic activity, oxidative stress.

Introduction

Cardiovascular diseases are the leading cause of death worldwide and the main concern for health authorities in developed countries. Dietary patterns and physical activity are the most recommended and important lifestyle changes to reduce risk factors. Particularly, hypertension is a multifactorial disease with systemic implications and one of the main risk factors for the development of cardiovascular diseases (Zhang et al., 2021). The etiology of hypertension begins in the renin-angiotensin system (RAS); thus, this system is mainly targeted for the regulation of blood pressure (Harrison-Bernard, 2009). Specifically, in RAS the angiotensin-I converting enzyme (ACE) hydrolyzes angiotensin I to release angiotensin-II (Ang II), a potent vasoconstrictor. Additionally to this, Ang II possesses other signaling mechanisms with important implications for physiological and pathophysiological effects (Rajagopalan et al., 1996).

In this sense, it has been reported that Ang II is related with the production of reactive oxygen species (ROS) (Masi et al., 2019). In healthy patients, homeostasis in cellular redox is reached by the balance between ROS and endogenous antioxidants. However, in several diseases such as hypertension, an enhanced production of ROS takes place, leading to an antioxidant imbalance and thus, developing an oxidative stress state (Yuan et al., 2012; Masi et al., 2019). Moreover, this oxidative stress may induce vascular endothelial dysfunction and reduce nitric oxide (NO) production (Yuan et al., 2012).

Thus, the regulation of blood pressure and neutralization of reactive oxygen species *in vivo* by food bioactives can be a good option to ameliorate and adjuvant in the treatment of hypertension. In this sense, rodent models have proven to be an invaluable tool for testing new possible treatments to lower blood pressure as well as for studying molecular mechanisms underlying essential hypertension (Jama et al., 2021). Overall, rodent hypertensive models may be genetic or induced. Usually, spontaneously hypertensive rats (SHR) (genetic) is the most commonly used hypertension model, followed by the induced hypertensive model with the of infusion Ang II (Jama et al., 2021). Inappropriate regulation of the renin angiotensin-aldosterone system (RAAS) in primary hypertension has made the Ang II rodent model a reliable model to study hypertension. Doses of Ang II usually range from 200 ng/kg/min to 1440 ng/kg/min over a period of 14– 28 days, and the effects on blood pressure (BP) may vary (Jama et al., 2021). However, there are advantages and disadvantages associated with this model. In this regard, although Ang II can produce immediate effects on blood pressure; nevertheless, the development of hypertension in humans takes years to

develop (Gomolak and Didion, 2014). Moreover, higher Ang II doses (*e.g.* >1000 ng/kg/min), induces a rapid increase in BP (Gomolak and Didion, 2014). On the other hand, repeated doses of <400 ng/kg/min, results in a gradual increase of BP and has been associated with end-organ damage, which mimics essential hypertension in humans (Kawada et al., 2002).

Furthermore, the infusion of Ang II in murine models stimulates the production of NADPH oxidases, which has been reported to enhance the $O_2^{\cdot-}$ anion production in the vasculature of kidneys, and thus, increasing reactive oxygen species (ROS) (López *et al.*, 2003). The conjunction of these factors lead to the imbalance of the first line of antioxidant defense with final implications in the increase of blood pressure, renal damage and endothelial dysfunction (Gomolak and Didion, 2014).

On the other hand, there is a growing body of evidence showing that fermented milks may act as adjuvants in the treatment of hypertension (Beltrán-Barrientos et al., 2016), either by inhibiting ACE, or by neutralizing reactive oxygen species or both (Beltrán-Barrientos et al., 2018a). In previous studies we reported the antihypertensive effect of fermented milk by *Lactococcus lactis* NRRL B-50571 (FM-571) in SHR and in clinical studies (Rodríguez-Figueroa et al., 2013ab; Beltrán-Barrientos *et al.*, 2018ab), where the effect was associated to the delivery of peptides with ACE inhibitory activity and an antioxidant effect. In particular, these studies were carried out when hypertension had already developed. Nevertheless, the potential protective effect over the development of hypertension has not yet been explored. Thus, the aim of the present work was to evaluate the potential effect of FM-571 as a preventive treatment in a murine model with induced hypertension by Ang II.

