UADY

POSGRADO INSTITUCIONAL EN CIENCIAS QUÍMICAS Y BIOQUÍMICAS

# FRACCIONES PEPTÍDICAS ANTIBACTERIANAS DE SEMILLAS DE CHÍA (*Salvia hispanica* L.) Y SU ESTABILIDAD A CONDICIONES DE PROCESAMIENTO DE LOS ALIMENTOS

TESIS

PRESENTADA POR

**ANAÍ LEÓN MADRAZO**

EN OPCIÓN AL GRADO DE

MAESTRA EN CIENCIAS QUÍMICAS Y BIOQUÍMICAS

MÉRIDA, YUCATÁN, MÉXICO

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UNIVERSIDAD AUTÓNOMA DE YUCATÁN

COORDINACIÓN GENERAL
DEL SISTEMA DE POSGRADO
INVESTIGACIÓN Y VINCULACIÓN

MAESTRÍA EN CIENCIAS
QUÍMICAS Y BIOQUÍMICAS

Mérida, Yucatán, 17 de septiembre de 2020
Oficio Núm: PICQB/195/2020

**Asunto:** Autorización de digitalización

La tesis "Fracciones peptídicas antibacterianas de semillas de chía (*Salvia hispanica* L.) y su estabilidad a condiciones de procesamiento de los alimentos" presentada por Anaí León Madrazo, en cumplimiento parcial de los requisitos para optar por el grado de Maestra en Ciencias Químicas y Bioquímicas, ha sido aprobada en cuanto a su contenido científico y en cuanto a lo establecido en el Manual de Procedimientos del Posgrado Institucional en Ciencias Químicas y Bioquímicas, por lo que se le autoriza la digitalización de los ejemplares correspondientes.

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| Dra. Ana Ly Arroyo Herrera | |
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**DRA. MAIRA RUBI SEGURA CAMPOS**
**COORDINADORA DEL POSGRADO INSTITUCIONAL**
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## AGRADECIMIENTOS

A Dios por bendecirme con vida, salud y fuerza necesarias para concluir este trabajo.

A mi directora de tesis, la Dra. Maira Rubi Segura Campos, por darme la oportunidad de ser parte de su equipo de investigación, por brindarme su apoyo en cada etapa de este proyecto y por motivarme siempre a mejorar y dar lo mejor de mí.

A los doctores, Fanny G. Concha Valdez, Ana Ly Arroyo Herrera y Domancar Orona Tamayo por sus valiosas aportaciones en el desarrollo de este trabajo, por compartir su experiencia y fomentar mi capacidad de análisis en los seminarios de maestría.

Al QFB. Alfredo B. Fuentes Ortíz, por transmitirme su conocimiento en técnicas de evaluación de actividad antibacteriana, así como también, al Dr. Rodrigo A. Rivera Solís, personal y compañeros del Laboratorio de Biotecnología y Microbiología de la Facultad de Ingeniería Química de la UADY, por capacitarme en el uso del equipo de electroforesis.

A la E.E. Alejandra González Tamayo, por sus innumerables asesorías en el área de estadística y por su apoyo en el análisis TOPSIS.

A mis padres y mis hermanos, por ser mi soporte en todo momento, por su amor incondicional y palabras de aliento.

A Eduardo Lezama, por acompañarme y darme alegría en los momentos difíciles de este proceso.

A mis colegas y amigos Edwin Martínez, Nidia Quintal, Juan Pablo Quintal, Jonatan Uuh, Josabet Uhh, Ulil Us, Sibele Fernandes, Carlos de la Cruz, William Rubio, Lillibeth Manzanilla, Ivan Chan, Alejandra Domínguez y Francisco Valenzuela por las experiencias compartidas y consejos para mejorar mi rendimiento académico y ponencias.

Este trabajo fue realizado en el Laboratorio de Ciencia de los Alimentos y el Laboratorio de Biotecnología y Microbiología de la Facultad de Ingeniería Química de la Universidad Autónoma de Yucatán, bajo la dirección de la Dra. Maira Rubi Segura Campos y formó parte del proyecto CYTED del Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo 119RT0567, con el apoyo de la beca nacional CONACYT 933185.

## RESUMEN

El estudio de los péptidos antimicrobianos (PAM) y su producción a partir de la hidrólisis de proteínas alimentarias ha cobrado relevancia en los últimos años debido a su potencial aplicación en la conservación de alimentos. Sin embargo, existen diversas fuentes vegetales de PAM que continúan sin ser exploradas; este es el caso de *Salvia hispanica* L. (chía). Por lo anterior, el objetivo de este estudio fue, evaluar el potencial antibacteriano de fracciones peptídicas de chía (FPC) obtenidas con un sistema secuencial Pepsina-Pancreatina® (PP), frente a bacterias asociadas a enfermedades de transmisión alimentaria. Para ello, el hidrolizado proteínico (HP) se sometió a ultrafiltración (UF) para obtener 3 FPC (F<1, F 1-3 y F 3-5 kDa), que se caracterizaron por Tricine-SDS-PAGE. Se determinó la actividad antimicrobiana (AM) en *Listeria monocytogenes* ATCC51414*, Bacillus subtilis* ATCC465*, Shigella flexneri* ATCC9748*, Staphylococcus aureus* ATCC25923, *Salmonella* Typhimurium ATCC51821, *Salmonella* Typhi, *Salmonella* Paratyphi ATCC9150, *Salmonella* Enteritidis ATCC13076 y *Escherichia coli* O157:H7 mediante difusión en disco y dilución en microplaca. Para evaluar la potencial aplicación de las FPC en sistemas alimentarios, se determinó su estabilidad a la temperatura, pH y proteasas. Mediante la Técnica de Orden de Preferencia por Similitud a la Solución Ideal (TOPSIS), se analizaron las secuencias contenidas en F<1 para hallar el péptido con mayor potencial antibacteriano. Los resultados mostraron que, el grado de hidrólisis (GH) obtenido con PP (33.79% ±2.14) generó péptidos de cadena corta. La UF produjo tres FPC que revelaron bandas de < 1.06 kDa en F <1; 1.6, 2 y 2.4 kDa en F 1-3, y 2.1, 2.5, 3.2 y 4.7 kDa, en F 3-5 mediante Tricine-SDS-PAGE. El valor de Concentración Mínima Inhibitoria (CMI) más bajo, se registró en F <1, con 635.47 ± 3.6532 µg/mL para *Listeria monocytogenes*. Debido a que F <1 presentó la mayor AM contra *L. monocytogenes,* se destaca su potencial aplicación en productos donde esta bacteria puede estar presente, como productos lácteos o cárnicos. F<1 conservó su AM en un rango de temperatura de 4-80°C y fue resistente a la exposición a pH 5-8. La AM se perdió tras la incubación con tripsina y pepsina. El análisis TOPSIS reveló que, la secuencia peptídica con mayor potencial antibacteriano es KLKKNL, a la cual podría atribuirse el efecto inhibitorio observado en F<1. Por su actividad inhibitoria de bacterias Gram-positivas y la resistencia a distintas condiciones de procesamiento, F<1 podría tener un papel importante en la conservación de los alimentos Se sugiere que la actividad antibacteriana de F<1 podría estar relacionada con la presencia de péptidos catiónicos de cadena corta, como KLKKNL.

## ABSTRACT

Antimicrobial peptides (PAM) research and their production through the hydrolysis of food proteins has gained relevance in recent years due to their potential application in food preservation. Despite this, there are several vegetal sources of PAM that remain unexplored, such as chia (*Salvia hispanica* L.). Therefore, the aim of this study was to evaluate the antimicrobial potential of chia peptide fractions (FPC) obtained with a Pepsin-Pancreatin® sequential system against food borne bacteria. For this purpose, the chia protein hydrolysate (HP) was subjected to ultrafiltration processes to yield 3 FPC (F<1, F 1-3 y F 3-5 kDa), which were characterized by Tricine-SDS-PAGE. Antibacterial activity was determined in *Listeria monocytogenes* ATCC51414*, Bacillus subtilis* ATCC465*, Shigella flexneri* ATCC9748*, Staphylococcus aureus* ATCC25923, *Salmonella* Typhimurium ATCC51821, *Salmonella* Typhi, *Salmonella* Paratyphi ATCC9150, *Salmonella* Enteritidis ATCC13076 and *Escherichia coli* O157:H7 by *in vitro* disk diffusion and microplate dilution tests in accordance with the provisions of the Clinical and Laboratory Standards Institute (CLSI). In order to assess the potential application of FPC in food systems, their pH, temperature and protease stability was evaluated. Sequences in F<1 were analyzed using The Technique for Order of Preference by Similarity to Ideal Solution (TOPSIS) to determine the peptide with the highest antimicrobial potential. The results showed that degree of hydrolysis (GH) obtained with the PP system (33.79% ± 2.14) was suitable to produce short chain peptides. Ultrafiltration allowed obtaining three FPC that revealed low intensity bands with molecular weights lower than 1.06 kDa in F<1; 1.6, 2 and 2.4 kDa in F 1-3; 2.1, 2.5, 3.2 and 4.7 kDa in F 3-5 using Tricine-SDS-PAGE. The lowest Minimum Inhibitory Concentration (CMI), was observed in F <1, with 635.47 ± 3.6532 µg/mL against *Listeria monocytogenes*. Since the highest antibacterial activity against *L. monocytogenes* was shown in F<1*,* it is remarked its potential application in food products where this bacterium is generally present such as those derived from milk and meat products. F<1 retained its antibacterial activity in a temperature range of 4-80°C and was resistant to exposure at pH 5-8. In contrast, the antibacterial activity of F<1 was lost after incubation with trypsin and pepsin. The TOPSIS analysis revealed that KLKKNL is the sequence with the highest antimicrobial potential and it could be exerting the inhibitory effect observed in F<1. Due to its inhibitory capacity against Gram-positive bacteria and its resistance to certain food processing conditions, F<1 could play a role in food preservation. The antimicrobial activity of F<1 is suggested to be related with the presence of short-chain cationic peptides, such as KLKKNL.

**ÍNDICE**

# INTRODUCCIÓN

El deterioro causado por microorganismos, reduce la vida de anaquel de los alimentos y aumenta el riesgo de enfermedades (Krepker *et al*. 2017). De acuerdo con la FAO (2019), un tercio de todos los alimentos producidos globalmente, se pierden dentro de la cadena de suministro entre el productor y el mercado, debido principalmente a contaminación microbiológica y deterioro, generando como consecuencia importantes pérdidas económicas (Elkhishin *et al*., 2017). Además, los alimentos contaminados con patógenos representan un grave peligro para la salud, tanto en países desarrollados como en vías de desarrollo (Zhao *et al*. 2016). Las enfermedades de transmisión alimentaria (ETA) asociadas a contaminación microbiológica, se atribuyen especialmente a bacterias Gram-negativas como *Salmonella* Typhi y *Escherichia coli*. También se han identificado otras bacterias Gram-positivas como *Staphylococcus aureus* y *Listeria monocytogenes*, involucradas en casos de ETA (Mostafa *et al*. 2018). Existe un amplio número de conservadores alimentarios utilizados como agentes antimicrobianos que protegen a los alimentos de la acción de bacterias, por lo tanto, prolongan la vida de anaquel del producto. Sin embargo, el uso de algunos conservadores está vinculado a efectos secundarios en la salud (Silva & Lidon, 2016) y su aplicación masiva en la industria alimentaria ha dado lugar al surgimiento de cepas resistentes a estos compuestos químicos (Mostafa *et al*. 2018). Debido a lo anterior, el desarrollo de nuevos agentes antibacterianos es una alternativa viable para disminuir los riesgos a la salud y las deficiencias económicas ocasionadas por la contaminación bacteriana de los alimentos. Se espera que, la demanda de agentes antimicrobianos aumente a medida que se ponga en evidencia la influencia negativa ejercida por los conservadores alimentarios usados actualmente sobre la salud de los consumidores (Pisoschi *et al*. 2018). En este sentido, existe un gran interés en el estudio de los PAM y su aplicación en la conservación de los alimentos, por su capacidad para inhibir el crecimiento de un amplio espectro de bacterias. Los PAM son un grupo abundante y diverso de moléculas producidas en una variedad de especies de invertebrados, plantas y animales; generalmente consisten de 10 a 50 residuos de aminoácidos y son parte de la defensa innata de los organismos (Liu, Wu, Hou, Li, & Sha, 2017). Su composición aminoacídica, carácter anfipático, carga catiónica y tamaño, les permite unirse e insertarse en la membrana de las bacterias y desestabilizarla (Sánchez and Vázquez 2017). Una amplia variedad estructural es exhibida por los PAM, pero es posible clasificarlos en dos conjuntos de acuerdo con las características de sus estructuras secundarias: péptidos α-helicoidales y péptidos conformados en lámina β. El mecanismo de acción de los PAM sugiere una disrupción de la membrana bacteriana, la cual puede atribuirse, entre otros factores, al carácter catiónico de estas moléculas debido a la presencia de residuos de lisina, arginina e histidina (Johnson C P Santos *et al*. 2018). Los aminoácidos hidrofóbicos también confieren actividad antimicrobiana ya que

mejoran la solubilidad de los lípidos y permeabilizan con facilidad la membrana celular (Dash and Ghosh 2017). Péptidos con potencial antibacteriano pueden ser generados mediante la hidrólisis controlada *in vitro* de proteínas alimentarias (Bojórquez *et al*. 2013), como pueden ser las proteínas vegetales. En el caso particular de las semillas, el contenido de proteína representa entre un 10 y 40% de su peso seco (Liu *et al*. 2017). Por esa razón, han sido utilizadas en diversos estudios para la obtención de HP y FP con actividad biológica. La actividad antibacteriana de FP del frijol lima (*Phaseolus lunatus*) obtenidas por hidrólisis enzimática con un sistema secuencial PP, fue estudiada por Bojórquez-Balam *et al*. (2013), siendo las FP con peso molecular <10 kDa las que presentaron un mayor efecto inhibitorio a una concentración mínima Inhibitoria (CMI) de 392.04 µg/mL para *S. aureus* y 993.17 µg/mL, en el caso de *Shigella flexneri*. Hwang *et al*. (2016), realizaron una hidrólisis enzimática de semillas de linaza (*Linum usitatissimum)* con una proteasa bacteriana de *Bacillus altitudinis* HK02. El HP fue ultrafiltrado para obtener fracciones de bajo peso molecular, de las cuales, la fracción <1 kDa inhibió el crecimiento de *Escherichia coli* y *Pseudomonas aeruginosa* a una concentración de 60 µg/mL. La CMI registrada en péptidos derivados de plantas a lo largo de diferentes estudios, se encuentran entre 8-100 µg/mL, sin observarse, en algunos casos, presencia de células vivas a partir de los 64 µg/mL (Cardillo *et al*. 2018).

