UNIVERSIDAD AUTÓNOMA DEL ESTADO DE HIDALGO

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DOCTORADO EN CIENCIAS DE LOS ALIMENTOS Y SALUD HUMANA

TESIS DOCTORAL

"Análisis de un hidrolizado de proteínas de suero fermentado por protocooperación de bacterias ácido lácticas: potencial nutracéutico en el tratamiento de diabetes tipo 2 e hipertensión arterial"

Para obtener el grado de Doctora en Ciencias de los Alimentos y Salud Humana

PRESENTA

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Por este medio se informa que el comité tutorial asignado a la M. en C. Laura Berenice Olvera Rosales con número de cuenta 232998, estudiante del Doctorado en Ciencias de los Alimentos y Salud Humana ha terminado el trabajo de tesis titulado "Análisis de un hidrolizado de proteínas de suero fermentado por protocooperación de bacterias ácido lácticas: potencial nutracéutico en el tratamiento de diabetes tipo 2 e hipertensión arterial", y por lo tanto se autoriza la impresión del documento en extenso propuesto por la estudiante después de haber sido revisado, analizado y evaluado de acuerdo a lo estipulado en el Artículo 73, VI del Reglamento General de Estudios de Posgrado.
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Por este medio se informa que la alumna Laura Berenice Olvera Rosales, estudiante del Doctorado en Ciencias de los Alimentos y Salud Humana concluyó el trabajo de tesis, publicando los siguientes artículos:

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Debido a lo anterior, la estudiante cumple con los requerimientos de egreso establecidos por el programa de posgrado, al contar con tres artículos aceptados en una revista indizada. Por lo que solicitamos a usted tenga a bien permitir al doctorando dar continuidad al proceso necesario que conlleve a la obtención del grado de Doctor en Ciencias de los Alimentos y Salud Humana.

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Abreviaturas

Abs Absorbancia
ACN Acetonitrilo
ANOVA Analysis of variance, análisis de varianza
BSA Bovine serum albumin, albúmina de suero bovino
Da Dalton(s)
DE Desviación estándar
DPPIV Dipeptidil peptidasa IV
ECA Enzima convertidora de angiotensina
g Gramo(s)
GIP Gastric inhibitory peptide, péptido gástrico inhibitorio
GLP Glucagon-like peptide, péptido tipo glucagón
h Hora(s)
kDa Kilodalton(s)
L Litro(s)
LAB Bacterias ácido lácticas
M Molar
mg Miligramos
Min Minuto(s)
mL Mililitro(s)
mM Milimolar
MS Mass spectrometry, espectrometría de masas
N Normal
NCBI National center for biotechnology information, centro nacional de información para la biotecnología
nm Nanómetros
No. Número
ºC Grados centígrados
OMS Organización Mundial de la Salud
PAGE Poliacrilamide gel electrophoresis, electroforésis en gel de poliacrilamida
pI Punto isoeléctrico
PM Peso molecular
ppm Partes por millón
rpm Revoluciones por minuto
s Segundo(s)
T2DM Diabetes Mellitus tipo 2
TOF Time of flight, tiempo de vuelo
Tris Tris(hidroximetil)aminometano
U Unidad(es)
ug Microgramos
uL Microlitro(s)
uM Micromolar
V Volt(s)
x *g* Fuerza centrífuga

Capítulo I.

Introducción al trabajo de investigación

Resumen

La Diabetes mellitus tipo 2 (T2DM) es un problema de salud mundial cuya fisiopatología se caracteriza por una condición de hiperglucemia, así como una producción insuficiente de insulina como resultado de resistencia a la misma (RI). Adicionalmente, en los pacientes diabéticos suele aparecer un cuadro de hipertensión, que es una comorbilidad muy común. De hecho, a nivel mundial, se reporta una prevalencia de hipertensión arterial en población diabética mayor del 50%. Es por ello que se han explorado fuentes naturales que ayuden en el tratamiento de estos padecimientos. Es así que, en los últimos años se ha reportado que las proteínas de suero de leche además de su valor nutricional desempeñan diversas funciones biológicas con efectos benéficos para la salud. Sin embargo, muchos de los compuestos que ejercen dichos efectos se encuentran encriptados dentro de las proteínas de suero y solamente pueden ser liberados mediante su fragmentación. Los compuestos liberados tras la fragmentación son denominados péptidos bioactivos. La síntesis de este tipo de péptidos puede llevarse a cabo mediante la acción de proteasas presentes en bacterias ácido lácticas (LAB).

De esta manera, el desarrollo del proyecto de investigación plantea seis capítulos. Siendo el primero de ellos, una introducción general al proyecto de investigación. El segundo capítulo, se centra en una revisión publicada en la revista Foods sobre el impacto de la microbiota intestinal en el estado salud/enfermedad y la importancia del consumo de prebióticos y probióticos en el establecimiento de la misma. El tercer capítulo, presenta una extensa revisión publicada en la revista Critical Reviews in Foods Science and Nutrition. En esta revisión se abordan aspectos generales del suero de leche y su potencial como fuente de péptidos bioactivos, describiendo las principales proteínas presentes en esta matriz láctea. De igual manera, se hace referencia a los principales métodos de obtención de péptidos bioactivos. Posteriormente, se describe cada una de las bioactividades reportadas a partir de péptidos provenientes de proteínas de suero de leche. Finalmente, se describen algunos de los principales productos en el mercado que contienen péptidos bioactivos de proteínas de

suero de leche. El cuarto capítulo, presenta los resultados publicados en la revista Foods, donde se presenta la construcción del perfil proteolítico de *L. rhamnosus* GG y *S. thermophilus* SY-102 integrando tres técnicas diferentes con la finalidad de determinar el grado de hidrólisis proteica y su potencial producción de fracciones peptídicas a partir de una fermentación de suero de leche. De igual manera, se determinó la inhibición de la enzima convertidora de angiotensina (ECA) con la finalidad de evaluar la actividad antihipertensiva de los péptidos generados durante la fermentación. El quinto capítulo, plantea los resultados sometidos a la revista *Dairy*. En este capítulo se presenta el crecimiento de las cepas durante la fermentación en suero de leche a partir de tres sistemas de fermentación. Los parámetros cinéticos son reportados, al igual que el perfil proteolítico y la actividad antidiabética de los péptidos generados durante la fermentación.

El perfil proteolítico observado indicó que la fermentación de suero de leche con LAB tiene un alto potencial en la producción de fracciones peptídicas de bajo peso molecular con bioactividad considerable. Por último, en el capítulo seis se presentan los resultados correspondientes a la separación por RP-HPLC de las fracciones peptídicas obtenidas a partir de la hidrólisis de proteínas de suero de leche por protocooperación de bacterias ácido-lácticas. Estos resultados han sido sometidos a la revista Journal of medicinal Food. La actividad antidiabética, antihipertensiva y antioxidante es reportada.

Abstract

Diabetes mellitus type 2 (T2DM) is a global health problem whose pathophysiology is characterized by a state of hyperglycemia and insufficient insulin production due to insulin resistance (IR). In addition, diabetics usually present with hypertension, which is a very common concomitant disease. Worldwide, the prevalence of arterial hypertension in the diabetic population is reported to be more than 50%. For this reason, natural sources have been explored to help treat these conditions. For example, in recent years it has been reported that whey proteins, in addition to their nutritional value, perform various biological functions with beneficial effects on health. However, many of the compounds that exert these effects are encoded in serum proteins and can only be released by their fragmentation. The compounds released after fragmentation are called bioactive peptides. The synthesis of this type of peptides can be done by the action of proteases found in lactic acid bacteria (LAB).

Thus, the development of the research project includes six chapters. The first of them is a general introduction to the research project. The second chapter deals with a review published in the journal Foods on the influence of the gut microbiota on health or disease status and the importance of consuming prebiotics and probiotics in producing this status. The third chapter presents a comprehensive review published in Critical Reviews in Foods Science and Nutrition. This review covers general aspects of whey and its potential as a source of bioactive peptides, and describes the major proteins in this milk matrix. Likewise, the main methods used to obtain bioactive peptides are discussed. Then, each of the bioactivities reported from peptides derived from milk whey proteins is described. Finally, some of the major products on the market containing bioactive peptides from whey proteins are described. The fourth chapter contains the results published in Foods, presenting the proteolytic profiling of L. rhamnosus GG and S. thermophilus SY-102, using three different techniques to determine the degree of protein hydrolysis and its potential. Similarly, angiotensin converting enzyme inhibition (ACE) was determined to evaluate the antihypertensive effect of the peptides produced during fermentation. The fifth chapter contains the results submitted to the journal *Dairy*. In this chapter, the growth of strains during fermentation in whey from three fermentation plants is presented. The kinetic parameters are reported, as well as the proteolytic profile and antidiabetic activity of the peptides produced during fermentation.

The observed proteolytic profile indicates that the fermentation of whey with lactic acid bacteria has a high potential for the production of low molecular weight peptide fractions with considerable bioactive activity. Finally, in the sixth chapter, the results of the separation of the peptide fractions obtained from the hydrolysis of whey proteins by the protocolling of lactic acid bacteria are presented by RP-HPLC. These results were submitted to the Journal of medicinal Food. Antidiabetic, antihypertensive and antioxidant effects are reported.

1. Introducción

De acuerdo a la Federación Internacional de Diabetes (FID), más de 425 millones de adultos padecen esta enfermedad, de los cuales los pacientes con Diabetes mellitus tipo 2 (T2DM) representan aproximadamente el 90%. Se estima que esta cifra aumente a 629 millones en 2045 (Cho *et al*., 2018). La T2DM es un problema de salud mundial cuya fisiopatología se caracteriza por una condición de hiperglucemia, así como una producción insuficiente de insulina como resultado de resistencia a insulina (RI) (Sun *et al*., 2020). Adicionalmente en los pacientes diabéticos suele aparecer un cuadro de hipertensión, una comorbilidad muy común. La prevalencia de hipertensión en la población diabética es de 1 a 3 veces mayor que en población sana, los estudios indican que los individuos diabéticos con hipertensión tienen mayor riesgo de sufrir otras complicaciones como retinopatía diabética, insuficiencia renal y enfermedad cardiovascular (Vargas-Uricoechea & Cáceres-Acosta, 2019).

El tratamiento convencional para la T2DM incluye fármacos como metformina, sulfonilurea, inhibidores de la α-glucosidasa e inhibidores de la DPP-IV. Para tratar la hipertensión se utilizan inhibidores de la ECA (IECA), bloqueadores del receptor de angiotensina, bloqueadores de canales de calcio dihidropiridínicos y diuréticos tiazídicos (Vargas-Uricoechea & Cáceres-Acosta, 2019); Sin embargo, el uso de algunos fármacos trae consigo diversos efectos secundarios gastrointestinales, riesgo de hipoglucemia, aumento de peso corporal, retención hídrica, así como complicaciones cardiovasculares (Gomez-Peralta *et al*.,2018; Flood, Bell, de la Cruz y Ginchereau-Sowell, 2017). Es por ello que hay una necesidad de explorar el desarrollo de nuevos tratamientos con efectos secundarios mínimos o encontrar terapias combinadas entre fármacos y suplementos que logren suprimir dichos efectos. En este sentido, la elaboración de suplementos alimenticios a partir de diversas materias primas como el suero de leche, cuyas proteínas se ha demostrado que tienen valiosas funciones biológicas (Chatterton, *et al*., 2006), resulta un área de

oportunidad. Diversos estudios han demostrado que al utilizar esta materia prima en conjunto con bacterias ácido lácticas se obtiene una amplia gama de péptidos con actividad biológica (Olvera-Rosales *et al*.,2022). Dichos péptidos son liberados tras la fragmentación de las proteínas de suero de leche mediante diversos métodos, incluyendo la fermentación microbiana (Daliri *et al*., 2018).

Péptidos con capacidad antihipertensiva y antidiabética se han obtenido tras la hidrólisis de proteínas de suero de leche, enfocándose en la inhibición de enzimas como la ECA y la DPP-IV (Olvera-Rosales *et al*., 2022). Los resultados han demostrado que estas secuencias tienen un alto porcentaje de inhibición de estas enzimas implicadas en los procesos tanto de vasoconstricción como de liberación de insulina y regulación de glucosa (Berger *et al*., 2018: Sultan *et al*., 2018).

Dada esta evidencia, el uso de péptidos con propiedades antihipertensivas y antidiabéticas representa un área de oportunidad en el tratamiento de enfermedades no transmisibles. Por lo tanto, este estudio tiene por objetivo evaluar el potencial nutracéutico de proteínas de suero de leche hidrolizadas por protocooperación de LAB a través de un análisis de bioactividad *in-vitro* para proponerlo como un coadyuvante en el tratamiento de hipertensión y T2DM.

1.2 Hipótesis

La hidrólisis de proteínas de suero de leche por protocooperación de LAB, conduce a la producción y liberación de péptidos de bajo peso molecular con alta actividad antihipertensiva y antidiabética.

1.3 Justificación

La T2DM corresponde a un trastorno metabólico caracterizado por la deficiencia en el efecto de la insulina. Este trastorno es causado por una alteración en la función endócrina del páncreas o en los tejidos efectores que pierden la sensibilidad a la insulina. A nivel mundial, la diabetes es considerada un grave problema de salud pública. Aproximadamente 415 millones de personas en el mundo lo manifiestan, siendo el 91% correspondiente a diabetes tipo 2. En México, se estima que entre 6.6 y 10 millones de personas padecen diabetes, siendo la primera causa de muerte entre las mujeres y la segunda entre los hombres.

El desarrollo de coadyuvantes a base de suero de leche, obtenidos a partir de fermentaciones microbianas, representa una alternativa para el uso de este residuo industrial de gran valor biológico y nutricional. En México el suero lácteo es subutilizado y destinado principalmente a la

alimentación animal. De igual manera, representa un problema ambiental al ser desechado en diversos cuerpos de agua. Sin embargo, se ha comprobado que las proteínas de suero de leche, tienen valiosas funciones biológicas entre las que se incluye su actividad antihipertensiva, antidiabética, antioxidante e inmunomoduladora. Estas propiedades hacen que los péptidos bioactivos sean potenciales coadyuvantes en el tratamiento y la prevención de ciertas enfermedades cardiovasculares, cáncer y diabetes.

Se ha probado científicamente que productos diseñados con la incorporación de péptidos bioactivos ejercen un efecto positivo en el organismo. Estas moléculas actúan disminuyendo los valores de diversos parámetros bioquímicos que son determinantes en patologías como diabetes e hipertensión.

Por ello, en la presente investigación se plantea la hidrólisis de proteínas de suero de leche como una alternativa para la producción de péptidos bioactivos; específicamente aquellos con propiedades antihipertensivas y antidiabéticas para coadyuvar en el tratamiento de diabetes tipo 2 e hipertensión arterial.

1.4 Objetivos

1.4.1 Objetivo general

Evaluar el potencial nutracéutico de proteínas de suero de leche hidrolizadas por protocooperación de bacterias ácido lácticas a través de un análisis de bioactividad *in-vitro* para proponerlo como un coadyuvante en el tratamiento de T2DM e hipertensión.

1.4.2 Objetivos específicos

1. Elaborar revisiones bibliográficas que aborden aspectos relevantes de la microbiota intestinal y péptidos bioactivos de proteína de suero de leche a través de una metabúsqueda, con la finalidad de profundizar en el papel de los mismos en la salud humana.
2. Desarrollar fermentaciones de suero de leche inoculado con *Lactobacillus rhamnosus* GG y *Streptococcus thermophilus* SY-102 para analizar el perfil proteolítico y los cambios fisicoquímicos del medio.
3. Determinar la producción de péptidos derivados a partir de la hidrólisis de proteínas de suero de leche durante la fermentación mediante el método del ácido trinitrobencensulfónico, electroforesis en gel de poliacrilamida Tris-Tricina-SDS-PAGE y SEC-HPLC.
4. Determinar el porcentaje de inhibición de la enzima convertidora de angiotensina y la dipeptidil peptidasa IV de los concentrados de proteína a través de ensayos enzimáticos para valorar su capacidad antihipertensiva y su potencial bioactivo en el tratamiento de diabetes tipo 2.
5. Evaluar la capacidad antihipertensiva, antidiabética y antioxidante de las fracciones peptídicas derivadas de fermentación por protocooperación de bacterias ácido lácticas.

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1.5 Diagrama metodológico

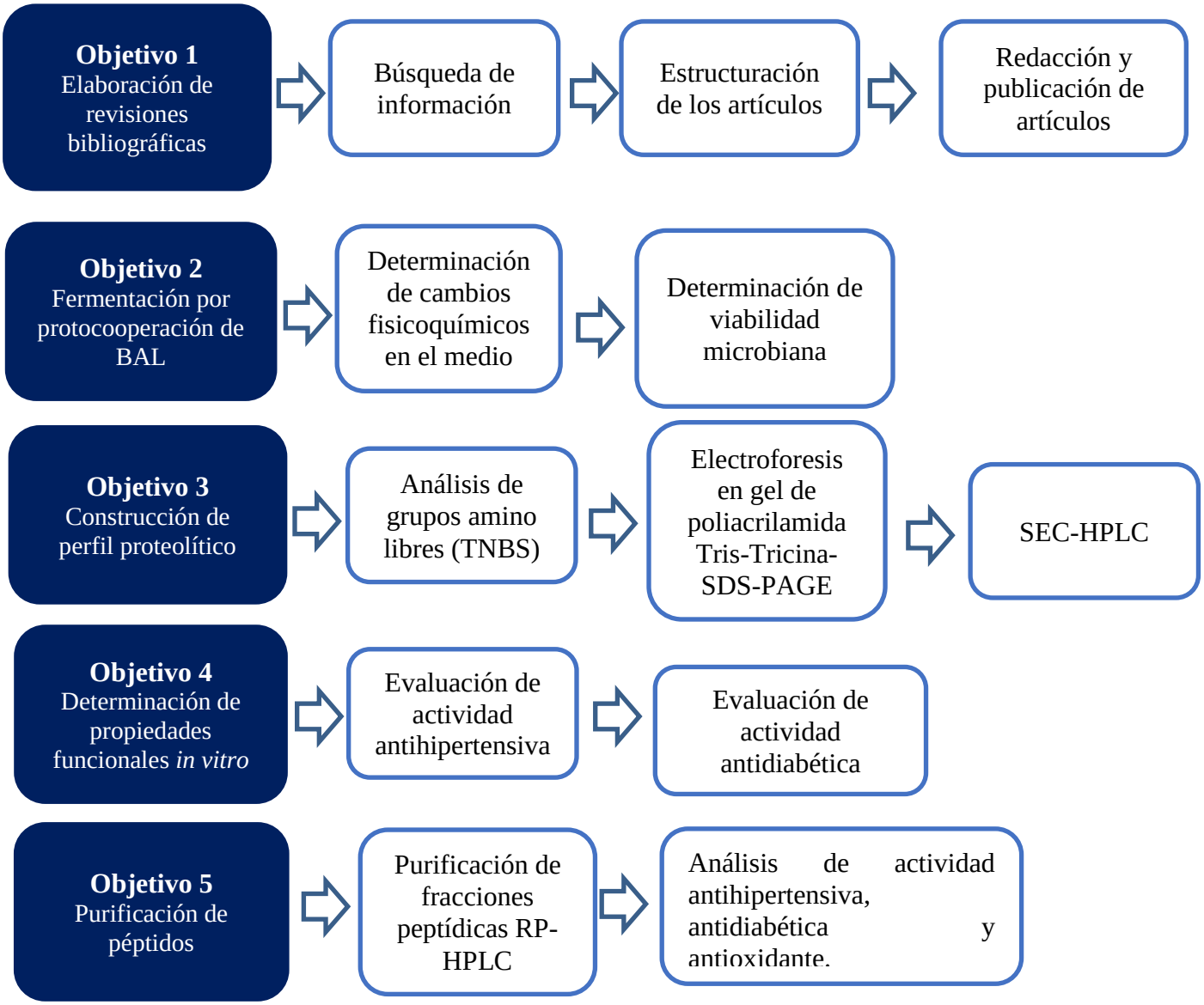

Figura 1. Diagrama metodológico

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Capítulo II.

Impact of the gut microbiota balance on the health-disease relation: importance of probiotics and prebiotics consumption

Introducción

En este capítulo, se realiza una revisión exhaustiva sobre la microbiota intestinal y el impacto de la misma sobre la salud humana. La información se ha publicado en la revista Foods. En primer plano, se resumen las características generales de la microbiota intestinal donde se incluye su composición. Se ha destacado que los principales filos que componen la microbiota sufren cambios en las diferentes etapas del desarrollo humano debido a diversos factores, entre los que se incluye la alimentación. De igual manera, en esta revisión se describen las principales funciones metabólicas de la microbiota intestinal, destacando su papel en la degradación de carbohidratos no digeribles, la producción de ácidos grasos de cadena corta, producción de cuerpos cetónicos y prevención en la colonización de microorganismos patógenos. De igual manera, en este capítulo se hace una recopilación del rol de la microbiota intestinal en enfermedades no transmisibles como diabetes, obesidad y síndrome metabólico, enfermedades gastrointestinales, enfermedades psiquiátricas y neurodegenerativas, así como diversas variantes de cáncer. Los mecanismos implicados en el desarrollo de estas enfermedades son descritos, haciendo énfasis en el eje microbiota-intestino-cerebro como un factor de suma importancia en la comunicación bidireccional entre el sistema nervioso, inmune, endócrino y la microbiota intestinal. Por último, se agrega información de algunos de los mecanismos en la modulación de la microbiota intestinal. Mismos que incluyen la incorporación de probióticos y prebióticos en la dieta como un factor importante en la regulación y mantenimiento de la microbiota intestinal. Diversos estudios *in vitro,* así como clínicos se incluyen en esta sección. Adicionalmente, se hace una descripción de los posibles mecanismos de estos elementos sobre la microbiota intestinal y la prevención y desarrollo de diversas enfermedades.

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Review

Impact of the Gut Microbiota Balance on the Health–Disease Relationship: The Importance of Consuming Probiotics and Prebiotics

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Abstract: Gut microbiota is a group of microorganisms that are deposited throughout the entire gastrointestinal tract. Currently, thanks to genomic tools, studies of gut microbiota have pointed towards the understanding of the metabolism of important bacteria that are not cultivable and their relationship with human homeostasis. Alterations in the composition of gut microbiota could explain, at least in part, some epidemics, such as diabetes and obesity. Likewise, dysbiosis has been associated with gastrointestinal disorders, neurodegenerative diseases, and even cancer. That is why several studies have recently been focused on the direct relationship that these types of conditions have with the specific composition of gut microbiota, as in the case of the microbiota–intestine–brain axis. In the same way, the control of microbiota is related to the diet. Therefore, this review highlights the importance of gut microbiota, from its composition to its relationship with the human health–disease condition, as well as emphasizes the effect of probiotic and prebiotic consumption on the balance of its composition.

Keywords: microbiota; microbiota–intestine–brain axis; probiotic; prebiotic

1. Introduction

The gut microbiota is a group of microorganisms that colonize the gastrointestinal tract, and is found in a higher proportion than the cells of the human body [1]. About half of the fecal mass constitutes the microorganisms that are essentially grouped into five phyla: Firmicutes, Bacteroidota, Verrucomicrobia, Actinobacteria, and Proteobacteria, with a 90% predominance of the first two [2]. Due to the diversity of microorganisms that makes them the most important environmental agent in the human body, various studies

indicate that gut microbiota is directly associated with both the health of the host and some diseases [3]. Despite the evidence on the relationship of gut microbiota with the parameters of various diseases, more research is needed to evaluate other factors, such as the interaction between host genetics, diet, and metabolism regulation [4].

Once the microbiota is established in an individual, it usually changes in a short time, and the changes are even generated in stages during the life of the human. Notwithstanding its high variability, the alteration of gut microbiota composition has huge implications in the pathogenesis of a wide range of diseases, from chronic gastrointestinal diseases to neurological disorders. The influence of gut microbiota in the development of diseases is so great that some investigations have shown alterations in the serotonergic neurotransmission of the central nervous system (CNS), which were secondary to gut microbiota imbalance (dysbiosis) [5]. For this reason, enormous efforts have been directed to reverse the effect of intestinal dysbiosis in neurodegenerative diseases.

One of the main factors affecting the concentration changes over specific microorganisms of the gut microbiota is the combination of diet with genetic factors [6]. It explains why, in addition to gastrointestinal diseases, the microbiota has a direct effect on the development of diseases such as type 2 diabetes mellitus (DM2) and obesity, which are part of the so-called metabolic syndrome [7]. Although it has been suggested that an adequate balance between Firmicutes and Bacteroidota is necessary to avoid the appearance of this type of disease, the studies are not conclusive in this regard [8]. However, gut dysbiosis is a preponderant factor in its development and control. Additionally, dysbiosis has been presented as a risk factor in the development of various types of cancer, and, in addition to the genetic factor, abnormal microbial translocation and molecular mimicry are directly related to this condition [9]. Even though the microorganisms of gut microbiota do not directly induce tumorigenesis, they could interact with the immune system to indirectly promote the proliferation of cancer cells [10].

That is why, knowing that diet has a significant impact on the establishment and composition of gut microbiota throughout life [11], this review aims to provide information about the relationship between the consumption of probiotics and prebiotics and the establishment of balanced gut microbiota. Moreover, the generalities of the microbiota and its importance in the establishment and development of chronic and degenerative diseases are disclosed. The relationship between the gut microbiota–brain axis, its importance in the development of psychiatric and neurodegenerative diseases, as well as its association with the development of brain cancer, have also been addressed. Finally, the use of fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) and their impact on the generation of beneficial populations in the digestive system, has also been mentioned in this review.

2. Human Gut Microbiota: Importance and Composition

The gut microbiota is a group of organisms that provides various benefits and imparts resistance to the colonization of new species, maintaining a symbiotic relationship with the host. However, an imbalance in this complex community could lead to recolonization by pathogenic microorganisms, causing inflammatory processes and the evolution of various diseases [12]. This suggests that gut microbiota maintains the homeostasis of the human intestine [13]. It provides various effects such as protection against pathogens, carbohydrate digestion, regulation of fat storage, production of essential vitamins, and modulation of the immune response, representing an environmental factor of great importance in human homeostasis [14].

The gut microbiota has prominent participation in various metabolic functions through the fermentation of indigestible carbohydrates and the production of short-chain fatty acids (SCFAs), with beneficial systemic effects [15]. Among the SCFAs, acetate, propionate, and butyrate are the most frequently produced [16]. These compounds appear to regulate mechanisms such as lipogenesis and cholesterol biosynthesis in the liver. Acetate is the SCFA achieving the highest concentration in plasma, which has been related to low plasma

insulin levels, propionate contributes to gluconeogenesis in the liver, and butyrate is used mainly as an energy source by the gut mucosa [17]. Additionally, gut microbiota allows the production of ketone bodies and carbon dioxide, as well as the regulation of energy homeostasis by stimulating intestinal enteroendocrine cells [17–19].

In addition to metabolic functions, gut microbiota contributes to the prevention of colonization of pathogenic microorganisms. This activity involves the production of bacteriocins or antimicrobial peptides [20]. Consequently, there is a competition for nutrients, which stimulates innate immunity through the secretion of IgA [21,22] and the activation of Toll-like cell receptors (TLCR). These compounds are capable of identifying molecular patterns associated with microorganisms that structurally include lipopolysaccharide of bacterial origin (LPS), lipoprotein, flagellin, and DNA of pathogens [15]. That is why the immunomodulatory activity exerted by the intestinal microbiota is involved in the interaction with cells of the immune system, participating in both the stimulation of innate immunity and the maturation and subsequent development of adaptive immunity [23,24].

Finally, the activity performed by the intestinal microbiota at the neurological level is crucial. This is carried out through bidirectional communication between the intestine and the brain through the enteric nervous system (ENS) [25]. Gut microbiota controls the ENS through the production, expression, and turnover of neurotransmitters and neurotrophic factors, the maintenance of the sensory barrier, the modulation of enteric sensory input, the production of bacterial metabolites, and the immune regulation of the mucosa [15,26]. Additionally, evidence indicates an association between mood disorders and dysbiosis [27]. This is due to the impact of neurotransmitters, such as serotonin and dopamine, originated by native gut microbiota, on brain alertness, mood control, memory, and the learning process of an individual [26,28].

Certainly, the success of the activities carried out by the intestinal microbiota depends to a great extent on its composition, which changes over time according to the different stages of an individual's life. This microbiota begins to appear from gestation and continues to develop in parallel with the host, fulfilling the necessary functions for the maintenance of homeostasis.

Gut Microbiota and Aging

Approximately 10^6–10^{14} microorganisms belonging to the domains eukaryotic, archaea, and bacteria colonize the human gastrointestinal tract. Gut microbiota is composed of approximately 1000 species, and the main phyla are Firmicutes and Bacteroidota, and, to a lesser extent, Fusobacteria, Cyanobacteria, Proteobacteria, Verrumicrobia, and Actinobacteria can also be found [29]. However, this composition undergoes changes over time (Figure 1) and the dominant bacterial phyla are different at each stage of human life [2].

Intestinal colonization begins in the gestational stage. Some studies have been able to determine the presence of various microorganisms in the placenta, umbilical cord, and amniotic fluid, Proteobacteria and Actinobacteria being the most predominant at this stage [30–32]. Later, during natural childbirth, newborns are colonized by taxa that originate in the mother's vagina, while newborns delivered by cesarean section will be colonized by microorganisms present on the skin [33]. After birth, the intestine is progressively colonized by various microbial strains. The first colonizing microorganisms generally belong to the enterococci and enterobacteria, followed by members of strict anaerobes genera, such as *Bifidobacterium*, *Clostridium*, and *Bacteroides* [34].

By the age of three, the microbiota has stabilized. However, bacterial strains will undergo significant fluctuations and changes over time, modifying their composition and gene expression. These modifications are due to anatomical, dietary, nutritional, and environmental alterations [6]. Additionally, the composition will also be determined by pathological disorders, such as gastrointestinal and systemic infections, as well as by the use of pharmacological agents, such as antibiotics, laxatives, prokinetics, and probiotics [35,36].

During older age, a restructuring in the intestinal microbiota has been observed. Claesson et al. [37] reported significant changes in subjects older than 65 years, specifically

an increase in the abundance of Bacteroidota and Proteobacteria. It has been hypothesized that alterations in the microbiota upon reaching an older age are mainly due to physiological changes in the gastrointestinal tract. Among these changes are the decrease in esophageal contractions and peristaltic movements, alteration in the gastric lining and fibrosis, the presence of low-grade chronic inflammation, and eating habits [38].

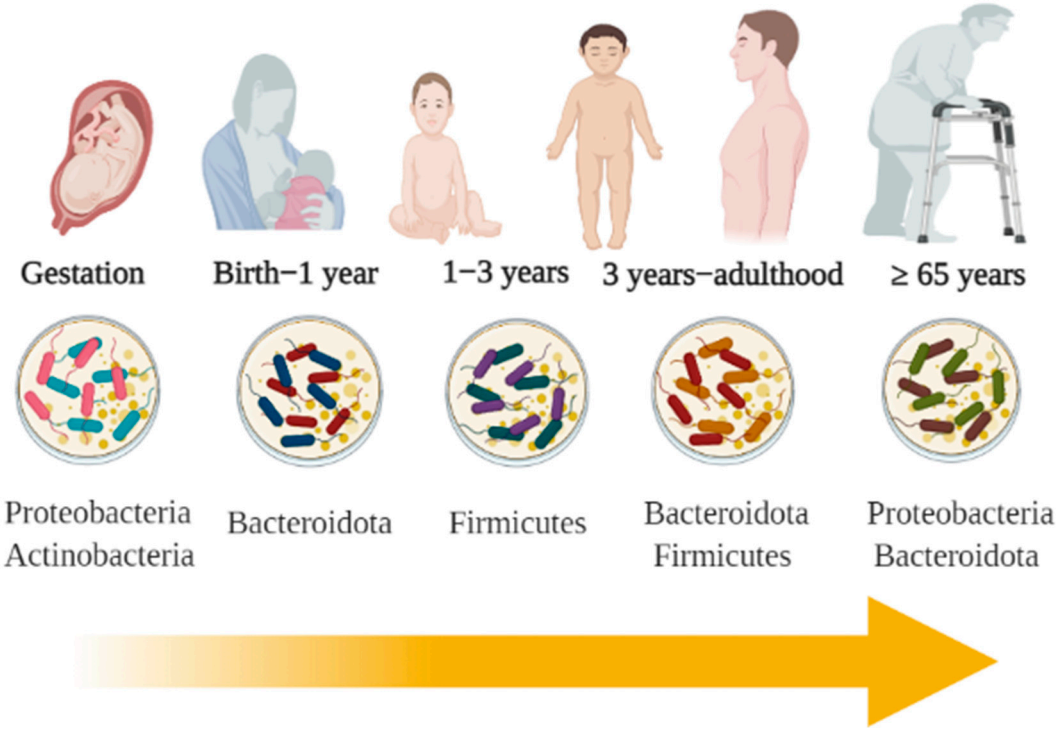

Figure 1. Dominant gut microbiota phyla in different life stages.

3. Role of Gut Microbiota in Human Health

3.1. Diabetes and Obesity (Metabolic Syndrome)

Diseases such as obesity and DM2, and, in general, metabolic syndrome are related to dysbiosis [7,35,39]. There are three mechanisms related to the microbiota and the development of these diseases. One of them is the type of carbon source for obtaining energy. The second is related to the modulation of some human genes and proteins, which are involved in the regulation of energy expenditure. Finally, the third mechanism is associated with the regulation of the levels of LPS of bacterial origin into the plasma, which can induce chronic subclinical inflammation. The last one leads to the development of insulin resistance through the activation of TLCR [40,41].

Most human studies indicate that an increase in the Firmicutes/Bacteroidota ratio is related to an increase in a low-grade inflammation state. Additionally, the little diversification of the intestinal microbiota is also associated with greater insulin resistance, inflammation, and adiposity [42,43]. This information is reinforced with metagenomic studies of the human intestinal microbiota relating not only obesity and insulin resistance, but also the increase of various markers, including TNF-α interleukin 6, and other proinflammatory cytokines [44–46].

Thus, the role of the intestinal microbiota in obesity and diabetes has also been demonstrated during the transplantation of fecal microbiota from lean human donors to obese human recipients [47]. Microbiota transplantation in these conditions increases insulin sensitivity and, therefore, there is better control of glycemic levels in subjects with metabolic syndrome [48]. Furthermore, a significant reduction in body weight in overweight and obese subjects is achieved through treatment with probiotics that could

colonize the gastrointestinal tract, as demonstrated by Kadooka et al. [49] by supplying *Lactobacillus gasseri* as a supplement.

In the specific case of diabetes, some studies have indicated changes in the composition or function of the intestinal microbiota in patients with this condition. It is known that dysbiosis in this pathology is related to a decreased population of butyrate-producing bacteria, such as *Faecalibacterium prausnitzii* and *Roseburia intestinalis* [50]. The intestinal production of SCFAs, such as butyrate, is related to the beneficial effect on peripheral tissues, such as the liver, and adipose and connective tissue, also improving insulin sensitivity [51]. In addition, there is an increase in the populations of opportunistic pathogens, such as *Escherichia coli*, *Bacteroides caccae*, *Erysipelatoclostridium ramosum*, *Clostridium symbiosum*, and *Clostridium hathewayi*, which have also been related to dysbiosis [52].

Metagenomic studies have revealed a direct relationship between dysbiosis and diseases such as DM2 and obesity, since a decrease in the population of butyrate-producing species of the genera *Clostridium*, *Fecalibacteria*, and *Roseburia*, belonging to the phylum Firmicutes, has been demonstrated [35,44,53]. Furthermore, the Firmicutes/Bacterioidetes relationship is altered by the increase not only of *Escherichia coli*, but also of the phylum in general [35,54,55]. These results demonstrate a link between microbiota and metabolic diseases such as obesity and diabetes, and pose an area of opportunity for the development of new therapeutic strategies.

3.2. Gastrointestinal Diseases

Despite the mutualistic relationship that exists between the microorganisms of the gut microbiota and the human, some bacteria can acquire virulence and change their symbiotic properties due to genetic, environmental, and dietary factors [56,57]. Various studies suggest that the alteration of gut microbiota, as well as its metabolic functions, are correlated with the appearance and progression of gastrointestinal diseases, such as severe diarrhea, celiac disease, and irritable bowel syndrome, among others [58–60].

Thus, the evolution of diseases such as intestinal inflammation is governed by complex interactions between several factors: environmental risks, host genetics, and the state of gut microbiota [61,62]. However, it is gut microbiota that has a direct effect when there is a reduction in the population of Firmicutes [56]. This fact is well documented through clinical studies that show that the diversity and richness of the microbiota are significantly reduced in patients with intestinal inflammation [56,58,63]. Furthermore, the pathogenesis of this disease is characterized by the accumulation of certain pathobionts, such as *Escherichia coli* and *Ruminococcus gnavus* [64,65].

On the other hand, in ulcerative colitis, which is characterized by inflammation and ulceration of the lining of the colon, an adaptation of microbial species such as *Bacteroides fragilis* and *Escherichia coli* has been observed. These microorganisms have evolved to acquire adherence to the mucosa of the ileum and degrade its walls [66–68]. Similarly, the presence of pathogenic microorganisms adhered to the intestinal wall of patients with irritable bowel syndrome has been manifested [67,69,70]. Furthermore, there is an increase in the Firmicutes/Bacteroidota ratio compared to that of healthy patients [70,71]. Specifically, there is a greater number of species of the family Ruminococcaceae and *Clostridium cluster* XIVa and a smaller number of *Bacteroides* [72].

In certain gastrointestinal diseases, dysbiosis has been related to genetic mutations [73]. For example, in the case of celiac disease, it has been reported that mutations present in genes involved in the secretion of intestinal mucus, the structure of associated bacteria, and a reduction in bacterial diversity and richness, influence the pathogenesis of the disease [74]. This disease presents a series of symptoms of chronic immune-mediated inflammation in the small intestine due to a lower abundance of *Bifidobacterium* spp. in the human intestine [75,76]. This marks an association with an increased risk for the development of autoimmune diseases [57].