Materials and Methods

Preparation of fermented milk

Fermented milk was prepared as described by Beltrán-Barrientos et al. (2018a). The strain *Lactococcus lactis* NRRL B-50571 was inoculated at 3% in broth M17 supplemented with sterile lactose (10%) and incubated at 30 ºC for 24 h; this procedure was made repeated twice to obtain a fresh culture. Reconstituted sterilized skim milk (10%; 110 ºC/10 min) was inoculated with fresh culture (3%) and incubated at 30 ºC for 12 h to obtain a fresh inoculum. Finally, fermented milk was prepared by the addition of the inoculum (3%) to pasteurized reconstituted skim milk (80 ºC/30

min) and incubated at 30 ºC for 48 h. Heat treatment (75 ºC/15 min) was applied after fermentation to inactive the bacteria followed by cooling in an ice bath. FM-571 was frozen at -20 ºC until used.

Murine model

Male Wistar rats (n = 28) with a mean weight of 235 ± 19.9 g were obtained from the Research Animal Facility at the University of Sonora (Hermosillo, Sonora, México). Rats were placed in individual stainless steel cages with water and a standard diet *ad libitum,* under 12 h cycles of light/dark at 22 ± 2 ºC and 50 ± 6% relative humidity. After a one-week of period of adaptation, rats were randomized into four groups (n=7): 1) FM-571, group administered with fermented milk FM-571; 2) Positive control, group administered with Captopril® (40 mg/kg body weight (BW)); 3) Negative control, hypertensive group; and 4) healthy control. The study protocol was approved by the Bioethics Committee of the Research Center for Food and Development (Spanish acronym, CIAD), Hermosillo, Sonora, Mexico (CE/017/2019).

Administration of treatments

Groups administered with FM-571 and Captopril® (40 mg/kg) (Positive control) had free access (*ad libitum*) during the first two weeks run-in period before the induction of hypertension and four weeks after induction. Captopril® was dissolved in purified water before administration. The Negative and Healthy controls had free access to purified water during the experiment.

Murine model of induced-hypertension

The induction of hypertension was carried out during the third week of intervention in two single intravenous (i.v.) doses (fifteen and twenty-first day) as previously reported by Kamkar-Del et al. (2020). Ang II (Sigma Aldrich, México City, México) was dissolved in sterile saline solution (0.9%; Pisa Laboratories, Jalisco, México) and 50 ng/kg BW were injected i.v. in the FM-571, Positive and Negative control groups. Healthy control group was also injected i.v. with the same volume without Ang II.

Systolic and diastolic blood pressure

After randomization, systolic and diastolic blood pressure (SBP and DBP; respectively) were taken each week. Blood pressure was measured in triplicate using a noninvasive blood pressure system

CODA® High Throughput System (Torrington, Connecticut, USA). After 7 weeks of treatment, rats were sacrificed and blood samples were collected to immediately obtain plasma and stored at -80 ºC until used for further assays.

ACE activity in plasma

ACE activity in plasma was measured according to the method reported by Cushman and Cheung (1970) with modifications. Hippuryl-L-histidyl-L-leucine (HHL) 2 mM was dissolved in a sodium borate buffer 100 mM (pH 8.3) containing 300 mM NaCl. Briefly, each sample (50 µL) of plasma was mixed with 50 µL of HHL and incubated for 30 min at 37 ºC. The reaction was stopped by the addition of 125 µL of HCl 1 mol/L. The formed hippuric acid was analyzed by reverse-phase (RP-HPLC) (Rendón-Rosales et al., 2022). Then, 10 µL was injected and analyzed on a Zorbax 300extend-C18 (4.6 x 250 mm, 5 µm) column in an Agilent 1260 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with OpenLAB Chromatography Data System version A.02.02 (Agilent Technologies, Germany) and the detector wavelength was set at 228 nm. The column was eluted (Flow 0.5 mL/min) with acetonitrile with 0.05% trifluoroacetic acid (TFA) and water with 0.05% of TFA and the area under the curve (AUC) of hippuric acid was determined. A standard curve of ACE from rabbit lung was carried out to interpolate the AUC of hippuric acid.

Catalase activity

Catalase activity in plasma was carried out according to the method reported by Baer and Sizer (1952). Hydrogen peroxide (30 mM) in a phosphate-borate solution (pH 7) was used as substrate and the decrease in its concentration at 37 ºC was monitored spectrophotometrically at 240 nm every 30 s for 5 min. One unit catalase activity was determined as the quantity of enzyme which removes 1 µM H_2O_2/min. Results were expressed as units/mg protein.

Glutathione peroxidase activity

Glutathione peroxidase activity was evaluated according to the method reported by Flohe and Gunzler (1984). Samples (20 µL) were placed on a microplate and incubated at 37 ºC for 10 min with 140 µL of a mixture containing PBS (100 mM in EDTA 1 mM, pH 7), Glutathione (GSH; 10 mM) and glutathione reductase (0.24 U/L in PBS 100 mM, pH 7) in a ratio 5:1:1. Afterwards, 20 µL of NADPH (1.5 mM in sodium bicarbonate 0.1%) were added and incubated at 37ºC for 3 min.