*Salvia hispanica* L. es una planta herbácea nativa del norte de Guatemala y el sur de México, cuyas pequeñas semillas destacan por su alto valor nutricional y funcional (Grancieri *et al*. 2019b). Su contenido de proteína (15- 25%) es mayor que el de otros granos tradicionales utilizados en la industria alimentaria (Ayerza and Coates 2011; Pereira da Silva *et al*. 2017), por lo tanto, representa una fuente prometedora de péptidos bioactivos (Coelho 2018). Segura-Campos *et al*. (2013), evaluaron la actividad antibacteriana de hidrolizados de chía obtenidos con un sistema secuencial Alcalasa-Flavourzyme®, utilizando tiempos de hidrólisis de 90 y 60 min. Ninguno de los hidrolizados exhibió actividad antimicrobiana contra las bacterias *Escherichia coli*, *Salmonella* Typhi, *Shigella flexneri*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis* y *Streptococcus agalactiae*. Por otra parte, Coelho *et al*. (2018), generaron hidrolizados proteínicos con actividad antibacteriana, a partir de una torta de chía parcialmente desgrasada, utilizando un sistema enzimático secuencial Alcalasa-Flavourzyme®, con tiempos de hidrólisis correspondientes a 60 y 180 min. *S. aureus* fue susceptible a estos hidrolizados a una CMI de 2.26 mg/mL y una Concentración Mínima Bactericida (CMB) de 5 mg/mL. No se observó susceptibilidad a los HP en *E. coli.* Diversos autores muestran el efecto antimicrobiano de PAM provenientes de plantas que se caracterizan por poseer pesos moleculares <10 kDa (Bojórquez *et al*. 2013; Cardillo *et al*. 2018; Hwang *et al*. 2016). Sin embargo, éstos no han sido estudiados en fracciones peptídicas de chía (FPC). Por lo anterior, el objetivo de este estudio fue evaluar el potencial antibacteriano de FPC obtenidas mediante un sistema enzimático secuencial PP, frente a bacterias Gram-positivas y Gram-negativas asociadas a ETA.

## HIPÓTESIS

Las fracciones peptídicas de *Salvia hispanica* L. obtenidas por ultrafiltración, inhiben el crecimiento de cepas bacterianas asociadas a enfermedades de transmisión alimentaria.

## JUSTIFICACIÓN

De acuerdo con la Organización Mundial de la Salud (2015), cada año las ETA afectan casi a 1 de cada 10 personas a nivel mundial. En México, las enfermedades infecciosas intestinales han sido un importante problema de salud desde 2011 (Diaz *et al*. 2018). Para tratar de controlar la proliferación de patógenos en alimentos, la industria ha indiscriminado el uso de los aditivos en alimentos, que se ha convertido en un factor indeseable para los consumidores en la actualidad. La limitación más grande de los conservadores alimentarios es su restricción a concentraciones que se han vuelto ineficaces ante el surgimiento de cepas cada vez más resistentes. La necesidad de expandir el espectro de la actividad antimicrobiana por encima de las dosis regulatoriamente aprobadas (Juneja, Dwivedi, and Sofos 2017) y las posibles connotaciones negativas a la salud, han causado que la comunidad científica explore otras alternativas para mejorar la calidad y el tiempo de vida de los alimentos, tal es el caso de los PAM.

Hidrolizados de chía, han demostrado tener diversos efectos biológicos entre los que se encuentra la actividad antimicrobiana. Su incorporación en alimentos ha sido estudiada no sólo por su funcionalidad tecnológica, sino también por sus beneficios a la salud. Su contenido de proteína puede ser aprovechado en subproductos de la extracción de aceites, lo que promueve una reducción del impacto ambiental y favorece el desarrollo de nuevos productos y mercados. Por otra parte, la utilización de un sistema enzimático PP para la hidrólisis proteínica, favorece la liberación de péptidos de bajo peso molecular con actividad biológica. En este contexto, la obtención de fracciones peptídicas de bajo peso molecular tiene un papel importante en las interacciones entre PAM y la membrana bacteriana. Por lo anterior, los péptidos de chía podrían presentar una potencial aplicación en la conservación de alimentos, siendo incorporados como antimicrobianos para prolongar su vida de anaquel.

## OBJETIVO GENERAL Y PARTICULARES

### Objetivo general

Evaluar la actividad antibacteriana de fracciones peptídicas de *Salvia hispanica* L., obtenidas de un sistema secuencial Pepsina-Pancreatina®, en la inhibición de bacterias Gram-positivas y Gram-negativas asociadas a enfermedades de transmisión alimentaria, así como su estabilidad a diferentes condiciones de procesamiento de los alimentos.

### Objetivos particulares

1. Determinar el grado de hidrólisis y el contenido proteínico del hidrolizado enzimático de *S. hispanica* L. obtenido con Pepsina-Pancreatina® y sus correspondientes fracciones peptídicas obtenidas por ultrafiltración, así como su perfil electroforético.
2. Evaluar la sensibilidad de bacterias Gram-positivas y Gram-negativas asociadas a enfermedades de transmisión alimentaria, a distintas concentraciones de las fracciones peptídicas de *S. hispanica* L.
3. Evaluar la estabilidad de las fracciones peptídicas de *S. hispanica* L. a diferentes condiciones de pH, temperatura y degradación por proteasas.
4. Identificar la secuencia peptídica con mayor potencial antibacteriano contenida en la fracción peptídica de *S. hispanica* L. más activa, mediante la Técnica de Orden de Preferencia por Similitud a la Solución Ideal.

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# Review of Antimicrobial Peptides as promoters of food safety: Limitations and possibilities within the food industry

## From Antimicrobial Peptides to Food preservation

Anaí León Madrazo and Maira Rubi Segura Campos

Facultad de Ingeniería Química, Universidad Autónoma de Yucatán. Periférico Norte Km. 33.5, Tablaje Catastral 13615, Col. Chuburná de Hidalgo Inn, 97203 Mérida, Yucatán, México. Corresponding author: maira.segura@correo.uady.mx

Antimicrobial peptides (AMPs) are a group of molecules that have been increasingly studied for their important role in the inhibition of bacterial agents. Their structure shows heterogeneity among the different types of AMPs. However, most of them stand out for their cationic nature and amphipathic character that enables them to interact with membrane components of bacterial cells. Some features such as high selectivity and thermostability have attracted the interest of the food industry towards the application of AMPs in food preservation due to their ability to inhibit foodborne pathogens and spoilage microorganisms. Despite this, there is a limited number of AMPs used as food preservatives nowadays. Therefore, this review aims to offer an overview of antimicrobial peptides, challenges, and potential applications in the food industry.

**Keywords:** Safety, quality, foods, peptides, applications

### Introduction

The rise in the outbreaks of more complex foodborne illness threatens population health and future economies. A large number of incidents have been reported in recent years, including the world's biggest listeriosis outbreak, which occurred in South-Africa during 2017-2018, leading to approximately 1000 people suffering from food poisoning and over 200 deaths (WHO, 2019a). Contaminated food contains harmful bacteria, viruses, and parasites that are capable to produce more than 200 different diseases, ranging from diarrhea to cancer. Hence, food safety and appropriate food supply must be assured (WHO, 2019b). The addition of preservatives is used for preventing or slowing down microbial growth in foods, which is the main cause of food spoilage and foodborne illness. Notwithstanding the extensive application of these antimicrobial agents, there is a lack of efficient and safe food preservatives, as a result of the emergence of resistant strains in response to the indiscriminate use of these additives (Keymanesh, Soltani, & Sardari, 2009).

El manuscrito fue enviado y aceptado por el Journal of Food Safety y se elaboró de acuerdo con las normas editoriales de dicha revista

Plant and animal-derived antimicrobials, lactic-acid bacteria and their antimicrobial metabolites such as bacteriocins, have shown the ability to inhibit lipid oxidation, color loss, prolongation of food shelf life and assurance of food safety (Pisoschi *et al*., 2018). AMPs are characterized by their low toxicity and thermal stability (Ebbensgaard *et al*., 2015; Said *et al*., 2018). These attributes are of relevance for food applications. They consist of 10-50 amino acid residues, classified into different groups depending on composition, size, and conformation. In general, when an antimicrobial peptide (AMP) is folded in a certain membrane or membrane-like environment, one part of the peptide is positively charged, consisting of arginine and lysine residues, while the other side contains a proportion of hydrophobic residues (Rai, Pandit, & Gaikwad, 2016). Natural preservatives, such as AMPs, are a viable alternative to address the problem of microbial resistance and to reduce the negative side effects on health caused by some synthetic compounds used as antimicrobial agents, while meeting the requirements of food safety, and exerting no negative impact on nutritional and sensory attributes of foodstuffs (Pisoschi *et al*., 2018).

**Microbiological risks in foods**

Food is rich in nutrients and suitable for the growth and reproduction of pathogens. Bacteria exist suspended in liquid food, usually living planktonically, or in solid and viscous food. They can easily adhere to the surface of food materials, food processing equipment, and the surface of pipelines (Zhao *et al*., 2017). Contamination of food can occur at many steps along the chain from farm to plate (Havelaar *et al*., 2015), for this reason, outbreak investigations play a key role in the prevention of foodborne pathogens diffusion, revealing the implicated food vehicles and the point where the contamination occurred (Schirone *et al*., 2019). An increasing number of multistate foodborne outbreaks have been reported (Table 1) and there are many factors involved in, including human demographics, consumer attitudes, changes in food processing and handling, as well as pathogen adaptation to new environments (Smith & Fratamico, 2018). Recent foodborne outbreaks have been frequently associated with *Escherichia coli*, *Listeria monocytogene*s, and *Salmonella enterica* serovars. *Salmonella* outbreaks were mostly linked to poultry (CDC 2018a, 2018b, 2018c, 2018d, 2019a), fruits, and vegetables (CDC 2018e, 2018f, 2018g, 2019b, 2019c, 2019d). *Salmonella* serotype Javiana has been responsible for outbreaks associated with vegetables in previous reports (Jackson *et al*., 2013). *E. coli* has been increasingly implicated with leafy greens (CDC 2018h, 2019e, 2020a, 2020b) and ground meat (CDC 2018i, 2019f, 2019g) while *L. monocytogenes* has caused foodborne outbreaks mainly related to meat products, such as sliced meat and ready-to-eat pork (CDC 2018j, 2018k, 2019h). The high incidence of foodborne outbreaks has resulted in several studies focused on the inactivation of *S. enterica*, *E. coli*, and *L. monocytogenes*, considering these pathogens as important targets in foods. (Gabriel *et al*., 2018; Rane, Bridges, & Wu, 2020; Roh *et al*., 2020; Shin *et al*., 2020; Trząskowska *et al*., 2018).