More in-depth studies are required to explore the therapeutic potential of the modulation of gut microbiota in the treatment of gastrointestinal diseases since, due to scientific

evidence, the phenotype of commensal bacteria can go from symbiotic to pathogenic in response to various risks factors [56]. These phenotypic alterations modify not only the host's immune system, but also impact the structure and diversity of gut microbiota, which leads to the development and/or progression of a greater diversity of gastrointestinal diseases [77–79].

3.3. Psychiatric and Neurodegenerative Diseases

The microbiota–intestine–brain axis (Figure 2) is a bidirectional communication network that includes the central nervous (CNS), autonomic nervous (ANS), and enteric nervous (ENS) systems. The immune system, the endocrine system, and the intestinal microbiota also belong to this axis [80]. Given the complexity of this network, possible intervention strategies have been explored, aimed at the dysbiosis of gut microbiota present in various neurological and psychiatric disorders, including the use of probiotic, prebiotic, and symbiotic foods [2].

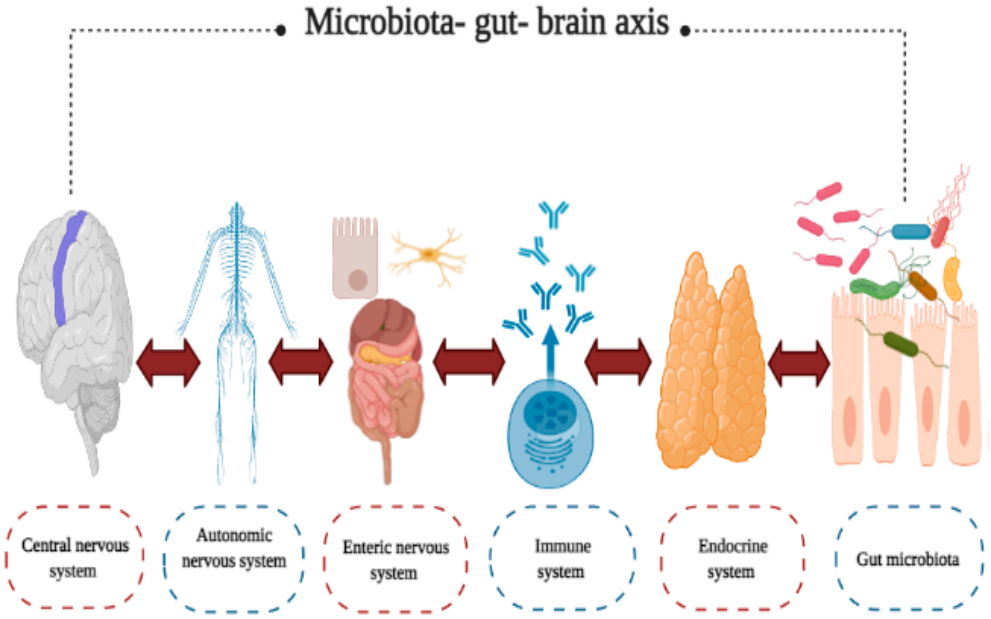

Figure 2. Microbiota–gut–brain axis, bidirectional communication network that includes the central nervous, autonomic nervous, enteric nervous, immune, and endocrine systems, and gut microbiota.

Some studies have pointed out that gut microbiota dysbiosis also plays an important role in psychiatric and neurological diseases, such as Alzheimer's, Parkinson's, autism, neurodegeneration, multiple sclerosis, anxiety, and depression [81,82]. Furthermore, various studies indicate that the intestinal microbiota influences the gut–brain system, triggering the symptoms that occur during a state of anxiety and stress [83]. Likewise, the intestinal microbiota seems to have an impact on pain tolerance mechanisms [84,85]. Similarly, it has been noted that gut microbiota is closely related to neurological functions, mood, and host behavior, as well as the circadian cycle [81]. However, the mechanisms by which this relationship is generated are still not entirely clear, but the concept of the "microbiota–gut–brain axis" has been intended to explain them [80]. Some of these mechanisms are as follows:

(1) Involvement of the vagus nerve. There is a connection between the ENS and the CNS that provides a direct communication pathway between gut microbiota and the CNS [86,87];
(2) Participation of the circulatory system. This system regulates the effects of various metabolites, such as neurotransmitters, hormones, and SCFA, that are produced by gut microbiota and impact on CNS functions [81];

(3) Regulation of signals and the synthesis of neurotransmitters. Gut microbiota apparently modulates the expression of central neurotransmitters and related receptors, and some species produce neurotransmitters, such as acetylcholine, dopamine, and adrenaline, or induce their synthesis [88–90];
(4) Production of SCFA. Gut microbiota is capable of modulating the maturation of the microglia and the permeability of the blood–brain barrier through the synthesis of SCFA [89,91,92];
(5) Immunomodulation. Gut microbiota influences the activation of peripheral immune cells that regulate CNS immune reactions [93,94].

Studies have shown differences between patients with neurological disorders and healthy controls [95]. In healthy individuals, an analysis of 16s rRNA from fecal microbiota showed that bacteria corresponding to the phyla Firmicutes and Bacteroidota have a higher proportion than that of the phyla Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia [96]. Additionally, microbial abundance and diversity are significantly reduced in patients with depression and anxiety disorders [95]. Thus, the concentration of the Lachnospiraceae and Ruminococcaceae families and the *Ruminococcus* and *Lactobacillus* genera is decreased [97]. This dysbiosis causes both microorganisms and the products of their metabolism to induce inflammation at the brain level through blood circulation and induce the production of various cytokines, such as IL-6, IL-1β, and TNF-α [95], which, in turn, modulate various brain processes that affect mood and behavior [98].

In the case of Parkinson's patients, gut microbiota alterations coincide with an aggravation of the condition [99–102], which is related to a lower concentration of *Prevotellaceace* species compared to the relative abundance of *Enterobacteriaceae* in the feces of these patients [103]. Likewise, a decrease in the *Prevotellaceae* population generates an increase in intestinal permeability and systemic exposure to LPS [87,104]. This endotoxin induces systemic inflammation by the production of proinflammatory cytokines that interact with TLCR and nuclear factor-kappa B (NF-κB). This mechanism is related to the progression of Parkinson's once LPS breaks the blood–brain barrier [105,106].

Another condition related to dysbiosis is Alzheimer's, a neurodegenerative disorder that leads to cognitive dysfunction [107]. An increase in intestinal permeability, as a consequence of dysbiosis, has, in turn, been associated with an increase in the concentration of various microorganisms, as well as products derived from their metabolism [108–110]. The released endotoxins are involved in neuroinflammation in patients with Alzheimer's by acting on the innate immune system [111,112]. In general, it has been observed that gut microbiota in people with this condition is represented by an increase in Proteobacteria and a decrease in SCFA-producing bacteria [113]. This imbalance leads to an increase in proinflammatory cytokines, such as TNF-α, IL-5, IL-6, IL-1β, and IL-8 [114]. Thus, the use of probiotics has been explored as an alternative to improve the cognitive functions of patients and the decrease in cytokines that lead to neuroinflammation [115–117].

3.4. Cancer

The role of the gut microbiota in the development of several types of cancer has been reported in recent years [118–120]. It has been highlighted that pathogenesis is not only attributed to genetic susceptibilities, but also to mechanisms that include abnormal microbial translocation, molecular mimicry, and dysregulation of local and systemic immunity [9]. In this context, it has been reported that some microorganisms belonging to gut microbiota have oncogenic effects or oncolytic activity in tumor cells. Approximately 20% of cancers are attributable to infectious agents, including bacteria [121]. In addition to this, it has been observed that there is a difference between healthy individuals and cancer patients in terms of population and microbial diversity present at the intestinal level [122].

The link between gut microbiota and carcinogenesis has been described, with an emphasis on bacterial metabolites. Thus, the main mechanisms of bacterial-mediated carcinogenesis are mainly based on the effects of the specific toxins or virulence factors produced [123,124]. Furthermore, microbial metabolites, such as polyamines and secondary

bile acids, are also involved in cancer cell proliferation and tumor induction through the β-catenin signaling pathway [125], epidermal growth factor receptor (EGFR) transactivation [126], and increased COX-2 activity [127].

That is why the immune system of the intestinal mucosa is related to cancer, in which its interaction with gut microbiota is considered a key factor in the maintenance of homeostasis [68,128]. Due to this interaction, there is an effect on the inhibition of bacterial adhesion and colonization, as well as the induction of cell differentiation [129]. In this sense, microbial species such as *Bacteroides fragilis* induce the differentiation of T cells CD4+ to regulatory T cells (Treg cells) [130], which are capable of secreting large amounts of anti-inflammatory cytokines, such as IL-10, and recognizing antigenic substances associated with the bacterial genera *Clostridium* and *Bacteroides* [131,132]. However, the intestinal microbiota not only has an immunomodulatory effect at the local level, but also the systemic level [128]. Metabolites produced by microorganisms enter the bloodstream and, therefore, affect the immune response in distant organs through interaction with TLCR [129].

Although the microorganisms of gut microbiota do not directly induce tumorigenesis, they could interact with the immune system to indirectly promote the proliferation of cancer cells [10]. Thus, a defective immune response increases the abundance of certain bacterial genera and triggers signaling pathways that lead to the transcription of oncogenes [10,128]. In addition to this, gut microbiota can indirectly promote cancer by inducing inflammation or immunosuppression through the production of cytokines [133,134]. Finally, the development of various malignant neoplasms, including some types of cancer (gastric, colorectal, pancreatic, breast, and brain cancer), is currently associated with variations in gut microbiota composition [135].

3.4.1. Colorectal Cancer

Some bacterial species related to the development of colorectal cancer, such as *Fusobacterium nucleatum*, *Peptostreptococcus anaerobius*, and *Bacteroides fragilis* enterotoxigenic, have been identified [136]. Moreover, a lower probability of survival in patients with colon cancer has been associated with *F. nucleatum* abundance [137] due to the induction of chemoresistance, which activates autophagy [134], leading to treatment failure or disease recurrence [128]. The species mentioned above induce tumor proliferation [138], promote inflammation [139], protect the tumor from the mechanisms exerted by the immune system [140], and cause damage to host cell DNA [141]. All these factors contribute to carcinogenesis. As in other types of cancer, in colorectal cancer, special emphasis has been placed on protein toxins produced by the intestinal microbiota [142]. The procarcinogenic effect of these toxins could be due to the direct attack on DNA, which leads to genomic instability or proliferation and induction of resistance to apoptosis in cancer derived from cellular signaling alterations [143].

3.4.2. Pancreatic Ductal Adenocarcinoma

This condition represents one of the most serious malignant neoplasms, with overall survival lower than 5 years [9]. Because surgical resection is often not possible, treatment is focused on chemotherapy. However, some patients could develop chemoresistance [144] associated with gut microbiota, which has a major impact on pancreatitis and pancreatic ductal adenocarcinoma [144,145].

In the same way that *Fusobacterium nucleatum* has been associated with colorectal cancer, it is currently known that this microorganism induces chemoresistance, autophagy, and inflammation in pancreatic carcinogenesis processes [134,146]. Additionally, in patients with this condition, an increase in Proteobacteria and Verrucomicrobia, and a decrease in Firmicutes and Bacteroidota have been observed, which are accompanied by the activation of inflammatory pathways in tumor tissues [147].

Furthermore, it has been observed that the presence of intratumoral pathogens and bacteria, such as *Acinetobacter*, *Aquabacterium*, *Oceanobacillus*, and *Rahnella*, are associated with a higher risk of presenting pancreatic ductal adenocarcinoma [148,149]. The develop-

ment of this disease involves the intestinal mucosa, epithelial and dendritic cells (DC), and different cells from the immune system [150]. The above-mentioned microorganisms are part of the gut microbiota and promote the development of adenocarcinoma through the release of a large number of metabolites [133] which interact with TLCR and also induce systemic inflammation and immune responses associated with pancreatic carcinogenesis and therapeutic resistance [151].

3.4.3. Breast Cancer

The most common type of cancer affecting women worldwide is breast cancer. More than 40,000 deaths per year occur, even though there has been significant progress in its diagnosis and treatment [152]. A strong link between dysbiosis and the appearance of neoplasms, including those of breast cancer, has been shown [124]. Thus, recent research has focused on the influence of gut microbiota on the development of breast cancer, beyond genetic, environmental, and lifestyle factors [153].

The differences in the concentration of *Bifidobacterium*, *Faecalibacterium prausnitzii*, and *Blautia* have been used as biomarkers associated with the clinical stage of breast tumors [154]. In addition, these differences are also associated with the body mass index of the patients. Indeed, it has been observed that overweight and obese women with breast tumors present lower concentrations of Firmicutes, *Faecalibacterium prausnitzii*, and *Blautia* spp., as well as *Akkermansia muciniphila* prevalence, compared to patients with normal weight [154,155].

Likewise, in postmenopausal women with breast cancer, there is an alteration in the composition of the fecal microbiota, as well as a lower microbial diversity [156]. Specifically, elevated levels of Clostridiaceae, Faecalibacterium, and Ruminococcaceae, as well as a decrease in the proportion of Dorea and Lachnospiraceae, have been reported [157]. Similarly, an increase in the population of species such as *Escherichia coli*, *Citrobacter koseri*, *Acinetobacter radioresistens*, *Salmonella enterica*, and *Fusobacterium nucleatum*, among others, has been observed [158]. However, some factors must be taken into account when determining the characteristics of the gut microbiota of patients with breast cancer, such as age, ethnicity, and geographic location [156].

3.4.4. Gastric Cancer

Gastric cancer is one of the most common neoplasms, which is characterized by acute and persistent inflammation [159]. In the same way, as in other types of cancer, the gut microbiota is related to the development of this disease and *Helicobacter pylori* is the main carcinogenic agent [160]. The mechanism of action of *H. pylori* to produce inflammation is associated with the degree of specific virulence of each strain [161]. The carcinogenesis process begins with genetic instability caused by the breaking of the host's DNA chain [162]. Similarly, the TLCR and NOD-like receptors that recognize the presence of *H. pylori* are also associated with the chronic carcinogenesis process [162].

Current data indicate that *H. pylori* infection produces a genotoxic effect through two possible mechanisms (Figure 3) [163]. First, immune cell infiltration, including neutrophils and macrophages, increases, leading to the production of reactive oxygen and nitrogen species (RONS) [164]. RONS cause damage in the DNA leading to single-strand breaks and increased expression of oncogenes [165]. Alternatively, the transcription factor NF-κB is activated by RONS, inducing the expression of oncogenes and cell cycle regulators [166]. In addition, this factor translocates to the nucleus, forming an NER protein complex (XPG and XPF) which cleaves the promoter regions of genes, impacting gene expression because of double-strand breaks [167].

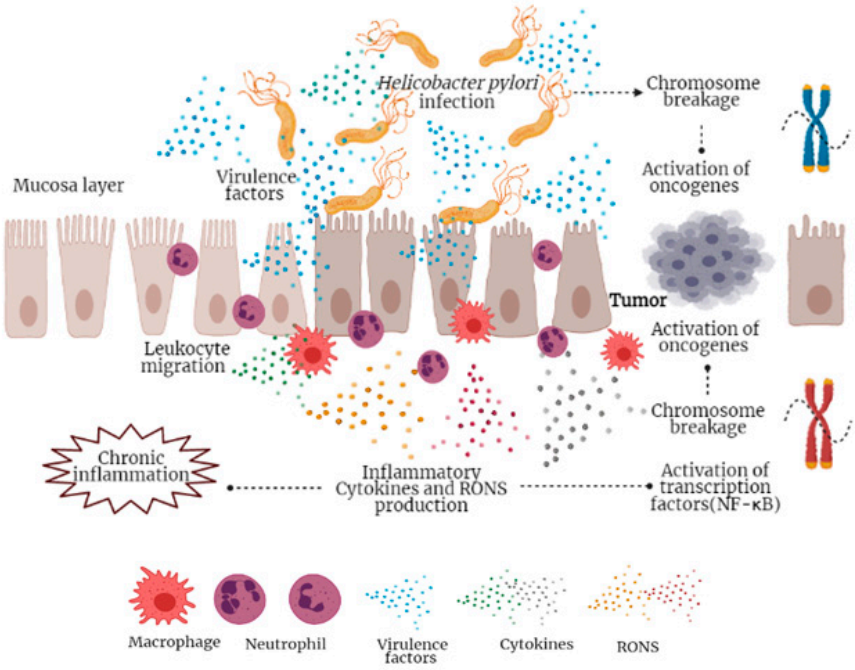

Figure 3. Mechanisms in the pathogenesis of *Helicobacter pylori*-associated gastric carcinogenesis.

Although gastric acidity serves as an important barrier that limits the entry of microbes into the gastrointestinal tract [159], *H. pylori* can survive the conditions present in the stomach, but its ability to colonize gastric glands is restricted due to the large amount of acid produced in these cavities [168]. However, concomitant inflammation and the presence of *H. pylori* increases damage in various regions of the stomach. The result of this damage is atrophy associated with the abundance of this microorganism, which is greater in gastric cancer than in gastritis and intestinal metaplasia [169,170].

Additionally, in subjects with *H. pylori* infection and precancerous gastric lesions, variations in the relative abundance of the dominant phyla, such as Bacteroidota, Firmicutes, and Proteobacteria, have been observed in fecal microbiota [171]. Other specific bacteria related to gastric carcinogenesis, such as *Peptostreptococcus stomatis*, *Slackia exigua*, *Parvimonas micra*, *Streptococcus anginosus*, and *Dialister pneumosintes*, have been studied, but *H. pylori* is the microorganism commonly associated with gastric cancer [172].

3.4.5. Brain Cancer

Gut microbiota and brain cancer association is a new topic that has gained interest in recent years [173]. This relationship can be explained by the mechanisms present in the microbiota–gut–brain axis, since it has been reported that they could influence the development or suppression of brain tumors [94].

Tryptophan is a substrate used by intestinal microorganisms to produce indoles. These molecules are involved in the signaling pathways between the gastrointestinal tract and the immune system [174]. This amino acid is metabolized in the kynurenine pathway, resulting in the biosynthesis of nicotinamide adenine dinucleotide and various neuroactive intermediates [173]. In this context, it has been reported that a dysregulation of the kynurenine pathway could contribute to the development of brain cancer by interrupting the antitumor immune response [175,176]. Likewise, gut microbiota could influence the brain tumor microenvironment through different mechanisms: (1) control of the expansion and activation of T cells [177]; (2) microglia [178–180]; (3) cytokine and arginine production, and tryptophan availability via kynurenine [179,181]; and (4) production of reactive oxygen species (ROS) and generation of antioxidants [119,180].

4. Modulation of Gut Microbiota through Diet

Diet is a preponderant factor that affects the establishment and composition of the gut microbiota throughout life [11], and changes during adulthood could affect intestinal homeostasis [182]. When there is a reduced dietary diversity and a lack of essential nutrients, a dysbiosis of gut microbiota occurs, leading to the appearance of various disorders [183]. The species that comprise the human gut microbiota require a wide range of nutrients and energy sources to promote growth, and they have a direct relationship with the effects associated with human health [182].

It has been highlighted that the intake of specific nutritional elements (carbon sources, nitrogen sources, growth factors, etc.) contributes to the diversification in the composition of the intestinal microbiota [184]. In this way, auxotrophies of some microorganisms that maintain the balance of the intestinal microbiota have been determined [185,186]. Marcobal et al. [187] point out that *Bifidobacterium infantis* and *Bacteroides thetaiotaomicron*, which are present in the gut microbiota of infants and adults, require oligosaccharides present in milk and the mucosa of the large intestine. This suggests that the particular carbon metabolism of each of the phyla plays a preponderant role in both the survival and long-term stability of gut microbiota.

The amount, type, and balance of proteins, carbohydrates, and fats of the diet greatly impact the gut microbiota [188], mainly due to the products from their degradation. This degradation causes the formation and release of SCFAs, phenols, indoles, and amines, with a wide range of physiological effects on the host [189].

It has been reported that a diet rich in prebiotics, such as inulin, oligofructose, and fructooligosaccharides, among other polysaccharides of plant origin, increase the growth of lactobacilli and bifidobacteria in gut microbiota [190]. Contrarily, the intake of a diet rich in simple carbohydrates and high in fat affects the abundance of Firmicutes phylum populations and decreases Bacteroidota, which, in some studies, has been related to obesity [191,192].

On the other hand, an animal-based diet is related to a greater microbial diversity increasing bile-tolerant bacteria and Bacteroidota, and decreasing Firmicutes [182]. The consumption of protein and fat of animal origin have also been linked to Bacteroidota phylum, while carbohydrates have been related to Bacteroidetes, as well as Firmicutes phylum, indicating an association with dietary patterns [193]. Additionally, the type of diet has an influence on intestinal transit time, which is faster with a plant-based diet than with an animal-based one [193,194]. Plant-based diets are a source of nondigestible fiber. It is generally accepted that the benefits of fiber intake on health are derived from laxation, increasing fecal bulking, and stool water content that stimulate mucus secretion and peristalsis [195].

Sprong et al. [196], in an in vivo study, observed a significant increase in the counts of bifidobacteria and lactobacilli when the diet included whey cheese or casein supplemented with threonine or cysteine. According to the aforementioned studies, gut microbiota composition can be positively modified through diet. Despite this, current food habits are characterized by no significant consumption of fruits, vegetables, and fish, leading to several health disorders, such as diabetes, hypertension, cardiovascular accidents, increased levels of cholesterol and triglycerides, greater insulin resistance, and inflammation, among others [197].

That is why there is an urgent need to develop efficient strategies aimed at reversing, preventing, and treating metabolic disorders associated with the dysbiosis process [198]. It can be achieved from a pharmacological and nutritional approach by incorporating prebiotics, probiotics, symbiotics, and other supplements into the diet. With their consumption, gut microbiota balance could be re-established or a healthy gut microbiota could be maintained when homeostasis has been lost due to an adverse condition [199].

4.1. Probiotics and Microbiota

The use of probiotic species has been relevant in the treatment of human and animal diseases due to their effect on the modulation of the intestinal microbiota. The potential that probiotic microorganisms represent has driven research for the production of probiotic foods and the modulation of gut microbiota, promoting their consumption [200].

The Food and Agriculture Organization and the World Health Organization define probiotics as strains of live microorganisms that confer beneficial effects on health when administered in specific amounts. The International Scientific Association for Probiotics and Prebiotics (ISAPP) supports this definition [201,202]. In recent years, a new terminology that provides a comprehensive approach to all the beneficial aspects of probiotics has been suggested. In this way, three main classes of probiotics have been proposed, including: (1) true probiotic (TP), to refer to a viable and active probiotic cell; (2) pseudo-probiotic (PP), which refers to a viable and inactive cell as a spore or vegetative body; and, (3) phantom probiotic (GP), to refer to a dead/nonviable cell that is intact or lysed [203]. According to this new terminology, a probiotic could be defined as a viable or nonviable microbial cell in a vegetative or spore state, intact or lysed, that is potentially healthy for the host [202].

Since the beginning of the 20th century, when the importance of the consumption of probiotic foods with a specific mixture of microorganisms began to be highlighted [199], studies on this type of food have been intensified in animal and human models. In this way, the beneficial effect of several species of specific strains, which have immunological, metabolic, and neuroendocrine activity, has been verified [199]. Certainly, the beneficial effects exerted by probiotic microorganisms are numerous, highlighting their effect on the development of microbiota that inhabits the organism (Table 1). Thus, it has been determined that the consumption of probiotic microorganisms helps to regulate intestinal homeostasis, maintaining an adequate balance between pathogens and bacteria necessary for the correct functioning of the organism [204].

Table 1. Probiotics and effects on gut microbiota.

Probiotic Strain	Model Description	Effects on Gut Microbiota	Source
Lactobacillus helveticus Bar13	Healthy male and female adults aged between 71 and 88 years. Consumption of 10^9 CFU/mL of *L. helveticus* Bar13 once a day for 30 days	No increase in clostridium cluster XI	[205]
Lactobacillus rhamnosus GG	Healthy adults (mean age of 42 years). Daily consumption of 250 mL milk-based fruit drink containing either *L. rhamnosus* GG (ATCC 53103, 6.2×10^7 CFU/mL) for three weeks	No significant impact on gut microbiota composition	[206]
Lactobacillus casei Zhang	Healthy adults consuming 10^6 CFU/mL of *L. paracasei* Zhang for 28 days	Difference in composition and diversity of intestinal microbiota compared to baseline	[207]
Lactobacillus paracasei DG	Healthy male and female adults aged between 23 and 55 years. The subjects consumed a capsule containing at least 24 billion viable *L. paracasei* DG cells for 4 weeks	Increase in proteobacteria and *Coprococcus*; decrease in *Blautia*	[208]
Lactobacillus salivarius UBLS22	Healthy young volunteers. Consumption of 2×10^9 CFU/mL of *L. salivarius* for 6 weeks	Increase in lactobacilli and decrease in *E. coli*	[209]
Lactobacillus casei NCDC 19	Male C57BL/6 mice (6–7 weeks old). Diet supplemented with 10^8 CFU/mL of *L. casei* NCDC 19 for 8 weeks	Increase in bifidobacteria population	[210]

Table 1. *Cont.*

Probiotic Strain	Model Description	Effects on Gut Microbiota	Source
Bifidobacterium animalis subsp. *Lactis*	Adult female monozygotic twin pairs consuming 4.9×10^7 CFU/mL of *B. animalis* subsp. *Lactis* for 7 weeks	No change in dominant microbiota	[211]
	Subjects with irritable bowel síndrome consuming 10^9 CFU/mL of *B. animalis* subsp. *Lactis* for 4 weeks	Increase in butyrate producing species and decrease in a pathobiont, *Bilophila wadsworthia*, abundance	[212]
Akkemansia muciniphila	Obese and Type 2 Diabetic mice fed with 2×10^8 CFU/0.2 mL of *A. muciniphila* for 4 weeks	Increase in the intestinal endocannabinoid content (acylglycerols)	[213]
Faecalibacterium prausnitzii	Adult male Sprague–Dawley (SD) rats with colitis consuming 10^9 CFU/mL of *F. prausnitzii* for 7 days	Induction of interleukin IL-10 production	[214]
Bacteroides uniformis CECT 7771	Obese adult male wild-type C57BL-6 mice consuming 5.0×10^8 CFU/mL of *B. uniformis* CECT 7771 for 7 weeks	Partial stabilization of the microbiota	[215]

Various genetic and molecular studies have made it possible to determine that probiotics exert beneficial effects through four main mechanisms (Figure 4): (1) the immunomodulation they exert in the host; (2) antagonism through the production of antimicrobial substances; (3) the inhibition of bacterial toxins; and (4) competition with pathogens by adhesion to the epithelium and by nutrients [216,217]. These mechanisms are relevant in the prophylaxis, treatment of infections, and maintenance of the host's intestinal microbiota [202].

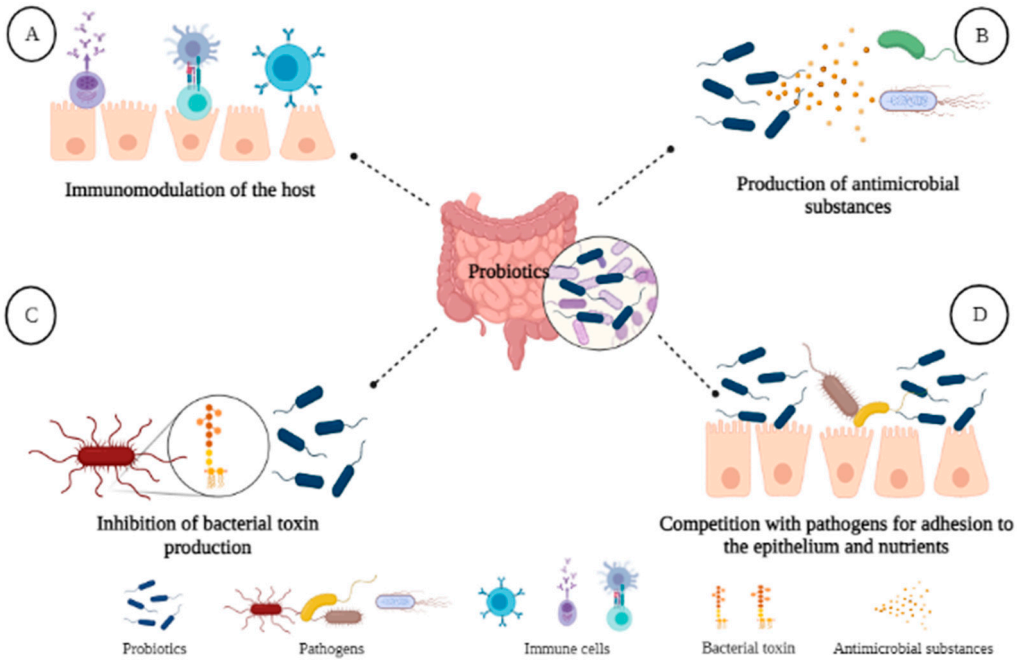

Figure 4. Action mechanisms of probiotics during gut colonization. (**A**) Human immunomodulation exerted by probiotics; (**B**) antagonism derived from production of antimicrobial substances; (**C**) inhibition of toxin action by probiotic metabolism; (**D**) control of pathogen adhesion on epithelium by a competition mechanism.

It is in the maintenance of the microbiota that probiotics play an important role in processes such as the absorption of cholesterol, the regulation of blood pressure, and glucose metabolism. [218–220]. In vivo studies have shown that the administration of

Bifidobacterium and *Lactobacillus* strains has a significant impact on the composition of gut microbiota [221–223]. Park et al. [224] supplemented a diet with the probiotic strains *Lactilactobacillus cruvatus* HY7601 and *Lactiplantibacillus plantarum* KY1032, observing a decrease in the concentration of proinflammatory genes, greater expression of genes related to the oxidation of fatty acids in the liver, thus, as relevant alterations in the diversity and function of the microbiota. However, in clinical studies, this evidence is not conclusive, which is why it has been suggested that the observed changes may only occur in microbial metabolism at the level of SCFAs production and not in the microbial population [225].

Contrarily to these observations, several studies have demonstrated that probiotics produce changes in specific bacterial communities. Plaza-Díaz et al. [226] observed that the ingestion of *L. rhamnosus*, *Lactobacillus paracasei*, or *Bifidobacterium* induces changes in the adult fecal bacterial population. Similarly, it has been observed that the administration of *Lactobacillus salivarius* Ls-33 modifies the populations of fecal bacteria in adolescents with obesity, including several groups of clostridia [227]. On the other hand, in patients with irritable bowel syndrome, considerable fluctuations in clostridium populations have been observed. These alterations are related to the decrease in gastrointestinal symptoms after ingestion of *B. animalis* subsp. *lactis*, which is also related to the decrease in the concentration of pathogenic bacteria and the modification of the colonic production of SCFAs [212,228].

Probiotics play important roles when they come into contact with the rest of the microbial communities, influencing the metabolism of other members of the host's microbiota [225]. An example of this is the bifidobacteria that metabolize a great diversity of carbohydrates, which come from the diet or the intestinal mucosa of the host and produce acetic and lactic acid in different proportions [229]. Specifically, *Bifidobacterium bifidum* increases its metabolism when it grows along with other species, such as *Bifidobacterium breve*, which enhances the catabolism of glycosylated compounds, such as mucin and 3-sialylactose [230,231].

Nevertheless, a modification of the intestinal microbiota through the consumption of probiotics is not the only way to produce a beneficial effect in the host. The probiotic effect can be manifested through the interaction with the immune system [232], but it is necessary to highlight the challenges and opportunities regarding the studies of probiotic microorganisms capable of generating long-term effects after modifying the intestinal microbiota. In this context, in addition to traditional health-promoting bacteria (*Bifidobacterium* and lactic acid bacteria), in recent years, the beneficial effect has been noted after the therapeutic use of next-generation probiotics [233]. This concept includes microorganisms such as *Akkermansia muciniphila* whose effect is associated with glucose metabolism, lipid metabolism, and intestinal immunity [234], for which it has been proposed as a target for immunotherapy in various types of cancer [233,235].

Recently, some authors have proved the benefits of consuming probiotics through clinical studies. Hou et al. [236] determined beneficial changes in the gut microbiota of healthy adults after the consumption of *Lactobacillus casei* Zhang. The effect was related to enterotypic changes due to the increment in concentration of beneficial microorganisms, inhibiting the growth of pathogenic ones. Such changes increased both the development of lactobacilli and the beneficial metabolic functions of gut microbiota. Similarly, the consumption of probiotic fermented milk and yogurt by healthy adults has shown a direct effect on the concentration changes of *Bifidobacterium* spp., especially *B. longum* [237]. The consumption of probiotic fermented milk also has proved to generate beneficial changes in obese patients [238]. Likewise, the inclusion of a mixture of eight probiotic bacteria (*Lactobacillus* spp., *Bifidobacterium*, and *Streptococcus thermophilus*) into the diet of obese people during 15 days generates changes in the concentration of bifidobacteria. The main effect is related to a decrease in *Collinisela*, a proinflammatory biomarker, and an increase in *Akkermansia* concentration, as well as an improvement in oxidative stress biomarkers [239]. Probiotic consumption, specifically *L. casei*, could improve the management of diarrhea in infants,

affecting both the modification of gut microbiota and the attenuation of inflammatory biomarkers [240].

Pharmacological treatments should not be replaced by probiotics, but their consumption could be incorporated into the diet during disease management to provide their well-documented beneficial effects. Thus, the development of probiotic foods containing vehicles for microorganisms that exert a benefit in the modulation of gut microbiota is an area of opportunity in the food science and technology field [241]. Of course, one of the most important challenges is to guarantee the survival of probiotic microorganisms in sufficient concentrations to reach the adequate amounts that promote changes in metabolic functions of gut microbiota.

4.2. Prebiotics and Microbiota

Prebiotics are currently defined by ISAPP as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" [242]. Among the best-known prebiotics are inulin, fructooligosaccharides, galactooligosaccharides, and lactulose [243]. These molecules are selectively fermented in the colon, conferring beneficial effects to the host, including stimulation of both growth and metabolic activity of various bacterial groups of the intestinal microbiota [244], mainly probiotics, which use prebiotics as a carbon source [242,245].

Recently, another type of compound including polyphenols has been proposed as a prebiotic since they meet the current definition previously mentioned. It appears that their beneficial effect on the host depends on the microbial utilization and the metabolites produced rather than on parent compounds. However, as studies on these emerging prebiotics are not yet conclusive, more evidence on the health benefits associated with polyphenols and probiotic interaction is needed [245].

Bacterial fermentation of prebiotics leads to the production of SCFAs, mainly butyrate, acetate, and propionate [246]. These acids have an impact on various cellular mechanisms, such as the activation of G-protein-coupled receptors and the inhibition of histone deacetylation [247]. Likewise, they act as a source of energy for colorectal tissues, exert an anti-inflammatory effect, and act as molecules that are related to the signaling pathways of the microbiota–intestine–brain axis [248]. In addition, other organic acids, such as formate, lactate, and succinate, decrease intestinal pH, preventing the growth of pathogenic bacteria [249].

Thus, it has been indicated that the consumption of prebiotics by healthy adults increases the concentration of *Bifidobacterium* spp. and *Lactobacillus* spp. in gut microbiota [250]. However, the composition of the microbiota after the consumption of certain prebiotics is not limited only to these taxa. Through the application of sequencing techniques and metagenomic analysis, it has been shown that prebiotics affect the entire composition of gut microbiota (Table 2) [251–253].

Table 2. Prebiotics and their effects on gut microbiota.

Prebiotic	Model description	Effects on gut microbiota	Source
Galacto-oligosaccharides (GOS)	Healthy adult volunteers. GOS daily dose of 2.4 g	Increase in *Bifidobacterium* and *Lactobacillus* abundance	[250]
Alpha-GOS, betha-GOS, Xylo-oligosaccharides (XOS), β-glucan, inuline	In vitro fermentation of standardized fecal sample from healthy adult volunteers. Positive control: 4 mg/mL of fructooligosaccharides. 5 different concentrations of each prebiotic assayed	β-glucan: Increase in Bacteroidotes (*Prevotella*) and Firmicutes (*Roseburia*) Alpha-GOS and XOS: Increase in bifidobacteria	[254]

Table 2. *Cont.*

Prebiotic	Model description	Effects on gut microbiota	Source
Bovine milk oligosaccharides (BMO)	BMO and lactose co-culture effect on *Bifidobacterium longum* subsp. *longum* metabolism and *Clostridium perfringens* inhibition	Decrease in *Clostridium perfringers* and increase in *Bifidobacterium*	[255]
Resistant starch type 4 (RST4)	Subjects with metabolic syndrome. 26 weeks treatment including two 12-week intervention periods, one for RST4 (30%, *v/v* in flour) and one for control flour	Increase mainly in *Bacteroides* and *Parabacteroides* spp. Microbial enrichment involved *Christensenella minuta*, recently identified in human feces	[256]
Resistant starch type 3	Pigs, 3 months old. The percentage of the prebiotic diet was increased in 20% increments until 100% was reached	Increase in *Prevotella*, *Ruminococcus*, and *Lachnospiraceae*	[257]
Resistant starch type 2 (RST2)	Male C57BL/6J mice (18–20 months old)	Increase in *Ruminococcus bromii*, *Eubacterium rectale*	[258]
	Healthy male and female human subjects aged between 23 and 38 years. RST2 daily dosis of 100 g for 2 weeks	*Bifidobacterium* spp., *Allemansia*, and *Allobacum* genera	[259]
β-glucan	Healthy human subjects. Barley β-glucans daily dose of 3 g for 2 months	Increase in *Prevotella*, *Roseburia*, and *Clostridium*, decrease in Firmicutes and Fusubacterium concentration	[260]

On the other hand, studies also suggest that beneficial changes in gut microbiota are maintained with continuous consumption of prebiotics. In addition, microbial diversification is dependent on the basal or indigenous microbiota, including the growth of specific species and the enzymatic capacity of some strains [248,261]. For example, resistant starch is considered a prebiotic, since it can be fermented in the colon, conferring beneficial metabolic effects. Among the most studied benefits, the following stand out: an increase in the turnover of bile salts, laxative effect, control of blood lipid levels, and a decrease in the postprandial glucose response [262]. In addition, this carbohydrate contributes to cell growth and proliferation by increasing the concentration of butyrate once it has been fermented in the intestine [263].