Thereafter, 20 μL BHT (Butylated hydroxytoluene) (12 mM) was added and the consumption of NADPH was monitored at 340 nm every 30 s during 5 min at 25 ºC. The decrease in NADPH concentration was calculated from the linear slope of absorption using the extinction coefficient of 6.22 $mM^{-1}cm^{-1}$. One unit glutathione peroxidase activity was determined as the quantity of enzyme which oxidized 1 μM NADPH/min. Results were expressed as units/mg protein.

Lipid peroxidation

Lipid peroxidation products were determined with the method of thiobarbituric acid, which measures malondialdehyde-reactive products (MDA) (Todorova et al., 2005). Samples (0.5 mL) were mixed with 0.5 mL of trichloroacetic acid (20%) and centrifuged at 2000 *xg* for 20 min. Afterwards, 750 μL of supernatant was mixed with 750 μL of thiobarbituric acid (0.67%) and heated at 96 ºC for 1 h. After cooling, the intensity of the pink color in the final product was determined at 532 nm. The MDA concentration was calculated using the Lambert-Beer equation and the extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1}.

Oxidative stress index

The oxidative stress index was calculated taking into account the ratio between lipid peroxidation products (MDA) and the antioxidant enzymes CAT and GPx (LPO/CAT + GPx) as previously reported by Ruas et al. (2008).

Statistical analysis

The study was a completely randomized design and the response variables were measured in triplicates. Data are expressed as mean ± standard deviation. One-way ANOVA was performed to compare treatments and the Tukey-Kramer test was used to compare means among treatments to establish significant differences using a p-value of 0.05. Data without normal distribution are presented as medians, analyzed by Kruskal-Wallis test and were considered significant when $p<0.05$.

Results and Discussion

Twenty-eight Wistar rats were randomized into four groups and no significant differences ($p>0.05$) between groups were observed in the clinical characteristics (weight, SBP, DBP and heart rate)

(**Table 1**). Several studies have reported the induction of hypertension after the administration of different repeated doses of Ang II with surgical minipumps, over different time periods (Jama et al., 2021). Also, Zubcevic et al. (2017) administered Ang II (36 μg/kg BW) via intraperitoneal to Wistar-Kyoto (WKY) rats and the increase in blood pressure returned to baseline within 1 h. Therefore, the increase in blood pressure and the duration of the effect is dependent on the via administration of the inductor agent (*e.g.* Ang II, dexamethasone) and the damage caused at the systemic level (Jama et al., 2021). In the present study, hypertension was induced after the administration of two doses of Ang II i.v. and this effect lasted three weeks.

Changes in SBP and DBP during seven weeks are depicted in **Figure 1**. Results showed that after two weeks of intervention, all groups showed a decrease in SBP and DBP; however, they were not significantly ($p>0.05$) different among them; thus, indicating that FM-571 did not show an effect in normotensive rats. In the third week, Ang II was administered to FM-571 and the control groups. SBP and DBP in the Negative control group significantly increased ($p<0.05$) and were significantly different from the Healthy control group. Interestingly, for FM-571 and the Positive control groups, SBP and DBP significantly decreased ($p<0.05$) and were significantly different ($p<0.05$) from the Negative control group from the fourth to the sixth-weeks of intervention. Moreover, after 7-weeks of intervention, the FM-571 and the Positive control groups maintained low SBP and DBP.

Table 1. Clinical characteristics of Wistar rats.

Groups	**Healthy control (Purified water)**	**Negative control (Purified water)**	**Positive control (Captopril)**	**FM-571**	**p-value**
Weight (kg)	278.14 ±14.47	277.7 ± 15.8	272.6 ± 10.9	266 ± 15.84	0.4
SBP (mmHg)	126.53 ± 16.76	125.5 ± 14.7	128.2 ± 23.1	125.19 ± 21.13	0.99
DBP (mmHg)	96.6 ± 17.78	88.8 ± 10.0	92.0 ± 16.9	94.1 ± 14.52	0.8
Heart rate (beats/min)	296 ± 60.55	365.9 ± 63.4	358.9 ± 130.4	341.6 ± 166.79	0.66

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FM-571: Fermented milk with *Lactococcus lactis* NRRL B-50571.