**Table 1.** Pathogens implicated in recent foodborne outbreaks (2018-2020) and associated foods.

| Food category | Pathogen | Reference |
|---|---|---|
| **Fruits** | | |
| Cut Fruit | *Salmonella* Javiana | (CDC 2020a) |
| Papayas | *Salmonella* Uganda | (CDC 2019d) |
| Pre-cut melon | *Salmonella* Carrau | (CDC 2019e) |
| | *Salmonella* Adelaide | (CDC 2018c) |
| **Vegetables and salads** | | |
| Clover sprouts | *Escherichia coli* O103 | (CDC 2020b) |
| Ready to eat chopped salad | *Escherichia coli* O157:H7 | (CDC 2020c) |
| Romaine lettuce | *Escherichia coli* O157:H7 | (CDC 2019f, 2020d) |
| Spring pasta salad | *Salmonella* Sandiego and *Salmonella* IIIb | (CDC 2018d) |
| Raw sprouts | *Salmonella* Montevideo | (CDC 2018e) |
| **Mushrooms** | | |
| Enoki mushroom | *Listeria monocytogenes* | (CDC 2020e) |
| **Cereals and grains** | | |
| Flour | *Escherichia coli* O26 | (CDC 2019k) |
| Wheat cereal | *Salmonella* Mbandaka | (CDC 2018h) |
| **Seed products** | | |
| Sesame seed paste | *Salmonella* Concord | (CDC 2019l, 2019m) |
| **Dried/frozen foods** | | |
| Dried coconut | *Salmonella* Typhimurium | (CDC 2018i) |
| Frozen shredded coconut | *Salmonella* I 4,[5],12:b:- and *Salmonella* Newport | (CDC 2018j) |
| **Egg and egg products** | | |
| Hard boiled eggs | *Listeria monocytogenes* | (CDC 2020f) |
| Shell eggs | *Salmonella* Enteritidis | (CDC 2018k) |
| | *Salmonella* Braenderup | (CDC 2018l) |
| **Meat** | | |
| Ground beef | *Salmonella Dublin* | (CDC 2019n) |
| | *Escherichia coli* O103 | (CDC 2019g) |
| | *Salmonella* Newport | (CDC 2018m) |
| | *Escherichia coli* O26 | (CDC 2018f) |
| Ground bison | *Escherichia coli* O103 and O121 | (CDC 2019h) |
| Sliced meats | *Listeria monocytogenes* | (CDC 2019i) |
| Ready-to-eat pork products | *Listeria monocytogenes* | (CDC 2019j) |

| | | |
|---|---|---|
| Ham | *Listeria monocytogenes* | (CDC 2018g) |
| **Poultry** | | |
| Ground turkey | *Salmonella* Schwarzengrund | (CDC 2019a) |
| Raw chicken products | *Salmonella* Infantis | (CDC 2019b) |
| Kosher chicken products | *Salmonella* I 4,[5],12:i:- | (CDC 2018a) |
| Raw turkey products | *Salmonella* Reading | (CDC 2019c) |
| Chicken Salad | *Salmonella* Typhimurium | (CDC 2018b) |
| **Dairy products** | | |
| Sliced cheese | *Listeria monocytogenes* | (CDC 2019i) |
| **Fish** | | |
| Frozen raw tuna | *Salmonella* Newport | (CDC 2019o) |
| **Shellfish** | | |
| Oysters | *Vibrio parahaemolyticus, Shigella flexneri,* STEC non-O157, *Vibrio albensis, Campylobacter lari,* | (CDC 2019p) |
| Crab meat | *Vibrio parahaemolyticus* | (CDC 2018n) |

Peptides and proteins with protective effects against *L. monocytogenes* could be used as natural preservative ingredients in meat products (Carvalho *et al*., 2018; Sant'Anna *et al*., 2013). *Triticum durum* Annexin 12 protein (TdAnn 12), possess a very high antioxidant and antimicrobial activity *in vitro,* its capacity to decrease the concentration *L. monocytogenes* was observed in beef meat, reducing the viable counts by a 2 log CFU/mL within 6 days under refrigeration conditions (Hsouna *et al*., 2020).

AMPs such as the sakacin C2 exhibits strong inhibitory activity against *E. coli* in milk and it dependens on the chemical composition and the additives supplemented to the product, as well as the conditions of processing. This bacteriocin may have the potential for inhibiting the growth of *E. coli* in milk and dairy products (Gao, Li, & Liu, 2013).

Food of vegetable origin have been evaluated as targets for application of enterocin AS-48 as a biopreservative. The application of washing treatments with enterocin AS-48 alone or in combination with other antimicrobial compounds was effective in the inactivation of *L. monocytogenes, Bacillus cereus,* and enteric bacteria in sprouts. In whole fruit pieces and sliced fruits (strawberries, raspberries, sliced melon, watermelon, pear, and kiwi), this protective effect against *E. coli* was also observed after treatment with enterocin AS-48 (Cobo-Molinos *et al*., 2005, 2008, 2009).

### Determinant features controlling antimicrobial activity of peptides

AMPs are highly heterogeneous in chain size, structure, and amino acid composition. Their size varies from 10-50 amino acid residues and their structures are diverse. However, some characteristics such as net positive charge and abundance of

hydrophobic amino acids, are quite frequent among AMPs (Pane *et al*., 2017). The key molecular features are determined by the amino acid sequence. Their composition is distinguished by being rich in lysine, arginine, tryptophan, and hydrophobic amino acids (Fjell *et al*., 2012). The cationic nature and unique geometry of arginine hydrogen bonds, in addition to the complex properties of tryptophan, enhance AMPs' activity. Their presence in the amino acid sequence increases the membrane binding ability of peptides; hydrogen bonds assist in the interaction with negatively charged surfaces (Jindal *et al*., 2014). Modification of AMPs' net charge has been used to enhance their activity, as evidenced in a study by Liu *et al*. (2019a). Their results showed that the positive charge was augmented via substitution of alanine for lysine in a peptide analogous to phylloseptin-PHa (PSPHa1). A rise in the antimicrobial activity of PSPHa1 was generated, highlighting the importance of AMPs' cationic charge in their membranolytic function. Studies approaching the design and synthesis of these molecules consider amphipathicity as a pivotal factor for the interaction of AMPs with microbial cell membranes. A balance between the hydrophobic and hydrophilic character of the peptide facilitates antibacterial activity and selectivity of AMPs, emphasizing a clearly amphipathic structure in an α-helical conformation (Zhong *et al*., 2020). This is supported by Rončević *et al*. (2019) in their study based on quantitative structure-activity relationship (QSAR) criteria for the evaluation of an algorithm that implements variations on peptides. The sequences were designed to position amino acids in a configuration where hydrophobic and polar residues did not disrupt amphipathicity, as it is known to correlate with potent membrane-directed activity. Otherwise, an excess of hydrophobic amino acids has been correlated with a strong hemolytic activity, because it obstructs the interaction between peptide and phospholipids of bacterial membranes, leading to a loss of selectivity (Ji *et al*., 2014; Tan *et al*., 2020). Although many AMPs have been discovered, it has been observed that the entire amino acid sequence is not always a requirement for an effective antimicrobial effect. According to previous reports small sequences of these natural peptides also exhibit antimicrobial activity, in some cases, higher than the original peptide. In this regard, the development of short-chain AMPs offers certain advantages such as ease of synthesis and lower reagent consumption. It is beneficial from an economic and environmental standpoint (Lyu *et al*., 2016; Strøm, Rekdal, & Svendsen, 2002a, 2002b).

There are extrinsic variables influencing AMPs' activity, including environmental factors, such as ionic strength and the presence of specific ions or solutes. At high salt concentrations, antimicrobial activity is decreased, and divalent cations stabilize bacterial membranes and compete with AMPs for the binding to anionic lipids (Sánchez-Gómez *et al*., 2008). Molecular characteristics of the target bacterial strain and its membrane composition are also relevant in AMPs' activity. Depending on the strain, the relative contribution of charge and hydrophobicity differs, consequently the optimal composition of an AMP can vary even among strains of the same species (Pane *et al*., 2017).

**Mechanisms of action**

The electrostatic interaction between the negatively charged phospholipids of the bacterial membrane and positively charged peptides is an important requirement in the mechanism of action of AMPs (Seyfi *et al*., 2019). The net negative charge is responsible for the initial adsorption of AMPs in the bacterial membrane, which is rich in anionic lipids; both Gram-positive and Gram-negative bacteria contain negatively charged membrane phospholipids, in addition, Gram-positive possess teichoic acid and Gram-negative have lipopolysaccharides (LPS), which are negatively charged substances (Feijó-Corrêa *et al*. 2019). Most accepted models to describe the AMP's mechanism of action are the barrel stave, toroidal pore and, carpet model.

In the barrel-stave model, AMPs initially bind to the membrane as a monomer, may undergo a conformational transition, and induce localized membrane thinning. After the threshold concentration is reached, the monomers oligomerize and become further inserted into the hydrophobic core of the membrane (Avci, Akbulut, & Ozkirimli, 2018; Ciumac *et al*., 2019). In this barrel-stave arrangement, the peptide chains aggregate laterally to form a cylinder superstructure, with their hydrophilic faces lining the water-filled lumen of the pore and their apolar residues pointing towards the membrane (Bocchinfuso *et al*., 2009). This mechanism has been associated with highly hydrophobic peptides, such as alamethicin, which has shown to exert a barrel-stave mechanism (Ageitos *et al*., 2017; Bertelsen *et al*., 2012; Pieta, Mirza, & Lipkowski, 2012). Among the known AMPs, only alamethicin, pardaxin and dermcidin displays pore formation under the barrel-stave model (Lee, Chen, & Huang, 2004; Ramamoorthy *et al*., 2010; Song *et al*., 2013).

The toroidal pore model has been associated with the formation of a distinctive pore-like structure where AMPs do not interact with one another, unlike the barrel-stave model, but cooperatively alter the local curvature of the lipid bilayer and promote the inward bending of lipids, leading to the formation of a toroid. The inner core of the pore is partly aligned by peptide and lipid heads (Kishore-Hazam, Goyal, & Ramakrishnan, 2019). 3D alignment of MSI-103, a peptide designed with a repeated heptameric sequence from peptidyl-glycylleucine-carboxyamide (PGLa), revealed that its antimicrobial activity is consistent with a toroidal pore model, in which both, peptides and lipids are aligned and stay in contact with the aqueous core of the pore (Strandberg *et al*., 2020). Another AMP that has been correlated with this model is LL-37, which can induce toroidal pores in lipid bilayers when its density is high enough on the surface. Pores induced by LL-37 have been described as dynamic and the monomers in the pores transfer dynamically among five transmembrane positions: the surface, the upper leaflet, the center, the lower leaflet, and the bottom (Li *et al*., 2016). However, some authors such as Zeth & Sancho-Vaello (2017), suggest that LL-37 generates fibril-like supramolecular structures on membranes, expressing a deviation of the toroidal pore model.

In the carpet model, peptides solubilize the membrane into micellar structures destabilizing it; peptides lying on the membranes reach a threshold concentration and spontaneously insert themselves in the barrel-stave model or form pores with peptide and lipids disposed alternately in the toroidal pore model (Torres *et al*., 2019). *Nicotiana alata* defensin 1 (NaD1) adopts a carpet-like configuration during the initial stages of its interaction with the target membrane (Järvå *et al*., 2018). According to Pfeil *et al*. (2018), Cecropin B, an AMP isolated from the moth *Hyalophora cecropia*, permeabilizes phospholipid bilayers through a carpet-like mechanism. Carretero *et al*. (2018) analyzed the peptide BP100 and its rationally designed analogous, they exhibited a carpet-like mechanism at high concentrations. At lower concentrations, peptides disturbed membrane organization, probably causing its thinning and increasing permeability. Oliva *et al*. (2019) characterized the binding of (P)GKY20 (modeled peptide from the Gly 271 to Ile 290 sequence in human thrombin) with biomembrane models. The results showed that (P)GKY20 selectively disrupted biomembranes resembling bacterial membranes through a carpet-like mechanism, using conformational changes and lipid segregation as key steps in membrane disruption.

**Application of AMPs in food preservation**

As other naturally produced antimicrobial agents, AMPs have gained prominence as a new generation of food preservatives that provides several advantages (Keymanesh *et al*., 2009). They are usually thermostable with good water solubility and broad-spectrum, some of them even have the ability to kill fungi and protozoa (Jiao *et al*., 2019). Organoleptic properties of food are affected to a lesser extent when compared to other antimicrobial agents such as oils and phenolic compounds. Although the majority of essential oils are classified as Generally Recognized As Safe (GRAS), there are concerns about their negative impact on sensory attributes (Chouliara *et al*., 2007; Gutierrez, Barry-Ryan, & Bourke, 2009; Lambert *et al*., 2001; Lv *et al*., 2011).

AMPs application in food preservation is still under review and continues in the process of overcoming technical limitations, such as large-scale production. AMP-producing lactic acid bacteria, such as *Lactobacillus fermentum*, can be applied as food preservatives since they are commonly used as starter cultures in fermented products and it can serve as cell factories for AMPs production. Besides the health-promoting benefits (probiotics) of *Lb. fermentum*, its proteinaceous compounds could perform the function of inhibiting contamination causing foodborne illnesses or food spoilage. Moreover, fermenticin produced by *Lb. fermentum* shows similar characteristics to other bacteriocins, including pH and temperature stability (Naghmouchi *et al*., 2019).

The spectrum of activity is a relevant aspect for AMPs incorporation in food, the selection should take into consideration the microorganisms that are generally present in the food product of interest and the AMP specificity towards them. AMPs such as defensins, which target lipid II, LTA, or lipid A at the bacterial membrane, are often active against a broad

series of Gram-positive and Gram-negative bacteria (Schmitt, Rosa, & Destoumieux-Garzón, 2016). In contrast, other AMPs show a narrow spectrum of activity, for example, it has been proven that peptides with abundant glycine residues specifically kill Gram-negative bacteria (Chou *et al*., 2019; Ilić *et al*., 2013). Evidence of this selectivity is also given by Xu *et al*. (2019), their work inquired into the antimicrobial activity of a low molecular weight (1.3-2.3 kDa) ε-Poly-L-lysine produced by a recombinant strain of *Streptomyces albulus*. Although this ε-Poly-L-lysine had a reduced inhibitory effect on bacteria, it had higher antimicrobial activity towards yeast, compared to the commonly used ε-Poly-L-lysine (3.2−4.5 kDa).