The metabolic effects, as well as the group of bacteria that are favored after the incorporation of starch, depending on the type of resistant starch that is consumed [264]. For example, type 4 resistant starches have been found to favor the growth of *Bacteroides* and *Parabacteroides* spp. in the intestine [256]. On the other hand, type 2 resistant starches increase the populations of *Ruminococcus bromii* and *Eubacterium rectole* spp. in humans [258], and *Bifidobacterium*, *Akkermansia*, and genera of *Allobaculum* in murine models [259]. Regarding type 3 resistant starches, several studies using animal and human models have shown that they favor the growth of beneficial bacterial populations, mainly SCFA-producing genera, such as *Prevotella*, *Ruminococcus*, *Lachnospiraceae*, *Veillonellaceae*, *Bulleidia*, and *Dialister* [257].

Alginate is another polysaccharide that has stood out for its prebiotic properties. Its consumption modifies the intestinal microbiota by increasing the relative abundance of microbial populations such as *Roseburia*, *Ruminococcus*, and *Lachnospira*, which are SCFA producers. Additionally, an increase in the concentration of bifidobacteria due to the consumption of alginate has been reported [257].

In the same way, an increase in the concentration of *Prevotella* and *Roseburia* has been observed by the in vitro fermentation of prebiotics such as inulin, galacto-oligosaccharides (GOS), and xylo-oligosaccharides (XOS) of corn and sugar cane with a high content of fiber and oat β-glucans. Furthermore, there is a concomitant increase in propionate production. Similarly, inulin, XOS, and GOS have a strong bifidogenic effect on the microbial compo-

sition and are precursors of the formation of butyrate by native microorganisms of the intestinal microbiota [254].

Other studies highlight the effects of barley β-glucans, which, when administered in low doses, significantly increase the count of bifidobacteria and lactobacilli in healthy subjects. Similarly, β-glucans from wheat flour and whole barley pasta increase the levels of *Roseburia hominis*, *Clostridium orbiscindens*, *Clostridium* sp., and *Ruminococcus* sp. in the gut microbiota. At the same time, a reduction in the levels of Firmicutes and Fusobacteria is observed, and it has been verified that the consumption of β-glucans affects the increase in the concentration of acids such as 2-methylpropanoic, acetic, butyric, and propionic [259].

In addition to prebiotic sources of plant origin, the effect of bovine milk oligosaccharides has also been evaluated, which, in combination with GOS, decrease the concentration of *Clostridium perfringens*, with a simultaneous increase in Bifidobacteria, lactate, and acetate [255]. Despite the results, some authors suggest that prebiotic supplementation does not always lead to a global change in the alpha or beta diversity of gut microbiota. However, a modification is observed in the abundance of certain bacterial taxa, such as *Ruminococcaceae* (*Clostridium cluster* IV), *Parabacteroides*, and *Phascolarctobacterium* [265].

Currently, the role of FODMAPs in the intestinal microbiota has been studied, noting that the low intake of these compounds is related to a reduction in the symptoms of various gastrointestinal diseases, including irritable bowel syndrome and inflammatory bowel disease [266,267]. Conversely, a diet high in FODMAPs seems to have an opposite effect to prebiotic supplementation, decreasing *Bifidobacterium* populations and increasing bacteria associated with dysbiosis [266]. However, more studies are needed to evaluate the effects of a diet high in FODMAPs on the composition of gut microbiota, focusing on the persistence of changes in microbial composition and adverse health effects.

5. Conclusions

The intestinal microbiota has been considered as another organ of the human body, with its own characteristics that make it essential in metabolic functioning. Due to these characteristics, dysbiosis has been implicated as one of the main factors in the development of diseases such as diabetes, obesity, cancer, or those related with neurodegenerative problems. Thus, the maintenance of the correct balance in the composition of taxa that form gut microbiota is of utmost importance. This review fulfills the objective of informing about the importance of the intestinal microbiota for the human's good health. At the same time, the consumption of probiotics and prebiotics directly affects both the maintenance of the composition's balance of microbiota and the prevention/management of dysbiosis. However, despite the studies carried out in this field, they are still not conclusive in the direct relationship of microbiota, such as the Firmicutes/Bacteroidota, in the development of diseases. This is clearly an area of opportunities and challenges that food science research should consider in order to determine in a conclusive way the role that diet plays in maintaining the good composition of microbiota and, likewise, the role that it plays within the health–disease state of the human.

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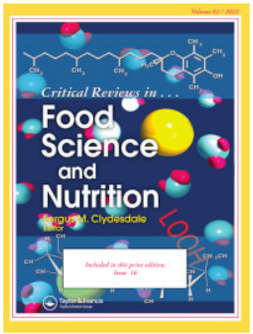
Capítulo III.

Bioactive peptides of whey: obtaining, activity, mechanism of action, and further applications

Introducción

Esta extensa revisión publicada en la revista Critical reviews in Food Science and Nutrition se centra en el estudio de los péptidos bioactivos obtenidos a partir de suero de leche. En la primera etapa de este capítulo se realiza una descripción del suero de leche como subproducto de la industria láctea. Posteriormente, se describe cada una de las proteínas presentes en el suero, indicando sus características generales, así como su actividad biológica. La revisión continúa haciendo una recopilación de los principales métodos empleados en la producción de péptidos bioactivos de proteínas de suero de leche. En esta sección, se realiza una descripción y análisis sobre técnicas de obtención de péptidos las cuales incluyen la hidrólisis enzimática, la hidrólisis química, la fermentación microbiana y el análisis *in sílico*.

De igual manera, en este capítulo se presentan las diferentes actividades biológicas que los péptidos provenientes de hidrólisis de proteínas de suero de leche pueden desempeñar. En este sentido, se describen péptidos con diversa actividad biológica. Los mecanismos de acción para cada una de las bioactividades se describen extensamente y se incluyen estudios in *vitro* e in *vivo*, así como un listado de las secuencias peptídicas reportadas. Por otro lado, se destaca la viabilidad de los péptidos mediante los mecanismos de absorción a través de la membrana intestinal siendo este un paso fundamental para determinar su bioactividad. Por último, se revisan algunos productos adicionados con péptidos bioactivos que actualmente se encuentran disponibles para su comercialización. Así mismo, se hace hincapié sobre las perspectivas a futuro en la obtención, caracterización y uso de péptidos de proteínas de suero de leche como una alternativa en el tratamiento de diversas enfermedades.



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REVIEW

Bioactive peptides of whey: obtaining, activity, mechanism of action, and further applications

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ABSTRACT

Bioactive peptides derived from diverse food proteins have been part of diverse investigations. Whey is a rich source of proteins and components related to biological activity. It is known that proteins have effects that promote health benefits. Peptides derived from whey proteins are currently widely studied. These bioactive peptides are amino acid sequences that are encrypted within the first structure of proteins, which required hydrolysis for their release. The hydrolysis could be through in vitro or in vivo enzymatic digestion and using microorganisms in fermented systems. The biological activities associated with bio-peptides include immunomodulatory properties, antibacterial, antihypertensive, antioxidant and opioid, etc. These functions are related to general conditions of health or reduced risk of certain chronic illnesses. To determine the suitability of these peptides/ingredients for applications in food technology, clinical studies are required to evaluate their bioavailability, health claims, and safety of them. This review aimed to describe the biological importance of whey proteins according to the incidence in human health, their role as bioactive peptides source, describing methods, and obtaining technics. In addition, the paper exposes biochemical mechanisms during the activity exerted by biopeptides of whey, and their application trends.

KEYWORDS

Bioactive peptide;
whey proteins;
antihypertensive peptide;
milk;
human health

1. Introduction

Whey is an industrial waste obtained from cheese manufacturing after casein coagulation (Mollea, Marmo, and Bosco 2013). It has been considered a serious environmental problem, due to the large amounts that are generated each year and that usually contaminate bodies of water (Kareb and Aïder 2019). That is why, strategies for processing this waste include lactose obtaining or manufacturing of protein concentrates (Kareb and Aïder 2019). However, due to the nutritional properties it has been used to obtain bioactive peptides from the hydrolysis of whey proteins, which have shown to exert important biological effects (Oriach et al. 2016).

Bioactive peptides are amino acid sequences that exert different biological effects, which are encrypted in proteins structure and could be released once those proteins are fragmented (Dullius, Goettert, and de Souza 2018). Fragmentation is carried out by different methods, including from the most common techniques such as enzymatic, chemical synthesis and fermentation, to the most innovative ones: bioinformatics tools focused on the prediction of peptides through in silico analysis (Corrêa et al. 2014; Fu and Lin 2017; Xia et al. 2020). In recent years, a large number of bioactive peptide sequences from whey proteins (antihypertensive, antithrombotic, antimicrobial, anticancer, opioid, antiviral, among others) (Onuh and Aluko 2019), have been reported for helping in the treatment of various diseases (Chakrabarti, Guha, and Majumder 2018).

This review provides information on the whey composition, making a specific description of the proteins that compose it and highlighting, not only its nutritional value, but also its biological importance. Similarly, the various means of obtaining peptides are discussed, describing enzymatic synthesis, chemical synthesis, fermentation as well as the use of bioinformatics programs that allow the prediction of peptide sequences with bioactive potential.

Similarly, a detailed description of the main peptide sequences obtained from whey proteins is presented, discussing the biological activity and the different mechanisms of action that these peptides exert, including in vitro and in vivo studies. Finally, some commercial products that have been designed with the incorporation of whey protein bioactive peptides are presented and future perspectives on new production trends and potential use of whey protein bioactive peptides in the treatment of various pathologies in humans.

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2. General characteristics of whey: Waste of the dairy industry

Whey is the liquid obtained as the main residue of the cheese industry (Mollea, Marmo, and Bosco 2013). It is estimated that approximately 8 to 9 L of whey are eliminated during the production of 1 to 2 Kg of cheese and worldwide more than 160 million tons of whey are discarded per year (Kareb and Aïder 2019). Despite being a waste with a high commercial value due to its chemical composition, only 50% of the production is industrially processed. The main products obtained from whey are protein and lactose concentrate. That is why the elimination of this residue has become a challenge for the dairy industry. (Hannibal et al. 2015; Kareb and Aïder 2019).

An alternative for whey valorization has recently been presented, by converting the lactose content into bioactive compounds such as lactosacrose, lactulose and galactoligasaccharides that can be used for their prebiotic effect (Oriach et al. 2016). On the other hand, whey proteins have attracted great interest for their valorization since, in addition to their nutritional properties; they are usually a potential source of bioactive peptides with a wide range of benefits for human health (Sebastián-Nicolas et al. 2021).

2.1. Whey composition

Whey contains approximately 93% of water and 50% of the total milk solids (Patel 2015; Kareb and Aïder 2019) and in general terms it has 96% of the total lactose present in milk, 25% of the protein and 8% of the fat (Yadav et al. 2015). The composition is variable due to the origin of obtaining the whey (Table 1). Within the protein fraction, β-lactoglobulin (35-65%) and α-lactalbumin (12-25%) are found, and to a lesser extent immunoglobulins (8%), serum albumins (5%) and lactoferrin (1%) (S. Patel 2015). It contains lactic acid and appreciable amounts of citric acid, as well as non-protein nitrogenous compounds (urea and uric acid) and complex of B vitamins (Dragone et al. 2009). In addition to these components, whey also has a large amount of minerals such as calcium, phosphorus, magnesium, sodium, and potassium. The concentration of other minerals such as zinc, iron, copper, and manganese depends on the origin of the milk (Yadav et al. 2015).

Although the coagulation processes of milk are usually different, the chemical composition of whey is similar. Milk solids reach 50% and approximately 20% correspond to proteins, mainly α-lactalbumin and β-lactoglobulin (Yadav et al. 2015). Sweet whey contains the caseinomacropeptide that is produced by enzymatic hydrolysis of κ-casein. Additionally, the amount of free amino acids varies depending on the degree of casein hydrolysis (Hulmi, Lockwood, and Stout 2010). Due to this, the content of free amino acids in sweet whey is higher than in milk (Kareb and Aïder 2019).

3. Whey proteins and bioactive implications

The main proteins present in whey are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), and immunoglobulins (IG) (Kareb and Aïder 2019). To a lesser extent, lactoferrin (LF), lactoperoxidase (LP), proteosapeptone (PP), and glycomacropeptide (GMP) are also found, the latter is released through the enzymatic hydrolysis of casein by plasmin and during cheese-making process, so it is only found in sweet whey (Carvalho, Prazeres, and Rivas 2013; Brandelli, Daroit, and Corrêa 2015).

These proteins have a globular structure with high solubility and are easily denatured by heat (Lievore et al. 2015), they have the ability to form intramolecular bonds through disulfide between sulfhydryl groups present in cysteine (Tavares and Malcata 2013). In addition to their nutritional value, due to their high content of essential amino acids, whey proteins perform various biological functions, which is why they represent an interest factor in various food applications (Madureira et al. 2010; Mollea, Marmo, and Bosco 2013).

3.1. β-Lactoglobulin (β-LG)

Whey contains about 3.2 g/L of β-LG (Mollea, Marmo, and Bosco 2013). The molecular weight is variable (18.2 to 18.3 kDa) and dimeric structure is found under specific conditions of pH (5.2-7) (Apenten, Khokhar, and Galani 2002), presenting a sulfhydryl group and two disulfide bonds (Walstra et al. 2005). The aminoacidic chain is composed by 162 units and factors such as ionic strength and pH, determine its solubility. This proteins is denatured at temperatures above 65° C (de Castro et al. 2017).

The structure of β-LG allows this protein to be a vehicle for hydrophobic compounds such as fat-soluble vitamins and lipids, therefore it plays a role in the metabolism of fatty acids (Modler 2009; Kareb and Aïder 2019). B-LG is a source of cysteine, an important amino acid for the

Table 1. Approximate physicochemical whey composition.

Component	Sweet whey (g/ L)	Acid whey (g/L)
Lactose	46 – 52	44-46
Protein	6 – 10	6-8
Fat	0- 5	0-5
Lactic acid	0.5	6.4
Calcium	0.6	1.2-1.6
Phosphorus	0.7	2-4.5
Magnesium	0.17	0.17
Sodium	1.1	1.1
Potassium	1	1

(Yadav et al. 2015).

synthesis of glutathione, an intracellular antioxidant that participates in immune and liver regulation and whose role has been studied in diseases such as Alzheimer's and cancer (Nongonierma, O'keeffe, and FitzGerald 2016).

3.2. α-Lactalbumin (α-LA)

α -LA is present in whey at concentration of 1.2 g/L (Mollea, Marmo, and Bosco 2013) and it is a small globular protein consisting of a single polypeptide chain of 123 amino acids with 8 cysteine residues, 4 residues of tryptophan and other essential amino acids (Chang and Li 2002). Its molecular weight is 14 kDa, (Hernández-Ledesma, Ramos, and Gómez-Ruiz 2011). It has a single strong Ca^{2+} binding site (Yadav et al. 2015), which is why it has served as a Ca^{2+} binding protein model (Permyakov 2020). In addition to the Ca^{2+} ion, α-LA bind to other cations such as Mg^{2+}, $Mn^{2+,}$ Na^{+}, and K^{+}. Similarly, it has several Zn^{2+} binding sites (Noyelle and Van Dael 2002). However, binding to Ca^{2+} is essential to maintain the protein conformation (Owusu 1992).

Because its high content of tryptophan, which acts as a precursor to the neurotransmitters serotonin and melatonin, α-LA can help improve mood, sleep, and cognitive performance (Markus, Olivier, and de Haan 2002; Park and Nam 2015). Other functions include its participation in lipid oxidation, mineral absorption and immunomodulatory activity by exerting a suppressive effect against increased release of pro-inflammatory cytokines and tumor necrosis factor-α (TNF-α) (Bouthegourd et al. 2002; Permyakov 2020). Due to its ability to solubilize and stabilize hydrophobic compounds, it acts as a vehicle for vitamin A and D (Permyakov 2020). Similarly, its antitumor activity stands out, formed by a complex by which it induces apoptosis in tumor cells without affecting other cell lines (Gustafsson et al. 2005; Tolin et al. 2010).

3.3. Bovine serum albumin (BSA)

BSA is found in a concentration of 0.4 g/L in whey (Mollea, Marmo, and Bosco 2013), this concentration is similar to that reported in breast milk (Yadav et al. 2015). This protein has a molecular weight of 66.26 kDa (Modler 2009). Its polypeptide chain is made up of 583 amino acids with 35 cysteine residues and 17 disulfide bridges (Walstra et al. 2005; Hernández-Ledesma, Ramos, and Gómez-Ruiz 2011).

This protein has the ability to reversibly bind to different ligands, which allows its use as both a fatty acid vehicle and its participation in lipid synthesis (Choi et al. 2002; Mollea, Marmo, and Bosco 2013). Furthermore, it can also be used as a source of essential amino acids such as tryptophan, valine and phenylalanine (Modler 2009). One of the best-known bioactivities of BSA is its ability to inhibit tumor growth thanks to the modulation of the activity of various growth regulatory factors (Lu et al. 2006). Similarly, its ability to protect lipids from phenol-induced oxidation has been reported (Tong et al. 2000).

3.4. Immunoglobulins (Igs)

Bovine serum immunoglobulins belong to a heterogeneous family of glycoproteins, the three main classes found in whey are IgA, IgM, and IgG; of the latter there are two subclasses IgG1 and IgG2 (Kumar et al. 2018). The proportion of immunoglobulins present in whey is around 0.7 g/L and IgG is the one with the highest concentration, reaching up to 80% (w/w) of the total (Yadav et al. 2015).

IgG occurs in monomeric while IgA and IgM occur in polymeric forms (Mollea, Marmo, and Bosco 2013). Among the biological activities of Igs is the neutralization of toxins and viruses since they can provide prophylactic protection against several infectious intestinal elements including rotavirus and *Helicobacter pylori*, acting in the modulation of the immune system by providing protection against diseases in the newborn by passive immunity (Oona et al. 1997; Sarker et al. 1998). Similarly, their activity as hypocholesterolemic and antihypertensive agents has been reported (Modler 2009; Mollea, Marmo, and Bosco 2013).

3.5. Lactoferrin (LF)

This glycoprotein belongs to the family of transfer proteins, it binds to iron and in this way makes possible a greater bioavailability of this mineral. The concentration in which it is found in whey is 0.1 g/L (Mollea, Marmo, and Bosco 2013; Yadav et al. 2015; Kareb and Aïder 2019). LF has a molecular weight of 76.50 kDa and consists of a single polypeptide chain of 700 amino acids, which can contain one to two carbohydrate chains (González-Chávez, Arévalo-Gallegos, and Rascón-Cruz 2009; Modler 2009).

This protein has been reported to have a wide range of biological functions, highlighting its antimicrobial, antifungal, antiviral and immunomodulatory activity (Lopez-Exposito and Recio 2008; Demers-Mathieu et al. 2013; Bruni et al. 2016). Similarly, it promotes cell differentiation, endothelial cell adhesion, and cytokine production (Cornish et al. 2004; Ulber et al. 2001). Furthermore, LF is part of the innate immunity mechanism participating in specific immune reactions since it represents one of the first lines of defense against pathogens (Legrand et al. 2005). In this sense, the ability to bind free iron makes LF have antimicrobial activity against iron-dependent pathogens such as *Escherichia coli*. Likewise, LF acts as an agonist for the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* due to its ability to donate iron (Sherman et al. 2004). Other functions include its antitumor activity (Fujita et al. 2004) and stimulation of cell growth and differentiation, especially in bone tissue (Cornish et al. 2004).

3.6. Glycomacropeptide (GMP)

GMP is the hydrophilic derivative of k-casein obtained after enzymatic coagulation of caseins in milk during cheese making by adding chymosin or by the action of pepsin during digestion (Foisy Sauvé et al. 2021). Its chain of 64 amino acids gives it a molecular weight of 6.8 kDa with an

isoelectric pH of 4.3 to 4.6 (Yadav et al. 2015; Foisy Sauvé et al. 2021). This fraction has been considered as a bioactive peptide which does not contain aromatic amino acids such as phenylalanine, tyrosine, tryptophan and neither histidine, cysteine and arginine, but it is highly glycosylated, sialylated and phosphorylated in response to post-translational modifications (Modler 2009; Rao, Sharma, and Rajput 2012; Kumar et al. 2018). These properties allow it to be soluble in acidic media and present high resistance to chemical and biological degradation, therefore, it remains stable in gastric transit, passes through the small intestine and is absorbed by the blood circulation (Foisy Sauvé et al. 2021).

It has numerous biological activities, such as the inhibitory effect on acid secretions, the ability to function directly as a neurotransmitter or indirectly by regulating the secretion of intestinal hormones (Thomä-Worringer, Sørensen, and López-Fandiño 2006). In addition to these activities, GMP promotes remineralization and prevents dental demineralization due to its antimicrobial and platelet aggregation inhibition properties (Santos, da Costa, and Garcia-Rojas 2018). Similarly, its effect has been reported in diseases such as obesity, hypertension and dyslipidemia due to its antioxidant, hypolipidemic, anti-inflammatory and immunomodulatory activity (Aimutis 2004; Foisy Sauvé et al. 2021).

3.7. Lactoperoxidase (LP)

Lactoperoxidase is a natural enzyme of the mammalian immune system, in whey it is found in a concentration of 0.03 g/L (Hernández-Ledesma, Ramos, and Gómez-Ruiz 2011; Mollea, Marmo, and Bosco 2013). The molecular mass of this enzyme is 78 kDa and it consists of a unique polypeptide chain of 612 amino acid residues that is bound to approximately 10% (w/w) of carbohydrate residues (Yadav et al. 2015).

The main biological activity of this protein is its antimicrobial action, which has been reported to inhibit gram-negative and catalase-positive microorganisms, such as pseudomonas, coliforms, salmonella, and shigella (Seifu, Buys, and Donkin 2005). Likewise, the inhibition of micro-organisms involved in the appearance of dental cavities has been explored (Sarr et al. 2018; Magacz et al. 2019). Furthermore, its antiviral properties against influenza (Shin et al., 2005) and its antifungal activity against *Aspergillus flavus*, *Trichoderma* spp, *Alternaria* spp, Pen*icillium chrysogenum* and *Claviceps* spp. (Seifu, Buys, and Donkin 2005; Mollea, Marmo, and Bosco 2013).

3.8. Proteose-peptone (PP)

Proteose-peptone has 4 components called component 3 (PP3), component 5, component 8-slow and component 8-fast (Krissansen 2007). While component 3 is derived from the fat globule membrane, the other three peptides are originated from β-casein due to the action of plasmin (Yadav et al. 2015). PP3 remains soluble when milk is heated at 95° C for 20 min under acidic conditions at pH 4.7 (Yadav et al. 2015; Moatsou and Sakkas 2019). Likewise, PP3 exhibits antimicrobial (Pedersen et al., 2012) and immunostimulatory properties (Sugahara et al. 2005). Specifically, PP3 has been studied for its possible protective action against cavities and the acidity produced in this condition (Grenby et al. 2001). Similarly, it has been reported that PP3 presents activity as a bifidogenic factor, observing an improvement in the increased concentration of *Bifidobacterium animalis* (Etienne, Girardet, and Linden 1994).

4. Production of bioactive peptides from whey proteins

Although the nutritional and functional properties of whey proteins have been reported (A. R. Madureira et al. 2010; Dullius, Goettert, and de Souza 2018), it is known that many of the compounds that exert the beneficial effects are found encrypted in the amino acid chain of proteins and are only released through their hydrolysis (Pihlanto-Leppälä 2000). The released compounds are called bioactive peptides, (Dullius, Goettert, and de Souza 2018) and these fractions are obtained through the action of digestive enzymes, as well as by the action of microbial proteases, including the contained in lactic acid bacteria (LAB) (Korhonen 2009; Corrêa et al. 2014; Xia et al. 2020).

However, in recent years new strategies have been developed to obtain bioactive peptides with high potential. These methodologies include chemical synthesis and the use of enzymes of plant origin (Hussein et al. 2020; Estévez et al. 2020; Mazorra-Manzano et al. 2020). Furthermore, *in silico* prediction was used as a bioinformatic tool to predict peptide sequences by simulating hydrolytic fractionation. In this way, the toxic, sensory, allergenic properties and penetration capacity in intestinal cells are predicted (Fu and Lin 2017). The main characteristics of obtaining bioactive peptides by enzymatic, chemical and fermentation synthesis are presented below. In the same way, the most relevant aspects in the prediction of peptides with biological activity through *in silico* analysis will be explained.

4.1. Enzymatic hydrolysis

The enzymatic hydrolysis of whey proteins is the most common technique for obtaining bioactive peptides (Aluko 2012; Sila and Bougatef 2016). Enzymes used must be specific, maintaining optimal hydrolysis conditions, which include pH, temperature, time, and enzyme/substrate ratio (Zhao and Ashaolu 2020).

In this context, gastrointestinal enzymes such as pepsin, pancreatin and trypsin have been used for the generation of peptides with inhibitory activity of dipeptidyl peptidase IV (DPPIV) (Jia et al. 2020) and angiotensin converting enzyme (ACE) (Chatterjee et al. 2015), bactericidal activity (Wang, He, et al. 2020), antihypertensive and antioxidant activity (Giromini et al. 2019), cytoprotective peptides (Ballatore et al. 2020), among others. In addition to these enzymes, alcalase has also been used to obtain peptides with antioxidant and antihypertensive activity (Athira et al. 2015; Barrón-Ayala et al. 2020). Recent research has shown that

the use of flavoenzymes leads to the obtaining of peptides that inhibit cellular DNA damage and lipid oxidation (Chiang et al. 2017; Giromini et al. 2019).

Another type of enzymes used is this were obtained from microorganisms such as lactic acid bacteria (LAB) (Guo et al. 2019). LAB proteases are usually purified in a simple way and the cultivation of microorganisms has a low cost, in addition, a great variety of specific bioactive peptides can be obtained (Corrêa et al. 2014; Liu et al. 2010; Hati et al. 2018).

However, in recent years the obtaining of bioactive whey protein peptides from enzymes of plant origin has also been explored. In this sense, the use of proteases of plant origin to obtain peptides with ACE inhibitory activity has been reported (Estévez et al. 2020; Mazorra-Manzano et al. 2020). Other new methodologies include the immobilization of enzymes, which allows both to generate products with a high degree of purity and to recycle enzymes. That is why it is considered an economically viable process to obtain bioactive peptides (Pedroche et al. 2007; Chmiel, Takors, and Weuster-Botz 2018; Yanwei Wang, Ai, et al. 2014; Y. Guo et al. 2019).

4.2. Chemical hydrolysis

Chemical hydrolysis of proteins is a widely used process, which has been defined as a simple and less expensive method compared to other methods from obtaining peptides (Ulug, Jahandideh, and Wu 2021). During the process, acidic or alkaline compounds are used to produce free peptides and amino acids (Wang et al. 2017). This leads to the obtaining of peptides with variable chemical composition that implies a low efficiency that sometimes results in toxic residues (Ulug, Jahandideh, and Wu 2021).In addition to this, the use of strong chemicals and solvents in extreme conditions as well as the temperature and pH conditions used have an impact on the nutritional and functional characteristics of the obtained peptides (Kristinsson and Rasco 2000; Wisuthiphaet, Klinchan, and Kongruang 2016).

On the other hand, chemical synthesis from free amino acids is commonly used on a laboratory scale through two variants, which correspond to the liquid phase and the solid phase. The former is used for the generation of short peptides, while the latter is common to synthesize peptides composed by 10 to 100 residues. However, its application to produce peptides at an industrial level is unfeasible (Madureira et al. 2010).

4.3. Fermentation

Microbial fermentation represents a cost-effective method for the production of bioactive peptides from whey proteins (Daliri et al. 2018). This method has been used for the development of various functional products (Pihlanto 2013; Hafeez et al. 2014) and its implementation using whey as a fermentation base with different microorganisms serves to obtain bioactive peptides (Raveschot et al. 2018). Diverse are the functionalities found in the bioactive peptides obtained from the fermentation of whey. Within these bioactivities are the antihypertensive and antioxidant activity (Madureira et al. 2013; Begunova et al. 2020), the antimicrobial (Taha et al. 2017; Ali et al. 2019), the antidiabetic (Sharma et al. 2021), the -inflammatory, and anti-hemolytic activity (Aguilar-Toalá et al. 2017).

LAB belonging to the genus *Lactobacillus* are commonly used for the production of peptides with biological activity (Hafeez et al. 2014). These microorganisms have great potential as producers of new bioactive peptides, thanks to the presence of different enzymes that are part of their proteolytic system (Patel and Hati 2018).

Despite the wide range of peptides generated by microbial fermentation, it has not been possible to exploit production at an industrial level due to the low yield obtained (Raveschot et al. 2018). However, microbial fermentation has been used in the production of fermented foods that have been studied as an alternative for consumption and its effect on human health has been evaluated (Taha et al. 2017; Kareb and Aïder 2019).

4.4. In silico prediction

In silico analysis is proposed as an alternative that allows a more systematic process design and the development of new methods that lead to the determination of peptides with biological activity by evaluating their physicochemical properties (Udenigwe 2014; Fu and Lin 2017; J. Tong et al. 2017).

Bioinformatics tools allow predicting peptide sequences through hydrolysis simulation including variables such as pH, time, temperature and enzyme: substrate ratio (Chatterjee et al. 2015; Dullius, Goettert, and de Souza 2018). In the same way, other characteristics are predicted, including its allergenic capacity, its degree of toxicity and its ability to penetrate intestinal cells and thus exert its effect (Minkiewicz et al. 2008; Iwaniak et al. 2016).

Currently, it is known that peptides that have a certain length, physicochemical properties and defined amino acid patterns, exert a specific biological activity (Dullius, Goettert, and de Souza 2018; Minkiewicz et al. 2008). Platforms such as BIOPEP, which is widely used, allow *in silico* hydrolysis by choosing among the 31 proteolytic enzymes available in its library, of which at least two can be evaluated at different pH (Nongonierma, O'keeffe, and FitzGerald 2016).

Additionally, this platform also allows evaluating the biological and physiological properties and the degree of allergenicity of the resulting peptides (Iwaniak et al. 2016). However, other platforms are commonly used in parallel to evaluate differences in peptide fragments or predict secondary structures of linear peptides, in this sense platforms such as REMUS and PEP-FOLD3 are found (Gasteiger et al. 2005; Lamiable et al. 2016). From *in silico* analysis using these platforms, bioactive peptides inhibitors of angiotensin converting enzyme (ACEI) and inhibitors of α-glucosidase have been predicted, as well as peptides with antimicrobial, anti-inflammatory and antioxidant activity

(Minkiewicz, Iwaniak, and Darewicz 2019; Ashraf et al. 2021).

5. Biological activity of peptides derived from whey proteins

Bioactive peptides represent protein fragments approximately two to twenty amino acids in length (Tulipano 2020). These sequences have a positive impact on various physiological functions and also have an effect on human health (Chakrabarti, Guha, and Majumder 2018). The activity of bioactive peptides is based on both the unique amino acid composition and sequence. In this sense, the bioactive peptides of whey proteins can be divided into different categories according to their physiological effect or the protein from which they are derived (Mann et al. 2019).

According to this, it has been possible to identify peptides with antihypertensive, antithrombotic, antioxidant, opioid, antimicrobial, cytomodulatory, and immunomodulatory activities, among others (Brandelli, Daroit, and Corrêa 2015; Onuh and Aluko 2019). In the following sections, a description of the characteristics of the main types of bioactive peptides derived from whey proteins is made, indicating their biological activity and mechanism of action, as well as the in vitro and in vivo studies that have been carried out. Table 2 shows some sequences of peptides derived from whey proteins and its action mechanisms.

5.1. Peptides with antihypertensive activity

Hypertension is a global pathological condition that increases the risk of cardiovascular disease and damage to vital organs, this condition causes the death of approximately 7.5 million people each year (Sur 2017). The conversion of angiotensin I to angiotensin II plays an important role in the regulation of blood pressure (Figure 1), since angiotensin II is a potent vasoconstrictor that in turn inactivates the vasodilator bradykinin (Madureira et al. 2010). In this context, the role of bioactive peptides to assist in the treatment of arterial hypertension has been explored (Sultan et al. 2018). Evidence point to on the effectiveness of angiotensin converting enzyme (ACE) inhibitor peptides to modulate the cardiovascular system (Tondo et al. 2020). Specifically, isoleucine-proline-proline (IPP), valine-proline-proline (VPP), and isoleucine-tryptophan (IW) peptides from whey proteins, lower blood pressure and reduce the activity of plasma RCT in both hypertensive and normotensive patients (Martin et al. 2018). The inhibitory activity of these peptides is strongly influenced by the sequence of amino acids present at their C-terminal side. The most potent inhibitor peptides of ACE contain hydrophobic amino acid residues such as phenylalanine, tryptophan, isoleucine, tyrosine, and proline. These amino acids interact with the ACE active site (Li et al. 2004).

Similarly, there is a relationship between the inhibitory activity of peptides and the proteolytic enzymes used to obtain them, it has been found that the use of pancreatic enzymes such as trypsin is a more efficient method for obtaining ACE inhibitor peptides (Naik et al. 2013; Ibrahim, Ahmed, and Miyata 2017). In vitro studies have reported peptides generated from α-LA and β-LG that possess ACE inhibitory properties (Tavares et al. 2011; Welderufael, Gibson, and Jauregi 2012; Tondo et al. 2020). Protein hydrolysis using digestive enzymes such as pepsin, trypsin, chymotrypsin, pancreatin or a combination of these proteases results in high levels of ACE inhibition (Brandelli, Daroit, and Corrêa 2015).

It has been observed that through simulated digestion, potential bioactive peptides with antihypertensive capacity are obtained (Giromini et al. 2019). In particular, it has been reported that, after the consumption of whey protein hydrolysates, there is a modulation of vasorelaxant activity in aortic rings of murine models (Ozorio et al. 2020). Similarly, (Hussein et al. 2020), carried out the identification and characterization of bioactive peptides with high potential for inhibition of ACE from the hydrolysis of whey protein concentrates using alcalase. In addition to this, a significant reduction in blood pressure was reported in a hypertensive rat model after the consumption of the hydrolyzed whey protein concentrate.

On the other hand, ACE inhibitor peptides obtained after whey fermentation using a large amount of LAB have also been reported. In this context peptides with high inhibitory capacity have been obtained from fermentation with *Pediococcus acidilactici* SDL1414, *Lactobacillus rhamnosus* JDFM6, *Lactobacillus brevis* SDL1411, *Enterococcus faecalis* and *Lactobacillus plantarum* QS670, demonstrating its effect at in vivo models of hypertensive rats, where the consumption of fermented whey led to a decrease in blood pressure (Daliri et al. 2018; Worsztynowicz, Białas, and Grajek 2020; Xia et al. 2020).

Obtaining and purifying proteases from various microorganisms has allowed their use to produce peptides with antihypertensive activity. *Bacillus* sp. P7 and *Lactobacillus helveticus* LB 10 immobilized in sodium alginate to hydrolyze sheep's milk whey protein, produce peptides with high ACE inhibition potential (Corrêa et al. 2014; Y. Guo et al.

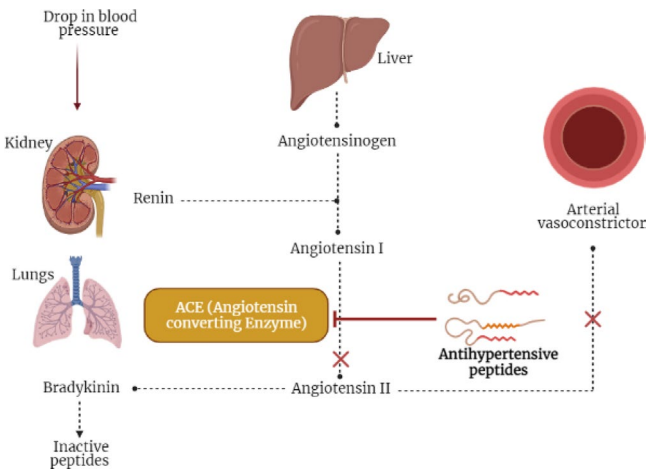

Figure 1. Mechanism of action of antihypertensive peptides. This type of peptides inhibit the angiotensin converting enzyme (ACE), in this way the conversion of Angiotensin I to Angiotensin II is not carried out and therefore the vasoconstrictor effect is suppressed.

Table 2. Bioactive peptides derived from whey proteins.