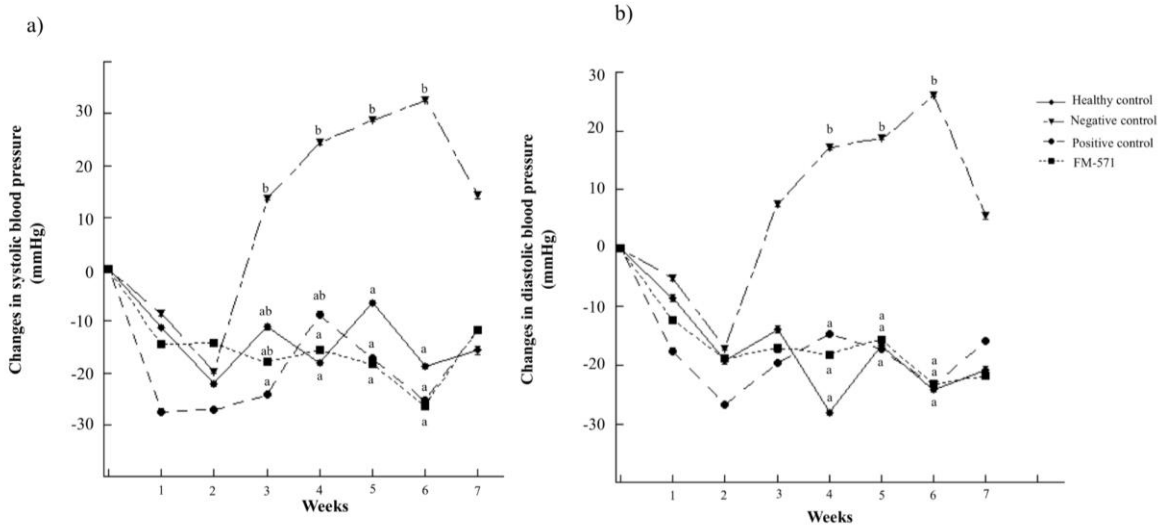

Figure 1. Changes in a) systolic and b) diastolic blood pressure (mmHg) from Wistar rats with induced hypertension administered with different treatments. Healthy and negative control: Purified water; Positive control: Captopril (40 mg/kg body weight); FM-571: Fermented milk with *Lactococcus lactis* NRRL B-50571. Data are presented as means ± SEM. Data points not sharing the same letter within a week were statistically different (p<0.05).

The antihypertensive effect of FM-571 has been previously reported in SHR (Rodríguez-Figueroa et al., 2013ab; Beltrán-Barrientos et al., 2018a) and prehypertensive subjects (Beltrán-Barrientos et al., 2018b). However, the antihypertensive effect of this fermented milk during the development of hypertension has not yet been reported. In fact, only few studies have reported the protective effect of fermented milks in the development of hypertension in the SHR model. In this regard, Sipola et al. (2001) reported that fermented milk with *Lactobacillus helveticus* and *Saccharomyces cerevisieae* significantly attenuated the development of hypertension in SHR. Similarly, after a long-term administration of a milk casein hydrolysate to SHR, the development of hypertension was also attenuated (Sánchez et al., 2011).

On the other hand, the antihypertensive effect of fermented milks or dairy peptides has been widely reported in SHR (García-Tejedor et al., 2015; Beltrán-Barrientos et al., 2016). Nevertheless, few studies have reported this effect in other hypertensive murine models. In this sense, El-Fattah et al. (2017) injected subcutaneously dexamethasone to Wistar rats to induce hypertension. Then, fermented milk with *Lactibacillus rhamnosus* B-1445, O-114, YC-X11 and *Lactibacillus helveticus* Lh-B 02 was administered and blood pressure was reduced. Similar to our findings, after

four weeks of treatment administration, blood pressure was reduced in all groups (El-Fattah et al., 2017). In fact, García-Tejedor et al. (2015) administered lactoferrin-derived peptides (RPYL and DPYKLRP) to hypertensive induced (Ang II) Wistar rats and results showed a blood pressure lowering effect.

As previously mentioned, it was reported that the antihypertensive effect of FM-571 in SHR was attributed to an antioxidant effect, nitric oxide production enhancement and ACE inhibition (Beltrán-Barrientos et al., 2018a). Therefore, the mechanistic antihypertensive effect of this fermented milk on this particular hypertensive induced model was investigated. Results showed no significant differences ($p>0.05$) between all groups in ACE activity in plasma. It has been reported that Ang II binds into AT1 receptor which enables sodium and water retention, enhances the production of aldosterone and vasoconstriction. Interestingly, milk-derived lactoferrin peptides have previously been demonstrated to block AT1 receptors in Ang II induced vasoconstriction in an *ex vivo* model (Fernandez-Musoles et al., 2014). However, further studies are needed to elucidate if FM-571 derived peptides may bind to AT1 receptors. Nevertheless, these results suggest that for this particular model of induced hypertension, the antihypertensive effect of this fermented milk was not associated with ACE inhibition. Hence, other potential pathways could be leading to the blood pressure lowering effect.