In this respect, despite the interest in the isolation and purification of AMPs, some research on peptide random mixtures has been conducted due to the diversity of sequences and structures observed in AMPs (Figure 1). A 20-mer random peptide mixture containing Lysine as the cationic component and either phenylalanine or leucine as the hydrophobic component, displayed strong antibacterial activity towards *E. coli, B. subtilis,* methicillin-resistant *Staphylococcus aureus,* vancomycin-resistant *Enterococcus faecium* and *L. monocytogenes* in a Minimum Inhibitory Concentration (MIC) range of 3-13 µg/ml. This finding provides evidence that AMPs can inhibit the growth of both Gram-negative and Gram-positive strains, as well as antibiotic resistant strains at very low concentrations (Amso and Hayouka 2019). In this way, peptide mixtures offer a broad spectrum of activity due to the variety of sequences that compose with the advantage of being less expensive. Stern Bauer & Hayouka (2018).

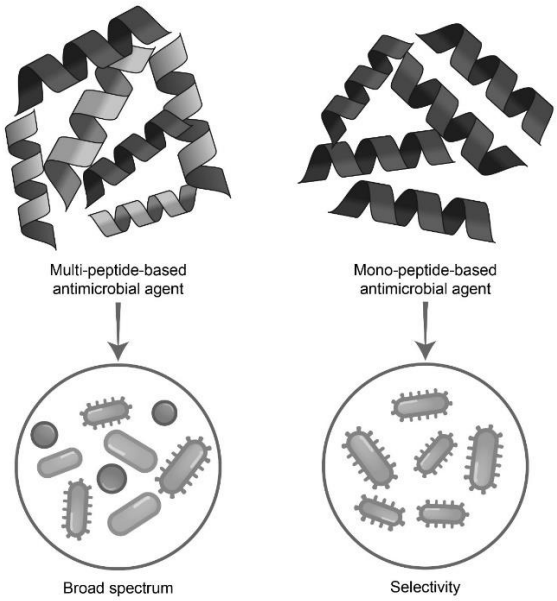


**Figure 1.** Differences in antibacterial activity of multi-peptide-based and mono-peptide-based antimicrobial agents.

In addition to antimicrobial activity, AMPs can present other activities of interest to the food industry, such as antioxidant activity. A study by Lima *et al*. (2019), reported both antibacterial and antioxidant activities in peptides from *Cynoscion guatucupa* protein hydrolysate obtained by enzymatic hydrolysis with Alcalase and Protamex. Peptide sequencing revealed that most abundant peptides in Alcalase hydrolysate (IELIEKPMGIF, RADLSRELEEISERL, DLAGRDLTDYLMKIL) and Protamex hydrolysate (LAGRDLTDYLMKIL, IITNWDDMEK, TALEEAEGTLEHEESKILR), share the presence of hydrophobic amino acids as phenylalanine, leucine, and tryptophan, which have been related with the interaction between peptides and bacterial membranes in the membranolytic activity of AMPs. They also reduced ~80% of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals in antioxidant activity assays. These findings provide evidence of the potential of AMPs to prolong the shelf life of food since they can contribute to the inhibition of lipid oxidation, as well as the growth of microbial agents.

Stability against factors that compromise antimicrobial activity, such as pH, temperature, and proteases, has been studied in different AMPs (Table 2). Some of the reported AMPs maintain antimicrobial activity in a wide pH range. A recombinant plectasin evaluated by Zhang *et al*. (2011), showed a strong inhibitory capacity on Gram-positive bacteria like *Staphylococcus aureus* in a pH range of 2-10, maintaining its antimicrobial activity even after exposure to a temperature of 100°C during 1 h.

Nevertheless, some peptides show stability at more specific pH ranges. In the case of Ano-D4 7–4C12, an analog of anoplin (AMP naturally isolated from the venomous sac of solitary spider wasps), antibacterial activity was observed after incubation at pH values between 6.2 and 8.2 (Zhong *et al*. 2020).

Modifying the sequence of AMPs in determined positions can confer tolerance to different pH conditions, for instance, the addition of histidine at the carboxyl terminus of a piscidin-like AMP enables a greater antimicrobial activity in an alkaline environment (pH 10.5) against *S. aureus* (Mao *et al*. 2013). In agreement with the Title 21 of the Code of Federal Regulations, low-acid foods are defined as those that have a finished equilibrium pH >4.6 and a water activity greater than 0.85, excluding alcoholic beverages, tomato, and tomato products. On the other hand, acidified foods are low-acid foods to which acids or acid foods have been added. They have a water activity greater than 0.85 and have a finished equilibrium pH of 4.6 or below. Acid foods are those that have a natural pH of 4.6 or below (21 CFR 113 2019; 21 CFR 114 2019). In this context, different AMPs could be applied to low-acid foods, as well as acid and acidified foods.

**Table 2.** Stability of antimicrobial peptides against proteases, heat and pH conditions.

| Peptide | Source | Sequence | Inhibitory concentration | pH | °C | Proteases | Mechanism of action | Reference |
|---|---|---|---|---|---|---|---|---|
| Plectasin | Recombinant (produced by *Pseudoplectania Nigrella* y expressed in *Pichia pastoris*) | GFGCNGPWDEDDMQC HNHCKSIKGYKGGYCAK GGFVCKCY | 2.56 µg/mL (Gram+) | 2-10 | 100 | Papain, pepsin | Cell wall precursor binding (lipid II) | (Zhang *et al*. 2011) |
| Ano-D4, 7–4$C_{12}$ | Synthetic (analogous to anoplin) | GLLKRIKTLL | 2-8 µM (Gram+ y Gram-) | 6.2-8.2 | ND | Trypsin | Plasma membrane disruption and intracellular action | (Zhong *et al*. 2020) |
| Pc-pis-His | Synthetic (based on Ps-pis, produced by *Pseudosciaena crocea*, with histidine addition) | IWGLIAHGVGHVGRLIHG LIRGH | 0.75-24 µM (Gram+ y Gram-) | 5.5-9.5 | 100 | ND | ND | (Mao *et al*. 2013) |
| CAM-W | Synthetic (Mutant of chimeric cecropin Cecropin A-melittin) | KWKLWKKIEKWGQGIGA VLKWLTTWL | 0.3 – 2.8 mg/mL (Gram+ y Gram-) | 2-9 | 20-90 | Trypsin pepsin, Human neutrophil elastase, *P. aeruginosa* elastase, V8 protease from *S. aureus*. | ND | (Ji *et al*. 2014) |
| C–L | Synthetic (Cecropin A and LL37 hybrid) | KWKLFKKIFKRIVQRIKDF LRN | 2.0 – 7.2 µg/mL (Gram+ y Gram-) | 4 – 11 | 80 | Trypsin, proteinase K | ND | (Wei *et al*. 2018) |
| HJH-1 | Synthetic (Analogue to P3, isolated from bovine erythrocytes) | KLLKHKLLVTLA | 6.25 - 50 µg/mL (Gram+ y Gram-) | 4-10 | 100 | ND | Membrane disruption | (Wang *et al*. 2018) |
| rHispidalin | Recombinant (hispidalin from *Benincasa hispida* expressed in *Pichia pastoris*) | MHHHHHHDDDDKSDYL NNNPLFPRYDIGNVELST AYRSFANQKAPGRLNQN WALTADYTYR | 40-60 µg/mL (Gram+ y Gram-) | 2-10 | 4-100 | Trypsin, proteinase K | Membrane disruption | (Meng *et al*. 2019) |
| LHP7 | Synthetic (hybrid peptide of LHP28 and plectasin) | LH28-(GPDGSGPDESGPDES)-plectasin LH28: FKCWRWQWRWKKLGA KVFKRLEKLFSKI Plectasin: GFGCNGPWDEDDMQC HNHCKSIKGYKGGYCAK GGFVCKCY | 0.023-11.66 µM (Gram+ y Gram-) | 2-10 | 4-90 | Pepsin, papain | ND | (Xi *et al*. 2013) |
| Cap18 | Rabbit neutrophils | GLRKRLRKFRNKIKEKLK KIGQKIQGLLPKLAPRTD Y | >32 µg/mL (Gram-) | ND | 90 | Susceptible to Trypsin, proteinase K | ND | (Ebbensgaard *et al*. 2015) |
| CF-14 | Catfish epidermal mucosa | RIVELTLPRVSVRL | 62.5 µg/mL (Gram-) | 4-12 | 80 | Pepsin, trypsin, proteinase K. | Intracellular action | (T. Li *et al*. 2019) |

The thermostability of AMPs is very important in food production since thermal treatments are generally used during food processing (Al-sahlany *et al*. 2020). Dairy products are subjected to thermal treatments with the primary aim of ensuring food safety and extending the product shelf life by destroying pathogenic microorganisms, reducing spoilage microorganisms, and inactivating enzymes. In the case of pasteurization,

thermal processing conditions vary widely, and industry parameters range from 70 to 85°C for 1 to 10 s in standard pasteurization while ultra-high-temperature processing (UHT), requires temperatures within 135°C to 150°C during 10 s (H. Liu *et al*., 2019b). The thermal resistance of an AMP produced by *Saccharomyces cerevisiae* was studied by Al-sahlany *et al*. (2020), the results showed that the peptide was stable in a temperature range from 50 to 90°C for 30 min. Antimicrobial activity was retained (93-95%) even after treatment at 100°C for 30 min. This thermal stability could be attributed to their nature and chemical structures, including primary structures with low molecular weight. A 30% reduction in antimicrobial activity was also exhibited at 120°C, suggesting that this activity could be lost during a UHT treatment. The peptide HJH-1 from bovine erythrocytes maintained its antibacterial activity against *E. coli* after heat treatment at 100°C for 30 min. Nonetheless, a slight reduction in activity could be observed as temperature increased. In this light, it could play an antibacterial role in foods that undergo thermal processing at temperatures <100°C as in standard pasteurization.

Industrial processes involving the use of proteases have been constantly introduced because proteolysis is a powerful tool in the modification of the properties of proteins in food systems, including changes in solubility, gelation, emulsifying and foaming characteristics, reduction of protein allergy, taste transformation or bioactive peptides liberation (Tavano 2013). In the dairy industry, proteases are used for milk coagulation, cheese ripening, and flavor development. Other applications in food processing are meat tenderization, gluten development, and dough preparations (Philipps-Wiemann 2018). A study conducted by Meng *et al*. (2019), proved that the exposure to proteases like pepsin and pancreatin in rHispidalin produced a reduction of the inhibition diameters by 50% in antibacterial activity assays against *S. aureus.* In contrast, rHispidalin was considered resistant to trypsin and proteinase K since treatment with these enzymes did not significantly alter their inhibition diameters. A decrease in the antimicrobial activity of the peptide Cap18 against *E. coli* was observed by Ebbensgaard *et al*. (2015) through an increase in MIC value from 8 to 16 µg/ml after incubation of Cap18 with proteinase K for 2 min. This variability in protease susceptibility can be explained by their proteolytic specificity of enzymes, an example of this is the capacity of trypsin and chymotrypsin to attack peptides at basic residues (lysine and arginine) and hydrophobic residues (tryptophan and phenylalanine), respectively (Kim *et al*. 2014). According to Koh *et al*. (2018), the stability of a peptide to enzymatic attack is related, besides its amino acid sequence, to its size, flexibility, and conformation.

AMPs stability has also been related to geometrical properties, such as radius of gyration, ovality, lipophilicity, surface area, and polar surface area. The distribution of the secondary structure has been determined as an important parameter for stability studies of peptides (Senthilkumar *et al*. 2017). Protegrin-1 was identified by Shruti & Rajasekaran

(2019) as a stable AMP and it was found that a high number of hydrogen bonds with distances less than 2.5 Å could significantly influence the stability of the peptide.

### Possible reactions of peptides within food systems

There are many molecules in foods that can react or interact with peptides, thereby, reducing the bioactivity of these peptides. This makes them highly susceptible to undergoing structural changes and reactions with the food matrix during product development (Udenigwe and Fogliano 2017). Nucleophilic reactivity of amino acids makes peptides inherently reactive (Brotzel and Mayr 2007). Peptides are subjected to modifications because they contain reactive groups such as free amino, carbonyl, and sulfur-containing functional groups. They can undergo backbone sidechain modification leading to changes in their native structure and formation of new compounds (Van Lancker, Adams, and De Kimpe 2011). In foods, Maillard reactions can occur by condensation of amino groups on peptides with carbonyl groups on reducing sugars, resulting in Schiff base formation and rearrangement to Amadori products (Lund and Ray 2017).

Apart from components naturally present in foods, proteins and peptides can interact with food additives, such as sodium nitrite, which is used in cured meat to enhance color but also as an antimicrobial agent (Kamdem and Tsopmo 2017). The pro-oxidant effect of sodium nitrite on proteins has been reported previously (Feng *et al*. 2016; Ozyurt and Otles 2020). Nitrite and ascorbate are common additives in dry fermented sausages and are used, among other reasons, to control the oxidative stability. Their effect on lipid oxidation is well-established in contrast to protein oxidation. The simultaneous addition of nitrite and ascorbate might increase the formation of carbonyl compounds in proteins. This increased protein carbonylation might alter the structure and functionality of proteins, compromising their technological properties (Berardo *et al*. 2016).

Hydrogen peroxide is a strong oxidant that is conventionally used as a chlorine-free bleaching and antimicrobial agent, permitted for direct contact with food by regulatory authorities in the United States and other jurisdictions (Ma *et al*. 2020). Residual hydrogen peroxide must be removed from foods, and the allowance level is 0.05% (w/w) (21 CFR 184.1366 2019). Residual hydrogen peroxide can generate hydroxyl radicals in the presence of radiation or metal ions such as iron or copper and can initiate the oxidative damage to proteins through the abstraction of a hydrogen atom from susceptible moieties in proteins and peptides (Hawkins and Davies 2001).