Biological activity	Mechanism of action	Origin	Amino acid sequence	Reference
Antihypertensive	Angiotensin converting enzyme (ACE) inhibition.	α-LA	IPP	(Martin et al. 2018)
			VPP	
			IW	
Antimicrobial	Pore formation, permeability induction and cell lysis.	α-LA	EQLTK GYGGVSLPEWVCTTF ALCSEK	(Pellegrini et al. 1999)
		β-LG	EIPTINT	(Ali et al. 2019)
		LF	IDALNENK	(Huertas Méndez et al. 2017)
			VLVLDTDYK	
			AASDISLLDAQSAPLR	
			RRWQWR	
Antidiabetic	Dipeptidyl peptidase IV (DPP-IV) inhibition.	α-LA	LAHKALCSEKL	(Nongonierma, O'keeffe, and FitzGerald 2016)
		α-LA	WLAHKAL	
	α-amylase (α-AAM) and α-glucosidase (α-AG) inhibition.	β-LG	WL	(Jia et al. 2020)
		β-LG	LDQWLCEKL	(Song et al. 2017)
		β-LG	ALGIILA TGMVAGGIMGLPA	(Ashraf et al. 2021)
			PAGNFLM	(Baba et al. 2021)
			PAVACCL	
			PPLPCHM	
			PAGNFLP	
			PVAAAPVM	
			PAGNFLMNGLMHR	
			PAVACCLPPLPCHM	
			MLPLMLPFTMGY	
			PAGNFLPPVAAAPVM	
Antioxidant	Lipid peroxidation inhibition, reactive oxygen species (ROS) inactivation and free radical scavenging. Metal ions chelation.	β-LG	MHIRL	(Hernández-Ledesma et al. 2007)
		β-LG	YVEEL	
		α-LA	WYSLAMAASDI	
		α-LA	ALMP	(Corrochano et al. 2019)
		BSA	GDLE	
			TKIPA	(Nongonierma and FitzGerald 2013)
			VEELKPT	
			WC	(Sadat et al. 2011)
			LDQW	
			INYW	Corrochano et al. 2019)
			VGIN	
			AVEGPK	
Anticancer	Antioxidant and immunomodulatory activity, angiogenesis inhibition, oncogen modulation, cancer cell apoptosis induction, β-glucuronidase inhibition.	β-LG	MKCLLLALAL	(Sah et al. 2015)
		α-LA	TCGAQALIVT	
		LF	QTMKGLDIQK	(Kimura, Sumiyoshi, and Kobayashi 2014)
			VAGTWYSLAM	
			AASDISLLDA	
			QSAPLRVYVE	
			MMSFVSLLLV	
			GILFHATQAE	
			QLTKCEVFRE	
			LKDLKGYGGV	
			SLPEWVCTTF	
			APRKN	
			VRWCT	
			ISQPE	
			WFKCR	
			RWQWR	
			MKKLG	
Anti-inflammatory	Inflammatory pathways inhibition, adipokines reduction, nitric oxide reléase inhibition, antiinflammatory interleukins production, bacterial lipopolysaccharides (LPS) binding.	β-LG	GTWYSL	
		LF	LSFNPTQL	
			MAASDISLL	
			AMAASDISLL	(Bamdad et al. 2017)
			DTDYKKYLLF	
			IIAEKTKIPAVF	
			DIQKVAGTWYSL	(Zong et al. 2019)
			ELKPTPEGDLEIL	
			LFP 20	
Antitrombotic	Fibrinogen to the thrombin IIa receptor binding inhibition.	α-LA	RGDGLF	(Fiat et al. 1993)
		LF	LRPVAAEIY	(Fan et al. 2019)
		LF	LRPVAAEIY (LF-LR)	(Xu et al. 2020)
Antiviral	Membrane receptor binding, viral gene expression suppression, virion inhibition, virus adsorption inhibition.	β-LG	ALMPHIR	(Çakır et al. 2021)
Antiviral		LF	IPAVFK	
		LF	FKC1RRWQWRMKKLGAPSITC1VRRAF	(Mistry et al. 2007)
			KANEGLTWNSLKDK	
			TGSCAFDEFFSQSCAPGADPKSR	
			TNGESTADWAKN	
			GKNGKNCPDKFC	(Scala et al. 2017)
			KSETKN	
			NDNTECLAKLGGRPTYEE	

(Continued)

Table 2. (Continued)

Biological activity	Mechanism of action	Origin	Amino acid sequence	Reference
Immunomodulatory	Lymphocyte proliferation, macrophages phagocytosis and antibodies synthesis stimulate. Proinflammatory cytokines regulation, bacterial endotoxin neutralization.	α-LA β-LG	LIVTQTMK EILLQK VLVLDTDYKKYLLF YLLF RELKDLK WLAHK IPAV	(Jacquot et al. 2010) (Oyama et al.2017)
Mineral chelating	Complexes mineral formation.	α-LA and β-LG	FD TAT EG	(Zhao et al. 2014) (Zhao et al. 2015) (Huang et al. 2015)
Osteoanabolic	Angiotensin converting enzyme (ACE) inhibition, proinflammatory cytokines suppression, gene expression induction.	β-LG β-LG	YVEEL YLLF IPP VPP	(Pandey, Kapila, and Kapila 2018) (Narva et al. 2004)
Hypocholesterolemic	Expression of the LDL receptor increased, bile salts binding.	β-LG β-LG	HIRL IIAEK	(Yamauchi, Ohinata, and Yoshikawa 2003) (Wakasa et al. 2011)
Opioid	Opioid receptors binding	α-LA β-LG	YGLF YLLF	(Antila et al. 1991) (Teschemacher, Koch, and Brantl 1997)

2019). However, one of the trends is the use of proteases of plant origin (Estévez et al. 2020; Mazorra-Manzano et al. 2020).

Clinical studies carried out in hypertensive patients have highlighted the effect of the consumption of whey protein hydrolysates in lowering blood pressure. Pins and Keenan (2019) reported that the intake of these compounds (20 g) obtained by enzymatic hydrolysis with porcine trypsin significantly reduced blood pressure in hypertensive patients. On the other hand, a study in overweight individuals showed that supplementation with whey protein hydrolysate (54 g) for 12 weeks promoted a decrease in blood pressure as a preventive measure to reduce the risk of hypertension (Pal, Ellis, and Ho 2010).

5.2. Peptides with antimicrobial activity

Whey proteins are an abundant resource for obtaining peptides with antimicrobial activity (Sah et al. 2018). Lactoferrin and its proteolytic fragments are the most studied in terms of antimicrobial capacity (Bruni et al. 2016), while the potential of β-LG and α-LA to produce bioactive antimicrobial peptides seems to be less explored territory, despite the fact that these proteins are the most abundant in serum (Hernández-Ledesma et al. 2014).

Bovine lactoferrin is part of the innate immune system and exhibits antimicrobial activity against a great diversity such as parasites, fungi, and Gram positive and negative bacteria (Farnaud and Evans 2003; García-Montoya et al. 2012). Derived from this protein, lactoferricin is produced after hydrolysis of LF by the action of gastric pepsin (Bellamy et al. 1992). The antimicrobial activity of lactoferricin has been reported, evaluating its antifungal effect against *Candida albicans*, antiparasitic against *Toxoplasma gondii* and *Eimeria stiedai*, and its antibacterial activity against *Escherichia coli*, *Bacillus cereus*, *Clostridium difficile*, *Klebsiella pneumoniae* and *Bacillus subtilis* among others has been reported (Farnaud and Evans 2003; Ulvatne et al. 2004; León-Calvijo et al. 2015).

Lactoferricin exhibits greater antimicrobial activity than lactoferrin, therefore it has been suggested that this peptide may be responsible for the antimicrobial activity of LF (Bellamy et al. 1992). Lactoferricin-derived peptides such as RRWQWR have been reported to exhibit high antibacterial activity against *E. coli* ATCC 11775 and *Salmonella enteritidis* ATCC 13076, while monomeric, cyclic, tetrameric, and palindromic peptides containing the RWQWR sequence exhibit high and specific activity against *E. coli* ATCC 11775 (Huertas Mendez et al. 2017).

It has been suggested that the mechanism by which lactoferricin exerts its antimicrobial effect is through electrostatic interactions between its positively charged residues and negatively charged molecules on the microbial surface, specifically lipopolysaccharides (LPS) causing breakdown of these compounds (Sah et al. 2018) (Figure 2). The hydrophobic residues of lactoferricin interact with the lipid bilayer, inducing an alteration of the membrane, which leads to permeability and finally to cell lysis (Haukland et al. 2001; Y. Liu et al. 2011; Sinha et al. 2013).

The hydrolysis of α-LA using trypsin and chymotrypsin produces antimicrobial peptides of great potential. Using trypsin, the peptides EQLTK, GYGGVSLPEWVCTTF and ALCSEK have been identified, while hydrolysis with chymotrypsin resulted in a different antimicrobial fragment identified as CKDDQNPH ISCDKF. These peptides exert their antimicrobial effect against *Bacillus subtilis*, *Staphylococcus epidermidis* and *Staphylococcus lentus* (Pellegrini et al. 1999). On the other hand, it has been reported that the hydrolysis of β-LG using trypsin produces antimicrobial peptides (IDALNENK, TPEVDDEALEK, KVAGT) with specific activity against Gram positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Listeria ivanovii* (Demers-Mathieu et al. 2013; Théolier et al. 2013).

The mechanism of action of antimicrobial peptides (Figure 2) derived from α-LA and β-LG seems to be

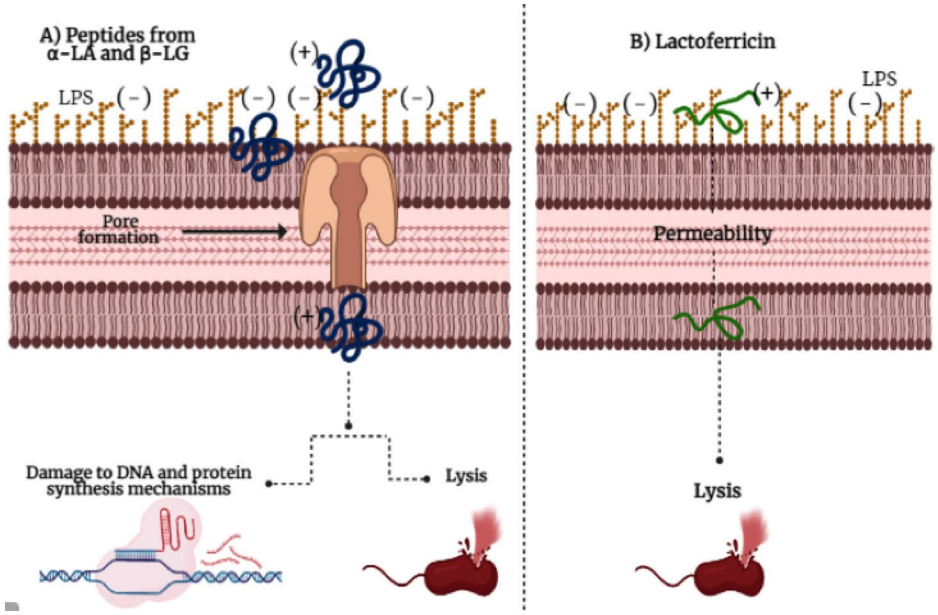

Figure 2. Mechanism of action of antimicrobial peptides from whey proteins. A) Mechanism of peptides derived from the hydrolysis of α-LA and β-LG. B) Mechanism of lactoferricin.

associated with the binding and adsorption of the bacterial membrane through negatively charged electrostatic interactions (Bahar and Ren 2013; Akalın 2014). Additionally, these peptides can cause breaks in the LPS layer, important components of the bacterial cell membrane, or bind to specific sites responsible for interactions with divalent cations such as Ca^{2+} and Mg^{2+} (Sah et al. 2018). Therefore, there is a distortion of the structure of the outer membrane that leads to permeability, allowing the passage of peptides and other molecules (da Silva and Teschke 2003). The amphipathic character of the peptide facilitates the insertion of hydrophobic compounds to the lipid bilayer of the bacterial cell membrane. For that reason, the formation of transient membranes and pores that affect both permeability and energy generation processes, resulting in rupture of plasmatic membrane and cell lysis (Oren, Hong, and Shai 1997; Demers-Mathieu et al. 2013; Sah et al. 2018). Similarly, these peptides could affect both the mechanisms of DNA and protein synthesis and the intracellular enzymatic activity (Benkerroum 2010; Bahar and Ren 2013; Akalın 2014).

Several studies have demonstrated the effect of peptides derived from whey proteins on the inhibition of bacterial growth after fermentation with LAB. It has been observed that *Lactobacillus helveticus* LH-2 and *Lactobacillus acidophilus* La-5 when grown in a medium with protein serum, specifically from β-LG, produce peptides (EIPTINT, IDALNENK, VLVLDTDYK, AASDISLLDAQSAPLR) against *Salmonella enterica* subsp. *enterica* serovar Typhimurium (Ali et al. 2019). Similarly, *Bacillus clausii* has made it possible to produce hydrolysates with antimicrobial effect against strains of *Salmonella typhimurium*, *Escherichia coli*, *Shigella flexneri*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Enterococcus faecalis* (Rochín-Medina et al. 2018). Additionally, it has been isolated whey peptides derived from whey proteins with activity against *Escherichia coli* ATCC 25922 (Wang, He, et al. 2020). Similarly, antimicrobial peptides with an effect on *Escherichia coli* and *Micrococcus luteus* have been identified from goat's milk whey proteins (Moreno-Montoro et al. 2017) and it has been highlighted that the peptides obtained from proteins Camel whey have a greater effect on the specific growth rate of *E. coli* Dh1α than those obtained from bovine whey protein (Salami et al. 2010).

5.3. Antidiabetic peptides

Type 2 diabetes mellitus (T2DM) is a pathophysiology that results from a condition of insulin resistance and insulin secretion dysfunction; as a consequence there is an increase in blood glucose concentrations (Jia et al. 2020). Various studies have indicated that peptides derived from whey protein hydrolysis have antidiabetic effects by controlling glucose concentrations and improving insulin sensitivity (Jia et al. 2020).

The mechanism of some of these peptides is related to the inhibition of enzymes that participate in the degradation of incretins responsible for stimulating insulin release and inhibiting glucagon secretion (Figure 3). One of the enzymes whose action is inhibited by peptides is the enzyme dipeptidyl peptidase IV (DPP-IV), a highly specific serine protease (Berger et al. 2018). This enzyme is responsible for the degradation of glucagon-like peptide (GLP-1), a polypeptide hormone secreted mainly by L cells in the ileum and colon (Jia et al. 2020). GLP-1 is a factor of great relevance in the maintenance of glucose homeostasis in the blood, since it promotes the secretion of insulin stimulated by glucose and pancreatic β cells, while it inhibits the secretion of glucagon from α cells pancreatic (Drucker and Nauck 2006). As a consequence of the degradation of GLP-1 by DPP-IV, the

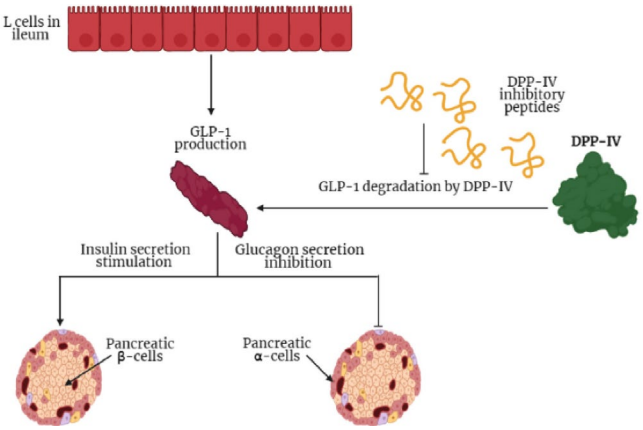

Figure 3. Mechanism of action of DPP-IV inhibitor peptides. GLP-1, produced in L cells of the ileum, stimulates insulin secretion by β-pancreatic cells at the same time as it inhibits glucagon secretion by α-pancreatic cells; DPP-IV degrades GLP-1 preventing these mechanisms from taking place, bioactive peptides inhibit DPP-IV and therefore the degradation of GLP-1.

ability to reduce postprandial blood glucose is significantly decreased; therefore the inhibition of the enzyme DPP-IV represents an alternative for the treatment of DMT2 by avoiding degradation of GLP-1.

Thus, it has been reported that the LAHKALCSEKL, WLAHKAL and WL peptides obtained from the hydrolysis of α-LA and β-LG with pepsin, trypsin, chymotrypsin, elastase and glutamyl endopeptidase, have strong inhibitory capacity of DPP-IV (Nongonierma, O'keeffe, and FitzGerald 2016). Specifically, it has been found that from the hydrolysis of α-LA with trypsin, a potent DPP-IV inhibitor peptide (LDQWLCEKL) is generated (Jia et al. 2020), while the hydrolysis of β-LG has allowed the obtention of IPAVF and IPAVFK, which are peptides with notable DPP-IV inhibitory activity (Silveira et al. 2013). In addition to cow whey, camel and mare whey has been of great interest for their antidiabetic properties. In the case of camel whey, it has been reported that peptides (PAGNFLM, PAVACCL, PPLPCHM, PAGNFLP, PVAAAPVM) are potent inhibitors of DPP-IV and also exert a positive effect on the activation of the insulin receptor in cell lines (Ashraf et al. 2021). Regarding mare's milk serum, two β-LG peptides (ALGIILA and TGMVAGGIMGLPA) with great inhibitory potential of DPP-IV have been identified, which were obtained after hydrolysis with papain, indicating that their activity improved even after in vitro simulated digestion. (Song et al. 2017). Likewise, it has been proven through simulated gastrointestinal digestion, that whey protein hydrolysates inhibit the activity of DPP-IV while intact serum stimulates the secretion of GLP-1 by enteroendocrine cells and therefore the insulin release, evidencing the glucoregulatory potential of these peptides in the enteroinsular axis (Power-Grant et al. 2015).

In addition to DPP-IV inhibitor peptides, other antidiabetic peptides have been studied for their ability to improve insulin sensitivity. Thus, peptides such as Wheylin-1, an anxiolytic-type dipeptide (MH) isolated from the hydrolysis of β-LG with thermolysin have been identified (Yamada et al. 2014). The antidiabetic effect of this dipeptide has been demonstrated in a T2DM model in KK-A^y mice. Wheylin-1 decreased blood glucose levels after a glucose tolerance test and increased insulin sensitivity by increasing the phosphorylation of Akt, a serine/threonine kinase that is activated in response to insulin in HepG2 liver cells and C2C12 muscle myotube cells (Ogiwara et al. 2018).

Similarly, the role of various peptides with the ability to inhibit the activity of the enzymes α-amylase (α-AAM), which hydrolyzes long-chain carbohydrates and α-glucosidase (α-AG), which catalyzes the cleavage of glucose from disaccharides (Yu et al. 2012). However, further investigation is required on the antidiabetic potential of α-AAM and α-AG inhibitor peptides at in vitro and in vivo models. Recently, the identification of novel peptides (PAGNFLMNGLMHR, PAVACCLPPLPCHM, MLPLMLPFTMGY, PAGNFLPPVAAAPVM) from camel whey protein hydrolysis has been reported (Baba et al. 2021). These peptides were identified as potential inhibitors of α-AAM and α-AG, due to the high number of binding sites and greater probability of binding with these enzymes (Baba et al. 2021). Furthermore, the inhibition of α-AAM and α-AG is considered an effective strategy for the control of diabetes by delaying glucose absorption (Z. Yu et al. 2012).

The effect of antidiabetic peptides has also been evidenced in clinical studies as reported by Sartorius et al. (2019). They evaluated the effect of consuming a whey protein hydrolysate in prediabetic patients. The patented compound (Pep2Dia®) was prepared by Ingredia S.A. (Arras CEDEX, France), which is a product of the hydrolysis mainly of α-LA and β-LG. This hydrolysate contained a dipeptide (PA) in a proportion of between 0.15-0.4% with inhibitory properties of α-AG and was supplied to patients in the form of a capsule, each containing 350 mg of hydrolysate that included 0.96 mg of peptide (Sartorius et al. 2019).

5.4. Antioxidant peptides

Oxidative stress is a condition caused by the increased production of reactive oxygen species (ROS), mainly anionic radicals such as superoxide $(O_2\bullet)^-$, singlet oxygen $(O_2\bullet)$, hydrogen dioxide $(HO_2\bullet)$, peroxide of hydrogen (H_2O_2) and hydroxyl radicals $(HO\bullet)$ (Madadlou and Abbaspourrad 2018). These elements are generated through normal reactions in the body, acting as signaling molecules and activating various survival pathways sensitive to stress conditions (Poljsak and Milisav 2014). However, an increase in the concentration of these molecules leads to cell damage, destruction of protein structures and DNA mutation (Tanzadehpanah, Asoodeh, and Chamani 2012), which leads to the initiation or progression of certain diseases (Moskovitz, Yim, and Boon Chock 2002).

Hydrolysis of whey proteins release peptides with antioxidant properties (Pihlanto 2006; Mann et al. 2019). The interest for the use of this type of peptides has increased because they are considered natural and safe antioxidants (Brandelli, Daroit, and Corrêa 2015). The antioxidant properties of peptides are related to their composition, structure and hydrophobicity (Chen et al. 1998). Thus, aromatic

amino acids such as Tyr, Trp, Met, Lys and His and amino acids that contain sulfide groups such as Cys have been determined to have great antioxidant potential by donating protons to electron-deficient radicals (Wang and De Mejia 2005; Sadat et al. 2011; Mann et al. 2019).

The mechanism of action of antioxidant peptides has not been fully elucidated (Mann et al. 2019) however, some researchers have pointed out that the mechanism may involve: 1) inhibition of lipid peroxidation by stopping the oxidation chain reaction of fats; 2) inactivation of reactive oxygen species (ROS) and free radical scavenging; 3) chelation of metal ions by eliminating traces of metals such as iron or copper that facilitate oxidation, and 4) combination of these mechanisms (Figure 4) (Moure, Domínguez, and Parajó 2006; Brandelli, Daroit, and Corrêa 2015; Mann et al. 2019). Antioxidant peptides have been reported to exert their effect through the intracellular conversion of cysteine to glutathione, protecting cells from ROS damage (Keri Marshall 2004).

Several in vitro studies have indicated that the hydrolysis of whey protein, using enzymes such as alcalase, produces peptides that improve antioxidant activity in liposome models. Reporting that the hydrolyzates of whey protein isolate show greater radical scavenging capacity, greater Cu^{+} chelating capacity and greater reducing power compared to non-hydrolyzed whey protein isolate (Bayram et al. 2008; Peng et al. 2010). Similarly, the hydrolysis of whey protein concentrate by trypsin has allowed the identification of strong antioxidant and cytoprotective peptides (ELKDLK and ALPMHIR), evidencing this effect in rat ileum cells (IEC-18) under stress conditions (Ballatore et al. 2020).

In addition to the use of digestive enzymes for the generation of antioxidant peptides, LAB have also been used. In this context, it has been reported that the fermentation of cow's milk whey with various strains of *Lactobacillus plantarum* has allowed obtaining peptides <1 kDa that exhibit better antioxidant activity compared to larger peptides (Aguilar-Toalá et al. 2017). Similarly, the fermentation of goat's milk whey with *Lactobacillus plantarum* C4 allows the obtention of this type of peptides (Moreno-Montoro et al. 2017).

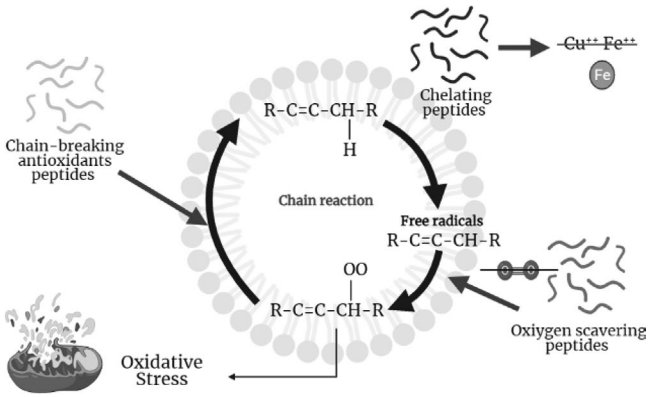

Figure 4. Mechanism of action of antioxidant peptides, these could participate in the breaking of the chain reaction of oxidation of fats, capture of free radicals and chelation of metal ions.

Different commercial enzyme preparations have been used to hydrolyze whey proteins. Actually, the hydrolysis of β-LG has allowed the identification of antioxidant sequences such as WY, WYS and WYSLAM as well as peptides of >3 kDa (MHIRL, YVEEL, WYSLAMAASDI) obtained by hydrolysis with Corollase PP (Hernández-Ledesma et al. 2005; Hernández-Ledesma et al. 2007). Similarly, sequences have been identified that participate in the chelation of specific metals such as the peptide TPEVDDEALEK, which participates in the chelation of iron (Cruz-Huerta et al. 2016). In the case of the hydrolysis of α-LA with DebitraseTM HYW20 has made it possible to identify a dipeptide (WC) with high antioxidant potential (Nongonierma and FitzGerald 2013). Similarly, the LDQW and INYW peptides derived from the hydrolysis of α-LA with thermolysin, are capable of inhibiting the ABTS radical 100% at 2.5 μM (Sadat et al. 2011).

The role of antioxidant peptides has also been explored in the context of the intestinal microbiota, since it has been reported that these types of peptides reverse the effects of intestinal homeostasis dysbiosis by regulating intestine ROS concentrations. The imbalance between ROS and antioxidant systems causes dysbiosis of the intestinal microbiota that has also been associated with neurological disorders (Dumitrescu et al. 2018; Cryan et al. 2019). Variation in intestinal metabolite concentrations in individuals has been observed to be associated with amino acid levels (N. Ma and Ma 2019). Thus, antioxidant peptides alter the structure of the microbial community by reducing the abundance of pathobionts such as *Escherichia coli* and *Clostridium perfringens* that generate high concentrations of ROS (Wang et al. 2019).

So, the consumption of α-LA hydrolysates in a mouse model (C57BL/6), led to an increase in the ratio of Bacteroidetes to Firmicutes (Li et al. 2019), while the consumption of serum peptides in Winstar rats and mice (C57BL/6) has led to a significant increase in the relative abundance of *Lactobacillus* spp., *Bifidobacterium* spp. (Monteiro et al. 2016; Y.-J. Yu et al. 2016). In addition, through simulated gastrointestinal digestion, the effect of antioxidant peptides has also been determined, reporting a release of whey peptides from β-LG (ALMP, GDLE, TKIPA, VEELKPT), α-LA (VGIN) and BSA (AVEGPK), which are transported through the intestinal barrier in Caco-2/HT-29 cells, inhibiting the formation of free radicals in muscle and liver cells (Corrochano et al. 2019).

5.5. Immunomodulatory peptides

The purpose of immunomodulatory peptides is to improve the innate immunity of the gastrointestinal mucosa during the first years of life and play a protective role in certain disorders associated with allergic reactions that, in turn, cause inflammation (Pérez-Cano et al. 2007). The hydrolysis of whey proteins has been shown to release peptides that change the immune system, thus exerting an immunomodulatory effect (Gauthier, Pouliot, and Saint-Sauveur 2006; Szwajkowska et al. 2011; Brandelli, Daroit, and Corrêa 2015).

Although the mechanism by which these types of peptides exert their effect is not completely clarified (Figure 5). It has been proposed that immunomodulatory peptides could modulate the immune response by stimulating the proliferation of lymphocytes and phagocytosis in macrophages, the synthesis of antibodies, the regulation of cytokine concentrations and even the neutralization of endotoxins of bacterial origin (LPS). LPS interact with toll-like cell receptors (TLRs) promoting the production of pro-inflammatory cytokines such as: tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 1β (IL-1β) and C-reactive protein (CRP) (Gill et al. 2000; Baylis et al. 2013; Adak and Khan 2019).

The consumption of whey hydrolysate has shown to stimulate the serum secretion of TGF-β1 lymphocytes, which is related with a significant increase in IgA immunoglobulin in mouse models infected with *E. coli* (Saint-Sauveur et al. 2009). Similarly, it has been reported that the peptides (LIVTQTMK, EILLQK, VLVLDTDYKKYLLF, YLLF, RELKDLK, WLAHK) obtained from the hydrolysis of β-LG and α-LA with trypsin or chymotrypsin stimulate lymphocyte proliferation and induce immunosuppressive effects on cytokine secretion (Jacquot et al. 2010). In addition, it has been observed that whey hydrolysates participate in the activation of TLR cell receptors and induce cytokine production in dendritic cells (Kiewiet et al. 2018).

The tetrapeptide IPAV from fraction 94-97 of β-LG exerts an anti-inflammatory effect in human intestinal Caco-2 cells, observing a reduction in the expression of IL-8 induced by TNF-α and an increase in the levels of phosphorylation of key cells and proteins involved in the suppression of inflammatory signaling in intestinal cells (Oyama et al. 2017). Other study revealed that after digestion of whey proteins, peptides produced reflected in a decrease in the plasma levels of IL-1α, IL-1β, IL-10 and TNF-α, as well as the ROS levels and cholesterol concentrations. Additionally, the levels of anti-inflammatory cytokines such as IL-2, IL-4, IL-7 and IL-8 increased significantly, while there was an important proliferation of lymphocytes, macrophages and monocytes in response to stimulation with different antigens (Badr et al. 2012).

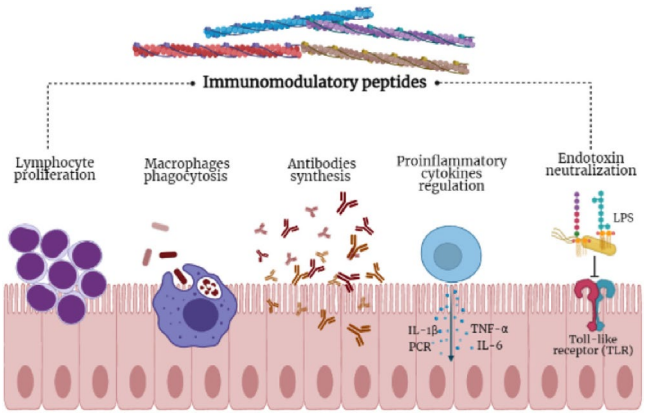

Figure 5. Mechanisms of action of immunomodulatory peptides. This type of peptides stimulate the proliferation of lymphocytes, phagocytosis in macrophages and the synthesis of antibodies. Likewise, they act by regulating the concentrations of pro-inflammatory cytokines and neutralizing endotoxins of bacterial origin (LPS), thus inhibiting the interaction with TLR receptors and the subsequent production of cytokines.

Araujo et al. (2017), evaluated the effect of goat's whey in a murine model of colitis, induced by 2,4-dinitrobenzenesulfonic acid. They observed that the consumption of whey at a concentration of 4 g/kg/day led to the release of peptides after digestion and in turn to an improvement of intestinal inflammation, specifically of the colon, as well as less infiltration of leukocytes. Likewise, a down regulation was observed in the gene expression of several pro-inflammatory markers such as IL-1β, IL-6, IL-17 and TNF-α. In addition, the consumption of goat whey increased the expression of proteins such as mucins, occludin proteins, and cytokine signaling suppressors (Araujo et al. 2017).

After in vivo digestion of the commercial whey hydrolysate Peptigen® IF-3090 by mice, a peptide (VRTPEVDDE) has been identified, which has demonstrated an immunomodulatory effect conferring by induction of the proliferation of $CD3^+$, $CD8^+$, $B220^+$, $CD19^+$, and $CD11b^+$ lymphocytes in the thymus and splenocytes. In addition to this, the growth and weight of the mouse pups was significantly higher in the mice fed with whey protein hydrolysate compared to the control groups (Takeda et al. 2020).

In clinical studies, an attenuation of pro-inflammatory cytokines has been reported in non-diabetic obese individuals when consuming 45 g of whey protein for 4 weeks (Holmer-Jensen et al. 2011). In the same way, the effect of the consumption of whey protein isolate (20 g/day) has been evaluated in specific diseases such as psoriasis, a chronic autoimmune disease that mainly affects the epidermal tissue, reporting the improvement of the conditions of patients by combating the severity of systemic inflammation (Prussick, Prussick, and Gutman 2013). The anti-inflammatory potential of whey protein was also evident in patients with cystic fibrosis who consumed whey protein (20 g/day) for 3 months, observing an increase of 46.6% with respect to the initial value in glutathione (GSH) levels of lymphocytes in the supplemented group, thus signaling a nutritional approach that may be helpful in maintaining optimal GSH levels and counteracting the harmful effects in the lungs of cystic fibrosis patients (Grey et al. 2003).

5.6. Anticancer peptides

Cancer is a term for a class of multifactorial diseases identified by the growth and spread of abnormal cells (Sah et al. 2015). The carcinogenesis process includes several stages, including increased signaling in the cell proliferation response, inhibition of suppression factors that regulate proliferation, invasion through metastasis, cancer cell immortality, induction of angiogenesis, and resistance to apoptosis (Hanahan and Weinberg 2011; Witsch, Sela, and Yarden 2010). These complex mechanisms have led to the search for pharmacological strategies that intervene in key signaling pathways in the carcinogenesis process.

Pharmacological therapies based on peptides have aroused interest in recent years. These compounds have low

cytotoxicity compared to drugs used in traditional chemotherapy (Barras and Widmann 2011). Certain peptides obtained through the hydrolysis of whey protein have anticancer properties (Kimura, Sumiyoshi, and Kobayashi 2014), and they act as specific signals that can inhibit the viability of cancer cells (Figure 6), mainly through the rupture of the cytoplasmic membrane by the formation of pores and by induction of apoptosis (Meisel 2005; Papo and Shai 2005).

The antitumor activity of peptides is due to different mechanisms such as inhibition of angiogenesis, modulation of oncogene expression, antioxidant and immunomodulatory activity (Kakde et al. 2011; Li and Cho 2012; Sah et al. 2015). Another mechanism that stands out is the inhibition of β-glucuronidase, a target enzyme for anticancer peptides (Sah et al. 2015).

In silico studies have allowed the identification of peptides with anticancer potential derived from the hydrolysis of β-LG (MKCLLLALAL, TCGAQALIVT, QTMKGLDIQK VAGTWYSLAM, AASDISLLDA, QSAPLRVYVE), α-LA (MMSFVSLLLV, GILFHATQAE, QLTKCEVFRE, LKDLKGYGGV, SLPEWVCTTF) and lactoferrin (APRKN, VRWCT, ISQPE, WFKCR, RWQWR, MKKLG), being lactoferricin the main peptide with anticancer activity (Sah et al. 2015). Lactoferricin has been observed to induce apoptosis of human T cells in leukemia and B lymphoma cells through the activation of caspases, which are implicated in cell death by apoptosis (Onishi et al. 2008; Furlong, Mader, and Hoskin 2010).

Additionally, the preventive effect of whey peptides has been studied in the context of skin cancer caused by ultraviolet B (UVB) light. Kimura, Sumiyoshi, and Kobayashi (2014), observed that the oral administration of whey peptides in doses of 200 and 400 mg/kg, twice a day reduces skin thickness and elasticity, preventing the formation of wrinkles and melanin granules in a model of furless mice, which were irradiated with UVB light for 17 weeks. Similarly, they reported that whey peptides were involved in the prevention of type IV collagen degradation, angiogenesis, proliferation, and DNA damage caused by UVB irradiation (Kimura, Sumiyoshi, and Kobayashi 2014).

Several in vivo studies on murine models highlight that the anticancer effect of peptides occurs after the consumption of whey protein. In female rat breast cancer models, the mammary glands of rats fed with whey protein have shown to have greater protection against endogenous DNA damage compared to rats fed with casein (Dave et al. 2006). In addition, with this type of supplementation, an increase in glutathione levels in the liver by 92% and a 47% decrease in tumor glutathione levels has been observed (Cheng et al. 2017).

Similarly, the effect of the consumption of whey protein hydrolysate (20% w/w) led to the inhibition of aberrant crypts in the colon in a mouse model with intestinal tumors (Xiao et al. 2006). A similar study showed that the consumption of Hilmar ™ 8380 whey protein hydrolysate in a dose of 2 mL/twice a week, resulted in less development of microscopic tumors in rats and therefore exhibits greater efficacy in prevention of colon cancer (Attaallah et al. 2012).

Human studies have also reported anticancer effects after the consumption of whey protein. In patients with colon cancer, the consumption of whey proteins in a dose of 7.8 g/day, prior to colorectal resection surgery, produced an increase in intestinal functionality after the operation (Gillis et al. 2016). In the case of breast cancer, it has been reported that a consumption of whey proteins in a dose of 20 g/2 times a day impacts on the reduction of fat mass at the same time that it considerably increases lean mass in patients with this type of cancer (Madzima et al. 2017). Similarly, supplementation with whey protein isolate (40 g/day) has been reported to significantly increase in albumin concentrations (2.9%), immunoglobulin G levels (4.8%) and glutathione concentrations (11.7%) in patients with colon, lung, rectal, stomach cancer and pancreatic cholangiocarcinoma (Bumrungpert et al. 2018). However, none of these studies have identified the sequences of the peptides generated after digestion of whey proteins and to which the anticancer

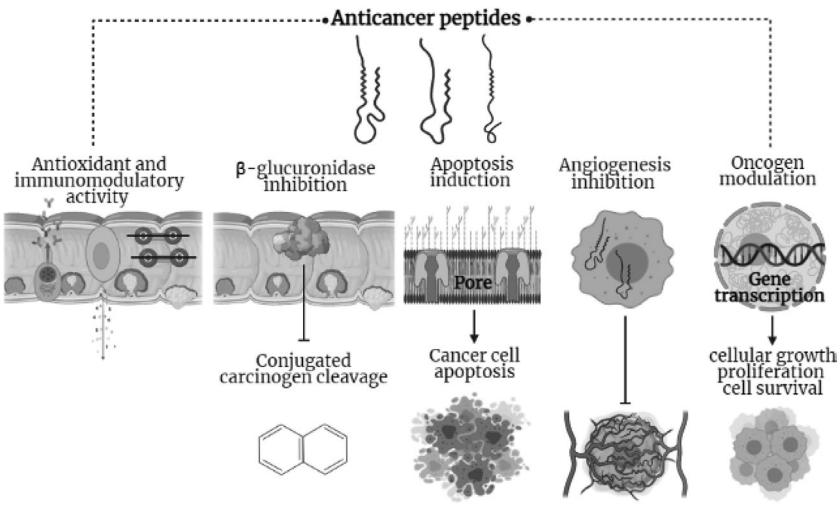

Figure 6. Main mechanisms of action of anticancer peptides, which include: 1) Antioxidant and immunomodulatory activity, 2) Inhibition of β-glucuronidase, an enzyme that participates in the cleavage of conjugated carcinogens, 3) Induction of apoptosis of cancer cells, 4) Inhibition of angiogenesis, and 5) Modulation of oncogene expression.

effect is attributed. Therefore, it is necessary to continue exploring the effect of peptides derived from whey proteins in the reduction of cancer.