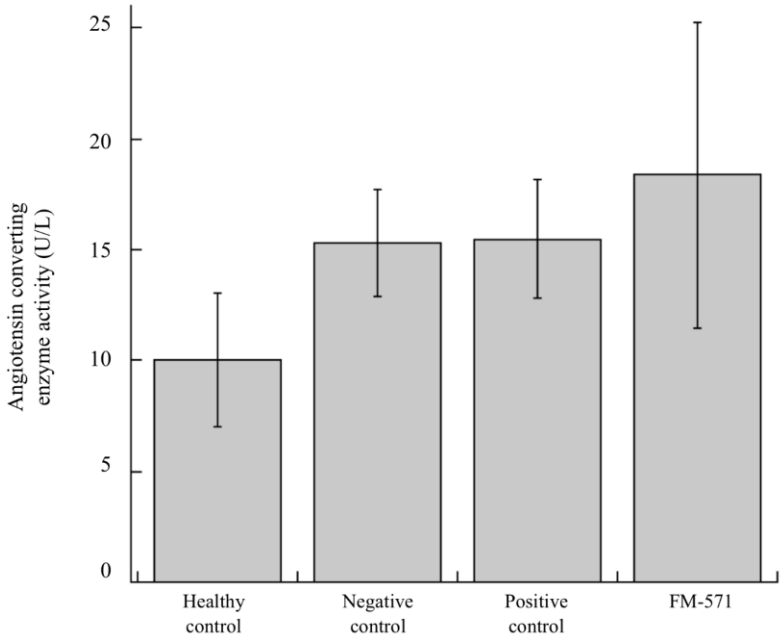

Figure 2. Angiotensin converting enzyme activity in plasma from Wistar rats with induced

hypertension administered with different treatments. Healthy and negative control: Purified water; Positive control: Captopril (40 mg/kg body weight); FM-571: Fermented milk with *Lactococcus lactis* NRRL B-50571. Data are presented as means ± SD.

It has been widely reported that there is a strong link between oxidative stress and hypertension in different hypertensive animal models (Griendling et al., 2021). In particular, endothelial dysfunction caused by oxidative stress contributes to an impaired nitric oxide/ROS balance, and thus, increasing vasoconstriction, oxidation and inflammation (Briones and Touyz, 2010). In Ang-II induced hypertension, NOX1 (a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX)), led to an increment of oxidative stress and blood pressure (Dikalova et al., 2005). Also, Ang-II induced hypertension enhances an impairment of endogenous antioxidant mechanisms (Lassegue *et al.,* 2003). Therefore, neutralization of ROS may be an alternative as an adjuvant in the treatment of hypertension. To elucidate the effect of treatments on the antioxidant system of the murine model of induced hypertension in this study, the activities of the antioxidant enzymes catalase (CAT), glutation peroxidase (GPx) and lipid peroxidation products (malondialdehyde, MDA) in plasma were determined.

In this regard, CAT activity in plasma from the FM-571 group was significantly ($p<0.001$) higher than the rest of the groups (**Figure 3**). In fact, CAT from the FM-571 group was 3- and 5-fold higher than the Negative and Positive control groups; respectively. Thus, these results showed that FM-571 enhanced CAT activity. Conversely, GPx was not significantly different ($p>0.05$) among groups (**Figure 4**). In fact, GPx from the FM-571 group was lower than Negative and Positive control groups. On the other hand, no significant differences ($p>0.05$) between groups were detected for MDA in plasma (**Figure 5**). Interestingly, after determining the oxidative stress index (balance between lipo peroxidation (MDA) and total antioxidant enzyme activity (CAT and GPx), results showed that FM-571 was significantly lower ($p<0.05$) than all groups (**Figure 6**).