Sulfur species are used as preservatives in the food industry by their antiseptic and antioxidant properties. They help to bleach pigments and eliminate unpleasant odors resulting from oxidation (Gabriele *et al*. 2018). Sulfites react with the disulfide bonds of cystine, peptides, and proteins to form S-sulfonates which are considered irreversibly bound forms. This sulfonation reaction is governed by the accessibility of the disulfide

groups (García-Alonso, Peña-Egido, and García-Moreno 2001). In raw and cooked meat products with sulfite added, losses in the content of sulfite and the formation of S-sulfonates were observed, this can be attributed to the generation of the above mentioned irreversibly bound forms (Peña-Egido, García-Alonso, and García-Moreno 2005).

Hence, less reactive matrices are imperative to preserve AMPs' integrity and stability. As suggested by Sun *et al*. (2020), fiber-rich food is a promising matrix due to its limited chemical reactivity. Fiber-rich foods include whole-grain bread and cereals or pseudocereals (wheat, maize, oats, rye, barley, triticale, millet, sorghum buckwheat, etc.), legumes and soy, fruits, vegetables, nuts and seeds (Miller Jones 2014). In contrast, food matrices with high reactivity, like most liquid-based food formulations should be avoided (Sarabandi, Gharehbeglou, and Jafari 2020).

### Active packaging and nanotechnologies

To limit interactions between AMPs and food components and to control their release, the development of active packaging has been introduced. Active packaging systems are developed to extend shelf life of foods and increase the period in which the food preserves its high quality (Gorrasi *et al*. 2020). An advantage of active packaging is the reduction or possible elimination of preservatives from food formulations due to the graduate migration of active compounds (Janjarasskul and Suppakul 2018). Lysozyme-incorporated packaging films were evaluated using free and complexed forms of lysozyme and it was found that complexation with polyacrylic acid was an effective tool to reduce its release rate, allowing sustained lysozyme release for extended periods (Peksen-Ozer *et al*., 2016). These observations suggest great potential of films incorporated with AMPs for food preservation and shelf-life extension.

Nanostructure materials have been studied for the protection of AMPs antimicrobial activity, for example, nanoparticles, nanoliposomes and nanofibers (Mahdavi, Mohammad, and Nouri 2019; Soto *et al*. 2016; Wu *et al*. 2017). In recent studies, AMPs have been loaded into nanocarriers in conjunction with other bioactive compounds. Nisin was successfully loaded into soy soluble polysaccharide (SSPS)-based nanocarriers, serving not only as an antimicrobial compound but also as a carrier material for loading curcumin as an antioxidant bioactive compound. The formation of these nanoparticles was mediated by the amphiphilic character and positive charge of nisin, resulting in multifunctional SSPS-based nanocarriers with antimicrobial and antioxidant activity, which also showed enhanced stability of nisin and curcumin (Luo *et al*. 2020). It should be emphasized here, that further investigation is needed to demonstrate the efficacy of these new technologies in real food systems. The peptide 1018K6 (bactenecin-derivative peptide) was covalently conjugated to a polyethylene terephthalate (PET) matrix to create a plastic packaging material with antimicrobial properties that was tested under real conditions using mozzarella cheese. It strongly reduced yeast and mold counts during the first 24 (Agrillo *et al*. 2019).

Biopolymers can be used to substitute non-biodegradable plastics with natural and eco-friendly materials, reducing the environmental impact and oil-dependence (Muller, González-Martínez, and Chiralt 2017). However, the poor stability, high sensitivity to environmental changes, weak mechanical and poor barrier properties of biodegradable polymers, such as starches, have led to the study of nanofillers for the improvement of physical, mechanical and barrier properties of these materials (Armentano *et al*. 2018; Esmaeili, Pircheraghi, and Bagheri 2017). Pediocin and nisin has been incorporated into corn starch films with antimicrobial activity against *L. monocytogenes* and *Clostridium perfringens*. The addition of halloysite clay improved mechanical and thermal performance (Meister-Meira *et al*. 2016). Controlled release by retention of the antimicrobial compounds in polymer matrices is also achieved by the use of nanofillers, due to the adsorptive capacity of nanoclays (Jamróz, Kulawik, and Kopel 2019).

**AMPs used in the food industry**

Nisin is the most important commercially available AMP approved as food preservative. It belongs to the lantibiotic class of bacteriocins produced by *Lactococcus lactis* subsp. *lactis* and has been declared by the Food and Drug Administration (FDA) as GRAS. Nisin is applied as a safe and natural food preservative in over 50 countries and can be used to preserve processed cheese, pasteurized dairy products and canned vegetables. More recent applications of nisin have been reported in high moisture products, hot baked flour products and pasteurized liquid eggs (Hwanhlem *et al*. 2017).

Ɛ-polylysine is a cationic AMP produced by *Streptomyces albulus*, and it is composed by 25-35 L-lysine linked through their carboxyl and Ɛ-amino groups rather than a conventional peptide bond. Ɛ-polylysine has a wide antibacterial spectrum of activity and has a lethal effect on Gram-positive and Gram-negative bacteria, yeast, mold and viruses. It shows good antibacterial effect against *E. coli* and *Salmonella*, pathogens that are difficult to control with other natural preservatives. Ɛ-polylysine has been generally used as food additive in Japan, Korea and other parts of the world. In the United States (U.S.), FDA has recognized it as a GRAS material (Luz *et al*. 2017).

Pediocin PA-1 is another bacteriocin used as a food preservative. It is composed of 44 amino acids and is produced by *Pediococcus acidilactici*. Pediocin PA-1 is on the market under the name ALTATM 2431. This AMP has been used to improve food shelf life mainly on ready-to-eat meat products, showing activity against *Listeria monocytogenes* growth (Johnson C P Santos *et al*. 2018).

## Food additive regulations

The definition of a food additive refers to a substance becoming a part of food “directly or indirectly” either becoming a component of food or used in the production of packaging material if it may be expected to become a component or to affect the characteristics of the packaged food, this is further described in the Code of Federal Regulations (21 CFR §170.3 2017). With antimicrobial food additives, to demonstrate that an antimicrobial agent achieves its intended technical effect it is necessary a detailed description of the antimicrobial effect and identification of any individual or groups of targeted microbes, a description of the conditions of use and any limitations on conditions of use, such as type of foods, proposed use level, the temperature range of use and method of application. Antimicrobial effect data, including full reports of the efficacy studies and directions and suggestions regarding the proposed use, are also required (FDA, 2008). The approval process is initiated as a food additive petition (FAP) or as a GRAS petition. A GRAS substance is neither safer nor less safe than an approved food additive, the difference is that, for a GRAS substance, there is common knowledge of safety within the expert community. This indicates that both types of approved substances meet the same standard of safety (Burdock and Carabin 2004). It is also important to note that U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) is responsible for determining the suitability of food additives in meat and poultry products (FDA, 2008). The highly rigorous evaluation AMPs must be subjected by regulatory agencies, could be a reason for its limited used in the food industry.

## Future perspectives of AMPs application in food industry

Food preservatives and physical treatments traditionally used in the food industry to prevent microbial growth may be associated with health negative effects and loss of nutritional and organoleptic characteristics of food. For this reason, identification and characterization of new antimicrobial such as AMPs have been of great importance, since it could contribute reducing the amount of synthetic additives in food products, as well as the intensity of physical treatments, to generate products that meet consumer demand, with the attribute of minimally processed. Despite the large number of studies conducted in this field, there are few AMPs authorized by regulatory agencies for food application. The need for studies on the behavior of these molecules within complex food systems is imperative since the effect of some food processing parameters on AMP activity, such as temperature and pH, has been studied separately. A closer approach to set specific conditions that must be simultaneously present during food processing is required to determine AMPs' applicability, stability, and possible reactions with food components. It should be emphasized that, even though multiple efforts have been introduced to make AMPs suitable for the food industry, the adaptation of the food industry for the inclusion of AMPs have not been well considered, this can imply less aggressive food processing, which can be beneficial to preserve the sensory and quality properties of foods.

## Conclusions

A great diversity of low molecular weight AMPs and generally positive net charge, with wide spectrum against bacteria, fungi and viruses, have been reported. Their amphipathicity allows them to be soluble in both aqueous and lipid-rich environments, this characteristic gives them the ability of permeabilizing microbial membranes. Its inhibitory capacity against foodborne pathogens has been supported by research, being effective in the elimination of a wide range of microorganisms. AMPs have also exhibited antimicrobial activity under different food processing conditions. Moreover, *in situ* studies, have demonstrated the inhibitory effects of AMPs in food models. However, there are still concerns about the integrity and stability of this molecules in real food systems. The increasing demand for new antimicrobial agents has led to the study of strategies such as chemical and amino acid sequence modifications, to develop AMPs from existing ones with the aim of granting more pronounced antimicrobial activity and greater stability. Incorporation of AMPs into food packaging have also been studied, to assure AMPs delivery into food surfaces gradually, limiting the direct exposure with food components that can lead to different reactions and formation of new compounds. Characterization of interactions between AMPs and food components, as well as possible newly formed compounds remains a challenge in this field. Further studies are needed to define the conditions of use, such as type of foods, proposed use level, the temperature range of use and method of application in order to get official approval for food applications.

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# Antibacterial peptide fractions from chia seeds (*Salvia hispanica* L.) and their stability to food processing conditions

## Antibacterial agents from chia

Anaí León Madrazo[1], Alfredo Benjamín Fuentes Ortíz[2] and Maira Rubi Segura Campos[1*]

[1]Facultad de Ingeniería Química, Universidad Autónoma de Yucatán. Periférico Norte Km. 33.5, Tablaje Catastral 13615, Col. Chuburná de Hidalgo Inn, 97203 Mérida, Yucatán, México. [2]Facultad de Química, Universidad Autónoma de Yucatán. Calle 43, No. 613 x calle 90. Col. Inalámbrica, 97069 Mérida, Yucatán, México.

*Corresponding author: maira.segura@correo.uady.mx

## Abstract

**Aim:** Evaluate the antibacterial activity and stability of chia peptide fractions (CPFs) for potential applications in food preservation.

**Methods and results:** CPFs (F <1, F 1-3, and F 3-5 kDa) were obtained by enzymatic hydrolysis of a protein-rich fraction and ultrafiltration. The electrophoretic profile of CPFs was determined by Tricine-SDS-PAGE and *in vitro* disk diffusion was performed against a set of foodborne bacteria to evaluate antibacterial activity. Minimum Inhibitory and Bactericidal Concentrations (MIC and MBC) of F <1 were determined. Stability under different conditions of pH, temperature, and proteolysis was assessed to investigate F <1 resistance to food processing. Only Gram-positive bacteria were susceptible to FPCs and the most active fraction was F <1. The lowest MIC was reported (635.4 ± 3.6 µg $mL^{-1}$) against *Listeria monocytogenes* and F <1 remained active after incubation at 4-80°C and a pH range of 5-8, but inactive after exposure to pepsin and trypsin. Sequences in F <1 were analyzed using The Technique for Order of Preference by Similarity to Ideal Solution (TOPSIS) determining that KLKKNL was the peptide with the highest antimicrobial potential observed in F <1.

**Conclusions:** F <1 exhibited features of interest for food preservation, such as antimicrobial activity and stability in a wide range of temperatures. The inhibitory effect of F <1 could be attributed to the peptide KLKKNL.

**Significance and impact of the study:** Due to its inhibitory capacity against Gram-positive bacteria and its resistance to certain food processing conditions, F <1 could be an alternative to chemical preservatives in the food industry.