5.7. Mineral chelating peptides

Anemia and osteoporosis are diseases characterized by a constant demineralization and they represent a continuous challenge since in the elderly population these problems represent reduction of the quality of life and an increase of socioeconomic costs (Lee et al. 2017). The prevalence of these disorders worldwide makes it considered a serious public health problem (Mann et al. 2019).

Calcium and iron are two of the most important minerals for proper cell function and which are lost in diseases whose pathophysiology is characterized by the lost of mineral concentration (Combet and Buckton 2015; Prashanth et al. 2015; Walters, Esfandi, and Tsopmo 2018). In addition, its deficiency is linked to some types of cancer and arterial hypertension (Power et al. 1999; Wang et al. 2008). On the other hand, iron participates in the process of cellular respiration, nucleic acid synthesis and gene expression (Lieu et al. 2001; Walters, Esfandi, and Tsopmo 2018) and in addition to anemia, its deficiency is related not to depression and poor nervous and muscular development in children (Umbreit 2005).

Food supplementation with mineral salts, contributes to the demineralization process. However, it represents a problem for the food industry since the addition of these components implies a change in the physical and sensory properties of food (Guo et al. 2014). In this context, metal chelating peptides from whey have been identified as potential functional elements capable of improving the bioavailability of calcium, iron and zinc (Guo et al. 2014).

The mechanism of action of this type of peptide is directly related to the net charge and the length of the side chain and the degree of complex formation with the different minerals will depend on this. Some studies have indicated that the iron-binding site corresponds mainly to the terminal carboxyl group of the peptide, although other binding sites such as phosphate groups in peptides have also been pointed out (Caetano-Silva et al. 2015; Chaud et al. 2002; Zhao et al. 2015; Sun et al. 2016). Similarly, amino acid residues found within peptide structures such as cysteine, serine, histidine, aspartate, and glutamate could serve as ligands for binding of transition metals (Sun et al. 2016). The structures of these amino acid residues could form a complex with peptides through electrostatic interactions and hydrogen bonds (Walters, Esfandi, and Tsopmo 2018).

In vitro studies have allowed the identification of peptides with high mineral chelating potential. An example of this is the dipeptide FD, purified from whey protein hydrolysate, this peptide shows specific calcium binding capacity and it has been identified that the amino and carboxyl groups of the purified peptide are the calcium binding sites. The calcium binding capacity of FD reached 73.34 μg/mg, thus it has the potential to be used as a calcium-binding ingredient in dietary supplements (Zhao et al. 2014). In the same way, the tripeptide TAT purified from whey protein hydrolysates has also shown a strong calcium binding capacity reaching 79.5 μg/mg and pointing out that the chelate binding sites are an oxygen atom of the carbonyl group and the nitrogen of the amino group or imino group (Zhao et al. 2015), while the dipeptide EG demonstrated a calcium binding capacity of 67.81 μg/mg, indicating the carboxyl and carbonyl groups as binding sites (Huang et al. 2015).

5.8. Osteanabolic peptides

Osteoporosis is defined as a skeletal disorder characterized by microarchitectural deterioration, low bone mass, and low mineral density as a result of increased resorption and decreased bone formation (Fukushima et al. 2010; Pandey, Kapila, and Kapila 2018). Furthermore, the net deterioration of bone structure leads to an increased risk of fracture (Giannoudis et al. 2007; Gorter et al. 2021). The prevalence of this disease increases with age, with postmenopausal women being more likely to be affected (Gorter et al. 2021). Worldwide, it is estimated that osteoporotic fractures will rise to 18 million by the year 2040, which is a serious public health problem since it is associated with high rates of mortality and morbidity (Yaacobi et al. 2017). Most of the drugs available for the treatment of osteoporosis have focused their effects on the inhibition of bone resorption. Thus, bisphosphonates, calcitonin, and selective estrogen receptor modulators have been the most widely used, despite the side effects and complications associated with their use (Armas and Recker 2012; Pandey, Kapila, and Kapila 2018; Antebi, Pelled, and Gazit 2014). However, the use of conventional drugs is insufficient in the treatment of this pathophysiology, being essential to improve the anabolic function of osteoblasts in terms of cell differentiation and proliferation (Pandey, Kapila, and Kapila 2018).

The effect of milk and whey proteins on bone formation has been demonstrated, highlighting the therapeutic role of these proteins through the activation of osteoblasts (Xu 2009; Mada et al. 2020). Various studies have indicated that peptides derived from the hydrolysis of whey proteins affect the proliferation and differentiation of primary osteoblasts. Similarly, these peptides exert an osteoprotective effect by suppressing the inflammatory state and improving bone formation markers (Pandey, Kapila, and Kapila 2018; Pandey, Kapila, and Kapila 2018) (Figure 7).

The mechanism of action of these types of peptides (Figure 8) is related to reproductive aging that occurs in menopause (oxidative stress and hypertension), conditions that increase the progression to osteoporosis (Popovic et al. 2011; Pandey, Kapila, and Kapila 2018). In this sense, antioxidant peptides and ACE inhibitors have been used in order to evaluate their effect on various osteoanabolic and osteoprotective parameters. In addition, ACE inhibitory peptides have been shown to prevent the formation of Angiotensin II, which has been demonstrated to decrease osteoblast differentiation (Pandey, Kapila, and Kapila 2018;

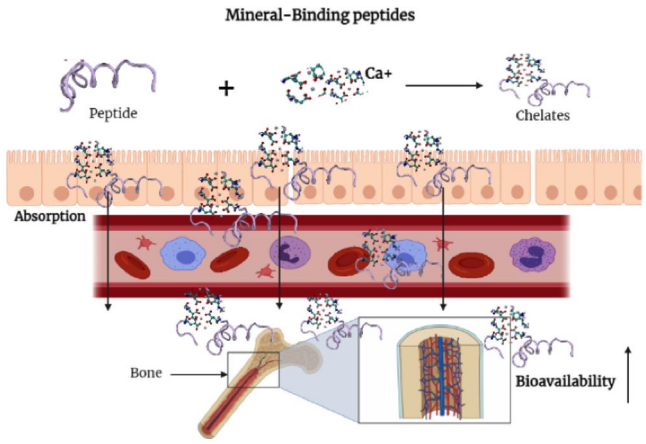

Figure 7. Mechanism of action of mineral chelating peptides. The bioavailability of minerals in the diet is affected by the basic intestinal environment. Chelating peptides could form complexes with minerals to improve their absorption and increase their bioavailability in bone tissues.

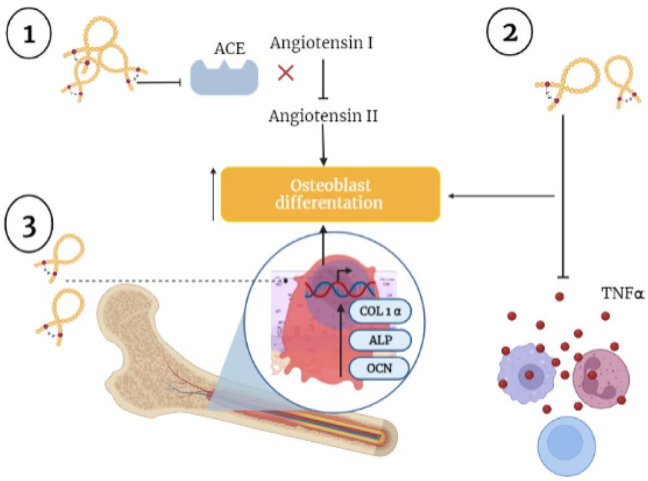

Figure 8. Mechanism of action of osteoanabolic peptides. This type of peptide act at different levels, with three main mechanisms of action. 1) Angiotensin Converting Enzyme (ACE) inhibitor peptides that regulate the conversion of Angiotensin I to Angiotensin II. The latter involved in the decrease in osteoblast differentiation, 2) Suppression of proinflammatory cytokines such as TNF-α, which leads to an increase in osteoblast differentiation, and 3) Induction of gene expression, specifically COL1α, ALP and OCN, important genes in the osteoblastogenesis process.

Pandey, Kapila, and Kapila 2018). Other mechanisms of action include the suppression of proinflammatory cytokines and the induction of increased gene expression, specifical genes such as COL1α, ALP, and OCN involved in the osteoblastogenesis process (Pandey, Kapila, and Kapila 2018).

In this sense, Pandey, Kapila, and Kapila (2018) have shown that the antioxidant peptide P2-YVEEL improves the proliferation and osteogenic differentiation of primary osteoblasts in vitro (Pandey, Kapila, and Kapila 2018). Likewise, it has been observed that the administration of antioxidant peptides (YVEEL) and ACE inhibitors (YLLF) improve altered bone formation, bone resorption, suppression of inflammatory cytokines such as TNF-α, and activation of remission in the parameters of altered trabecular microarchitectures in an OVX rat model of postmenopause (Pandey, Kapila, and Kapila 2018). Similarly, Kruger et al. (2005) reported in an OVX rat model that femoral bone density increased considerably after being supplemented for four months with serum peptide fractions.

Peptides derived from whey fermentation with lactic acid bacteria have also been tested in bone cells. Results have shown that the peptides IPP and VPP increase bone formation in cultured osteoblasts in vitro (Narva et al. 2004). Despite the in vitro and in vivo evidence, clinical studies are necessary to accurately assess the effect of these peptides on various bone parameters.

5.9. Hypocholesterolemic peptides

Atherogenic dyslipidemia is a pathophysiology characterized by an increase in plasma cholesterol concentrations and specifically low-density lipoproteins (LDL) (El Khoury and Anderson 2013). The increase in the concentrations fatty acids, phospholipids and triglycerides are considered an important risk factor in the pathogenesis of cardiovascular diseases, obesity, metabolic syndrome, diabetes and cerebrovascular accident (Nasri 2017; Jain Kishor et al. 2007).

Plasma cholesterol concentrations, especially LDL, are strongly influenced by diet, biosynthesis, absorption, and secretion of cholesterol (El Khoury and Anderson 2013). It has been observed that a 5-6% reduction in serum LDL cholesterol levels is associated in turn with a 7-12% reduction in the risk of manifesting coronary artery disease (Sirtori, Anderson, and Arnoldi 2007). In addition to use drugs, treatment include dietary approaches that help the modulation of lipid (Nasri 2017). In this sense, it has been reported peptides derived from whey with the hypocholesterolemic effect (Peighambardoust et al. 2021).

It has been suggested that the mechanistic of these kinds of peptides could improve blood lipid concentration levels. The atherogenic plasma profile is changed to a cardioprotective plasma profile by inducing LDL receptor expression, improving at the same time, bile acid synthesis and decreasing the absorption of steroids in the intestine (Erdmann, Cheung, and Schröder 2008; Ooi et al. 2015; Peighambardoust et al. 2021). Furthermore, hypocholesterolemic peptides bind to bile acids due to the hydrophobic interaction with tetracyclic ring structures, which allows a greater fecal excretion of cholesterol since the interactions between peptides and micellar bile salts depend on the hydrophobic/hydrophilic nature of peptides (Figure 9) (Howard and Udenigwe 2013; Guerin et al. 2016).

Studies have reported that whey peptides with hypocholesterolemic activity are mainly the product of the hydrolysis of β-LG. Thus, sequences such as the HIRL peptide have been reported to reduce LDL and serum total cholesterol in hyperlipidemic mice mediated by neurotensin (NT2), dopamine receptors (D2) and stimulated bile acid secretion (Yamauchi, Ohinata, and Yoshikawa 2003). Similarly, another peptide derived from β-LG is lactostatin, a pentapeptide (IIAEK) whose effect has shown greater hypocholesterolemic activity than β-sitosterol, which is the drug commonly used to treat hypercholesterolemia.

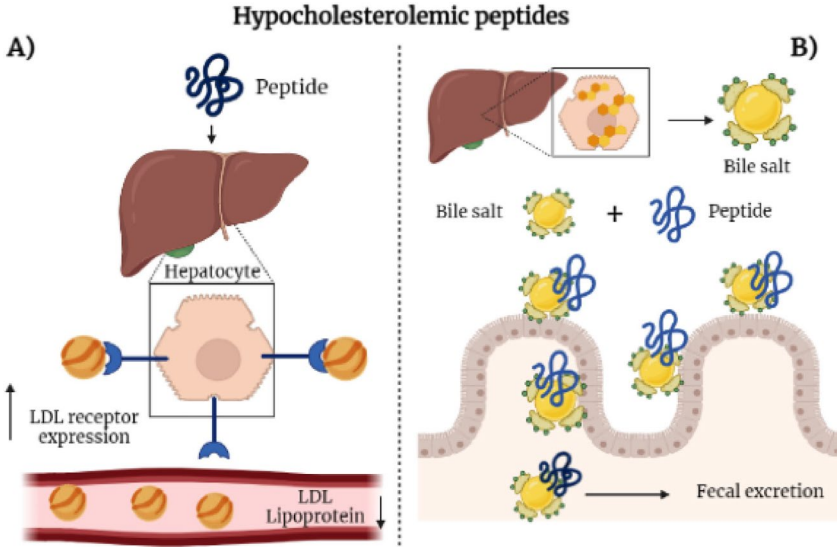

Figure 9. Mechanism of action of hypocholesterolemic peptides. A) Peptides promote increased expression of the LDL receptor in hepatocytes, once the lipoprotein binds to the receptor, LDL concentrations in the blood decrease. B) The cholesterol metabolism is carried out in the hepatocytes resulting in soluble bile salts, the peptides bind to these bile salts which allows a greater fecal excretion of cholesterol.

Lactostatin-expressing transgenic rice seeds were administered orally for three days at a dose of 300 mg/kg body weight/day, resulting in hypocholesterolemic activity in rats, decreasing serum LDL concentrations and increasing high-density lipoprotein (HDL) concentrations, which is considered beneficial cholesterol due to its cardioprotective effect (Wakasa et al. 2011). These results are consistent with those obtained by Nagaoka et al. (2001), who reported that lactostatin (IIAEK) can influence serum cholesterol levels and exhibit greater hypocholesterolemic activity compared to that of β-sitosterol, in animal studies, since a considerable decrease in serum and liver cholesterol levels has been observed.

Although human studies are scarce, reported results indicate that digestive hydrolysis of whey proteins releases peptides with hypocholesterolemic activity. It has been observed in non-diabetic obese individuals, obsessed postmenopausal women and subjects with type 2 diabetes mellitus that the supplementation of a high-fat meal plus 45 g of whey protein leads to a reduction in the postprandial concentration of lipids, mainly triglycerides (Mortensen et al. 2009; Pal, Ellis, and Ho 2010; Holmer-Jensen et al. 2013).

5.10. Opioid peptides

The responses to sleep apnea, stress and anxiety are related disorders; this leads to the appearance of various diseases (Tyagi et al. 2020). Specifically, prolonged or persistent stress contributes to the elevation of cortisone and decrease of levels of serotonin, dopamine and other neurotransmitters (Tyagi et al. 2020). In addition to this, pain represents one of the most common reasons why patients seek medical attention, however, pharmacological treatments are limited (Tavares et al. 2013). Drugs commonly used for pain management include opioids such as morphine and codeine (Baldo and Pham 2020). But, these drugs have shown different secondary effects in patients, such as constipation, addiction, sedation, respiratory depression and tolerance, among others (Liu and Wang 2012).

Peptides with opioid activity represent an alternative of treatment because the secondary effects are usually null and their production is relatively inexpensive. Opioid peptides are defined as enkephalins that have affinity for a specific opioid receptors (μ, κ, δ) (Pihlanto-Leppälä 2000; Garg, Nurgali, and Kumar Mishra 2016). The activation of these receptors by opioid ligands (Figure 10) leads to the inhibition of the enzyme adenylate cyclase. This enzyme is implied in concentrations reduction of intracellular cyclic AMP (cAMP), allowing the opening of the channels of K^+ causing cell hyperpolarization at the postsynaptic level which inhibits the opening of voltage-gated Ca^{++} channels, thus reducing the release of neurotransmitters at the presynaptic level which in turn allows a reduction in neuronal excitability and therefore an analgesic effect (Harkouk et al. 2018).

Regarding studies on opioid peptides derived from whey proteins, it has been reported that peptide sequences bind mainly to μ receptors, which are related to more powerful antinociceptive effects (Garg, Nurgali, and Kumar Mishra 2016). In this context, sequences derived from α-LA (YGLF) and β-LG (YLLF) whose opioid receptor is μ acting as agonists have been reported (Antila et al. 1991; Teschemacher, Koch, and Brantl 1997). These peptides have been observed to exert a continuous opioid property in terms of receptor binding and smooth muscle effect when they are evaluated in guinea pig ileum and rat brain homogenates (Antila et al. 1991). The β-LG-derived tetrapeptide called β-lactotensin (HIAL) has been reported as a peptide capable of inducing contraction of the longitudinal muscle of the guinea pig ileum in the absence of electrical and agonist stimulation (Pihlanto-Leppälä et al. 1997).

The antinociceptive effect of a mixture of peptide concentrate (PepC) obtained after hydrolysis of whey protein with cardosines of *Cynara cardunculus* has been evaluated

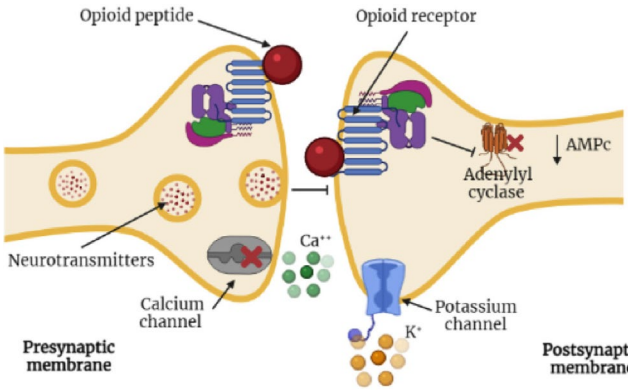

Figure 10. Mechanism of action of opioid peptides. The binding of peptide to the opioid receptor leads to the inhibition of adenylate cyclase decreasing the concentrations of intracellular cAMP which causes the opening of potassium channels and leading to a cell hyperpolarization at the postsynaptic level and the inhibition of the opening of calcium channels voltage-dependent, which limits the release of neurotransmitters at the presynaptic level and therefore reducing neuronal excitability and producing an analgesic effect.

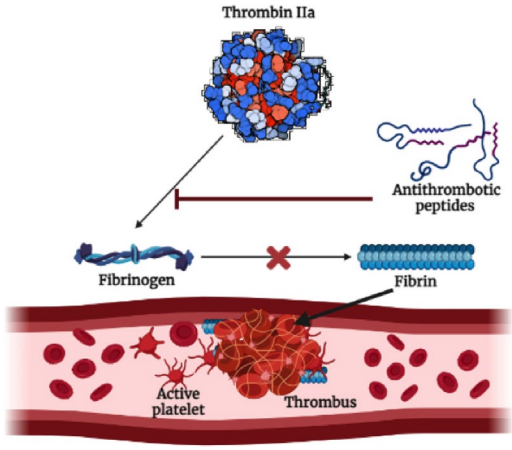

Figure 11. Mechanism of action of antithrombotic peptides. Thrombin IIa participates in the activation of platelets and in the conversion of fibrinogen to fibrin, which forms a network that reinforces the platelet plug and therefore the formation of the thrombus. Antithrombotic peptides have the ability to inhibit the binding of fibrinogen to the thrombin IIa receptor, preventing platelet coaggregation and therefore the thrombus formation.

at in vivo models through contortion tests, hot plate and formalin in mice, observing that PepC seems to possess activities limited peripheral antinociceptives (Tavares et al. 2013). Despite these results, the role of opioid peptides from whey proteins has not been fully elucidated since both in vitro and in vivo studies are scarce to date.

5.11. Antithrombotic peptides

Cardiovascular diseases include myocardial infarction, unstable angina, and stroke (Yang et al. 2017; Rafiq et al. 2017; Cheng et al. 2019). Ischemic heart disease caused by thrombosis tops the list of leading causes of death worldwide, increasing at worrying levels each year (Jasuja et al. 2012). The thrombosis process involves two main mechanisms, the activation of platelets and through interaction with coagulation factors (Kastelowitz et al. 2017; Cheng et al. 2019). The result of the interaction of both mechanisms is the formation of a thrombus (Majluf-Cruz and Espinosa-Larrañaga 2007).

In this context, emphasis is placed on an important coagulation factor in the thrombus formation process, thrombin (IIa), which is a serine protease whose main function is the activation of platelets, strengthening the coagulation system and therefore the production of greater amount of thrombin. Similarly, thrombin (IIa) is responsible for converting fibrinogen to fibrin and activates factors XIII and XIIIa, producing a fibrin mesh that reinforces the platelet plug (Jung et al. 2007). The mechanism of action of antithrombotic peptides (Figure 11) focuses on the similarity of their structure with the 400-411 fragment of the fibrinogen g chain. The peptide has the ability to inhibit the binding of fibrinogen with thrombin, avoiding thus platelet aggregation and the consequent production of fibrin; therefore thrombus formation does not take place (Carrasco and Guerra 2010).

The main whey protein reported as a source for obtaining antithrombotic peptides is lactoferrin. Thus, sequences such as RGDX have been identified from the hydrolysis of lactoferrin using pepsin (Qian et al. 1995). Recently it has been described that the peptide LRPVAAEIY derived from lactoferrin has a potential antithrombotic activity testing in an in vivo model of mice (Fan et al. 2019). In addition to the above, it has also been described that the mechanism of action of the LRPVAAEIY (LF-LR) peptide is based on prolonging the coagulation time of fibrinogen by affecting the conformation of thrombin binding to its active site (Xu et al. 2020). Additionally, the gastrointestinal digestion of α-LA derive on the peptide sequence RGDGLF with antithrombotic properties (Fiat et al. 1993).

5.12. Antiviral peptides

Viruses are one of the main causes of human disease, representing a serious global public health problem (Vilas Boas et al. 2019). The development of drugs and vaccines that lead to the neutralization of various viruses is a challenge due to the high mutation rates of these biosystems (Mahmoud 2016). The treatment of various viral diseases focuses on the use of antiviral drugs (Lou, Sun, and Rao 2014). However, in recent years there has been a bet on other elements such as peptides with antiviral properties, since they can act selectively and specifically, in addition to the fact that the probability of appearance of side effects and resistance to treatment is lower compared to the Routine antiviral drugs (Vilas Boas et al. 2019). Antiviral peptides have been studied in the context of diseases such as hepatitis, herpes, influenza, HIV, and recently SARS-CoV-2 (COVID-19)(Çakır et al. 2021).

The mechanism of action of these peptides is usually diverse (Figure 12), depending on the nature of the peptides and the type of virus studied. However, it has been described that there are at least three main mechanisms, which include inhibition of the binding and fusion of the virus with the cell membrane, breaking of the viral envelope and inhibition of virus replication (Skalickova et al. 2015). These molecules

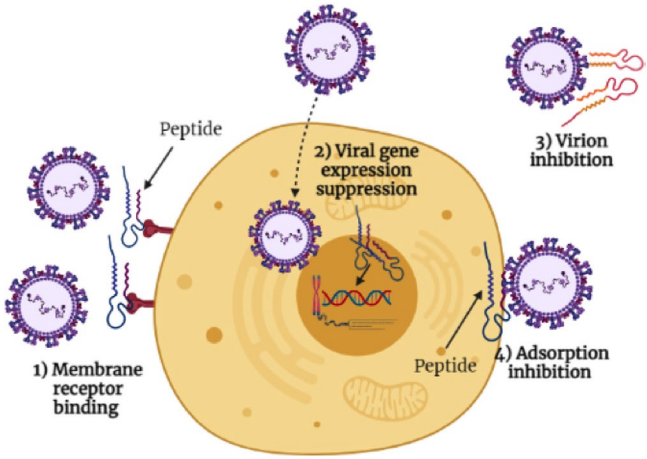

Figure 12. Mechanisms of action of antiviral peptides. This type of peptides act according to 1) Competing for binding to the membrane receptor, 2) Interfering in the replication of the genetic material of the virus and suppressing the expression of viral genes, 3) Directly inhibiting the viral particle by breaking its envelope, 4) inhibiting virus adsorption.

can be called virucidal when they act directly on the virion, breaking its envelope or compete for the binding receptor on the cell membrane, inhibiting virus adsorption (Galdiero et al. 2013). In the same way, this type of peptides can act in other stages of the viral cycle, which leads to the suppression of the expression of viral genes (Qureshi, Thakur, and Kumar 2013).

According to studies carried out with whey protein as a source of antiviral peptides, lactoferrin derivatives have been the most studied peptides, evaluating their effect on various viruses. The identification of the peptides KANEGLTWNSLKDK, TGSCAFDEFFSQSCAPGADPKSR, TNGESTADWAKN, GKNGKNCPDKFC, KSETKN, NDNTECLAKLGGRPTYEE, with broad anti-influenza activity and capable of inhibiting viral infection in a picomolar concentration range has been reported. The mechanism that these peptides employ is preventing both influenza virus hemagglutination and cellular infection (Scala et al. 2017). The antiviral effect of lactoferricin has also been reported on viruses such as human cytomegalovirus and herpes simplex, showing that these peptides inhibit the entry of the virus into fibroblasts in in vitro studies (Andersen et al. 2001; Andersen, Jenssen, and Gutteberg 2003).

Similarly, the effect of lactoferricin on human papillomavirus (HPV) has been reported, showing that bovine lactoferricin (FKC1RRWQWRMKKLGAPSITC1VRRAF) has an inhibitory effect on HPV infection and antiviral activity (Mistry et al. 2007). On the other hand, lactoferricin has also shown to exert a certain antiviral effect on the human immunodeficiency virus (HIV), by targeting the entry process of the virus into host cells (Berkhout et al. 2002). Regarding in vivo studies, more information is still required, however, it has been reported that lactoferricin block the virus in infected mouse models, indicating a potent in vivo antiviral effect (Shestakov et al. 2012).

On the other hand, the 2019 coronavirus disease (COVID-19) outbreak caused by the severe acute respiratory syndrome coronavirus (SARS-CoV-2) has made the search for new methods to manage the virus become a priority, since there is still no specific drug against this disease. However, the development of these methods is aimed at neutralizing the virus and inhibiting the membrane receptors of host cells (Çakır et al. 2021). In this context, peptides derived from β-LG (ALMPHIR, IPAVFK), with the potential to inactivate the virus and cell receptors have been identified from *in silico* analysis. In this study, peptides were obtained by simulated hydrolysis of goat milk serum, using trypsin. Subsequently, the effects of the peptides on SARS-CoV-2 and host cells were similarly identified by *in silico* analysis (Çakır et al. 2021). Despite the results obtained, which these peptides are pointed out as strong candidates in the treatment of SARS-CoV-2, future in vitro and in vivo studies are necessary to corroborate the effect of these peptides in biological models.

5.13. Anti-inflammatory peptides

The inflammatory response is the activation and production of inflammatory mediators such as interleukins (IL-6, IL-8), cytokines such as tumor necrosis factor-α (TNF-α), and growth factors such as interferon-γ (INF-γ) (Wu et al. 2014; La Manna et al. 2018; Guha and Majumder 2019). Pro-inflammatory mediators interact with various cellular components to amplify the inflammatory response (Guha and Majumder 2019). However, an alteration in the regulation of this process leads to an excessive production of inflammatory mediators, which has been associated with the progression of various diseases (Okin and Medzhitov 2012; La Manna et al. 2018). The treatment indicated in disorders that involve an inflammatory process includes the use of drugs with high degree of toxicity, low selectivity and specificity, as well as a large number of side effects (Lau and Dunn 2018).

In recent years, the use of peptides with anti-inflammatory activity has represented a promising alternative in the treatment of diseases with chronic and acute inflammatory processes, since the advantages of their use include their high potency and selectivity, low toxicity and low accumulation in tissues (La Manna et al. 2017; La Manna et al. 2018). Some of the mechanisms by which these peptides exert their effect are the inhibition of the inflammatory pathway NF-κB or the MAPK pathway, two of the main pathways involved in chronic inflammation (Guha and Majumder 2019). Likewise, these peptides can induce the reduction of adipokine levels and the inhibition of nitric oxide (NO) release, as well as stimulate the production of anti-inflammatory mediators such as IL-4 and IL-10. Another mechanism includes the binding to bacterial lipopolysaccharides (LPS), which are related to inflammatory processes by interacting with toll-like receptors (TLRs) (Chakrabarti and Wu 2015; Piccolomini et al. 2012; La Manna et al. 2018)). These mechanism are represented in Figure 13.

Some studies of anti-inflammatory peptides derived from whey have focused on the effect they have when binding to LPS. As previously described, LPS plays an important role in various inflammatory processes, since by binding to

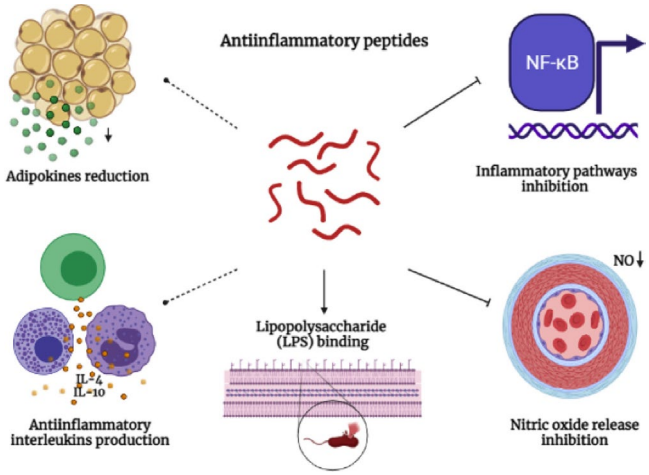

Figure 13. Main mechanisms of action of anti-inflammatory peptides. This type of peptides could act by inhibiting the main routes of inflammation such as the NF-κB pathway, in the same way they inhibit the release of nitric oxide whose accumulation leads to chronic inflammation. Other mechanisms include its participation in the reduction of adipokines and the production of anti-inflammatory interleukins, as well as the binding to LPS causing damage to the bacterial cell structure and inhibition of the inflammatory response that is triggered as a consequence of the interaction of LPS with TLR receptors.

toll-like cell receptors (TLRs) they trigger an inflammatory response by releasing pro-inflammatory mediators. However, when peptides bind to LPS, they in turn inhibit their binding to toll-like cell receptors (TLRs) and therefore the inflammatory response. In this sense, it has been observed that the lactoferrin-derived LFP 20 peptide could defend against the systemic inflammatory response activated by LPS in mice by modulating immune homeostasis (Zong et al. 2019).

Similarly, it has been reported that peptides released from in vitro whey digestion are capable of attenuating inflammatory responses in cystic fibrosis respiratory epithelial cells (CFTE29o) by suppressing IL-8 production induced by TNF-α and stimulated by LPS, which denotes an effect on the Toll-like receptor (TLR) pathway, because there is a reduced binding of LPS to this receptor (Iskandar et al. 2013). The DQWL peptide isolated from whey protein has been reported as an anti-inflammatory peptide by observing its ability to significantly inhibit the activation of nuclear factor-κB, IL-1β, cyclooxygenase-2, and expression of TNF-α mRNA in a LPS-induced inflammatory response model in mouse macrophages (Ma et al. 2016).

The anti-inflammatory properties of peptides derived from β-LG (GTWYSL, LSFNPTQL, MAASDISLL, AMAASDISLL, DTDYKKYLLF, IIAEKTKIPAVF, DIQKVAGTWYSL, ELKPTPEGDLEIL) have been evidenced in cells of macrophage stimulated by LPS, reporting a reduction of nitric oxide production and decrese in the synthesis of pro-inflammatory cytokines such as TNF-α and IL-1β (Bamdad et al. 2017). Additionally, it has been reported that diets based on whey protein, which bring about the release of peptides, suppress the release of pro-inflammatory cytokines and damage by oxidative stress in murine models of sepsis and cirrhosis (Jobara et al. 2014; Tsutsumi et al. 2015). However, clinical studies in humans are necessary to evaluate the effect of this type of peptides in various diseases associated with chronic inflammatory processes.

6. Bioavailability of whey peptides

One of the most important challenges in the field of bioactive peptide research is to ensure the bioavailability of these sequences and thus guarantee their effect on health. Gastrointestinal proteases have a direct effect on the hydrolysis of peptide sequences. However, to ensure the biological effect of bioactive peptides, it is necessary to verify the resistance to the action of intestinal enzymes and the absorption into the blood serum (Fitzgeral et al., 2004). In this sense, peptide bioavailability studies have been carried out due to concerns about the absorption capacity and susceptibility to the decomposition of these sequences into inactive fragments. This stability should ensure that the ingested peptides arrive intact at the desired cellular sites of action (Bhandari et al. 2020). It has been observed that some peptides that have shown in-vitro activity are not necessarily bioavailable. This is the case of a study carried out by Boelsma and Kloek (2010), who determined that two peptides derived from whey proteins showed antihypertensive activity in-vitro, but only one of them showed a potent inhibitory effect on ACE and reduction of blood pressure.

Efforts have been made to identify peptides derived from bioavailable whey proteins to be used in turn to increase the absorption of minerals essential for human health. This is how peptides have been obtained by in vitro digestion, finding that the WPI peptide achieved an increase of 45 to 100% in the bioavailability of iron (Caetano-Silva et al. 2018). Furthermore, Wang, Ai, et al. (2014) studied hydrolysates of β-lactoglobulin and α-lactalbumin complexed with iron and evaluated the absorption of the metal in in-vitro tests, retention of Fe in cells, and Fe transported by Caco-2 cells. The authors reported high absorption of iron from the synthesized complexes, verifying the ability of these complexes to resist the gastrointestinal tract. Likewise, Wang, He, et al. (2020) determined the degree of Zn absorption when bound to peptides derived from the hydrolysis of whey proteins with trypsin, pancreatin, and papain. Additionally, these authors indicated resistance of the peptide fractions complexed with Zn to the action of the enzymatic activity of the gastrointestinal tract. Fe and Zn complexation studies with whey-derived peptides are a field of research with the greatest expectations in the alternative treatment of anemia and mineral deficiency (Caetano-Silva, et al., 2021).

On the other hand, iron-peptide complexes have been considered a promising source to increase the bioavailability of this mineral, in this sense it has been observed that peptides isolate from protein hydrolyzed with pancreatin (TPEVDDE, VRTPEVDDE, DDDLTDDI, FKDLGEEH) increase iron solubility at pH 7.0, from 0% to almost 100%, which also remain stable under simulated gastric digestion conditions (50.8–89.4%). Similarly, it has been reported that the binding site of these peptides with iron corresponds to the carboxyl groups (Caetano-Silva et al. 2015).

Intestinal digestion has been shown to release a selection of high sulfur whey peptides. These peptides are transported through the Caco-2/HT-29 intestinal barrier, inhibiting the formation of free radicals in muscle and liver cells (Corrochano et al. 2019). Likewise, whey proteins are known to provide peptides with potential antioxidant activity. This is how fractions (f) of β-lactoglobulin resistant to the gastrointestinal tract have been identified: f(41–58), f(92–100), f(126–138), and f(149–154). In the case of α-lactalbumin, the identified fractions are 4: f(17–27), f(63–68), f(80–90), and f(97–102). Five from bovine serum albumin (BSA) have been found: f(11–18), f(107–114), f(219–224), f(489–495), and f(514–518). But, the most abundant fractions are those derived from lactoferrin: f(67–77), f(140–145), f(216–228), f(289–295), f(309–318), f(332 – 337), and f(592–594) (Giblin et al. 2019). Finally, peptides with opioid capacity and ACE inhibitors have been reported in a study carried out at the jejunum level. The majority (72%) of the whey-derived peptides detected were from β-lactoglobulin and were generally larger in size (nine to fifteen amino acids) compared to casein-derived peptides (six to nine amino acids) (Horner, Drummond, and Brennan 2016).

The mechanism of peptide absorption (Figure 14) is through expressed transporters in the intestine (PepT1), which transport small peptides while oligopeptides are passively transported through hydrophobic regions of the epithelial membrane (Darewicz et al., 2011). In both cases, the process is dependent on the specific properties of each peptide. In the case of PepT1, it prefers to transport apolar hydrophobic peptides with a neutral charge (Gleeson, Brayden, and Ryan 2018; Xu et al. 2017; Lin et al. 2017). In the case of the paracellular pathway (TJ), it tends to transport hydrophilic, neutral, or negatively charged peptides of small size and low molecular weight (Quirós et al. 2008; Xu et al., 2019). Large, hydrophobic, positively charged peptides tend to be transported by transcytosis (Regazzo et al. 2010; Komin et al. 2017; Ding et al., 2018) whereas highly lipid-soluble peptides are readily transported by passive transcellular diffusion. Xu et al., 2019). Peptides resistant to intestinal peptidases are transported into the bloodstream

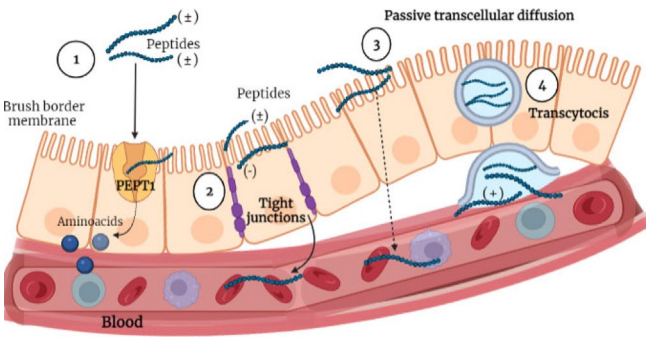

Figure 14. Mechanism of peptide absorption through the intestinal membrane. 1) Peptides of neutral charge and hydrophobic nature can be transported by the intestinal receptor PEP1, 2) The paracellular route (Tight junctions) tends to transport hydrophilic, neutral or negatively charged peptides. 3) Peptides with high lipid solubility are transported by passive transcellular diffusion. 4) Peptides of high molecular weight and positive charge will be transported by transcytosis through vesicles.

in micromolar concentrations, so when they remain intact they remain bioactive for several minutes or hours (Xu et al., 2019).