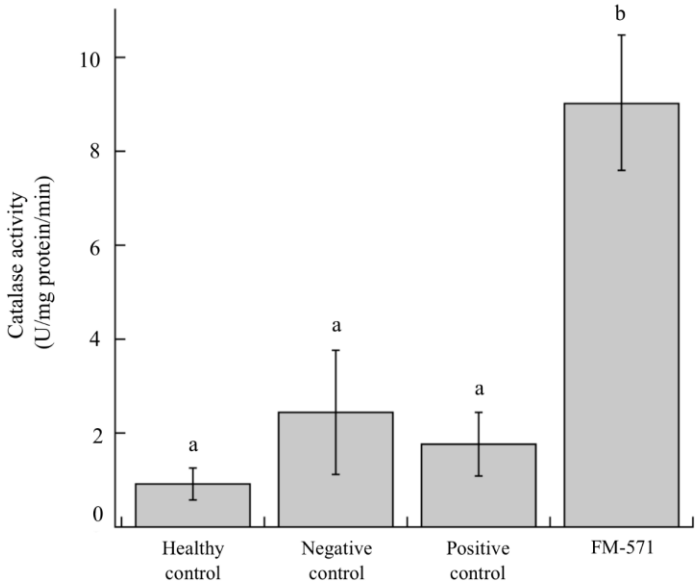

Figure 3. Catalase activity in plasma from Wistar rats with induced hypertension administered with different treatments. Healthy and negative control: Purified water; Positive control: Captopril (40 mg/kg body weight); FM-571: Fermented milk with *Lactococcus lactis* NRRL B-50571. Data are presented as means ± SD. Data not sharing the same letter were statistically different (p<0.05).

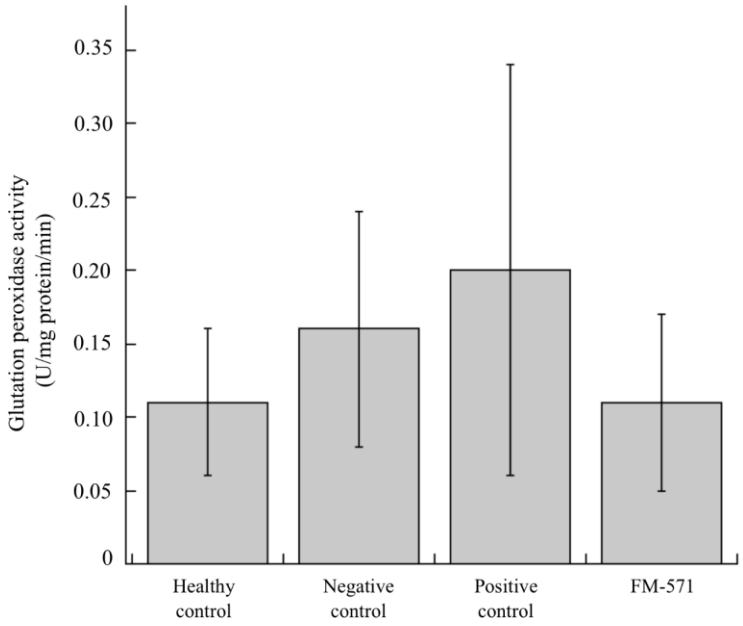

Figure 4. Glutation peroxidase activity in plasma from Wistar rats with induced hypertension administered with different treatments. Healthy and negative control: Purified water; Positive

control: Captopril (40 mg/kg body weight); FM-571: Fermented milk with *Lactococcus lactis* NRRL B-50571. Data are presented as means ± SD.

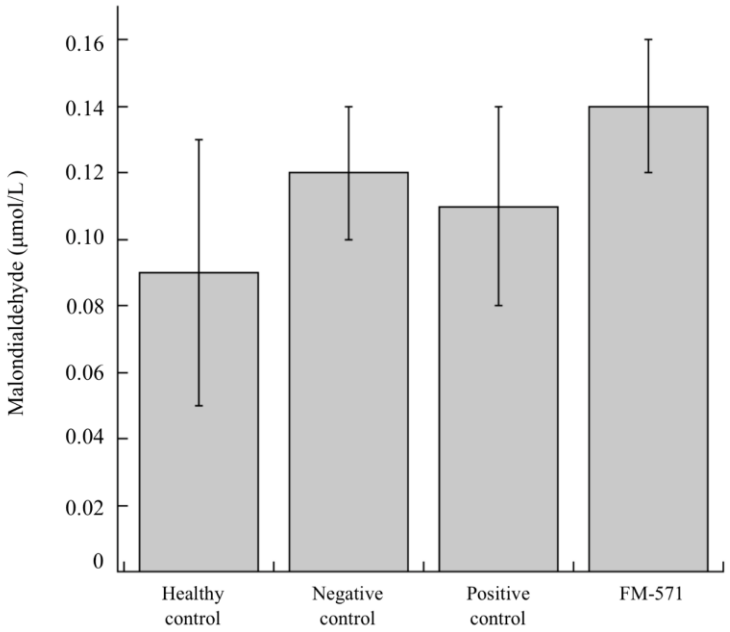

Figure 5. Malondialdehyde levels in plasma from Wistar rats with induced hypertension administered with different treatments. Healthy and negative control: Purified water; Positive control: Captopril (40 mg/kg body weight); FM-571: Fermented milk with *Lactococcus lactis* NRRL B-50571. Data are presented as means ± SD.