**Keywords:** Chia seeds, peptide fractions, antibacterial activity, stability, food preservation, foodborne bacteria

## Introduction

Microbial spoilage caused by pathogenic microorganisms reduces the shelf-life of foods and increases the risk of foodborne illnesses (Krepker *et al*. 2017). In low-income countries, food loss is mostly attributed to microbial contamination. Improvements in food preservation are necessary to reduce food waste and limit foodborne diseases which each year cause over hundreds of thousands of deaths (WHO 2015). Most food poisoning reports are associated with bacterial contamination, especially Gram-negative bacteria like *Salmonella* Typhi, *Escherichia coli*, and *Pseudomonas aeruginosa*. Other Gram-positive bacteria including *Staphylococcus aureus* and *Bacillus cereus* have been also identified as the causal agents of foodborne diseases or food spoilage (Mostafa *et al*. 2018). Food additives are used as antimicrobial agents to extend the shelf life of food by shielding them against deterioration caused by microorganisms, however, they have been associated with health side effects (Silva and Lidon 2016). Additionally, in the last decades, it has become apparent that long-term sub-lethal exposure to these antimicrobial agents can exert a selective pressure leading to the emergence of microbial strains with reduced susceptibility to these antimicrobials, which can persistently colonize food-processing environments and contaminate food (Oniciuc *et al*. 2019). Consequently, the demand for natural antimicrobial alternatives is expected to increase steadily as the negative influence exerted by some synthetic preservatives on the consumers' health has been demonstrated (Pisoschi *et al*. 2018). In this respect, there is an increasing interest in antimicrobial peptides (AMPs) research and their application in food preservation due to their inhibitory capacity against a broad spectrum of bacteria. AMPs are an abundant and diverse group of molecules produced in a variety of plant and animal species; they consist generally from 10 to 50 amino acid residues and are part of the innate immune response of organisms (Liu *et al*. 2017). Their amino acid composition amphipathic character, cationic charge, and size allows them to interact with microbial membranes and destabilize it (Sánchez and Vázquez 2017). AMPs exhibit a broad structural variety, but it is possible to categorize them into two groups according to their secondary structures: α-helical and β-sheet conformed peptides. The mechanism of action of PAMs suggests a disruption of bacterial membranes, which can be attributed to the cationic character of these molecules given by the presence of lysine, arginine, and histidine residues (Johnson C.P. Santos *et al*. 2018). Hydrophobic amino acids also confer antimicrobial activity since they improve the solubility of lipids and easily pass through the cell membrane (Dash and Ghosh 2017). Peptides with antimicrobial potential can be produced through *in vitro* controlled hydrolysis of food proteins such as plant proteins (Bojórquez *et al*. 2013). The seeds of flowering plants usually accumulate large quantities of seed protein varying from 10% (in cereals) to 40% (in certain legumes and oilseeds) of the dry weight (Liu *et al*. 2017). For these reasons, seeds have been studied for the obtention of hydrolysates and peptide fractions (PFs) with biological activity. Antibacterial activity of peptide fractions (PFs) from lima bean (*Phaseolus lunatus*) obtained by enzymatic hydrolysis with a sequential pepsin-pancreatin system, was studied by Bojórquez *et al.* (2013), the low molecular weight PFs (<10 kDa) presented the greatest

inhibitory effect at a MIC of 392.04 µg $mL^{-1}$ against *Staphylococcus aureus* and 993.17 µg $mL^{-1}$ for *Shigella flexneri*. Hwang *et al.* (2016) performed enzymatic hydrolysis of flaxseed (*Linum usitatissimum*) with a bacterial protease from *Bacillus altitudinis* HK02, the <1 kDa fraction inhibited the growth of *Escherichia coli* and *Pseudomonas aeruginosa* at 60 µg $mL^{-1}$. The MIC value registered in plant-derived peptides among different studies is found between 8-100 µg $mL^{-1}$ (Cardillo *et al*. 2018).

Peptides can be also obtained from residual wastes generated by agro-industries, allowing the valorization of residues with the advantage of lower production costs and no negative environmental impacts involved, thus being of great economic relevance. An example of these agro-industrial wastes are seed cakes from oiled plants, such as chia (*Salvia hispanica* L) (Beltrán-Ramírez et al. 2019). *S. hispanica* L. is an herbaceous plant, native to northern Guatemala and southern Mexico (Grancieri *et al*. 2019b). Its protein content (15-25 %) is greater than other traditional grains used in the food industry (Ayerza and Coates 2011; Pereira da Silva *et al*. 2017) and therefore it represents a promising source of bioactive peptides (Coelho 2018). Segura-Campos *et al.* (2013), evaluated the antibacterial activity of chia hydrolysates obtained with a sequential alcalase-flavourzyme system during 90 and 60 min. Antibacterial activity was not displayed in chia hydrolysates against *Escherichia coli*, *Salmonella* Typhi, *Shigella flexneri*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis* y *Streptococcus agalactiae*. On the other hand, Coelho *et al.* (2018), obtained antibacterial protein hydrolysates from partially defatted chia flour, using enzymatic hydrolysis with alcalase-flavourzyme during 60 and 180 min, respectively. *S. aureus* was susceptible to chia hydrolysates at a CMI of 2.26 mg $mL^{-1}$ and CMB of 5 µg $mL^{-1}$. The inhibition of *E. coli* was not observed. Some studies show the antimicrobial effect of plant-derived plants with low molecular weights (< 10 kDa) (Bojórquez *et al*. 2013; Cardillo *et al*. 2018; Hwang *et al*. 2016). However, antimicrobial activity has not been investigated in chia peptide fractions. Therefore, this study aimed to evaluate the antibacterial activity of chia peptide fractions (CPFs) and their stability for potential applications in food preservation.

## Materials and methods

### *Plant material and reagents*

Chia seeds were harvested in January 2017 from Jalisco State, Mexico. They were identified as seeds of *Salvia hispanica* L. and voucher specimen (identification number 69494) was deposited in the herbarium of the Center of Scientific Investigation of Yucatan (CICY). All chemicals were of reagent grade purchased from Sigma Chemical Co., Merck, and J.T. Baker.

### *Bacterial strains and culture media*

Bacterial strains used in this study were selected due to their relation to foodborne diseases and they were provided from the culture collection in the Biotechnology and Microbiology Lab of the Autonomous University of Yucatan: *Listeria monocytogenes* ATCC51414*, Bacillus subtilis* ATCC465*, Shigella flexneri* ATCC9748*, Staphylococcus aureus* ATCC25923, *Salmonella* Typhimurium ATCC51821, *Salmonella* Typhi, *Salmonella* Paratyphi ATCC9150, *Salmonella* Enteritidis ATCC13076 y *Escherichia coli* O157:H7. Bacterial strains were maintained at -20°C in BHI containing 40% glycerol (v/v). Disk diffusion and microplate dilution assays were performed in Mueller Hinton agar and BHI broth, purchased from MCD LAB.

### *Obtention of degummed and defatted chia flour*

Impurities and hulls were removed to extract mucilage and oil from chia seeds according to the methodology reported by Salazar *et al.* (2020) with some modifications. Degummed flour was obtained removing mucilage from seeds in a suspension with distilled water (1:40 w/v) with continuous agitation during 90 min at room temperature. Seeds were dried in a Fisher Scientific stove for 24 h at 50°C, then seeds were passed through a Thomas-Wiley mill to obtain a coarse grind and pressed with an 8 tons TRUPPER hydraulic jack to extract chia oil. The resulting thick flour was subjected to exhaustive oil extraction using the Soxhlet method with hexane as solvent at 70°C for 2h. To ensure oil removal the Soxhlet procedure was repeated. The flour was passed through a 0.5 mm mesh and in a Ro-tap sieve shaking system for 30 min with a 140 µm mesh to yield a chia protein-rich flour (CPRF). The percentage of humidity was determined as described by the AOAC gravimetric method (925.09) with the following equation: $\%H = [(P_i - P_2)\ P_m^{-1}]\ 100$, where $P_i$: constant weight of the crucible with the sample, $P_2$: weight of the crucible with dry sample and $P_m$: exact weight of the sample.

### *Obtention of hydrolysate and peptide fractions*

Enzymatic hydrolysis and ultrafiltration were performed as reported by Martínez Leo and Segura Campos, (2020). For the hydrolysis of CPRF, an enzymatic pepsin-pancreatin sequential system was applied for 90 min. A substrate concentration of 4% and an enzyme-substrate ratio of 1:10 was used. The protein-rich fraction was adjusted to pH 2 using 6 N HCl and hydrolyzed by treatment with pepsin (Sigma) for 45 min, followed by treatment with pancreatin (Sigma) for another 45 min, the temperature was kept at 37°C. Hydrolysis was stopped by heating to 80°C for 30 min, followed by centrifuging (Hermle Z300K centrifuge) at 3350 x *g* for 20 min at a temperature of 4°C to remove the insoluble portion. Subsequently, degree of hydrolysis (DH) was calculated by determining free amino groups with o-phthaldialdehyde (OPA) following the spectrophotometric method by Nielsen *et al.*, (2001) through the equation: $DH: (h\ h_{tot}^{-1})\ 100$; where $h_{tot}$ is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed

bonds. Chia protein hydrolysate underwent an ultrafiltration process using three membranes (1, 3, and 5 kDa). The hydrolysate was first passed through a 1 kDa cutoff membrane, then, the retentate was filtered with the 3 kDa cutoff membrane and the same process was performed with the 5 kDa cutoff membrane. Permeated fractions of 3 different molecular ranges were collected in sterile containers and denominated F <1, F 1-3, and F 3-5. Protein concentration of chia seeds hydrolysate and peptide fractions was determined by colorimetric assay with Folin-Ciocalteau reagent (Sigma) using bovine serum albumin (Sigma) as standard.

### *Tricine-SDS-PAGE*

Tricine-SDS-PAGE was performed as described by Schägger (2006), in a vertical gel unit Mini-PROTEAN tetra cell from BioRad. Migration was carried out in a 16% separating gel added with 6M urea. Spacer and stacking gel concentrations were 10% and 4%, respectively. Low molecular weight standards (1.06 – 26.6 kDa) from the Ultra-Low Range Molecular Weight Marker Sigma kit were applied. The electrophoretic run was conducted at 30V through the stacking gel and constant 90V until the tracking dye (Coomassie brilliant blue G250) reached the bottom of the gel. The gel was incubated for 30 min in a fixing solution (50% methanol, 10% acetic acid, and 100 mM ammonium acetate), then it was stained in a 0.025% Coomassie brilliant blue and 10% acetic acid solution. The gel was destained in 10% acid acetic solution with 15-60 min incubations and was transferred to water to remove acetic acid. The relative molecular weights of the resolved peptide fractions were compared with those of molecular weight markers, using the curve of molecular weight standards and relative migration distance.

### *Antibacterial activity*

Antibacterial susceptibility to CPFs was assessed using *in vitro* disk diffusion and Minimum Inhibitory Concentration and Minimum Bactericidal Concentration in microplate dilution, following criteria described by the Clinical and Laboratory Standards Institute (CLSI).

#### *Inoculum preparation*

To reactivate bacteria from stock culture, 50 µL of the bacterial sample was transferred to 5mL of BHI broth and incubated 24h at 37°C. Bacteria were transferred from BHI to Mueller-Hinton solid agar to obtain fresh colonies. Plates were incubated at 37°C for 24h in an inverted position. The top of 3-5 colonies was touched with a sterile loop and transferred into a tube containing 5 mL of sterile saline (0.85% NaCl) until a turbidity equivalent to a 0.5 McFarland standard (approximately 1-2 x $10^8$ CFU $mL^{-1}$ for *E. coli* ATCC 25922) was achieved.

***Disk diffusion test***

Bacterial suspensions with a 0.5 McFarland equivalent turbidity were inoculated in 100 mm Petri dishes with Mueller Hinton agar by streaking a sterile swab over the entire agar surface. To ensure an even distribution of the inoculum, the procedure was repeated by streaking two more times, rotating the plate approximately 60° each time. Subsequently, disks of 6 mm diameter were placed onto the surface of the inoculated agar plate. Each disk was pressed down and distributed on the agar surface. Disks were loaded with 10 µL of each CPF at different concentrations (500, 250, and 125 µg $mL^{-1}$). As a positive control for Gram-positive bacteria 500 µg $mL^{-1}$ penicillin was employed, while 500 µg $mL^{-1}$ gentamicin was used for Gram-negative bacteria. Benzoic acid (1%) was also applied as a control since this is a preservative added in the food industry and distilled water was used as a negative control. Inverted inoculated plates were incubated for 24 h at 37°C. After incubation, inhibition zones were measured with a Vernier. Bacterial susceptibly to CPFs was determined as follows: no inhibition zone (-), diameter <10mm was considered inactive (+), diameter between 10 and 13 mm was considered partially active (++) and diameter >14 mm was considered active (+++) (Valgas *et al*. 2007).

***Minimum Inhibitory Concentration and Minimum Bactericidal Concentration***

MIC and MBC values were determined in the FPC with the greatest antibacterial activity against susceptible strains, following the method reported by Coelho *et al.* (2018) with adjustments. Sterile 96 well microplates (Costar) containing 100 µL of BHI broth, 100 µL of FPCs (1000, 850, 700, 550, 400, 250 µg $mL^{-1}$) and 100 µL of bacterial inoculum (80 µL of BHI broth and 20 µL of sterile saline with bacteria adjusted to a 0.5 McFarland equivalent). The inhibition of bacterial growth was evaluated through registered absorbance at 620 nm in a Multiskan Plus microplate reader (Thermo Electron Corporation) after incubation for 24 h at 37°C. To calculate CMI values, the absorbance of wells with CPFs at different concentrations, positive control (well containing BHI broth), and negative control (well with bacterial inoculum) was considered. CMI was established as the concentration at which 50% of bacterial growth reduction was observed in culture media with CPFs. For MBC determination, 15 µL from wells exhibiting bacterial growth inhibition were extracted and inoculated on Mueller Hinton agar plates. Inverted plates were incubated for 24 h at a temperature of 37°C. CMB was considered as the concentration at which bacterial growth was not observed.

## *Temperature, pH and protease effect on antibacterial activity*

Stability assays were carried out as proposed by Essig *et al.* (2014) with adjustments, to investigate the resistance of F <1 under different food processing conditions, such as temperature, pH, and proteolysis. Thermal stability was tested in PBS buffer, pH 7.4, at 4, 25, 60, 80, 100°C for 1 h of incubation. The pH stability was determined after incubation

for 1h at room temperature in buffer solutions of different pH values including: 250 mM KCl/HCl (pH 2), 100 mM sodium acetate (pH 4 and 5), 200mM sodium phosphate (pH 6), 100 mM Tris-HCl (pH 8) and 100 mM carbonate-bicarbonate (pH 10). The effect of the proteases trypsin and pepsin on the activity of F <1 was evaluated by incubation with respective proteases at 37°C for 3 h in a reaction mixture containing a ratio of 1:10 (w/w) of protease to F <1. For pepsin, the reaction was realized in 250 mM KCl/HCl buffer, pH 2, and for trypsin, in 100 mM Tris-HCl buffer, pH 8. After incubation, the samples were centrifuged at 12,000 x *g*, and the antibacterial activity of the supernatant was tested by disk diffusion on susceptible bacteria. As a positive control, a disk was loaded with a stock solution of F <1 at a concentration of 700 µg $mL^{-1}$. To ensure that buffers themselves were not contributing to any inhibition, they were used as a negative control.