Knowles et al. (2019) have found 31 peptides from the hydrolysis of whey proteins with the ability to resist passage through the digestive tract. Of these peptides, the authors tested, through assays with C2C12 cell lines, the 6 with the highest bioavailability (TKIPA, NLPPL, PVPQ, VGIN, VAGT, and KVPQ). The capacity of these fractions in the protection of cells due to cell damage by free radicals was verified. In contrast, other studies have revealed that sequences derived from peptide fractionation due to intestinal peptidases, with a high inhibitory capacity of DPP-IV, have less activity than the precursor peptide from B-lactoglobulin (WPI) (Lacroix et al. 2017).

Thus, the challenge to transport bioactive peptides is hydrolysis in the epithelial cells of the intestine and plasma. Furthermore, the bioactivities of these peptides can be hampered by concentrations so small that they reach their target. Therefore, it is necessary to design peptides or search for those that have a high affinity for PepT1 or high bioavailability with the aim of designing functional foods. Furthermore, the molecular mechanisms by which bioactive peptides cross the intestinal epithelium and exert bioactivities in target tissues need to be investigated at the cellular and molecular level. Further studies in humans are required and effective and safe strategies should be explored to improve the bioavailability of whey-derived peptide fractions using a combination of chemical and molecular biological techniques.

7. Commercial products added with bioactive whey peptides

Due to the great diversity of biological functions that bioactive peptides from whey perform, research has focused on the development of safe commercial products, added with this type of elements. In this way, it is intended that the consumption of these products be an adjunct in the treatment of various diseases, since, as described, the mechanisms of action exerted by bioactive peptides impact on different signaling pathways related to progression of diseases.

Most of the research on foods added with bioactive peptides has focused on dairy products such as fermented milk, cheese and yogurt (Barrón-Ayala et al. 2020; Seppo et al. 2003). However, there is currently another range of commercial products added with bioactive peptides and recommended for their effect on various biological parameters. In this context, products such as BioZate® (Davisco, USA) have been pointed out, a hydrolyzed whey protein isolate with more than 80% pure protein and containing peptides derived from β-LG, recommended for lowering blood pressure (Korhonen 2009; Dullius, Goettert, and de Souza 2018); BioPureGMP ™ (Davisco, USA), a product that contains 86% GMP glycomacropeptide, recommended for the prevention of dental caries, correct blood clotting, antibacterial, antiviral and to produce a satiety effect reducing appetite (Chungchunlam et al. 2014; Dullius, Goettert, and de Souza 2018).

Similarly, commercial products include Vivinal® ALPHA (Borculo Domo Ingredients (BDI) the Netherlands), a product rich in α-LA that is derived in peptides with relaxing and sleep-inducing effects, Praventin ™ (DMV International, the Netherlands) , a hydrolyzate of whey proteins, mainly lactoferrin. Praventin ™ is marketed as a dietary supplement in capsule presentation and recommended as an adjuvant in the treatment of acne (Dullius, Goettert, and de Souza 2018). Other products derived from whey and marketed as dietary supplements added with bioactive peptides, include Dermylex ™ (Advitec, Inc., Canada) in tablet form, recommended for the treatment of psoriasis. Hilmar ™ 8390 (Hilmar Ingredients, USA) which is recommended for its antihypertensive/antidiabetic properties and NOP-47 ™, used as an anti-inflammatory (Glanbia Nutritionals, USA) (Korhonen and Pihlanto 2006; Korhonen 2009; Ballard et al. 2009; Dullius, Goettert, and de Souza 2018).

However, not only this type of product has been designed with the incorporation of bioactive whey peptides, in recent years it has opted for the development of meat and sausage products. As a first step in the development of functional meat products, whey protein hydrolysates have been added to the pork sausage formula, resulting in an increase in antihypertensive potential, which does not decrease despite storage conditions. The incorporation of whey hydrolysates in pork sausages could be an option to provide antihypertensive peptides through the consumption of foods of animal origin (Barrón-Ayala et al. 2020).

8. Perspectives on the use and production of bioactive peptides

The wide variety of biological functions that peptides from whey proteins could perform has increased the interest for their use as adjuvants in the treatment of various diseases. However, the limitations for its therapeutic use have focused on its short plasma half-life and low bioavailability due to the presence of digestive enzymes responsible for breaking the amide bonds of ingested proteins and that are also effective to cleave the same types of linkages in peptides (Lau and Dunn 2018). However, research on these molecules has been expanding in recent years since, as described above, bioactive peptides tend to have greater absorption, no side effects, and inexpensive production compared to drugs commonly used in the treatment of diseases.

Bioactive peptides have shown therapeutic promise in both preclinical in vitro studies with murine models of various diseases such as diabetes, cancer, hypertension, dyslipidemia, and in clinical studies with delimited populations. However, that kind of studies remains limited and the results are often inconsistent. In addition, it is necessary to describe the physiological mechanisms that these peptides use to exert their therapeutic effect analyzing the behavior of these molecules. In this case, parameters such as the administration and necessary dose for them to exert the desired effect, the period of consumption, the product formulation mechanisms, and the preventive and corrective effects must also be carefully evaluated, since their approval and consequent commercialization will depend on this.

Research in recent years has concentrated efforts and has focused on increasing the stability of peptides in the gastrointestinal tract through certain strategies that include modification or substitution of the terminal amino or carboxyl group, modification of the peptide structure, formulation of nanoparticles, increase in molecular mass, nanoencapsulation and use of liposomes for encapsulation of peptides (Yao et al. 2018; Rezaei et al. 2020). The stability of the peptides through the gastrointestinal tract is a relevant aspect that would undoubtedly allow the effects of the peptides to be more powerful (Lau and Dunn 2018).

Likewise, there is a growing interest in the production of bioactive peptides using novel and unconventional technologies. In this sense, the new trends in peptide production include high hydrostatic pressure, ultrasound, ohmic heating, pulsed electric fields, microwave-assisted extractions and subcritical water hydrolysis, a method which the water is at a temperature between 100° C and 374° C and a pressure lower than 22 MPa (Bamdad et al. 2017; Ulug, Jahandideh, and Wu 2021). However, some of these methods are still being combined with enzymatic hydrolysis. The new trend toward the use of novel technologies in the production of bioactive peptides continues to gain momentum because they are environmentally friendly, innovative and sustainable (Ulug, Jahandideh, and Wu 2021).

9. Conclusion

The use of bioactive peptides derived from food proteins in the development of functional foods is a current and highly relevant topic. However, within current and previous research there are challenges that must be met and opportunities that open up new fields of research. Among the most important challenges is the massive production, either biogenic or synthetic, of this type of compound. Production processes seem to be expensive and this is a challenge in process engineering. On the other hand, research has led to the wide range of bioactivities that these peptides exert, especially in the field of human health. It is well known that according to current data, diseases such as COVID-19 could be treated with antiviral, antihypertensive or antithrombotic peptides. Thus, this enhances an opportunity in the investigation of the biochemical mechanisms of action of this type of peptide and its implication in its potential use in the treatment of this type of disease. Considering this review, it is very likely that a residue from the food industry such as whey is an important source of peptides with various bioactivities, which could be obtained through any hydrolytic method.

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Capítulo IV.

Whey Fermentation by Lacticaseibacillus rhamnosus GG and Streptococcus thermophilus SY-102: Proteolytic Profile and ACE-Inhibitory Activity

Introducción

La hidrólisis de proteínas de suero de leche a partir de fermentación microbiana ha resultado ser un método con gran potencial para la obtención de péptidos con alta actividad antihipertensiva. Los resultados de este capítulo han sido sometidos a la revista Foods y se analiza en primer lugar el patrón de crecimiento microbiano a partir de fermentaciones de suero de leche con *S. thermophilus* SY-102 y *L. rhamnosus* GG cultivados de manera independiente y en cocultivo. Se hace énfasis en la protocooperación de bacterias ácido lácticas como una interacción que permite la obtención de una mayor concentración y diversidad de fracciones peptídicas con considerable actividad biológica. De igual manera, se presenta una evaluación de los cambios fisicoquímicos en el medio durante la fermentación mediante la determinación de la acidificación del medio de cultivo (Cambios de pH) y se establece una relación entre estos resultados y el patrón de crecimiento microbiano realizando una comparación entre los cultivos independientes y el cocultivo. Por otro lado, se determina la producción de fracciones peptídicas de bajo peso molecular mediante la construcción del perfil proteolítico a partir de los cultivos independientes y cocultivo. Para este efecto, se emplean tres técnicas principales: 1) Determinación de grupos amino libres mediante la técnica TNBS para conocer la concentración de péptidos producidos; 2) Electroforesis en gel de poliacrilamida (SDS-PAGE) para determinar tanto el origen del fraccionamiento como las fracciones peptídicas y 3) Análisis SEC-HPLC utilizando una columna de exclusión por tamaño, con el objetivo de determinar el peso molecular de los péptidos separados, especialmente los de bajo peso molecular. Los resultados del perfil proteolítico son relacionados con los obtenidos en una última fase donde se evalúa el potencial antihipertensivo de las fracciones peptídicas obtenidas a partir de la fermentación de los cultivos independientes y el cocultivo, determinando la actividad inhibitoria de la enzima convertidora de angiotensina (ECA).

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ACE-Inhibitory Activity of Whey Proteins Fractions Derived of Fermentation by *Lacticaseibacillus rhamnosus* GG and *Streptococcus thermophilus* SY-102

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Abstract: Many studies have reported the benefits of probiotic microorganisms and the production of angiotensin-converting enzyme (ACE) inhibitors. Determining the proteolytic and ACE inhibition capacities during whey fermentation was the goal of the study. *Lacticaseibacillus rhamnosus* GG, *Streptococcus thermophilus* SY-102, and both bacteria together were initially inoculated into whey, reaching an initial concentration of 10^8 CFU per milliliter in each fermentation system. Through the use of TNBS, SDS-PAGE, and SEC-HPLC methods, the proteolytic profile was examined. An in vitro investigation was performed to test the ACE inhibition capacity. With *S. thermophilus*, the logarithmic phase of microbial development was shorter than with *L. rhamnosus* (6 and 12 h, respectively). The logarithmic phase in the co-culture fermentation, however, was extended to 24 h. There were no significant differences in pH between the fermentations. However, the co-culture had a greater concentration of protein hydrolysis (453 ± 0.06 μg/mL), as indicated by the amount of free amino groups. Similarly, this fermentation produced more low molecular weight peptides. The higher inhibition activity, which increased at the conclusion of the fermentation with the co-culture and reached 53.42%, was influenced by the higher peptide synthesis. These findings highlighted the significance of creating useful co-culture products.

Keywords: probiotic; whey fermentation; ACE inhibition; antihypertensive peptides; bioactive peptides

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1. Introduction

After casein coagulation, whey, a byproduct of the dairy industry, is removed during the cheesemaking procedure [1]. It is estimated that more than 160 million tonnes of whey are wasted annually in the word. Only a small portion, however, is processed to produce other by-products [2]. Because it poses a severe environmental problem, the elimination of this waste has become an ongoing challenge for the industry [2,3].

According to numerous studies, whey proteins are frequently a potential source of bioactive peptides, which have beneficial effects on human health [4,5]. However, these sequences need to be released following protein hydrolysis to have a biological impact [6]. Due to its effectiveness and profitability, microbial fermentation is one of the traditional processes used to produce peptides [7–10]. In this way, peptide sequences with various biological activities have been obtained by using a variety of microbial strains [11]. As

a result, these microorganisms' proteolytic mechanism encourage the release of peptide sequences [12].

Often peptides exert their effects through a variety of different mechanisms, one of which is their antihypertensive activity linked to the inhibition of angiotensin-converting enzyme (ACE) [13,14]. Angiotensin I is converted into angiotensin II by this enzyme which is a potent vasoconstrictor and the key regulator of blood pressure [15]. The description of sequences that inhibit ACE activity and hence produce an antihypertensive impact has been made possible by research into bioactive peptides [16]. Since whey was fermented with *L. rhamnosus* GG and *S. thermophilus* SY-102 in monoculture and with both bacteria for co-culture fermentation, the study's goal was to examine the synthesis of low molecular weight peptides and the inhibitory potential of ACE.

2. Materials and Methods

2.1. Culture Preparation

Bacteria were collected from Iztapalapa campus of the Universidad Autónoma Metropolitana's Food Biotechnology Laboratory. They were cultured in MRS broth and incubated at 42 °C for 24 h. Then, 9 mL of powdered whey solution (10% (*w*/*v*) obtained from Dairy Gold Co-Operative Society Ltd., Cork, Ireland) that had previously been heat-treated at 90 °C during 10 min were added to one milliliter of the inoculated broth. At 42 °C, it was incubated for 24 h. An amount of 100 mL of a 10% (*w*/*v*) powdered whey solution that had been pasteurized (90 °C for 10 min) received 1 mL of this solution. In the same way, each microorganism underwent independent conditioning. The solution was cooled down after 24 h of incubation at 42 °C. The solution obtained was the starter. A viable count was carried out to ascertain the initial bacterial concentration before the fermentations.

2.2. Fermentation

Three systems—inoculation with *Lacticaseibacillus rhamnosus* GG, inoculation with *Streptococcus thermophilus* SY-102, and finally inoculation with both microorganisms—were set up for fermentation. Each microorganism was inoculated into each system to reach initially about 1×10^8 CFU per milliliter of bacteria calculated from the starter culture. In the co-culture case, the initial count of bacteria was inoculated equally (to reach 1×10^4 CFU per milliliter of each microorganism). Whey powder containing 10% (*w*/*v*) was used to make the fermentation solution. Prior to inoculation, this solution was heat-treated at 90 °C during 10 min. A total of 48 h were spent incubating the samples at 42 °C. Aliquots were obtained every three hours and seeded on MRS agar plates using the microdrop technique to test the vitality of the microorganisms [17]. The samples were diluted from 1×10^{-1} to $1 \times 10^{-8,}$ incubating for 48 h at 42 °C. Using an Eppendorf centrifuge, samples were spun at 24,600× *g* at 4 °C for 10 min to separate both coagulated proteins and biomass. The centrifuged samples' supernatants were kept in a freezer at −4 °C for further examination. Each sample was examined three times.

2.3. Proteolytic Profile Analysis

By means of three distinct experiments, the proteolytic profile was characterized. Peptides concentration was determined using the technique of TNBS, and the peptides released during fermentation were separated using Tris-Tricine polyacrylamide gel electrophoresis (SDS-Tris-Tricine-PAGE). Finally, an HPLC analysis utilizing a size exclusion column was performed to detect the presence of peptides, particularly low molecular weight peptides.

2.3.1. Analysis of Free Amino Groups

Using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method, the free amino groups produced by whey fermentation were identified. In foil-wrapped test tubes, 125 µL of material was combined with 1 mL of 0.21 M phosphate buffer, pH 8.2. The tubes were kept at 50 °C in the dark for one hour. After 60 min, the reaction was finished by adding 2 mL of 0.1 N hydrochloric acid. The samples were then compared to the control in a

spectrophotometer at a wavelength of 340 nm. Deionized water generated the control, applying a concentration curve of glycine (0.05 to 0.25 mg/mL).

2.3.2. Peptide Separation by Tris-Tricine-SDS-PAGE

The approach suggested by Schägger and Von Jagow [18] was applied while taking González-Olivares et al. [19]'s adjustments into consideration. Using the Bradford method, the samples' protein concentration was normalized at 150 ppm, and 20 µL of the sample were charged. Using a gel (16.5% T) made from a 30% T solution (19:1 acrylamide:bisacrylamide ratio and 5% crosslinker, Bio-Rad, Hercules, CA, USA), electrophoresis was carried out. Gel-Doc Software (Bio-Rad, Hercules, CA, USA) was used to evaluate the gels after they had been stained using Coomassie Blue G-250 (Bio-Rad, Hercules, CA, USA).

2.3.3. Separation of Peptides by SEC-HPLC

A SRT-SEC-150 SEPAX Technologies, Inc (USA) column (300 mm × 7.6 mm × 5 µm) with an exclusion range of 0. 5–150 kDa was used to separate the peptides produced during whey fermentation. A 60-min isocratic separation using a mobile phase of 0.1 M KH_2PO_4/K_2HPO_4 buffer with a pH of 6.8 was performed at 0.5 mL min^{-1} of flow rate. The target analytes were detected at a wavelength of 220 nm. The analysis was conducted in an HPLC from Perkin Elmer Series 200 connected to a manual injection system (20 µL) and a UV-vis detection system (190–380 nm).

2.4. ACE Inhibitory Activity

With some modifications from Cushman et al. [20], the inhibitory effect of ACE-I (EC 3.4.15.1; Sigma-Aldrich, Saint Louis, USA) was assessed spectrophotometrically. Hippuric-histidyl-leucine (HHL; Sigma-Aldrich, Saint Louis, MO, USA) was dissolved at a concentration of 5 mM in sodium borate buffer (0.1 M, pH 8.3 with 0.3 M sodium chloride). Then, 100 µL of the substrate and 40 µL of the sample (AbsM) were combined with 10 µL of ACE (EC 3.4,15.1, 5.1U/mg; Sigma-Aldrich). At 37 °C, the reaction was run for 75 min. Hippuric acid was produced and quantified at 220 nm in a Power Wave XS UV-Biotek spectrometer (KC Junior software, Kansas, MO, USA) after extraction with ethyl acetate. Then it was resuspended using deionized water and measured. The same treatment was performed for a 100% of enzymatic activity sample (AbsC) prepared with 40 µL of borate buffer instead and a 0% of enzymatic activity sample (AbsB) prepared with 50 µL of borate buffer and 100 µL of the substrate (HHL). ACE inhibitory activity was calculated using the formula based on the absorbance obtained from the measurements.

2.5. Statistical Analysis

$$\text{ACE inhibition\%} = [(\text{AbsC} - \text{AbsM})/(\text{AbsC} - \text{AbsB})] \times 100$$

Results were analyzed by one-way ANOVA ($p = 0.05$) and a post hoc Tukey test by using the NCSS statistical software (NCSS 2007, v.0, Kaysville, UT, USA, 2007).

3. Results and Discussion

3.1. Growth during Fermentation

The fermentation results revealed their growth differed when microorganisms were grown in monoculture versus co-culture. It was observed that *L. rhamnosus* GG had better growth in monoculture. In contrast, the growth of *S. thermophilus* SY-102 did not reflect this behavior since it showed a shorter lag phase, and the logarithmic phase was reached during the first 6 h of fermentation. In contrast, *L. rhamnosus* GG showed a longer lag phase, reaching its logarithmic phase within 12 h of fermentation (Figure 1). These results are similar to those observed in other studies, where, in general, *Lactobacillus* species grow much better in monoculture compared to *S. thermophilus* SY-102 [21].

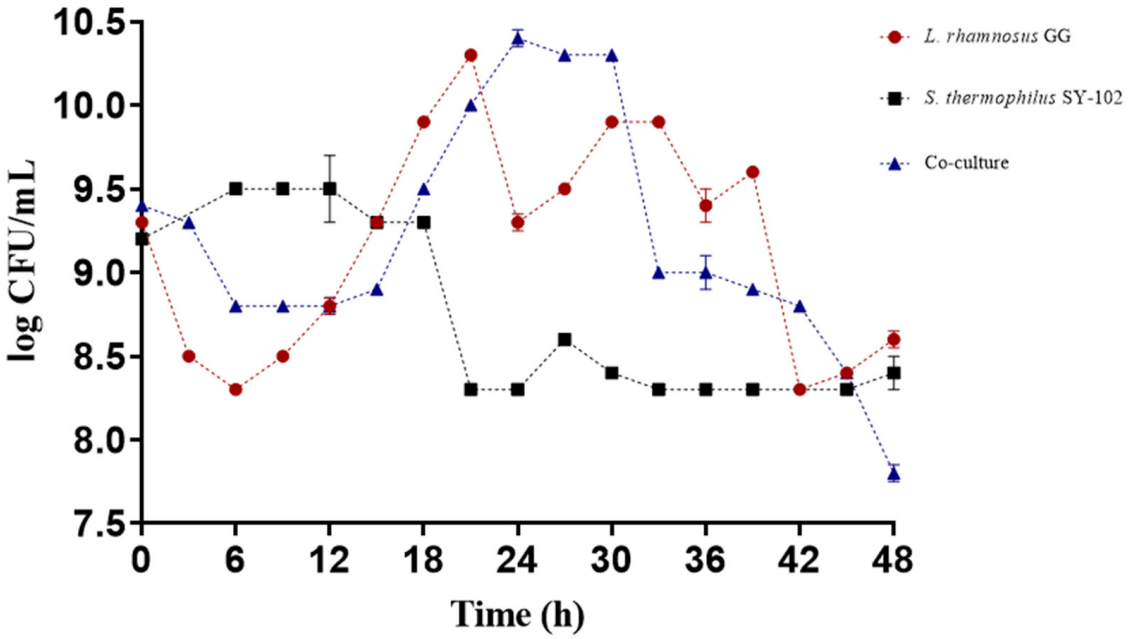

Figure 1. Growth during whey fermentation by *Lacticaseibacillus rhamnosus* GG ● and *Streptococcus thermophilus* SY-102 in monoculture ■ and co-culture ▲.

There is a synergistic effect in the co-culture, since it is known that in this type of culture the microorganisms develop a proto-cooperation relationship, releasing and exchanging metabolites necessary for optimal growth [19]. Specifically, *L. rhamnosus* GG expresses its extracellular proteinase PrtR to utilize whey proteins and provide a nitrogen source for *S. thermophilus* ssp. and itself. *S. thermophilus* ssp., on the other hand, could provide *Lactobacillus* species with formic acid, folic acid, and carbon dioxide [22,23]. In addition, it synthesizes some amino acids and expresses its cell-wall-associated proteinase (PrtS) [24].

The synergistic effect between *L. rhamnosus* GG and *S. thermophilus* ssp. impacts the growth of microorganisms and the acidification of the medium [23]. This mutualistic and proto-cooperative relationship allows for the environmental conditions necessary for the growth of both strains. However, there is competition for carbon and nitrogen sources [12].

3.2. pH Changes of the Medium during the Time of Fermentation

The pH progressively decreased during the time of fermentation, reaching a pH of 4.5 in the case of *L. rhamnosus* GG and 4.8 for *S. thermophilus* SY-102 in monoculture. In the co-culture, the pH of the medium reached a value of 4.8 (Figure 2).

It is known that the pH decreases due to the production of lactic acid as a product of the conversion of lactose and the generation of biomass. Additionally, the differences between each system are attributed to each microorganism's ability to use the carbon source. In some studies, it has been observed that the co-culture of these microorganisms leads to much faster medium acidification, reaching a considerably more acidic pH than a monoculture [25]. However, in this case, the pH did not decrease as drastically in co-culture. The reason for this behavior may be due to the amino groups that are released during the fermentation process, the generation of biomass itself, and the relationship of competition for the carbon source between the microorganisms.

Additionally, it is well known that the combination of *S. thermophilus* ssp. with some species of probiotics, such as *L. delbrueckii* subsp. *bulgaricus*, inhibits the rate of lactic acid production compared to monoculture [26]. Similarly, each of the microorganisms has a specific proteolytic system. This proteolytic capacity strongly influences the auxotrophies of each strain [27]. It is known that Lactobacillus species tend to adapt better to more acidic media since they modulate their intracellular pH [28]. In addition, *L. rhamnosus* GG is frequently used as a probiotic microorganism as it has a great capacity for resistance to

acidic pH, colonizing, and adhering to the epithelial cells of the gastrointestinal tract [29]. On the contrary, *S. thermophilus* SY-102, despite being a lactic acid bacterium, grows much better during the first stages of fermentation, when the pH of the medium is not yet so acidic. Therefore, towards the last hours of fermentation, the culture would be dominated by *L. rhamnosus* GG.

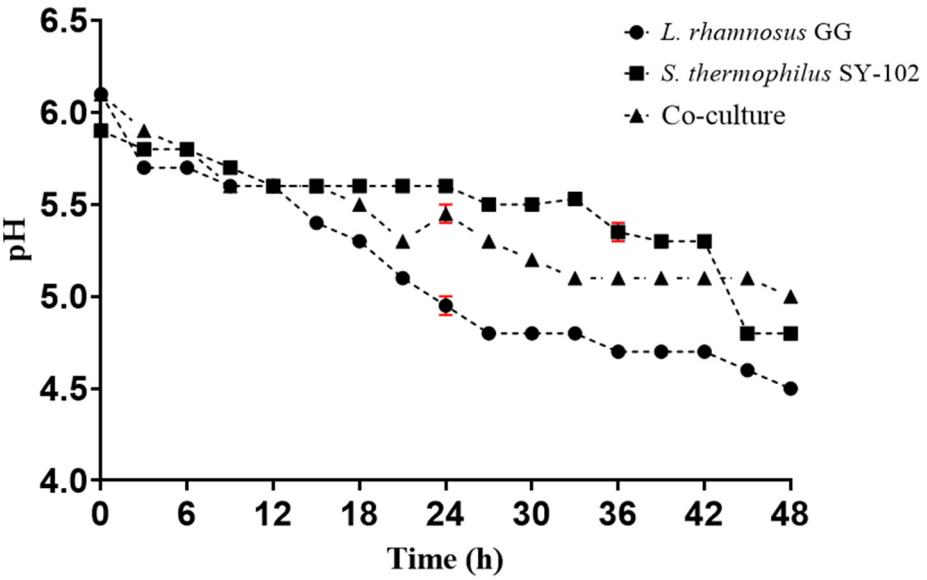

Figure 2. pH changes during whey fermentation by *Lacticaseibacillus rhamnosus* GG ● and *Streptococcus thermophilus* SY-102 in monoculture ■ and co-culture ▲.

3.3. Proteolysis

3.3.1. Determination of Free Amino Groups by Technique of TNBS

The release of amino groups during fermentation, was different for both monoculture and co-culture systems (Table 1). The behavior observed in the monoculture of both strains was using the peptides available in the whey (time 0). Subsequently, starting from this concentration of amino groups, a decrease was observed towards 12 h of fermentation (from 367.28 ± 0.02 to 209.18 ± 0.02 µg/mL for *L. rhamnosus* GG and from 423.31 ± 0.13 to 167.52 ± 0.05 µg/mL for *S. thermophilus* SY-102). At the end of fermentation, this concentration increases again, due to the proteolytic capacity of the strains to release amino groups into the medium.

Table 1. Concentration of free amino groups from the fermentation of whey with *L. rhamnosus* GG, *S. thermophilus* SY-102, and both microorganism in co-culture. The mean ± standard deviation (SD) expressed the results of three replicates.

Time of Fermentation (h)	Free Amino Groups µg/mL		
	***L. rhamnosus* GG**	***S. thermophilus* SY-102**	***L. rhamnosus* GG + *S. thermophilus* SY-102**
0	366.91 ± 0.02 ^{aAB}	463.43 ± 0.13 ^{aA}	226.59 ± 0.15 ^{aAB}
9	277.67 ± 0.01 ^{abA}	264.63 ± 0.02 ^{bA}	285.28 ± 0.13 ^{aA}
12	208.97 ± 0.02 ^{abcA}	167.34 ± 0.05 ^{bcA}	363.32 ± 0.02 ^{aB}
15	239.85 ± 0.02 ^{abcA}	255.06 ± 0.00 ^{bA}	253.76 ± 0.20 ^{aA}
18	277.23 ± 0.07 ^{abA}	350.60 ± 0.03 ^{abdA}	268.97 ± 0.17 ^{aA}
21	293.54 ± 0.05 ^{abA}	323.54 ± 0.04 ^{abAC}	452.67 ± 0.06 ^{aB}

Lowercase letters compare means between times of the same fermentation system. Uppercase letters compare means between fermentation systems with the same time of fermentation. According to Tukey's test, the same letter did not present a significant difference ($p < 0.05$).

The concentration of free amino groups depends on each microorganism's proteolytic system. It has been observed that *S. thermophilus* SY-102 in monoculture has a higher proteolytic activity in the early stages of fermentation, which decreases in the final stages [25]. However, once this microorganism satisfies its needs for amino acids such as methionine and glutamine, it releases those unnecessary amino acids and peptides into the medium [21]. In this sense, an accumulation in the medium of peptides with pyroglutamic acid and cysteine has been observed, which are not preferred by *S. thermophilus* ssp. [21]. On the contrary, *L. rhamnosus* GG requires peptides that contain cysteine, serine, arginine, proline, and glutamine [29].

In the co-culture case, a greater accumulation of amino groups could be observed towards the end of the fermentation (21 h) compared to the monocultures. The concentrations in the co-culture had a slight variation during the fermentation. However, in the last period of the logarithmic phase, they reached a maximum of 453.13 $\pm$ 0.06 µg/mL. This same behavior has been reported in other studies, where it has been observed that the concentrations of amino groups are higher in co-culture than in monocultures using the same strains [25].

Since there is a synergistic effect, the proto-cooperation interaction between the strains determines a higher concentration of amino groups [25]. Furthermore, the proteolytic systems of each strain play an important role in the initial proteolysis. In these systems, the *Lactobacillus* species have a higher initial proteolytic activity than the *Streptococcus* species [23]. This activity is determined by the protease associated with its cell wall, which initiates the hydrolysis of proteins to satisfy its auxotrophies and release peptides and free amino acids that could be used by *S. thermophilus* ssp. [30].

In contrast, the initial hydrolysis rate in *S. thermophilus* ssp. is usually very low since most hydrolysis is carried out by its system of intracellular peptidases and aminopeptidases [31]. Thus, it has been documented that the growth of *S. thermophilus* ssp. depends on the other microorganism's metabolism when it is in co-culture. The proteolytic activity continues until the end of fermentation when the amino acids necessary for its growth have been obtained [27].

3.3.2. Peptide Separation by Tris-Tricine-SDS-PAGE

Peptide separation analysis by electrophoresis showed an accumulation of peptides smaller than 10 kDa in the three systems studied (Figure 3). However, in the case of monocultures, the accumulation was higher in the culture with *L. rhamnosus* GG compared to *S. thermophilus* SY-102. This is associated with the proteolytic system of each species. In the case of *L. rhamnosus* GG, protein hydrolysis is mostly carried out through the proteinase associated with its cell wall (PrtR). Subsequently, hydrolysis continues through its system of intracellular peptidases and aminopeptidases. By contrast, at the beginning of fermentation *S. thermophilus* SY-102 shows higher activity due to its proteinase (PrtS). This activity decreases as the fermentation time increases since hydrolysis is mostly carried out by its intracellular peptidase system [25,27].

As has been pointed out, the proto-cooperation relationship between the microorganisms has a synergistic effect, which allows a greater accumulation of low molecular weight peptides in the co-culture system. In this case, the PrtR of *L. rhamnosus* GG begins protein hydrolysis, releasing amino acids necessary for *S. thermophilus* SY-102. However, the latter has specific auxotrophies, so they are satisfied through their proteolytic system. Peptides that are not needed by both species are excreted through their cell wall and released into the medium. Therefore, the peptides accumulated in the medium will be found in higher concentrations in the co-culture system.

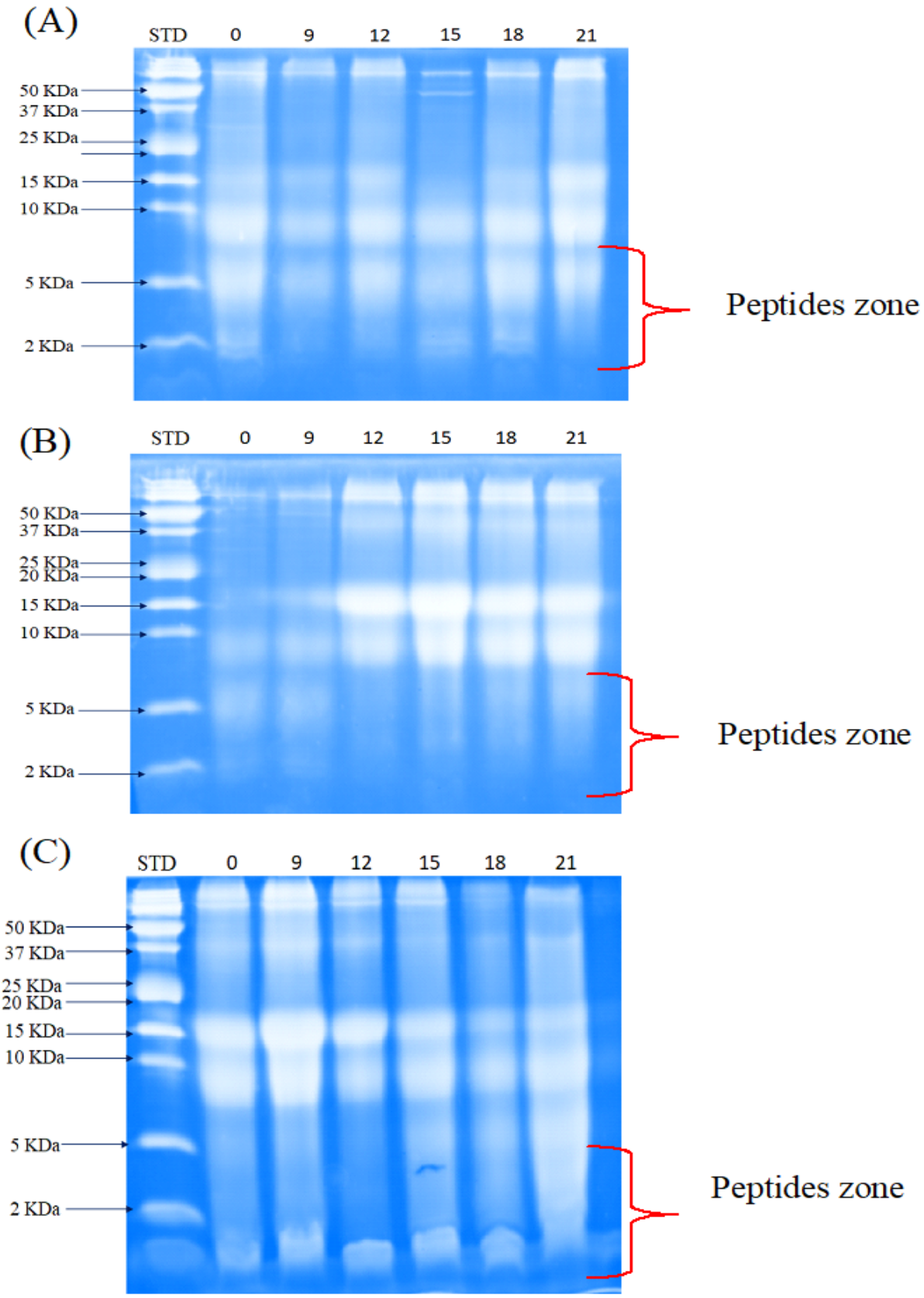

Figure 3. Peptide separation by SDS-PAGE in whey fermented by *L. rhamnosus* GG (**A**), *S. thermophilus* SY-102 (**B**), and co-culture (**C**). (STD) peptides standard. Fermentation time (hours) 0–21.

Increasing the concentration of small peptides is interesting in this study because it has been reported that peptide size and biological activity are related. Various studies have reported peptides less than 10 kDa with antihypertensive and antidiabetic activity [7,25,32].

3.3.3. Separation of Peptides by SEC-HPLC

All fractions of peptides (beginning, middle, and end of the fermentation) were analyzed. In all three systems, a decrease in the concentration of the main globular proteins (α-lactalbumin (α-LA) and β-lactoglobulin (β-LG)) was observed. Similarly, the accumulation of small peptide fractions towards the end of fermentation (21 h) was determined. In the case of *L. rhamnosus* GG (Figure 4A), two protein fractions of 13.63 kDa and 9.43 kDa were observed at the beginning of fermentation, corresponding to whey proteins (α-LA and β-LG) with a retention time of 22 and 25 min, respectively. These fractions remained constant during the fermentation process. Towards the end of the process (21 h), the formation of a new peptide fraction of 6.9 kDa with a retention time of 27 min was detected.

In the fermentation with *S. thermophilus* SY-102 there was a decrease in the concentration of the fractions corresponding to whey proteins (Figure 4B). Likewise, two protein fractions were observed at the beginning of fermentation. The first fraction observed was 32.08 kDa, which could be the product of the association of proteins that form polymers under certain conditions of temperature and pH. The second fraction, with a molecular weight of 13.63 kDa, disappears around 21 h of fermentation. At the end of the fermentation time (21 h), two fractions of 12.15 kDa and 7.5 kDa were obtained, with a retention time of 23 and 27 min, respectively. Even though this monoculture produced low molecular weight peptide fractions, fermentation with *L. rhamnosus* GG increased their accumulation. This coincides with the results obtained in the separation by electrophoresis.

The differences in the proteolytic systems of each strain play an important role in the accumulation of peptide fractions. Intracellular peptidases reduce peptide fractions within the cell, discarding those with amino acids that are not needed [33]. However, the initial proteolysis also gives rise to free fractions in the medium. In addition, each of the strains has different auxotrophies, so the preference for these fractions will differ according to the amino acids necessary for each strain. In this sense, most of the proteolytic process of *L. rhamnosus* GG is carried out by the proteinase linked to its cell wall, PrtR. In contrast, for *S. thermophilus* ssp. this process is carried out mainly by intracellular peptidases [34].

In the case of the co-culture (Figure 4C), it was observed that the greatest accumulation of small peptide fractions appeared at the end of the fermentation process (21 h). At the beginning of the fermentation, two fractions were observed, one of nine and the other of 5.11 kDa. However, these fractions could be found naturally in the medium since the thermal process of whey treatment causes it to be partially hydrolyzed. On the other hand, a change in the concentration of the peptide fractions can be observed as the fermentation time increases. In this way, at time 7 (21 h), the accumulation of peptide fractions of 9.9 kDa was observed, with a retention time of 24.7 min.