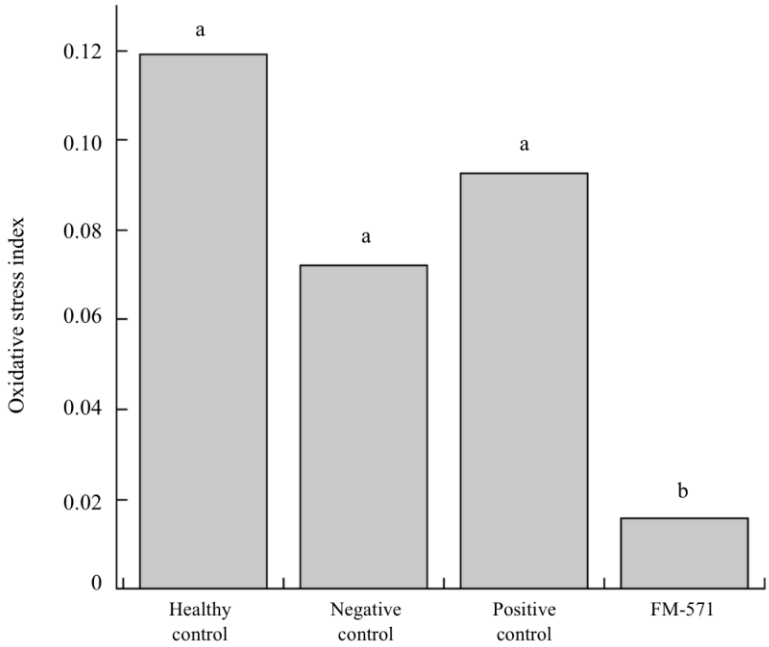

Figure 6. Oxidative stress index in plasma from Wistar rats with induced hypertension administered with different treatments. Healthy and negative control: Purified water; Positive control: Captopril (40 mg/kg body weight); FM-571: Fermented milk with *Lactococcus lactis* NRRL B-50571. Data are presented as median, and were analyzed by non-parametric test (Kruskal-Wallis $p<0.05$). Data not sharing the same letter were statistically different ($p<0.05$).

It has been evidenced that different sources of bioactive peptides may modulate oxidative stress and oxidative stress biomarkers (*e.g.* lipid peroxidation), as well as antioxidant response (*e.g.* enhancement of antioxidant enzymes) (Qiao et al., 2021). In this regard, a peptide from buffalo casein reduced MDA levels and stimulated the production of CAT, superoxide dismutase (SOD) and Gpx (Sowmya et al., 2019). Other studies with peptides from soybean (Zhang et al., 2019a), beef shank (Lee and Hur, 2019), corn gluten (Wang et al., 2016) were able to enhance CAT activity in specific cell lines exposed to different oxidative stress stimulations. Additionally, *in vivo* studies with different animal models have reported the enhancement of different antioxidant enzymes. In particular, Fang et al. (2018) administered Manchurian walnuts (*Juglans mandshurica Maxim.*) hydrolysate to healthy mice under exercise conditions. Results showed that this hydrolysate increased CAT, GPx and SOD activities; and lowered lipid peroxidation. Also, Awad et al. (2016) reported that when a milk protein concentrate was administered to healthy and diabetic rats, CAT, SOD and GPx were increased. Furthermore, alcalase hydrolyzed oyster (*Crassostrea rivularis*) increased activities of SOD, CAT and reduced MDA levels in normal mice (Zhang et al., 2019b). Additionally, El-Fattah et al. (2017) reported that when fermented milk was administered to rats with induced hypertension, SOD, CAT and total antioxidant activities were higher than in the control groups.

Moreover, derived or synthethized peptides from milk (Tonolo et al., 2020), soybean (Zhang et al., 2019a), microalgae (Je et al., 2015) and perrilla (*Perilla frutescens L. Britton*) seed protein hydrolysates (Yang et al., 2018) have also reported to decrease lipid peroxidation levels in different cell lines exposed to oxidative stress. Also, *in vivo* studies have shown a decrease of lipid peroxidation after the administration of peptides or hydrolysates from potato (*Solanum tuberosum*) to SHR (Tsai et al., 2020). In fact, a previous study showed that after the administration of FM-571 to SHR, CAT and GPx were higher than the negative control group. Furthermore, MDA and oxidative stress index were significantly lower than the negative control (Beltrán-Barrientos *et al.*,

2018a).