### *Multi-criteria analysis for determination of potential antimicrobial peptide sequences in F<1*

Data from unpublished work (2020) was used for the determination of potential antimicrobial peptide sequences in F <1. The analysis was performed following the Technique for Order of Preference by Similarity to Ideal Solution (TOPSIS) according to Yoon and Kim (2017). This multi-criteria decision analysis method is based on the concept that the chosen alternative has the longest geometric distance from the negative ideal solution and the shortest geometric distance from the positive ideal solution. In the first step, a decision matrix was established (1067 peptide sequence x 7 physicochemical indicators). The normalized decision matrix and the weighted normalized decision matrix were calculated. Subsequently, the positive and negative ideal solutions were determined and the Euclidean distance between them was measured. The relative closeness (Rc) to the ideal solution was calculated and the alternatives were ranked based on Rc values (0-1). The alternative closest to 1 was considered the best alternative.

### *Statistical analysis*

All the experiments were performed in triplicate (n =3) and results were expressed as the mean ± standard deviation and evaluated by one-way analysis of variance (ANOVA) ($p < 0.05$). Tukey's post hoc test was used to establish statistical differences between CPFs treatments. Statistical software Statgraphics Centurion XVI.II and GraphPad Prism 8 were employed to analyze data.

## Results

### *Degummed and defatted flour*

From the removal of mucilage and oil from chia seeds together with grinding and sieving procedures, a CPRF was obtained, showing a percentage of humidity of 8.4% ± 0.04 and protein content of 75.28% ± 1.08.

### *Chia protein hydrolysate and peptide fractions*

Enzymatic hydrolysis with a sequential pepsin-pancreatin system of CPRF yielded a chia protein hydrolysate (CPH) with a resulting degree of hydrolysis of 33.79% ± 2.14. Fractionation was achieved with ultrafiltration of CPH, producing 3 CPFs. Their protein contents are presented in Table 1.

**Table 1.** Protein content of CPH and CPF.

| CPH/CPF | Protein content (mg $mL^{-1}$) |
|---|---|
| CPH | $0.507 \pm 0.02^{a}$ |
| F < 1 kDa | $0.074 \pm 0.02^{b}$ |
| F 1-3 kDa | $0.069 \pm 0.01^{b}$ |
| F 3-5 kDa | $0.059 \pm 0.02^{c}$ |

Data expressed as mean ± standard deviation (n=3). $^{a-c}$Different letters represent significant statistical differences between chia protein derivatives with a one-way ANOVA and a Tukey's post hoc test ($p < 0.05$).

### *Tricine-SDS-PAGE*

The tricine-SDS-PAGE analysis revealed CPFs' molecular weight distribution which is shown in Figure 1. Bands in F <1 were below 1.06 kDa, while F 1-3 revealed three low-intensity bands of 1.6, 2 y 2.4 kDa. F 3-5 exhibited the presence of 4 blurred bands of 2.1, 2.5, 3.2, and 4.7 kDa.

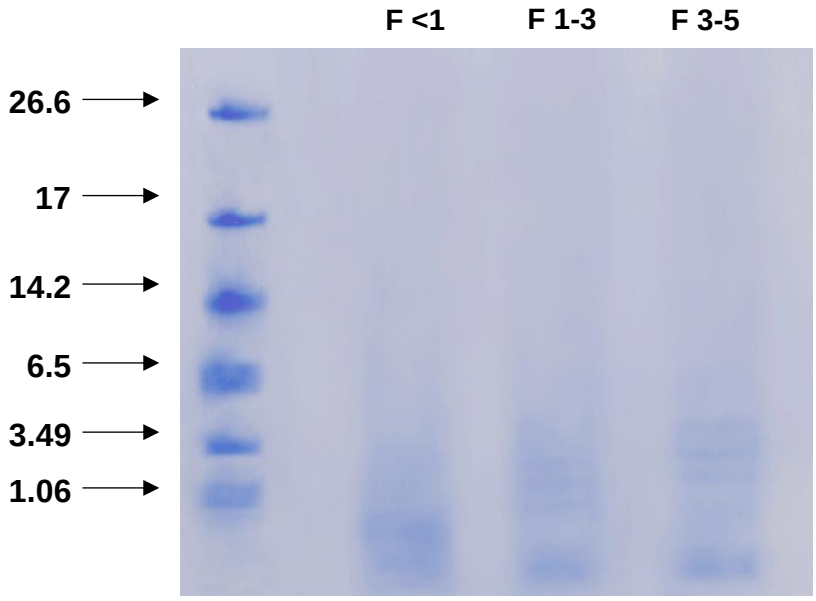


**Figure 1.** Tricine-SDS-PAGE of F <1, F 1-3 and F 3-5 separated by ultrafiltration.

## *Antibacterial activity*

### *Disk diffusion test*

Antibacterial activity of FPCs was evaluated trough disk diffusion assay. F <1, F 1-3, and F 3-5 were considered inactive against all Gram-negative bacteria tested (*Shigella flexneri*, *Salmonella* Typhimurium, *Salmonella* Typhi, *Salmonella* Paratyphi, *Salmonella* Enteritidis and *Escherichia coli* O:157:H7). Gram-positive bacterial susceptibility to FPCs can be observed in *Staphylococcus aureus, Bacillus subtilis, and Listeria monocytogenes.* The degree of inhibition was categorized according to the inhibition diameter generated by the gradient diffusion of CPF to the agar surface, results are presented in Table 2. F <1 was considered active at the highest concentration tested (500 µg $mL^{-1}$) against *Staphylococcus aureus* and *Bacillus subtilis* due to the inhibition diameter was greater than 14 mm. At the same concentration, F<1 was considered partially active in the inhibition of *Listeria monocytogenes* growth. However, at a concentration of 250 µg $mL^{-1}$, F <1 was classified as inactive. F 1-3 was considered partially active against the three gram-positive test strains at a concentration of 500 µg $mL^{-1}$, contrarily, it was shown inactive at inferior concentrations against *Listeria monocytogenes* since inhibition diameter registered was less than 10 mm. F 3-5 exhibited bacterial inhibition with insufficient diameter to be considered active at the highest concentration against all strains under study. At inferior concentrations (250 and 125 µg $mL^{-1}$), inhibition halos were reported between 7 and 9 mm.

**Table 2.** Degree of inhibition of bacterial growth produced by CPFs.

| Treatment | | Bacteria | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | SA | BS | LM | EC | ST | STM | SP | SE | SF |
| | C+ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ |
| | C- | - | - | - | - | - | - | - | - | - |
| | BA | - | - | - | - | - | - | - | - | - |
| | $C_1$ | - | - | - | - | - | - | - | - | - |
| F <1 | $C_2$ | ++ | ++ | + | - | - | - | - | - | - |
| | $C_3$ | +++ | +++ | ++ | - | - | - | - | - | - |
| | $C_1$ | - | - | - | - | - | - | - | - | - |
| F 1-3 | $C_2$ | ++ | ++ | + | - | - | - | - | - | - |
| | $C_3$ | ++ | ++ | ++ | - | - | - | - | - | - |
| | $C_1$ | - | - | - | - | - | - | - | - | - |
| F 3-5 | $C_2$ | - | - | - | - | - | - | - | - | - |
| | $C_3$ | + | + | + | - | - | - | - | - | - |

No inhibition zone (-), diameter <10mm “Inactive” (+), diameter between 10 and 13 mm “partially active” (++) and diameter >14 mm “active” (+++) (Valgas *et al*., 2007). Bacterial strains: SA (*Staphylococcus aureus*), BS (*Bacillus subtilis*), LM (*Listeria monocytogenes*), EC (*Escherichia coli*), ST (*Salmonella Typhi*), STM (*Salmonella Typhimurium*), SP (*Salmonella Parathyphi*), SE (*Salmonella Enteritidis*), SF (*Shigella flexneri*). C+ (500 µg $mL^{-1}$ Penicillin/Gentamicin), C- (distilled water), BA (1% Benzoic acid), C1 (500 µg $mL^{-1}$), C2 (250 µg $mL^{-1}$), C3 (125 µg $mL^{-1}$).

The greatest inhibition degrees were achieved at a concentration of 500 µg $mL^{-1}$ on Gram-positive bacteria. *Staphylococcus aureus* registered inhibition halos of 17.332 ± 0.58 mm (F <1), 14.667 ± 0.57 mm (F 1-3) and 7.333 ± 0.57 mm (F 3-5). *Bacillus subtilis* showed more susceptibility than other Gram-positive strains tested, with inhibition diameters of 17.668 ± 0.57 mm (F <1), 16.666 ± 0.05 mm (F 1-3) and 8.233 ± 0.54 mm (F 3-5). FPCs

were less active against *Listeria monocytogenes* than *S. aureus* and *B. subtilis*. Benzoic acid, commonly used in the food industry as a preservative in flavored soft drinks, fruit and vegetables pickled or candied, marmalade, jams and jellies, confectionery, products based on fish and eggs, cooked shrimp, sauces, prepared salads, condiment and spices and cooked beets (Silva and Lidon 2016), was not active against any of the bacterial strains Gram-positive and Gram-negative analyzed in this study. It was possibly due to the low diffusivity of benzoic acid to the agar surface since, agglomeration of crystals restricted its complete diffusivity to the culture media (Adarme-Vega and Rincones-Lizarazo 2008).

### *Minimum Inhibitory and Minimum Bactericidal concentrations*

MIC and MBC values of F <1, F 1-3, and F 3-5 against strains classified as susceptible (Gram-positive), were detected by microplate dilution. The results are shown in Table 3.

**Table 3.** MIC and MBC values of CPFs

| CPF | Minimum Inhibitory Concentration (µg $mL^{-1}$) | | |
|---|---|---|---|
| | SA | BS | LM |
| F <1 | 662.69 ± 5.27[b] | 642.82 ± 4.28[b] | 635.47 ± 3.65[b] |
| F 1-3 | 818.60 ± 2.50[a] | 807.89 ± 3.05[a] | 818.94 ± 2.85[a] |
| F 3-5 | ND | ND | ND |
| | Minimum Bactericidal Concentration (µg $mL^{-1}$) | | |
| F <1 | ND | ND | ND |
| F 1-3 | ND | ND | ND |
| F 3-5 | ND | ND | ND |

Mean ± standard deviation (n=3). [a-b]Different letters represent significant statistical differences between CPFs with a one-way ANOVA and a Tukey's post hoc test ($p < 0.05$). ND: not detected.

### *Effect of temperature, pH and protease on antibacterial activity*

Generally, conventional processing temperatures of foods are between 80 and 121°C (Kabak 2009). For this reason, an important condition for antimicrobial peptides application in the food industry is thermostability, which was determined after incubation of F <1 at 4, 25, 60, 80, and 100°C for 1 h. Inhibition diameters produced by F <1 are

presented in Figure 2. The highest inhibition diameters were 17.91 ± 0.19, 19.22 ± 0.22 and 18.54 ± 0.35 mm for *L. monocytogenes, B. subtilis,* and *S. aureus* respectively, which samples were incubated at 4°C. Data registered no significant statistical difference between diameters generated by F <1 incubated at 4°C and the control, there were no differences either between samples incubated at 25°C and 60°C. According to Valgas *et al.* (2007), inhibition diameters greater than 14 mm, classify F <1 samples as active after incubation at a temperature range from 4 to 80°C. Significant loss of F <1 antibacterial activity was not observed until after 80°C and more than 50% of antibacterial activity was registered in samples incubated at 100°C, at this temperature, inhibition diameters were less than 10 mm, therefore, classified as inactive.

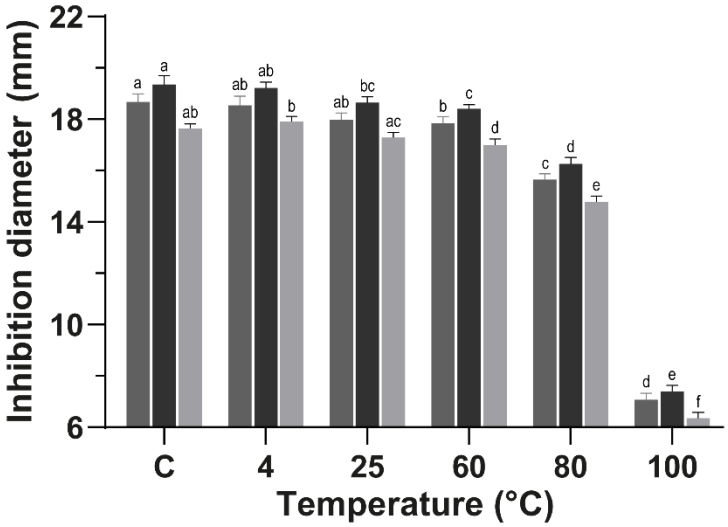


**Figure 2.** Effect of temperature on antibacterial activity of F<1 against (▉) *S. aureus,* (▉) *B. subtilis* (▉) *L. monocytogenes*. [a-f]Different letters represent significant statistical differences between bars of the same color with a one-way ANOVA and a Tukey's post hoc test ($p < 0.05$).