These results coincide with evidence reported by Sebastián-Nicolás et al. [25], which they observed a greater accumulation of peptide fractions from milk fermented with *L. rhamnosus* GG and *S. thermophilus* SY-102 in co-culture. Similarly, in the co-culture fermentation, the proteolytic systems of each strain will act specifically to obtain the amino acids necessary for their metabolism. This microorganism has a series of intracellular peptidases, among which *pep*S stands out. This endopeptidase breaks the oligopeptides and releases them into the medium fractions that frequently contain hydrophobic residues and aromatic amino acids [27]. The latter has been related to an inhibition of the ACE, which leads to an antihypertensive effect [23].

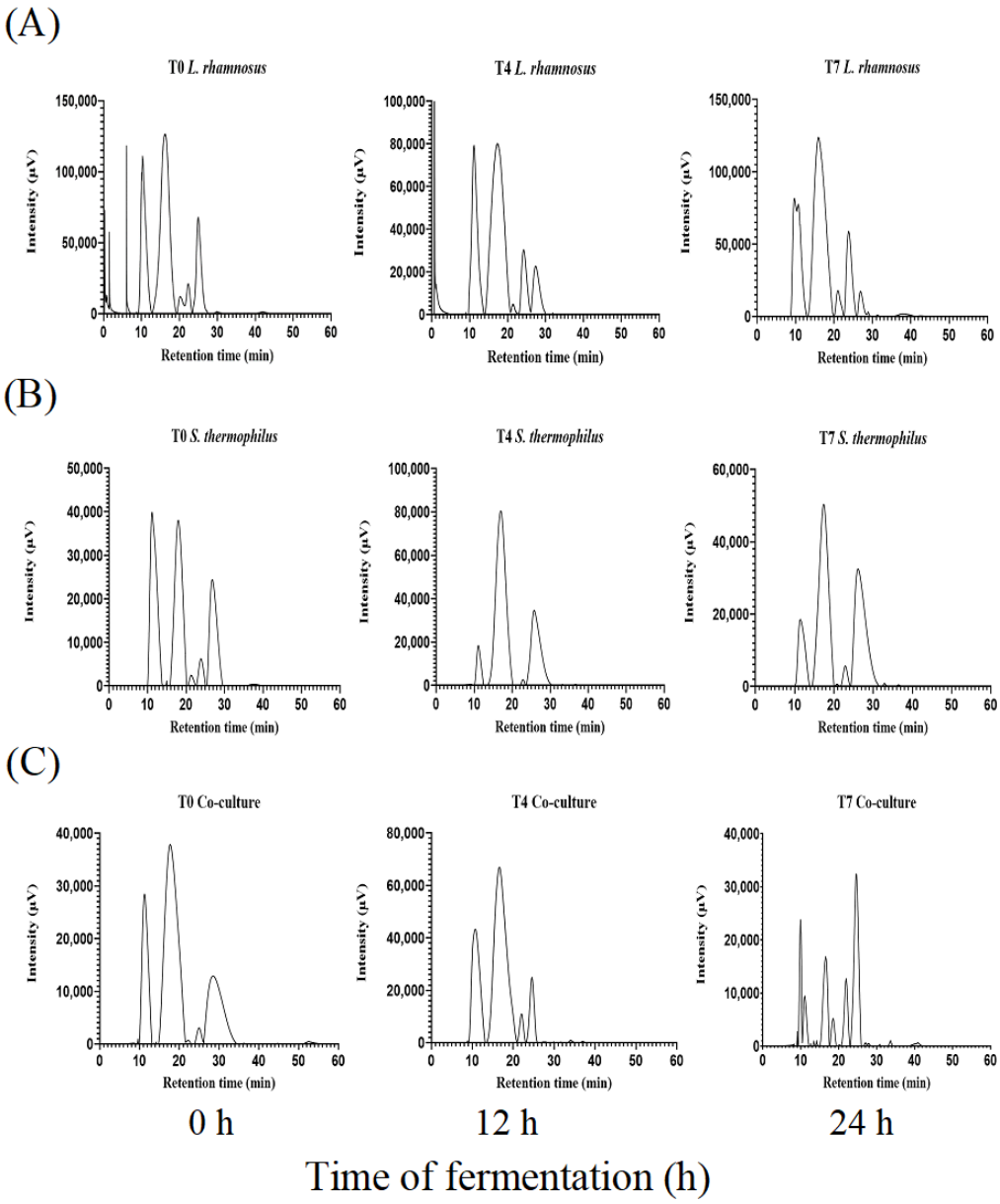

Figure 4. Peptide separation by SEC-HPLC of whey fermented by *L. rhamnosus* GG (**A**), *S. thermophilus* SY-102 (**B**), and co-culture (**C**). Beginning (0 h), middle (12 h), and end (21 h) of fermentation.

3.4. Determination of ACE Inhibition

According to the results obtained from the ACE inhibitory activity, a gradual increase in ACE inhibition was observed for the case of *L. rhamnosus* GG in monoculture. At the initial stage of fermentation (Figure 5), 25.81% of inhibition percentage was observed. Subsequently, this percentage increased to 28.23% at 12 h of fermentation, reaching an inhibition percentage of 42.34% at the end of 21 h. It is known that this microorganism needs aromatic amino acids at the beginning of fermentation to cover its metabolic needs [35]. This could explain the small increase in ACE inhibition towards the end of the first 12 h fermentation. However, once it has enough aromatic amino acids, it releases unnecessary ones into the medium, increasing the percentage of inhibition towards 21 h of fermentation.

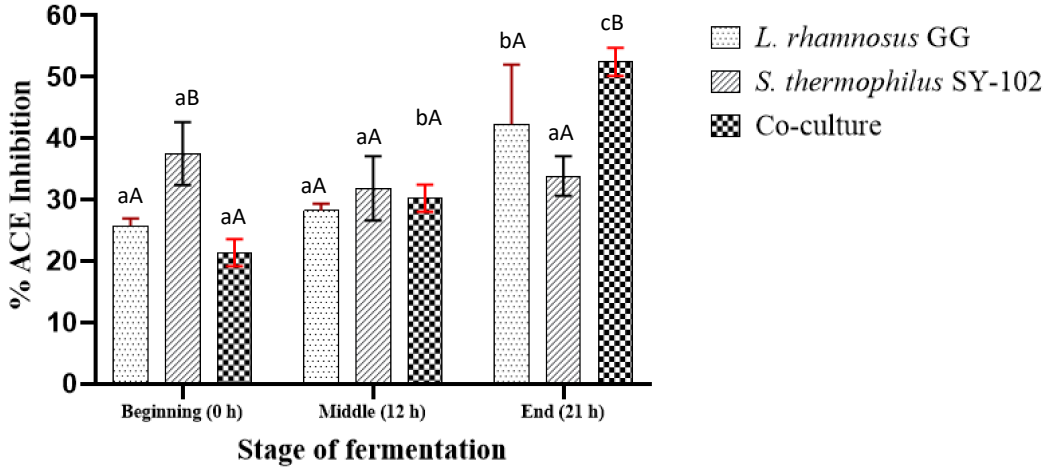

Figure 5. Inhibitory activity of ACE by peptidic fractions of whey fermented by *L. rhamnosus* GG, *S. thermophilus* SY-102, and co-culture. Lowercase letters compare means between times of the same fermentation system. Uppercase letters compare means between fermentation systems with the same time of fermentation.

In the case of *S. thermophilus* SY-102 in monoculture, a decrease in the percentage of ACE inhibition from 37.5% to 31.85% was observed. Subsequently, this percentage increases to 33.87% after 21 h of fermentation, reflecting a slight increase in inhibitory activity. It is known that *S. thermophilus* ssp. does not prefer aromatic amino acids, and peptide accumulation is imminent. However, the fact that it did not reach a higher inhibition percentage could be related to the results observed in its growth, which was not superior to *L. rhamnosus* GG. According to the electrophoresis results, its proteolytic capacity to produce low molecular weight peptides was lower than that of *L. rhamnosus* GG monoculture and co-culture. This could be related to a lower ACE inhibitory activity, as the accumulation of peptides containing aromatic amino acids was not encouraged during fermentation.

An opposite effect could be observed from the co-culture. The percentage of ACE inhibition gradually increased from 21.37% to 52.42% by 21 h of fermentation. This result is consistent with those observed in the proteolytic profile of the co-culture, in which there was a higher concentration of free amino groups and peptide fractions of low molecular weight. It has been reported that symbiotic growth in co-culture systems leads to a wide variety of bioactive peptides and a higher concentration of them. The differences in the proteolytic systems of the strains favor the accumulation of peptides in the medium and increase the antihypertensive activity [25,36].

Various studies have pointed out the close relationship between the structure and bioactivity of peptides [37,38]. In the case of ACE inhibition, it has been pointed out that inhibitors contain hydrophobic amino acids in their structure [23]. Specifically, antihypertensive activity is strongly associated with the sequence present at the C-terminal site [5]. Thus, peptides with amino acid residues such as tryptophan, proline, phenylalanine, and isoleucine tend to be more potent in ACE inhibition by interacting with its active site [39].

Using microorganisms in co-culture to ferment certain protein matrices, such as whey, is an opportunity to produce antihypertensive peptides. This increases the possibility of generating fermented dairy products with functional potential, especially due to the advantages of using microorganisms.

4. Conclusions

The combination of both lactic acid bacteria studied in whey fermentation increases the growth of both microorganisms through a proto-cooperative relationship. Similarly, a co-culture system favors the strains' proteolytic activity, increasing the concentration of low molecular weight peptide fractions. Small peptides and, most likely, aromatic amino acids

in the structure could explain the antihypertensive activity observed in this study. Likewise, this activity increases when using a co-culture system compared to a monoculture.

Microbial fermentations involving the proto-cooperation of lactic acid bacteria represent a promising source of peptides with health-promoting properties. The sequences derived from the fractionation of whey proteins represent an important field of study to evaluate their role in various pathologies and diseases.

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Capítulo V.

Proteolytic system differences of lactic acid bacteria affect the release of encrypted DPP-IV inhibitory peptides in whey proteins

Los resultados de este capítulo han sido sometidos a la revista *Dairy*. En este apartado se concluye que la hidrólisis de proteínas de suero de leche por fermentación microbiana, conduce a la producción y liberación de péptidos con amplia actividad biológica, incluyendo actividad antidiabética por inhibición de la enzima DPP-IV. Para esta evaluación se establecieron tres sistemas de fermentación de suero de leche, el primero inoculando *S. thermophilus* SY-102, el segundo inoculado con *L. rhamnosus* GG y el tercero inoculando ambos microorganismos para establecer un cocultivo. Posteriormente, se realizó la determinación de crecimiento microbiano utilizando parámetros cinéticos que permitieran evaluar las diferencias metabólicas de las bacterias ácido lácticas. De igual manera, se establece la relación entre el crecimiento de las cepas y las concentraciones de ácido láctico producido durante la fermentación. Así mismo, se presenta la caracterización del perfil proteolítico a través de la determinación de grupos amino libres mediante la técnica TNBS para conocer la concentración de péptidos producidos y SDS-PAGE para separar los péptidos liberados durante la fermentación y obtener el perfil de pesos moleculares de los mismos. Posteriormente, se presenta la evaluación de la actividad inhibitoria de la DPP-IV y el potencial antidiabético de las fracciones peptídicas obtenidas a partir de la hidrólisis de proteínas de suero de leche. Así mismo, se discute y hace énfasis en las diferencias entre el sistema proteolítico de cada cepa y cómo es que estas tienen una fuerte influencia sobre la producción y liberación de péptidos con actividad inhibitoria de la DPP-IV. Por último, se enfatiza el uso de proteínas de suero de leche como un área de oportunidad para la obtención de péptidos con actividad antidiabética que permitan coadyuvar en el tratamiento de T2DM.

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Differences in the proteolytic system of lactic acid bacteria affect the release of DPP-IV inhibitory peptides from whey proteins

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Abstract: Peptide fractions from whey proteins may have biological functions, such as antidiabetic, which inhibit the DPP-IV enzyme. Lactic acid bacteria could release them. In this work, the antidiabetic capacity of peptides from whey proteins after hydrolysis by *Lactobacillus rhamnosus* GG and *Streptococcus thermophilus* SY-102 was analyzed. A whey solution of 10% was fermented with these lactic bacteria in monoculture and coculture, analyzing kinetic parameters and the proteolytic profile, using the TNBS technique for free amino groups determination and Tris-tricine polyacrylamide gel electrophoresis. An in-vitro inhibition assay of the DPP-IV enzyme was used. The kinetic parameters showed a higher duplication rate in the monoculture with *L. rhamnosus* than in the coculture, which was related to lactic acid production. Co-culture does not have the highest production of free amino groups and peptides. Still, peptides with lower molecular weight (>2kDa) were found and showed a high DPP-IV inhibitory capacity that was maintained from the middle of the fermentation to the end (55.4 %). In comparison, the monoculture of *L. rhamnosus* increased from 0 to 63.3 %. This demonstrates that the proteolytic capacity and the proteolytic system of each lactic acid bacteria determine the structure of released peptides.

Keywords: probiotic, bioactive peptides, proteolytic system, DPP-IV inhibition, whey

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1. Introduction

In recent years, inhibition of the enzyme Dipeptidyl Peptidase IV (DPP-IV) has been pointed out as a new alternative in the treatment of Diabetes Mellitus II (DM2)[1]. This enzyme is found on the surface of various cells and in the bloodstream [2]. DPP-IV hydrolyzes incretins important in regulating postprandial blood glucose levels [3]. These incretins are glucose-dependent insulinotropic peptides (GIP) and glucagon-like polypeptides (GLP-1). The production of both incretins takes place in the small intestine [4], and subsequently, they act on the pancreatic cells, stimulating insulin production in the β cells. In contrast, α cells inhibited glucagon secretion [5]. However, these incretins lose their bioactive properties when DPP-IV degrades them before they act at the pancreatic

cell level. Inhibition of the enzyme DPP-IV turns out to be a target point in the regulation of glucose concentrations. [6]. In this sense, using bioactive peptides as adjuvants in treating DM2 has been the subject of interest [7]. The mechanism of action that gives these peptides their antidiabetic effect is centered on inhibiting the DPP-IV enzyme. This inhibition results in the increase in the half-life of GIP and GLP-1 with their subsequent activity in pancreatic cells [5]. Recently, obtaining DPP-IV inhibitory peptides from various food matrices has been explored. Thus, whey proteins have been pointed out as a potential source for producing peptides with considerable antidiabetic activity [8]. It has been reported that the enzymatic hydrolysis of α-LA and β-LG leads to obtaining peptides (LDQWLCEKL, IPAVF, IPAVFK) with high DPP-IV inhibitory activity [7,9]. Similarly, using lactic acid bacteria to hydrolyze milk whey proteins has been considered a profitable alternative in obtaining peptides with antihypertensive, antioxidant, and anticholesterolemic activity, among others [8]. However, the antidiabetic activity of peptides obtained by microbial fermentation has been little studied. Therefore, the objective of this article was to determine the inhibitory capacity of the enzyme Dipeptidyl Peptidase IV (DPP-IV) of peptides generated by fermentation of milk whey with *Streptococcus thermophilus* SY-102, *Lacticaseibacillus rhamnosus* GG and both bacteria in co-culture.

2. Materials and Methods

2.1 Culture preparation

The *Streptococcus thermophilus* SY-102 and *Lacticaseibacillus rhamnosus* GG were obtained from the Food Biotechnology Laboratory of the Universidad Autónoma Metropolitana campus Iztapalapa. The microorganisms were previously conditioned in MRS broth and were incubated for 24 h at 42 °C. Subsequently, one milliliter of the inoculated broth was added to 9 mL of powdered whey solution (10% (w/v) Dairy Gold) previously pasteurized at 90 °C for 10 min. It was incubated for 24 h at 42 °C. Then, 1 mL was added to an Erlenmeyer flask with 100 mL of a 10% (w/v) powdered whey solution previously pasteurized (90 °C for 10 minutes). Each microorganism was conditioned in the same way independently. After incubating for 24 h at 42 °C, the solution was refrigerated. This solution was used as a starter culture, performing a viable count before each inoculation to determine the inoculum concentration in each fermentation.

2.2 Fermentation

Three systems were prepared to carry out the fermentations. The first system was inoculated with *Lacticaseibacillus rhamnosus* GG, the second with *Streptococcus thermophilus* SY-102, and the third inoculated with both microorganisms. The initial concentration of bacteria was approximately 1×10^8 CFU for each of the systems. The fermentation solution was prepared with 10% (w/v) powdered whey previously pasteurized (90 °C for 10 min). The solutions were incubated at 42 °C, sampling every 3 hours. Titratable acidity and microbial viability were determined. Subsequently, the samples were centrifuged at 24,600 × g for 10 min at 4 °C (Eppendorf) to eliminate biomass and high molecular weight proteins. The supernatants of the centrifuged samples were stored at -4 °C for later analysis. All samples were analyzed in triplicate.

2.3 Kinetic parameters

Kinetic parameters were calculated to evaluate the metabolic differences of lactic acid bacteria. The growth rate (μ) was calculated according to equation 1 (Eq. 1). Equations 2 and 3 were used to determine the generation time (g) and the growth constant (K). The initial (N0) and final (Nx) concentration of biomass corresponded to the time interval selected in the logarithmic phase, which were t0 and tx, respectively.

$$\ln(Nx) - \ln(N0) = \mu(tx - t0) \quad (1)$$

$$g = \ln(2)/\mu \quad (2)$$

$$K = 1/g \quad (3)$$

2.4 Proteolytic profile analysis

The characterization of the proteolytic profile was carried out through two different studies. First, free amino groups were determined using the TNBS technique to analyze the concentration of peptides produced. Subsequently, Tris-Tricine polyacrylamide gel electrophoresis (SDS-Tris-Tricine-PAGE) was performed to separate the peptides released during fermentation and obtain their molecular weight profile.

2.4.1 Free amino groups analysis

The free amino groups derived from whey fermentation were determined using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) technique. A 125 µL volume of sample was mixed with 1 mL of 0.21 M phosphate buffer solution, pH 8.2, in amber test tubes. Subsequently, 1 mL of 0.10% TNBS (Sigma Aldrich) was added to 0.21 M phosphate buffer, pH 8.2, followed by vortexing. The tubes were incubated for 1 h at 50 °C in the dark. The reaction was stopped after 60 min, adding 2 mL of 0.1 N hydrochloric acid. Finally, the samples were read in a spectrophotometer at a wavelength of 340 nm against the control. The control was prepared with deionized water, and a glycine concentration curve (0.05 to 0.25 mg/mL) was used.

2.4.2 Tris-Tricine Polyacrylamide Electrophoresis (Tris-Tricine-SDS-PAGE)

To perform polyacrylamide gel electrophoresis, the method proposed by Schägger and Von Jagow [10] was used, considering the modifications proposed by González-Olivares et al [11]. The protein concentration of the samples was standardized to 150 ppm by analyzing with the Bradford method. Electrophoresis was performed on a 16.5% T gel from a 30% T solution (19:1 acrylamide:bisacrylamide ratio and 5% crosslinker (Bio-Rad, Hercules, CA-USA). Gels were stained with Comassie Blue G-250 (Bio-Rad, Hercules, CA, USA) and analyzed with Gel-Doc Software (BioRad, Hercules, CA, USA).

2.5 DPP-IV Inhibitory Activity

The inhibitory effect of DPP-IV (D4943-1VL; Sigma-Aldrich, St. Louis, USA) was evaluated spectrophotometrically according to the technique of Nongonierma et al.[12] with some modifications. The substrate (Gly-Pro-p-nitroanilide; Sigma-Aldrich, St. Louis, USA) was dissolved in Tris-HCl buffer (pH 8) at a substrate concentration of 1.6 mM. Subsequently, 100 µL of the sample (AbsM) were added to 100 µL of substrate and incubated at 37 °C for 10 min. Following this incubation, 200 µL of DPP-IV (1U/mL; Sigma-Aldrich, St. Louis, USA) were added. The reaction was conducted for 60 min at 37 °C, finishing by adding 400 µL of 0.1 M potassium carbonate. The same treatment was followed for preparing the positive control (AbsB), adding 100 µL of Tris-HCl buffer instead of the sample volume. The negative control (AbsC) was prepared by adding 100 µL of the sample, 300 µL of Tris-HCl buffer (pH 8), and 400 µL of 0.1 M potassium carbonate. Finally, all the samples were read at 405 nm in a spectrometer. Power Wave XS UV-Biotek (KC Junior software, Kansas, MO, USA). The DPP-IV inhibitory activity was calculated using the following formula based on the absorbance of each measurement:

$$\% \text{ DPP-IV inhibition} = ((AbsB - (AbsM - AbsC)) / AbsB) \times 100$$

2.6 Statistic analysis

Results were analyzed by one-way ANOVA (p = 0.05) and by Tukey's test using the NCSS statistical software (NCSS 2007, v.0, Kaysville, UT, USA, 2007).

3. Results and Discussion

3.1 Microbial Growth and Lactic Acid Production

The results obtained from the fermentation demonstrated that the growth of the microorganisms between the independent cultures and the coculture was different. In the monoculture of L. rhamnosus, greater growth was observed, reaching its logarithmic phase close to 12 h of fermentation. On the contrary, in the monoculture of S. thermophilus a shorter lac phase was observed, reaching its logarithmic phase around 6 h of fermentation (Table 1). In this sense, other authors have reported that lactobacilli species in monocultures tend to present higher growth than streptococcus species [13]. A correlation was observed between growth and the concentration of lactic acid produced. Thus, lactic acid concentrations were higher in the culture of *L. rhamnosus* GG compared to the culture of *S. thermophilus* SY-102.

Table 1. Microbial growth, lactic acid concentration and kinetic parameters during fermentation with *L. rhamnosus* GG, *S. thermophilus* SY-102 and co-culture.

	***L. rhamnosus* GG**		***S. thermophilus* SY-102**		**Coculture**	
Time (h)	log CFU	lactic acid (g/L)	log CFU	lactic acid (g/L)	log CFU	lactic acid (g/L)
0	9.3 ± 0.00	1.44 ± 0.00	9.2 ± 0.00	1.25 ± 0.00	9.4 ± 0.00	1.62 ± 0.00
3	8.5 ± 0.00	1.62 ± 0.00	8.6 ± 0.00	1.44 ± 0.00	9.3 ± 0.00	1.98 ± 0.00
6	8.3 ± 0.00	1.62 ± 0.00	9.5 ± 0.00	1.44 ± 0.00	8.8 ± 0.00	2.16 ± 0.00
9	8.5 ± 0.00	2.16 ± 0.00	9.5 ± 0.00	1.44 ± 0.00	8.8 ± 0.00	2.34 ± 0.00
12	8.8 ± 0.10	2.07 ± 0.09	9.5 ± 0.20	1.62 ± 0.00	8.8 ± 0.10	2.52 ± 0.00
15	9.3 ± 0.00	2.34 ± 0.00	9.3 ± 0.00	1.62 ± 0.00	8.9 ± 0.00	2.52 ± 0.00
18	9.9 ± 0.00	2.70 ± 0.00	9.3 ± 0.00	1.62 ± 0.00	9.5 ± 0.00	3.00 ± 0.00
21	10.3± 0.00	3.20 ± 0.00	8.3 ± 0.00	1.98 ± 0.00	10.0± 0.00	3.78 ± 0.00
24	9.3 ± 0.10	3.74 ± 0.04	8.3 ± 0.00	1.98 ± 0.00	10.4± 0.10	2.61 ± 0.09
27	9.5 ± 0.00	4.10 ± 0.00	8.6 ± 0.00	2.34 ± 0.00	10.3± 0.00	2.52 ± 0.00
30	9.9 ± 0.00	4.50 ± 0.00	8.4 ± 0.00	2.34 ± 0.00	10.3± 0.00	2.80 ± 0.00
33	9.9 ± 0.00	4.50 ± 0.00	8.3 ± 0.00	2.52 ± 0.00	9.0 ± 0.00	2.80 ± 0.00
36	9.4 ± 0.10	5.20 ± 0.00	8.3 ± 0.00	2.61 ± 0.09	9.0 ± 0.10	3.24 ± 0.00
kinetic parameters						
Growth rate (μ)	0.322 h^{-1}		0.123 h^{-1}		0.233 h^{-1}	
Generation time (g)	2.14		5.61		2.97	
Growth rate constant (k)	0.467 g/h		0.178 g/h		0.336 g/h	

The metabolism rate of lactic acid bacteria is determined by the lactic acid production capacity, especially for homofermentative microorganisms, as is the case of those tested in this study. Thus, as observed in Table 1, the highest production of lactic acid was found in the monoculture with *L. rhamnosus* GG (5.20±0.0), followed by co-culture (3.24±0.0) and finally, the system of monoculture with *S. thermophilus* SY-102 (2.61±0.09). These results are closely related to the growth or duplication rate (◎), which followed the same trend as lactic acid production and generation time (g). Likewise, the growth constant was perfectly related to each parameter studied. These kinetic parameters are intended to somehow explain the metabolic behavior of microorganisms according to the environment in which they are found. Castañeda-Ovando et al. [14] related the growth of lactic bacteria in monoculture and co-culture to determine the fundamental differences in the process. Thus, the two main metabolic identification parameters are proteolytic capacity and lactic

acid production. In this case, it has been observed that coculture systems are more effective in duplication, generation time, and lactic acid production.

3.2 Free amino groups analysis by TNBS

The concentration of free amino groups after fermentation differed for the three systems evaluated. In the case of cultures of *L. rhamnosus* GG and *S. thermophilus* SY-102 independently, a decrease in the concentration of free amino groups was observed after 12 hours of fermentation, which started from 367.28 ± 0.02 to 209.18 ± 0.02 µg/mL for *L. rhamnosus* GG and from 423.31 ± 0.13 to 167.52 ± 005 µg/mL for *S. thermophilus* SY-102. Subsequently, around 21 h of fermentation, the concentration of amino groups increases again due to the proteolytic activity of each strain. The concentration of amino groups for *L. rhamnosus* GG was 293.83 ± 0.05, while for *S. thermophilus* SY-102 it was 323.87 ± 0.04 at the end of fermentation (Figure 1).

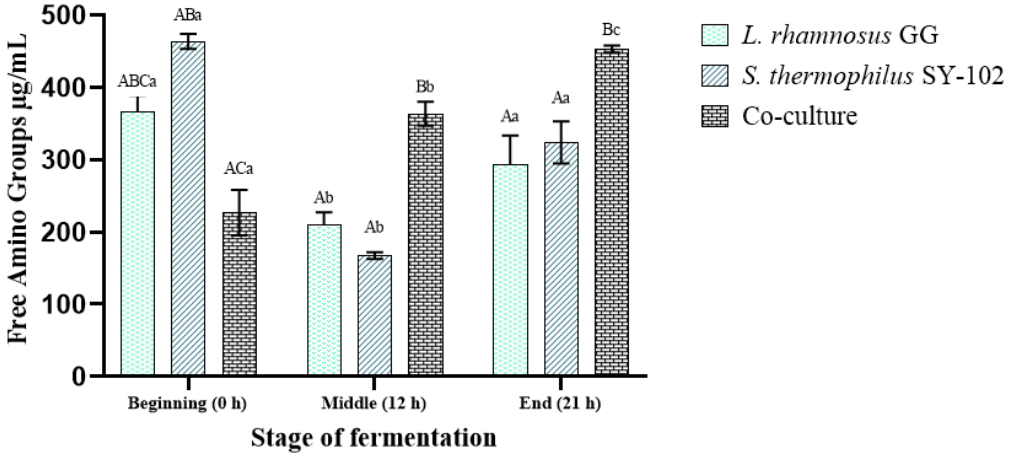

Figure 1. Production of amino groups released during the fermentation of whey with *L. rhamnosus* GG, *S. thermophilus* SY-102 and both microorganisms in co-culture.
The results are the average of three determinations ± the standard deviation. a-b Lower case letters indicate comparison of means between times of the same fermentation system. A-C Upper case letters mean comparison of means between fermentation systems with the same fermentation time. Samples with the same letter did not present significant difference using Tukey's test ($P < 0.05$).

It has been reported that in lactic acid bacteria, proteolytic activity and protein hydrolysis patterns vary considerably between strains [13,14]. These differences could be due to multiple factors, among which the following have been pointed out: 1) Changes and mutations in the genes involved in the expression of the proteinase linked to the cell wall and 2) Conditions in which the enzymatic activity is carried out [16]. Therefore, the concentration of amino groups and the amino acids released during fermentation will depend on the auxotrophies of each microorganism. In this sense, various studies have indicated that *S. thermophilus* strains require the incorporation of amino acids such as methionine and glutamine, thus satisfying their auxotrophies while releasing peptides and amino acids to the medium not metabolically necessary [16, 17].

Similarly, the activity of the proteolytic system of this microorganism varies in the different stages of fermentation. Thus, it has been observed that during the early stages of fermentation, the proteolytic activity of *S. thermophilus* species is higher and decreases as the fermentation time elapses. Therefore, it can satisfy its glutamine and methionine requirements at the beginning of fermentation. Similarly, it favors the accumulation in the medium of amino acids such as cysteine and glutamic acid, which are not necessary [13]. On the other hand, *L. rhamnosus* GG presents auxotrophies that differ from those of *S.*

thermophilus. An example of this is the use of amino acids such as cysteine. For *L. rhamnosus* GG, cysteine is an essential amino acid for survival, while for *S. thermophilus* species it does not represent a metabolic requirement [13]. In addition, *L. rhamnosus* GG typically utilizes a wide range of amino acids, including serine, glutamine, proline, and arginine [19]. However, the behavior and proteolytic activity of these strains in monoculture vary when they are in co-culture [18].

In the case of coculture fermentation, the final concentration of released amino groups was higher (453.13±0.06 μg/mL) compared to independent cultures. This trend has been previously reported from milk fermentation using the same strains [18]. Some studies have indicated that in a coculture medium, there is a protocooperation relationship between both microorganisms [20]. This interaction entails a synergistic effect that favors the increase in the concentrations of free amino groups and obtaining a greater diversity of peptides [19,20]. Thus, the initial proteolysis is related to the increase in amino groups at the end of fermentation and to the type of released peptides. In addition, the biological activity presented in a released peptide is always correlated with the composition of the peptide chain [18]. In this type of system, it has been observed that the initial proteolytic activity is higher in lactobacilli species [20]. The protease associated with its cell wall begins with the hydrolysis of proteins to satisfy its auxotrophies [21]. Subsequently, it excretes amino acids that are not necessary for it into the medium, which are used by *S. thermophilus* species[22].

In the case of *S. thermophilus* species, the initial proteolytic activity is relatively low when found in coculture. This microorganism will initially use the amino acids excreted by *L. rhamnosus* GG, so the protease associated with its cell wall will not be as active as that associated with L. rhamnosus. Ultimately, protein hydrolysis will continue via its system of intracellular peptidases and aminopeptidases to cover its specific auxotrophies [18].

3.3 Analysis of profile peptides by Tris-Tricine-SDS-PAGE

The gels obtained from electrophoresis were analyzed using the Gel-Doc Software (BioRad, Hercules, CA, USA). This way, the electropherograms corresponding to the monocultures and co-cultures were obtained (Figure 2). Low molecular weight peptides (<10 kDa) were obtained for the three fermentation systems. However, due to the specific proteolytic activity of each of the microorganisms, a different profile was presented in each case [23]. Thus, in monoculture fermentations (Figure 2-A, 2-B), a greater accumulation of peptides was observed in the *L. rhamnosus* GG system towards the end of the fermentation (21 h).

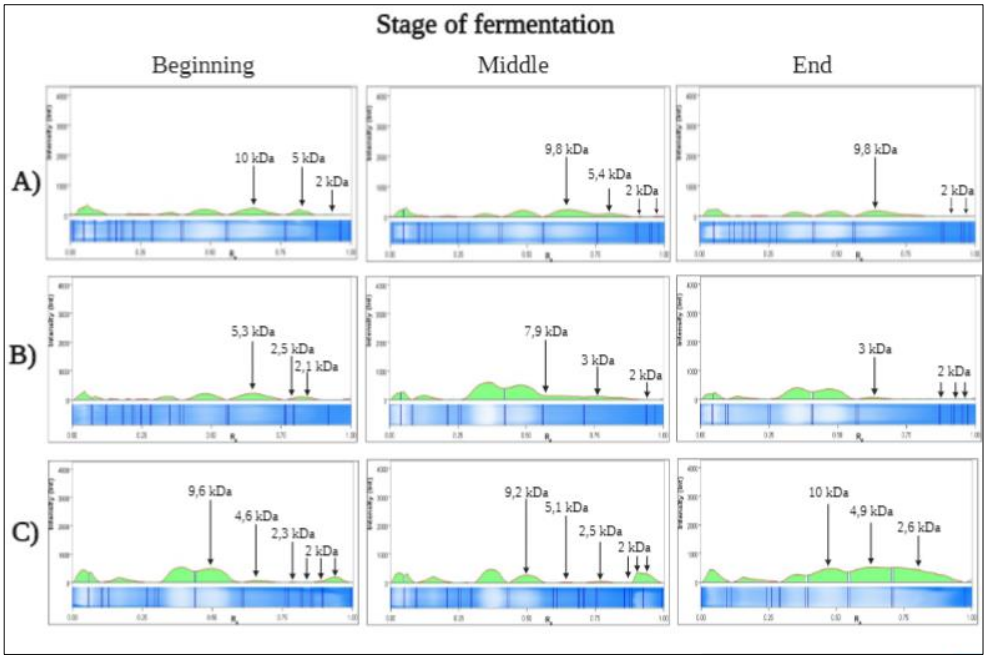

Figure 2. Separation of peptides by SDS-PAGE from whey fermented by *L. rhamnosus* GG (A), *S. thermophilus* SY-102 (B) and coculture (C). Start (0h), middle (12h), end (21h) of fermentation.

These results coincide with what has been observed in other studies that indicate a higher rate of hydrolysis from lactobacilli cultures compared to streptococcus cultures [16, 17]. The production of peptides between 4 and 36 amino acids by L. rhamnosus is carried out from the first fractionation of the proteins by the proteinase linked to its cell wall (PrtR) [21]. Peptides generated by the action of PrtR are incorporated through a system of oligopeptide (Opp), dipeptide, and tripeptide (DtpT, Dpp) transporters [23]. Subsequently, these peptides are broken down into amino acids by a series of intracellular peptidases and aminopeptidases. Once their auxotrophies are satisfied, the unused peptides and amino acids will be excreted into the medium.

Contrary to *L. rhamnosus* GG where protein hydrolysis is carried out mostly by PrtR, in *S. thermophilus* species hydrolysis is carried out mainly by the action of the intracellular peptidase complex (Pep O, Pep X, Pep S, Pep N, Pep C) [17]. For this reason, both the hydrolysis rate and the excretion of short-chain peptides are lower. Compared to lactobacilli species, streptococci have higher amino acid requirements, so initial proteolytic activity should be higher [17]. Therefore, at the beginning of fermentation, the proteinase associated with the cell wall of S. thermophilus (PrtS) has a high activity rate to supply the necessary amino acids from the proteins available in the medium [22]. However, the activity of this proteinase decreases as the fermentation time elapses.

The concentrations of low molecular weight peptides obtained from the coculture were higher than those observed in monocultures (Figure 2-C). These results are related to the results obtained in the analysis of free amino groups. Coculture systems tend to show a certain synergy that increases the concentration, size, and composition of the peptides obtained [20]. This represents an important factor related to the biological activity that peptides can exert since the bioactivity of certain peptides is related to both the weight and the amino acid composition of the peptide chain [24]. Due to the particularities of the proteolytic system of each strain, it is expected that these have a direct impact on the production of bioactive peptides, which is also closely linked to the structure of the proteolytic system and its mode of action (Figure 3).

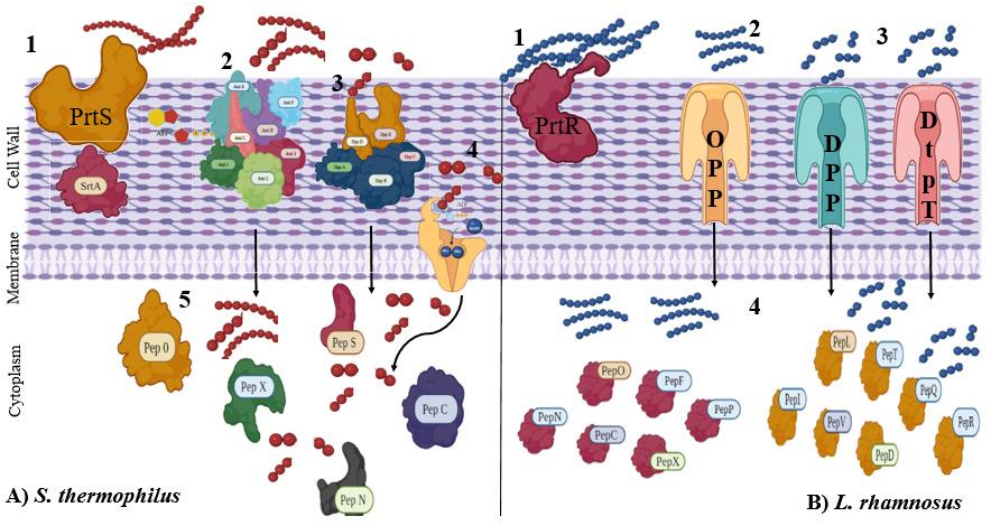

Fig 3. Proteolytic system of *S. thermophilus* speies and *L. rhamnosus* GG. The proteinase linked to their cell wall, their oligopeptide, dipeptide, and tripeptide transport system, as well as the intracellular peptidase complex, are the main differences in the proteolytic systems of these microorganisms. A) Protein hydrolysis in *S. thermophilus* begins with proteinase linked to its cell wall (Prts) (1) generating long-chain oligopeptides, dipeptides, and tripeptides. Subsequently, the oligopeptides are transported into the cell through a system of operons (2) (Ami 1, Ami 2, Ami 3, Ami C, Ami D, Ami E). The dipeptides and tripeptides will be integrated into the cell using a Dpp (3) system (Dpp A, Dpp B, Dpp C, Dpp D, Dpp E) or an NADH (4) proton pump system with ATP expenditure. Once the peptides are in the cytoplasm, most of the hydrolysis will be carried out by their system of endopeptidases and intracellular aminopeptidases (5) (Pep O, Pep X, Pep S, Pep N, Pep C). B) In *L. rhamnosus*, proteolysis is carried out mainly through the action of its proteinase (1) (PrtR). The oligopeptides generated will be transported through the (2) OPP system, while the dipeptides and tripeptides will be transported through the (3) DPP and DtPT systems. Finally, the peptides will be degraded from the intracellular endopeptidases and amino peptidases complex (4) (PepN, PepO, PepC, PepF, PepX, PepP, PepL, PepT, PepI, PepV, PepD, PepQ, PepR). Once the strains have covered their auxotrophies, they will release into the medium those peptide sequences that are not necessary for them.