Although the mechanisms underlying the enhancement of enzymatic antioxidant activities have not yet been fully elucidated, several approaches have been described. In this sense, different studies have shown that the modulation of the antioxidant response may be through the activation of Nrf2 transcription factor with specific peptides from milk (Tonolo et al., 2020), potato (Tsai et al., 2020) or watermelon seed protein hydrolysates (Wen et al., 2019). In fact, molecular modeling studies have shown that the activation of Nrf2 signaling pathway may be due to the disruption of Keap1-Nrf2 interaction from peptides and thereafter, the binding to the Nrf2 binding site (Wen et al., 2019; Fernando et al., 2020; Tonolo et al., 2020; Tsai et al., 2020; Wang et al., 2020). Furthermore, peptides may also help to decrease oxidative stress and maintain a balance in redox homeostasis through antioxidant activity by scavenging free radicals and chelating transition metals (Qiao et al., 2021). However, further studies are needed to elucidate if FM-571 may be involved in this reported mechanism.

On the other hand, it is noteworthy to mention that gut microbiota also plays an important role in the regulation of several body systems, and may contribute to the development of several diseases (Guo et al., 2021). Also, gut microbiota may be modulated through lifestyle changes. In this regard, dairy fermented products may provide beneficial effects on gut microbiota and health-related metabolites (Bellikci-Koyu et al., 2019). Although changes in gut microbiota were not determined in this study, it has been proposed that high abundances of Verrucomicrobiota and *Akkermansiaceae* may be related to greater antioxidant profile and antihypertensive effects (Everard et al., 2013; Gómez-Contreras et al., 2023). Thus, results in the present study may suggest that changes in gut microbiota may have also participated in the antioxidant and antihypertensive effects; nevertheless, further studies are necessary to determine these associations.

Conclusion

In the present study, the effect of FM-571 as a preventive treatment in an induced hypertension murine model with angiotensin II was shown. The results suggest that the antihypertensive effect may be due to the enhancement of the primary antioxidant defense system and the decrease in the oxidative stress index during the administration of FM-571. Also, contrary to previous studies where FM-571 was administered to SHR, the antihypertensive effect of this fermented milk in the model of induced hypertension of this study, was not associated with ACE inhibition. Hence, FM-

571 may be administered as an adjuvant in the prevention of hypertension.

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5. CONCLUSIONES GENERALES

El efecto antihipertensivo de FM-571 en ratas SHR fue evidenciado a través de diferentes mecanismos, inhibición de ECA, efecto antioxidante y producción de óxido nítrico (Beltrán-Barrientos et al., 2018a); éste fue atribuido a péptidos bioactivos derivados de la fermentación y digestión de la leche. Los hallazgos del presente estudio evidenciaron la bioaccesibilidad y biodisponibilidad de péptidos inhibidores de ECA de FM-571. En este sentido, se identificaron cien nuevos péptidos potencialmente inhibidores de ECA en el compartimento serosal después de digestión gastrointestinal simulada y absorción *ex vivo*. En particular, 14 péptidos presentaron la más alta afinidad de unión al sitio activo de ECA mediante análisis *in silico.* Por lo tanto, estos péptidos podrían estar asociados con el efecto antihipertensivo *in vivo*.

Por otro lado, los resultados del presente estudio evidenciaron que FM-571 ejerce efecto protector como tratamiento preventivo al desarrollo de hipertensión en un modelo murino de hipertensión inducida. Adicionalmente, se observó efecto antihipertensivo prolongado durante 7 semanas. Los resultados sugieren que el efecto antihipertensivo fue a través del mejoramiento del sistema de defensa antioxidante primario evidenciado con una alta actividad de catalasa y disminución del índice de estrés oxidativo durante la administración de FM-571.

Además, es importante mencionar el importante rol que la microbiota intestinal tiene en el funcionamiento de los diferentes sistemas del cuerpo; además, de su contribución en el desarrollo de diferentes enfermedades tales como la hipertensión arterial. Diversos estudios han evidenciado que los cambios en el estilo de vida pudieran modular la microbiota intestinal, tales como el consumo de alimentos lácteos fermentados. Aunque los cambios en la microbiota intestinal no fueron determinados en el presente proyecto, ha sido propuesto que altas abundancias en Verrucomicrobiota and *Akkermansiaceae* están relacionados con un alto perfil antioxidante y efecto antihipertensivo. Por lo anterior, los resultados en el estudio de prevención del desarrollo de hipertensión en modelo murino pudieran sugerir que se presentaron cambios en la microbiota intestinal, los cuales pudieran participar en los efectos antioxidante y antihipertensivo; no obstante, futuras investigaciones son necesarias para determinar estas asociaciones.

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