In a coculture system, the *L. rhamnosus* GG PrtR begins protein hydrolysis to satisfy its auxotrophies. The activity of the proteolytic system of *L. rhamnosus* GG will allow the release of amino acids that are essential for the development of *S. thermophilus* species in the medium, making evident the protocooperation relationship between both species [25]. In this sense, *L. rhamnosus* GG will prefer amino acids such as cysteine, serine, or proline, while *S. thermophilus* species cover their auxotrophies with amino acids such as methionine and glutamine [18, 23]. Studies have highlighted that these amino acids are promoters for activating oligopeptide transport systems in all lactic acid bacteria. That is why more than a system of competition for nutrients, a balance is established by the protocooperation activated in each fermentation [26]. In the end, the peptides and amino acids that are not used are excreted through the cell wall of both microorganisms and released into the medium [27].

Various studies have reported that coculture systems increase the rate of proteolysis, as well as the concentration and diversity of peptides in the medium [16,17,26]. Similarly, it has been observed that peptides obtained from cocultures have greater biological activity than those obtained from independent cultures [18]. The peptides obtained from the

coculture in this study weighted ≥ 2 kDa, which is related to that reported by other authors who point out a relationship between peptides of smaller size and greater biological activity [28].

3.4 DPP-IV inhibitory activity

The inhibition of DPP-IV by the action of the peptides generated during the fermentation was evaluated. At the beginning of fermentation, no inhibitory activity of the enzyme was presented in any of the three systems evaluated (Figure 4), even though whey naturally contains some peptides due to the heat treatment to which it is exposed [29]. However, it could be observed that this activity increases as the fermentation time elapses. This could be directly related to the proteolytic activity of the strains, attributing the inhibitory activity of DPP-IV solely to the peptides generated from microbial metabolism. The results showed a 46.9% inhibition percentage for the *L.rhamnosus* GG monoculture after 12 hours of fermentation. On the contrary, for the monoculture of *S. thermophilus* SY-102 there was no inhibitory activity of DPP-IV. This same trend was observed towards the end of fermentation (21 h), where the inhibitory activity of DPP-IV only reached 2.35% inhibition.

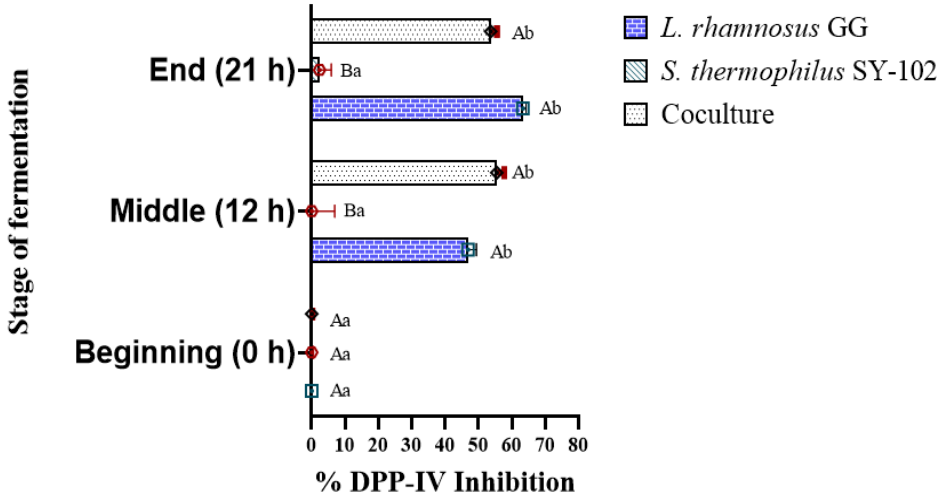

Figure 4. DPP-IV inhibitory activity by peptide fractions derived from 10% whey, fermented with L. rhamnosus GG, S. thermophilus and coculture of both microorganisms.

The results are the average of three determinations ± the standard deviation. a-b Lower case letters indicate comparison of means between times of the same fermentation system. A-C Upper case letters mean comparison of means between fermentation systems with the same fermentation time. Samples with the same letter did not present significant difference using Tukey's test ($P < 0.05$).

This could be related to the results observed in the determination of free amino groups and the electrophoresis analysis since the proteolytic capacity of *S. thermophilus* SY-102 was lower than *L. rhamnosus* GG. It has been reported that lactobacilli species tend to have a higher rate of proteolytic activity, producing higher concentrations of peptides with considerable biological activity [19]. Likewise, it is known that the proteolytic activity of *S. thermophilus* spp favors the accumulation of peptides with aromatic amino acids such as phenylalanine, tryptophan or tyrosine [25]. This has been related to other bioactivities, such as angiotensin-converting enzyme (ACE) inhibition. However, the results observed in this study could indicate that the peptides generated from the culture with *S. thermophilus* spp have no DPP-IV inhibitory activity, thus ruling out multiple biological activities.

On the other hand, in the case of monoculture with *L. rhamnosus* GG, a progressive increase in inhibitory activity was observed. After the start of fermentation, where no

activity was recorded, an increase from 46.9% (12 h) to 63.3% (21 h) of DPP-IV inhibition was observed. These results agree with the proteolytic activity of the strain, which was higher. This is consistent with the results observed from the analysis of free amino groups and electrophoresis. Different proteinases linked to the cell wall and their hydrolytic specificities give lactobacilli strains a great capacity to generate peptides of different molecular weights and sequences [16].

Similarly, a high percentage of DPP-IV inhibition was observed in the coculture. In this case, an inhibition percentage of 55.49% was obtained at 12 h of fermentation. Subsequently, at 21 h of fermentation, a slight decrease in activity was observed, reaching a percentage of 53.69%. These results were consistent with what was observed in the electrophoresis analysis, where the accumulation of low molecular weight peptides was higher compared to cocultures. The synergistic effect in coculture systems leads to the accumulation a wide variety of peptides [13]. Thus, it has been observed that the biological activity of peptides increases in coculture systems [30] due to the release into the medium of those peptides and amino acids that are unnecessary for microbial development [30].

Additionally, the activity of the specific intracellular peptidase of *S. thermophilus* spp favors the excretion of peptides with phenylalanine in their structure [31,32]. On the other hand, the proteolytic system of *L. rhamnosus* GG will favor the accumulation in the medium of peptides with amino acids such as proline, valine, or isoleucine [16]. In this sense, it has been reported that DPP-IV inhibitors contain in their structure amino acids such as proline, alanine, valine, leucine, and phenylalanine [1,30]. These peptides bind to the active site of DPP-IV competitively. However, some molecular docking studies point to the binding of inhibitors to DPP-IV outside of its active site through non-competitive actions [3]. Likewise, it has been reported that the inhibition mechanism associated with these peptides could be due to preventing the dimeric form of DPP-IV [3,33]. Despite the release of aromatic amino acids by *S. thermophilus* spp, it is known that the position of these amino acids in the peptide chain is one of the limitations for the peptide to present biological activity [34].

With the results found in this study, the importance of coculture fermentations has been demonstrated for the release of peptide sequences with a biological activity that increases the technological value of the inoculums in lactic fermentations, opening a very important field of study in the processing of functional and nutraceutical food, taking advantage of the differences in proteolytic systems.

5. Conclusions

The use of lactic acid bacteria in the fermentation of whey favors the production of peptides with antidiabetic activity. The combination of *L. rhamnosus* GG and *S. thermophilus* SY-102 in a coculture system promotes a greater proteolytic activity of the strains, increasing the concentration of low molecular weight peptide fractions. The inhibitory activity of DPP-IV by the action of the peptides obtained from the monoculture of *L. rhamnosus* GG and coculture increased during the fermentation time. The results obtained from this study indicate that *L. rhamnosus* GG in monoculture and a coculture system with *S.thermophilus* SY-102 generates peptides with high DPP-IV inhibitory activity (>50%). The inhibition could be related to the structure and composition of the peptides generated during fermentation by the action of the proteolytic system of each strain. Obtaining peptides with antidiabetic activity from microbial fermentations represents a viable alternative to assist in treating Diabetes mellitus II. The hydrolysis of whey proteins by lactic acid bacteria fermentation is an area of opportunity in the field of human health. Molecular coupling studies will be necessary to elucidate the interaction between the generated peptides

and DPP-IV, helping develop new products added with bioactive peptides that help treat various pathologies.

Supplementary Materials: not applicable

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Capítulo VI.

Promising medical function of protein whey hydrolysates by acid lactic fermentation: in vitro Study

Los resultados de este capítulo han sido sometidos a revisión en la Journal of Medicinal Food. En esta sección, se presentan los resultados correspondientes a las las fracciones peptídicas derivadas de la hidrólisis de proteínas de suero de leche mediante fermentación por protocooperación de bacterias ácido lácticas. Tras la fermentación de suero de leche utilizando *L. rhamnosus* GG y *S. thermophilus* SY-102, se realizó la separación de los péptidos derivados a través de RP-HPLC. De esta manera, se identificaron dos fracciones de carácter hidrofóbico. Posteriormente, se evaluó su capacidad bioactiva multifuncional al determinar la actividad antihipertensiva, antidiabética y antioxidante, reportando la IC50 en cada caso. Para la determinación de la capacidad antihipertensiva, se evaluó la actividad inhibitoria de la enzima convertidora de angiotensina (ACE), reportando valores de IC50 con alto potencial antihipertensivo. Por otro lado, se determinó la actividad antidiabética a través de la evaluación de la actividad inhibitoria de la Dipeptidil peptidasa IV. Finalmente, se determinó la actividad antioxidante a través de FRAP y DPPH.

La discusión se centra en la naturaleza de las fracciones obtenidas por RP-HPLC, señalando la relación de su carácter hidrofóbico con los aminoácidos que pudieran estar presentes en su estructura. De igual manera, se infiere una relación entre la secuencia de aminoácidos presente en la estructura de las fracciones obtenidas y su potencial bioactivo. Por otro lado, se establecen los patrones que los péptidos multifuncionales presentan para poder efectuar una bioactividad múltiple. Finalmente, se describe la relación de este tipo de péptidos en el tratamiento de enfermedades crónico- degenerativas como la Diabetes mellitus y la hipertensión arterial, haciendo énfasis en sus mecanismos de acción.

Los resultados demuestran que la hidrólisis de proteína de suero por protocooperación de bacterias ácido lácticas, deriva en la producción de fracciones peptídicas con propiedades multifuncionales.

Promising medical function of multifunctional protein whey hydrolysates by acid lactic fermentation: *in-vitro* study

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ABSTRACT

Whey is a by-product of the dairy industry that contains proteins that are a potential source of bioactive peptides and have high nutritional value. This study evaluated peptide fractions' antihypertensive, antidiabetic, and antioxidant activity capacity derived from lactic fermentation. The fractions evaluated were those separated by RP-HPLC obtained after fermentation of whey by protocooperation of *L. rhamnsosus* GG and *S. thermophilus* SY-102. The results show that the fermentation of whey proteins by protocooperation of lactic acid bacteria allows the release of peptide fractions with high bioactive potential.

Keywords: whey proteins; antihypertensive activity; antidiabetic activity; bioactive peptides

INTRODUCCIÓN

Milk whey is a polluting waste, a by-product of the dairy industry during cheese making[1]. However, this dairy matrix has a high nutritional value due to its high protein content (around 25%) [2]. For this reason, it is considered a potential source of bioactive peptides [3]. This type of protein fractions are encrypted in proteins, and when released, they perform various functions at the physiological level [4]. In this way, the bioactive peptides obtained could help treat various chronic-degenerative diseases, including Type 2 Diabetes mellitus and 3 arterial hypertension. These pathophysiologies are commonly present in the same clinical group. The mechanisms related to both conditions include damage at the macrovascular level, increased sodium retention at the renal level, and increased sympathetic nervous system activity due to hyperinsulinemia [5]. As a result, arterial hypertension is high in diabetic patients [6].

The different side effects of conventional drugs have led to the search for new alternatives that help treat these conditions [7,8]. In this way, bioactive peptides have proven to be an option with great therapeutic potential [3]. Hydrolysis of whey proteins by microbial fermentation has been widely used to obtain bioactive peptides [9]. For this reason, this study aimed to evaluate the antihypertensive, antidiabetic, and antioxidant capacity of peptide fractions derived from the hydrolysis of whey proteins after fermentation by protocooperation of lactic acid bacteria.

MATERIALS AND METHODS

Strain conditioning

Streptococcus thermophilus SY-102 and *Lacticaseibacillus rhamnosus* GG were obtained from the Food Biotechnology Laboratory of the Universidad Autónoma Metropolitana campus Iztapalapa. The microorganisms were conditioned in MRS broth and incubated for 24 h at 42 °C. Subsequently, one milliliter of the inoculated broth was added to 9 mL of powdered whey solution (10% (w/v) Dairy Gold) previously pasteurized at 90 °C for 10 min. It was incubated for 24 h at 42 °C. Each microorganism was conditioned in the same way independently.

Fermentation

A system was prepared with both microorganisms in a 10% (w/v) powdered whey solution for fermentation. This solution was pasteurized at 90 °C for 10 min before inoculation. The initial concentration of bacteria was approximately 1×10^8 CFU. The samples were incubated at 42 °C for 21 h. Subsequently, these were centrifuged at 24,600 × *g* for 10 min at 4 °C to eliminate biomass and high molecular weight proteins. The supernatants of the centrifuged samples were stored at -4 °C for later analysis. All samples were analyzed in triplicate.

Separation of peptides by RP-HPLC

Peptides were separated by reverse phase chromatography (RP-HPLC). The separation was performed on an HPLC system (Waters, USA) with a photodiode array detector (Spectra System Thermo Scientific, USA). A C8 column (250mm x 4.6mm x 5mm; Waters) was used. For the elution of the peptides, a linear gradient from 100% to 0 was followed in 56 minutes. Two solvents were used, solvent A (water with acetonitrile [ACN] 98:2) with trifluoroacetic acid (TFA) (650 µL per liter of solvent) against solvent B (water with ACN 35:65; with 650 µL of TFA per liter of solvent) in a flow of 1 mL/min. Separation was performed at 40 °C, and detection was performed at 280 nm. Fractions were manually collected every minute, evaluating the amount of protein using the Bradford technique [10].

Determination of antihypertensive activity

The determination of the antihypertensive activity by inhibition of the angiotensin-converting enzyme (ACE-I), with some modifications, was evaluated spectrophotometrically according to Cushman et al. [11]. The substrate, hippuryl-histidyl-

leucine (HHL; Sigma-Aldrich, St. Louis, USA), was dissolved in sodium borate buffer (0.1 M, pH 8.3 with 0.3 M sodium chloride) at a 5 mM substrate concentration. Subsequently, 20 µL of ECA (EC 3,4,15.1, 5.1U/mg; Sigma-Aldrich) were added to 200 µL of the substrate and 80 µL of the sample (AbsM). The reaction was carried out for 80 min at 37 °C. Subsequently, 250 µL of 0.1 N HCl were added. The entire reaction volume was taken to test tubes, and 1.7 mL of ethyl acetate were added. Then, 800 µL of the organic phase were taken and evaporated at 80 °C. 500 µL of water, 300 µL of pyridine, and 150 µL of Benzenesulfonil were added. Finally, it was shaken by inversion, and the absorbance at 410 nm was measured in a Power Wave XS UV-Biotek spectrometer (KC Junior software, Kansas, MO, USA). The same treatment was performed for a control sample (Abs100) with 80 µL of borate buffer instead of the sample. The ACE inhibitory activity was determined using the following formula according to the absorbance of each measurement:

% ACE inhibition = [(Abs100−AbsM)/ (Abs100)] × 100

Determination of antidiabetic activity

The antidiabetic activity was determined by evaluating the inhibitory effect of DPP-IV (D4943-1VL; Sigma-Aldrich, Saint Louis, USA) according to the technique of Nongonierma et al.[12] with some modifications. The substrate (Gly-Pro-p-nitroanilide; Sigma-Aldrich, St. Louis, USA) was dissolved in Tris-HCl buffer (pH 8) at a substrate concentration of 1.6 mM. Subsequently, 100 µL of the sample (AbsM) were added to 100 µL of the substrate, incubating at 37 °C for 10 min. After incubation, 200 µL of DPP-IV (1U/mL; Sigma-Aldrich, St. Louis, USA) were added. The reaction was carried out for 60 min at 37 °C. To terminate the reaction, 400 µL of 0.1 M potassium carbonate were added. The same treatment was followed for the positive control (AbsB) preparation but added 100 µL of Tris-HCl buffer instead of the sample volume. The negative control (AbsC) was prepared by adding 100 µL of sample, 300 µL of Tris-HCl buffer (pH 8), and 400 µL of 0.1 M potassium carbonate. Finally, all the samples were read at 405 nm in a spectrometer. Power Wave XS UV-Biotek (KC Junior software, Kansas, MO, USA). The DPP-IV inhibitory activity was calculated using the following formula according to the absorbance of each measurement:

$$\% \text{ DPP-IV inhibition} = \left(\frac{Abs\ B - (AbsM - AbsC)}{Abs\ B} \right) x100$$

Antioxidant activity

DPPH

The antioxidant activity of DPPH was determined according to the methodology proposed by Brand-Williams et al. [13] with some modifications. In tubes protected from light, 2.9 mL of 2.2-Diphenyl-l-picrylhydrazyl (DPPH) (0.1 mM dissolved in methanol) and 0.1 mL of the sample were added. The reaction was carried out in the dark for 50 min. After this time, the absorbance of the sample (SAbs) at 515 nm was measured (Genesys 10S UV VIS, Thermo Scientific), using methanol as blank (BAbs). The control (CAbs) consisted of 0.1 mL methanol and 2.9 mL DPPH. All determinations were performed in triplicate.

The percentage of remaining DPPH was calculated using the following formula:

$$\% \text{ DPPH remaining} = (SAbs - BAbs)/CAbs) \times 100$$

FRAP

The determination of antioxidant activity by FRAP was carried out following the methodology proposed by Benzie & Strain [14] with some modifications. The FRAP reagent was prepared from an acetate buffer (300 mM pH 3.6), a 10 mM TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) solution, prepared in 40 mM HCl and a 20 mM $FeCl_3$.6H2O ($Fe^{3+)}$ solution. All solutions were incubated at 37 °C for 15 min. Samples were measured at 593 nm using only the FRAP reagent as a blank. The antioxidant capacity of the samples was determined using the same procedure as the standard solutions, replacing the Fe^{2+} solution with 250 μL of each sample. The entire procedure was performed in triplicate. The values obtained for each sample were reported as equivalent mg of Fe^{2+} per 100 mL of sample.

Statistic analysis

Results were analyzed by one-way ANOVA (p = 0.05) and by Tukey's test using the NCSS statistical software (NCSS 2007, v.0, Kaysville, UT, USA, 2007).

RESULTS

Separation of peptide fractions by RP-HPLC

From the hydrolysis of milk whey proteins by protocooperation of lactic acid bacteria, two fractions were obtained by RP-HPLC (Fig. 1). The retention times of these fractions were 25.76 min for fraction 1 (F1) and 26.49 min for fraction 2 (F2). The protein content for both collected fractions was determined, observing a concentration of 102.16 mcg/mL (F1) and 140.5 mcg/mL (F2). The bioactivity of these fractions was evaluated by determining the IC_{50} in the ACE inhibition assays and DPP-IV inhibition. Likewise, the antioxidant activity was evaluated through the DPPH and FRAP assay.

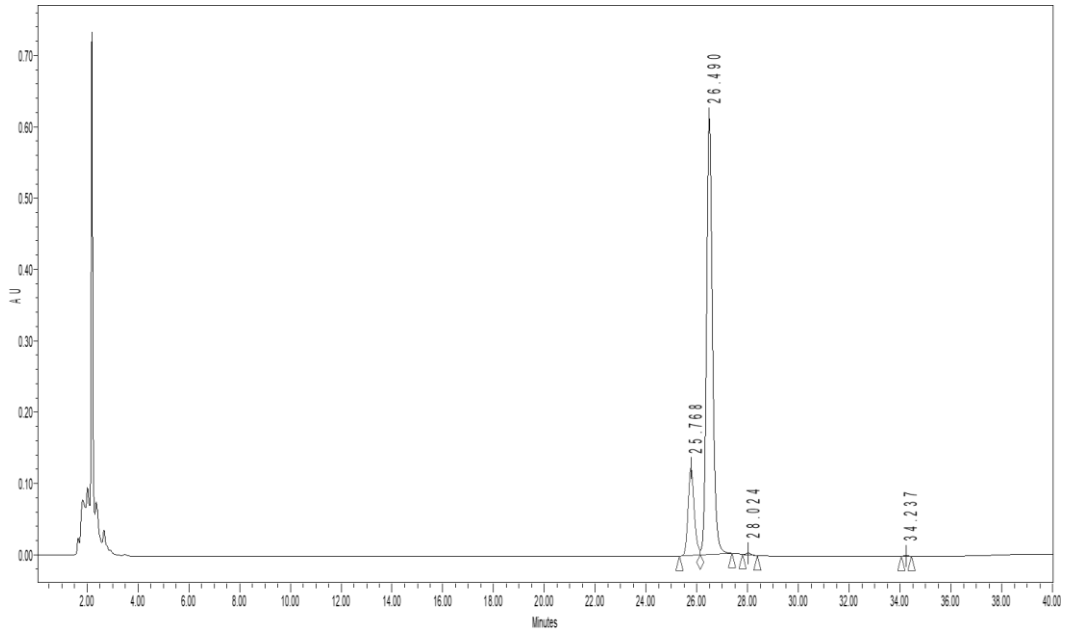

FIG 1. Separation by RP-HPLC of whey proteins hydrolyzed fractions by microbial fermentation.

Antihypertensive and antidiabetic activity

For the antihypertensive potential, the IC_{50} of the fractions obtained by fermentation was calculated (Fig. 2). The results showed IC_{50} values of 106.60 mcg/mL for F1 and 168.6 mcg/mL for F2. Likewise, there was a statistically significant difference ($p<0.05$) between the ACE inhibitory activity of F1 and F2. These results are similar to those previously reported in other studies where microbial fermentation obtained IC_{50} values from whey fractions ranging between 0.14 and 0.07 mg/mL [15] . Likewise, peptides derived from β- lactoglobulin have been reported with high ACE inhibitory activity,

favorably comparing this activity with that presented with conventional drugs such as captopril [16].

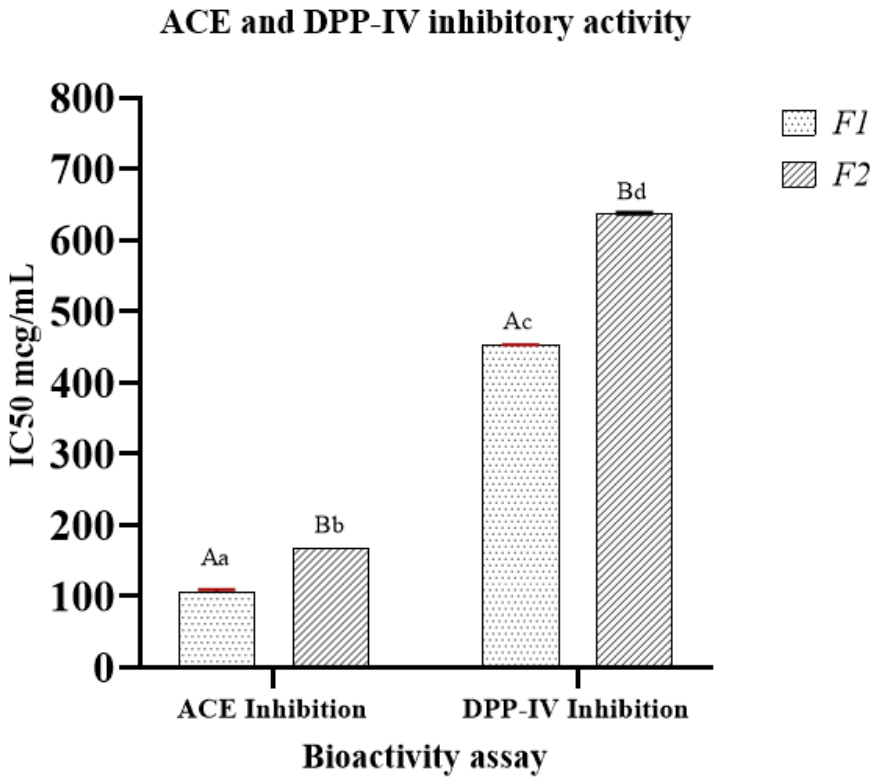

FIG 2. Bioactivities of whey protein fractions (Angiotensin I-Converting Enzyme and Dipeptidyl peptidase IV inhibitory activity (IC50, mcg/mL). Lowercase letters compare means between bioactivities. Uppercase letters compare means between the same bioactivity.

Similarly, the IC_{50} values for DPP-IV inhibition showed 452.54 mcg/mL results for F1 and 637.74 mcg/mL for F2. Likewise, there was a statistically significant difference between both fractions. These results are similar to those previously reported regarding DPP-IV inhibition which IC_{50} values of 0.55 mg/mL were observed from whey hydrolysates, specifically β-lactoglobulin [17].

Antioxidant activity

Regarding the antioxidant activity, two tests were carried out. For the antioxidant activity by DPPH, the IC_{50} values for F1 were 428.75 mcg/mL and 80.72 mcg/mL for F2. On the other hand, for the antioxidant activity reported by FRAP, results correspond to 0.0022 mg eq. Fe^{2+}/100mL for F1 and 0.076 mg eq. Fe^{2+}/100mL for F2. These results are consistent with those obtained in other studies in which the antioxidant activity of peptide

fractions obtained from whey proteins has been evaluated. In this context, the reported IC_{50} values were 0.45 ± 0.02 mg/mL from fractions derived from α-lactalbumin [18]. Despite the considerable values of antioxidant activity observed from the DPPH test, these were not related to the values reported by FRAP, which were lower.

TABLE 1. Antioxidant activity of whey protein fractions. DPPH (IC_{50} mcg/mL) and FRAP (mg eq. Fe^{2+}/100mL).

	Antioxidant activity		
Fraction	DPPH IC_{50} mcg/mL	FRAP mg eq. Fe^{2+}/100mL	Peptide concentration mcg/mL
F1	418.75	0.0022	102.16
F2	80.72	0.076	140.5

DISCUSION

Bioactive peptides from whey have been shown to have high nutraceutical and pharmaceutical potential [3]. In this sense, these bioactive fractions help treat various chronic diseases [19]. Multiple bioactive peptides are usually released during fermentation processes [20]. In this work, it was observed that the fractions released from whey fermentation present a multifunctional potential. Multifunctional peptides represent a challenging area of opportunity [21]. However, various studies have highlighted that the peptides released after fermentation not only exert a unique bioactivity [22]. On the contrary, it has been observed that these sequences have multifunctional potential [3]. In this context, the fractions obtained in this investigation showed multifunctional activity when their antihypertensive, antidiabetic, and antioxidant capacity was evaluated. According to the retention time, the fractions collected by RP-HPLC presented a hydrophobic character. This characteristic would allow for determining the nature of the amino acids present in the fractions obtained. Several studies have indicated that the peptides with the greatest ACE inhibitor potential contain hydrophobic amino acids such as proline and histidine [16,23]. Likewise, it has been reported that the binding of peptides to ACE is strongly related to the C-terminal sequence [24]. Thus, one of the common characteristics of ACE inhibitors is the presence of branched-chain aromatic amino acids

[23], or with residues of proline, hydroxyproline, phenylalanine, tryptophan, and tyrosine [16, 25, 26].

Similarly, regarding the enzyme DPP-IV, it has been reported that inhibitors may contain hydrophobic amino acids such as isoleucine, proline, and methionine [12]. But, it has been indicated that inhibitors of this enzyme may contain amino acid residues such as valine, leucine , phenylalanine [27, 28], with proline in position 2 of the amino terminus being one of the preferred substrates of DPP-IV [21]. According to molecular coupling analysis, it has been indicated that this type of inhibitor binds to DPP-IV competitively to the active site and outside the active site, preventing the dimeric form of the enzyme [29, 30]. On the other hand, the presence of hydrophobic amino acids in antioxidant peptide sequences derived from whey proteins has also been highlighted [31]. Specifically, peptides derived from β-lactoglobulin have been shown to have high antioxidant capacity [32]. This is related to histidine, methionine, tryptophan, and tyrosine content [32, 33].

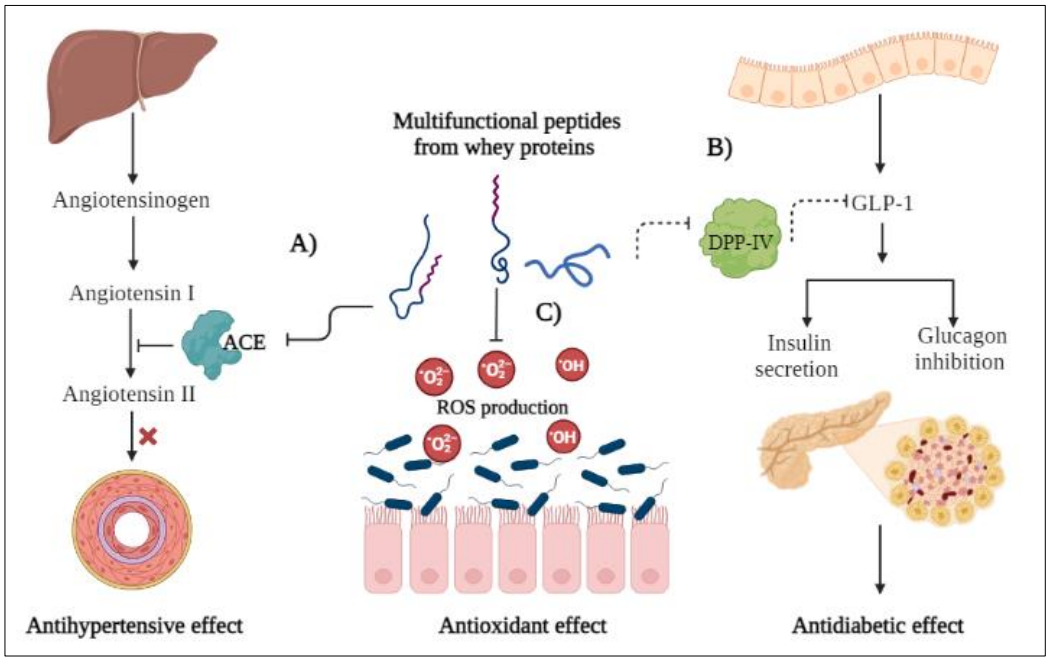

FIG 3. Mechanism of action of multifunctional peptides derived from whey protein. A) Antihypertensive peptides act by inhibiting angiotensin-converting enzyme (ACE). The switch from angiotensin I to angiotensin II is prevented by inhibiting this enzyme. Therefore, a vasoconstrictor effect is not carried out. B) Peptides with antidiabetic effect exert their bioactivity by inhibiting the enzyme Dipeptidyl peptidase-IV (DPP-IV). This enzyme participates in the degradation of the GLP-1 incretin, which stimulates insulin secretion and inhibits glucagon secretion under normal conditions. However, since DPP-IV degrades it, it cannot exert these effects. C) Antioxidant peptides can reverse the effects of dysbiosis by regulating the concentrations of reactive oxygen species (ROS) in the intestine. Similarly, they modify the structure of the microbial community by reducing the abundance of ROS-generating species.

Bioactive peptides' impact on chronic-degenerative diseases is directly related to their mechanism of action (Fig. 3) [3]. In Diabetes and hypertension, peptides have been observed to inhibit enzymes such as ACE and DPP -IV [34, 35]. These enzymes have a real impact on regulating blood pressure and releasing glucose and insulin [23, 29].

Likewise, antioxidant peptides play an important role as adjuvants in this pathophysiology type [36]. In this case, antioxidant peptides exert their effect by inactivating reactive oxygen species (ROS), breaking the lipid oxidation chain, or chelating metal ions [36, 37]. However, in recent years the impact of these peptides in regulating intestinal microbiota [38]. It is well known that dysbiosis in the intestinal microbiota leads to a pro-inflammatory state and insulin resistance characteristic of diseases such as Diabetes mellitus and arterial hypertension [39, 40]. Antioxidant peptides can reverse the effects of dysbiosis on the intestinal microbiota by regulating ROS concentrations in the intestine [3]. Likewise, these peptides modify the microbial community by reducing the abundance of microorganisms that generate high concentrations of ROS [41].

The IC_{50} of the ACE inhibitory activity reported in this investigation was 106.60 mcg/mL for F1 and 168.6 mcg/mL for F2. These results were even lower than the results reported by other authors. Therefore, they present higher ACE inhibitory activity. Konrad et al. [17] reported IC_{50} values below 0.65 mg/mL and 0.8 mg/mL from 3 kDa whey protein fractions derived from hydrolysis with a vegetable serine protease. On the other hand, Vermeisser et al. [15] reported IC_{50} values of 0.14 to 0.07 mg/ml from whey fractions derived by microbial fermentation. The difference between these results could be due to the protein hydrolysis process. It has been observed that protein hydrolysis from microbial fermentation generates a great diversity of peptides with a high bioactive potential [20].

On the other hand, it has been reported that ACE inhibitor peptides can simultaneously inhibit DPP-IV [17]. This is closely related to the sequence of amino acids in its structure. As previously pointed out, the hydrophobic nature of the amino acids in their structure is a common pattern in these peptides. The IC_{50} values corresponding to DPP-IV inhibition were 452.54 mcg/mL for F1 and 637.74 mcg/mL. These results are similar to those reported by other authors who have indicated IC_{50} values of 0.55 mg/mL and 0.07 mg/mL from fractions derived from WPC-80 and β-LG respectively [17]. Similarly, IC_{50} values of

0.075 and 1.28 mg/mL for WPI and β-lactoglobulin hydrolysates have been reported, which denotes the potential of whey proteins in releasing peptides with considerable biological activity [42]. However, it has been pointed out that the differences in the inhibitory potential of these peptides depend on their physicochemical characteristics and the protein content of the whey used [17].

The antioxidant activity of the fractions evaluated in this investigation may also be related to the amino acid sequence in its structure. In addition to inhibiting ACE and DPP-IV, some antihypertensive peptides may exert other bioactivities, such as antioxidant capacity. Protocooperation fermentation of lactic acid bacteria allows the release of fractions with high biological potential [43]. The microorganisms satisfy their auxotrophies without there necessarily being a competition between them [44]. In the case of *L. rhamnosus* and *S. thermophilus*, the protocooperation relationship allows the release of a great diversity of peptides with multiple bioactivities [43]. Each has specific nutritional requirements of nitrogen covered by their proteolytic system [45]. In this way, once these auxotrophies are covered, they will release into the medium those sequences that are not necessary for them [46]. Thus, peptides with hydrophobic amino acid sequences could be found in the medium, responsible for antihypertensive, antidiabetic, and antioxidant activity. Although the antioxidant activity reported by FRAP was not representative, the activity reported by DPPH shows the potential of these fractions to exert antioxidant bioactivity. IC_{50} values of 0.45 ± 0.02 mg/mL have been reported from peptides derived from the hydrolysis of α-lactalbumin.

These values are similar to what was reported in the present investigation, in which the IC_{50} values for antioxidant activity were 428.75 mcg/mL and 80.72 mcg/mL. The multifunctional activity of peptides is related to their amino acid composition, sequence, and size. Thus, smaller peptides exert multiple bioactivities such as antidiabetic, antioxidant, and antihypertensive capacity [47]. In this sense, the results of this research denote the multifunctional potential of the fractions derived by the hydrolysis of whey proteins through microbial fermentation.

CONCLUSION

The results of the present study demonstrate that the hydrolysis of whey protein by protocooperation of lactic acid bacteria leads to the production of peptide fractions with multifunctional properties. The fractions obtained by RP-HPLC suggest hydrophobic properties related to its antidiabetic, antihypertensive, and antioxidant potential. Likewise, these results suggest obtaining potential multifunctional peptides. In this way, they can help treat diseases such as Diabetes mellitus and arterial hypertension, simultaneously exerting antioxidant activity. However, more research is required to fully elucidate the mechanisms of action of these peptides. Similarly, human clinical studies are necessary to confirm the efficiency and evaluate the absorption and bioavailability of peptides derived from whey protein.

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Conclusiones generales

- Existe una relación de protocooperación entre *L. rhamnosus* GG y *S. thermophilus* SY-102 cuando estos microorganismos crecen en cocultivo. El efecto sinérgico presente en este sistema tiene relación con el tipo de péptidos generados durante la fermentación.

- Los patrones proteolíticos de cada cepa favorecen la acumulación de péptidos de bajo peso molecular a partir de un sistema de cocultivo.

- La actividad inhibitoria de la ECA y DPP-IV se incrementa con el uso de dos microorganismos fermentando el suero de leche en protocooperación

- Los péptidos con actividad antihipertensiva y antidiabética provienen de fracciones con carácter no polar lo cual se relaciona con los aminoácidos presentes en la estructura de péptidos que ya han sido reportados previamente.

- La fermentación de proteínas de suero de leche por acción de bacterias ácido lácticas representa un área de oportunidad importante para la obtención de péptidos con alta actividad antihipertensiva y antidiabética que coadyuven en el tratamiento de DM2 e hipertensión arterial.