Another factor influencing the stability of antimicrobial peptides is pH (Holcapkova *et al*. 2018). Results on pH stability assays after incubation at pH 2, 4, 5, 6, 8 and 10 (Figure 3) revealed that the greatest inhibition diameters were reported at pH 8 with inhibition zones of 18.92 ± 0.22, 19.12 ± 0.35 and 18.04 ± 0.18 mm against *S. aureus, B. subtilis,* and *L. monocytogenes* respectively. A significant statistical difference was not observed between F <1 incubated at pH 8 and control. On the other hand, the antibacterial activity of F <1 incubated at pH 2 was considered null since inhibition was not observed and it was inactive after incubation at pH 7 because it produced diameters <10 mm. Consequently, F <1 was stable in a pH range from 5 to 8 with inhibition diameters >14

mm. Inhibition diameters were notably reduced in F <1 incubated at pH 10 with values between 10 and 13 mm, classifying as partially active.

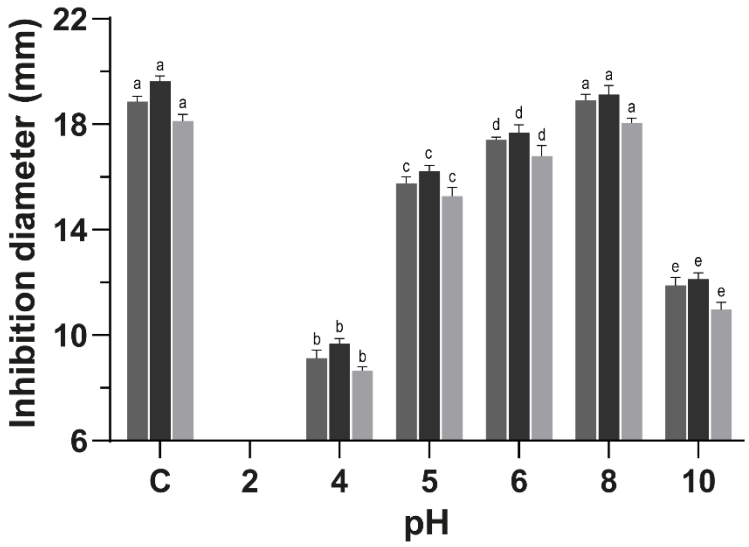


**Figure 3.** Effect of pH on the antibacterial activity of F <1 against (▉) *S. aureus,* (▉) *B. subtilis* (▉) *L. monocytogenes*. [a-e]Different letters represent significant statistical differences between bars of the same color, with a one-way ANOVA and a Tukey's post hoc test ($p < 0.05$).

Protease susceptibility of F <1 was analyzed after incubation with pepsin and trypsin for 3 h. Results showed significant statistical differences between inhibition diameters of F <1 incubated with proteases and control (Figure 4). Samples treated with trypsin were partially active since they produced inhibition diameters between 10 and 13 mm against *S. aureus* and *B. subtilis*. However, they were considered inactive in the inhibition of *L. monocytogenes* because the inhibition halo was less than 10 mm. F <1 treated with pepsin exhibited no inhibition zones against any bacteria.

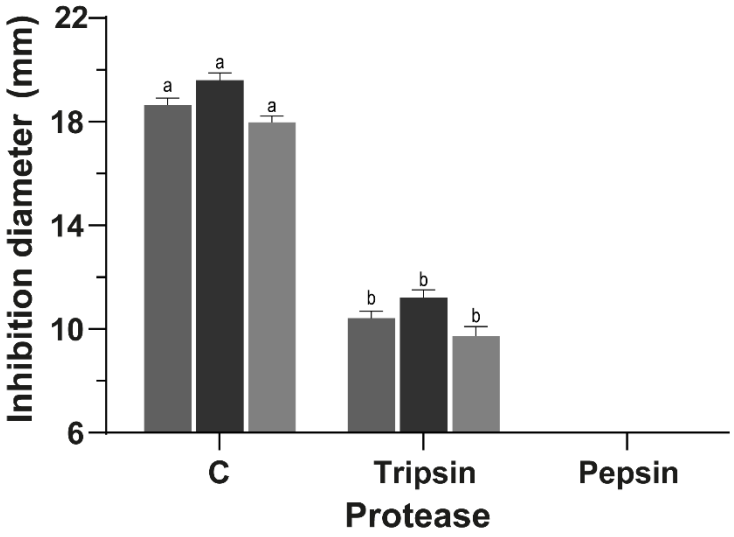


**Figure 4.** Effect of proteases on antibacterial activity of F <1 against (■) *S. aureus,* (■) *B. subtilis* (■) *L. monocytogenes*. [a-b]Different letters represent significant statistical differences between bars of the same color, with a one-way ANOVA and a Tukey's post hoc test ($p < 0.05$).

***Multi-criteria analysis for determination of potential antimicrobial peptide sequences in F<1***

To evaluate the antimicrobial potential of peptide sequences in F <1, they were ranked by the TOPSIS approach (Yoon, Kyung, 2017) using the following criteria: 1) chain length (5 - 10 amino acids); 2) net charge (+3 – +9); 3) hydrophobicity (30 – 50%); 4) the presence of arginine, tryptophan or lysine; 5) arginine: tryptophan proportion (4:5); 6) hydrophobic amino acids (isoleucine, valine, phenylalanine, tyrosine, tryptophan) on position 5, 7 or 9; and 7) presence of lysine as either N- or C- terminal (Aguilar-Toalá, Deering, and Liceaga 2020; Kim *et al*. 2014; Mikut *et al*. 2016; Yan *et al*. 2020). The analysis showed that both sequences KLKKNL and KKYRVF were ranked at the top in the antimicrobial sequences' assessment, followed by MLKSKR, MSKAKPGRSM, and SVVAKAPVGKR. There was no significant statistical difference ($p<0.05$) in the Rc values of KLKKNL and KKYRVF ranked as the alternatives with the nearest distance to the ideal positive solution and the farthest distance to the negative ideal solution. Through an alignment of sequences, a comparison with all sequences stored in the Antimicrobial Peptide Database (APD) was performed and it was found a higher percentage of similarity with one or more peptides in the APD for KLKKNL than KKYRVF. A 45.45% percentage of similarity was presented between KLKKNL and a short cecropin A-melittin hybrid peptide (KKLFKKILKFL) named BP100 (Badosa *et al*. 2007). The same

percentage of similarity is shared with L5K5W (KKLLKWLKKLL), a de novo AMP with a single tryptophan at the critical amphipathic interface (Kang *et al*. 2009). These observations altogether suggest that KLKKNL could be the peptide sequence exerting the antimicrobial effect observed in F <1.

## Discussion

Chia seed oil is considered one of the most essential characteristics of the plant and it has demonstrated promising antioxidant, antibacterial and antiviral activities against various microorganisms (Elshafie *et al*. 2018). Nevertheless, there are few studies on antibacterial properties of proteins and protein derivatives from chia seeds (Segura-Campos *et al.*, 2013; Coelho *et al.*, 2018; Aguilar-Toalá *et al*., 2020). In order to obtain chia protein hydrolysates through enzymatic reactions, protein-rich substrates are needed. The degummed procedure using water suspension allowed to remove soluble fiber, reducing the percentage of non-proteinaceous compounds in PRCF. Exhaustive oil extraction also contributed significantly to the obtention of high levels of proteins in PRCF. This is supported by research conducted by Segura-Campos *et al.* (2013) for the obtention of a chia protein-rich fraction, it showed that increasing protein content is promoted by the reduction of fiber and lipid content. On the other hand, CPH obtained with sequential pepsin-pancreatin enzymatic hydrolysis showed a degree of hydrolysis (DH) greater than 10%, which classified CPH as an extensive hydrolysate according to Vioque *et al*. (2001). DH herein reported is highest than 25% obtained by Coelho *et al.* (2018), with a sequential alcalase-flavourzyme system, from a protein-rich fraction obtained from partially defatted chia flour. This difference could be attributed to the remaining lipids in flour. A higher DH was registered by Segura-Campos *et al.*, (2013) for chia hydrolysates (43.8%) using alcalase-flavourzyme, in a period of 150 min. Variations in DH are influenced by enzymatic systems and obtention procedures of CPRFs that have repercussions on the content of protein that will be available for enzymatic proteolysis. Pepsin-pancreatin enzymatic system for 90 min used in this study, achieved an extensive DH in lower reaction time than above mentioned with alcalase-flavourzyme. Generation of extensive DHs facilitates the production of hydrolysates with potential biological activities, as antibacterial activity. The importance of extensive hydrolysates resides on the release of short chain peptides with antibacterial potential. Fractionation according to molecular weight was possible through ultrafiltration. In this work, the protein content of CPFs (F <1. F 1-3, F 3-5 kDa), was dependent on specific molecular weight peptide abundance in CPH.

CPFs revealed low molecular weight bands in Tricine-SDS-PAGE, corroborating the effectiveness of enzymatic hydrolysis, as well as the ultrafiltration process. Cotabarren *et al.* (2019), reported the molecular weights of chia crude protein extracts and their hydrolysates at different times of hydrolysis. Results revealed, mainly 10 and 75 kDa

molecular weight bands, which is within the molecular weight range reported by Grancieri *et al.* (2019) in rich-protein chia flour (8-114.6 kDa). The definition of ~50 kDa bands dropped after 30 min of hydrolysis. After 90 min, a decrease of intensity in ~20 kDa bands was observed, as well as the formation of new bands with molecular weights of ~20 kDa. This demonstrates that a 90 min hydrolysis process with proteases, as reported in the present study, can achieve the release of low molecular weight peptides from chia seeds.

The results obtained in this work showed that molecular weight bands were distributed according to the molecular weight cutoff determined by ultrafiltration membranes. Membranes were used in ascendant order (from 1 to 5 kDa), which allowed the yielding of low molecular weight peptides selectively. Contrarily, Herrera Chalé *et al.* (2015), studied peptide fractions from *Mucuna pruriens* hydrolysates using ultrafiltration membranes in descendant order. Electrophoretic profiles exhibited the presence of low molecular weight peptides in fractions of 5-10, 3-5 and 1-3 kDa, that were not observed in <1 kDa fraction. This could be attributed to the ability of peptides with a molecular weight lower than 1 kDa of passing through membranes with larger cutoffs (10, 5, and 3 kDa) during the first stages of the ultrafiltration process. Therefore, such peptides were distributed in fractions with molecular weights greater than 1 kDa. In consequence, ultrafiltration membrane order promoted the low concentration of this low molecular weight peptides in <1 kDa fraction, which difficulted their detection with SDS-PAGE. Limitations in the detection of short chain peptides with SDS-PAGE was also reported by Orona-Tamayo *et al.* (2015), who conducted the digestion of chia proteins with pepsin and a mixture of trypsin and pancreatin. Extensive hydrolysis released low molecular weight peptides that were difficult to resolve in polyacrylamide gels. However, molecular weight distribution could be observed. The molecular weight distribution of CPFs observed in this study, allowed to consider ultrafiltration a useful procedure in the generation of peptide fractions within specific molecular weight ranges.

Inhibition of Gram-positive bacteria generated by FPCs in disk diffusion tests was compatible with results reported by Coelho *et al.* (2018) in chia protein hydrolysates, which were inactive in the inhibition of Gram-negative *E. coli* O157:H7 and active against Gram positive *S. aureus*. The same behavior was observed in the AMP PTP-7, which was able to inhibit Gram-positive bacteria, such as *S. aureus*, but it was not active against Gram-negative tested strains (Kharidia and Liang 2011). More susceptibility in Gram-positive bacteria compared to Gram-negative could be explained through structural differences between them. Gram-negative bacteria differ from Gram-positive bacteria in the structure of the cell wall. This results in differences in the penetration and retention of chemical agents. Gram-negative bacteria have an envelope, consisting of three principal layers: the outer membrane, the peptidoglycan cell wall, and the cytoplasmic or inner membrane. Gram-positive bacteria generally lack the outer membrane. The main function of the outer membrane is to serve as a permeability barrier, excluding antibacterial compounds from penetrating the cell. This feature is one of the main factors contributing

to the intrinsic antibiotic resistance observed in Gram-negative bacteria (Exner *et al*. 2017). Since generally reported mechanisms of action in AMPs, involves membrane interaction (Sivieri *et al*. 2017), structural characteristics of cell wall have an important role in the establishment of antibacterial activity. In comparison with Gram-negative bacteria, Gram-positive have a higher proportion of negatively charged membrane components, such as phosphatidylglycerol (Malanovic and Lohner 2016), this feature increases the electrostatic attraction between positively charged antibacterial peptides and bacterial membranes. In this regard, less susceptibility to FPCs observed in *L. monocytogenes* could be attributed to variations in fatty acids composition of the cellular membrane. This can interfere with antibacterial activity due to a less negatively charged bacterial surface, as reported by Martínez and Rodríguez (2005) in *L. monocytogenes* strains with low susceptibility to nisin that showed a low ratio of pentadecylic:margaric acid. On the other hand, CMI values registered in F <1 and were lower than reported by Coelho *et al.* (2018), for chia protein hydrolysates (2.26 mg $mL^{-1}$) against *S. aureus*. Differences could derive from the enzymatic systems used for hydrolysis, and the composition of the chia protein hydrolysate and peptide fraction, since a hydrolysate contains a mixture of different peptides and the peptide fraction is selectively designed to contain peptides of specific molecular weights. CMB was not detected at the concentrations tested in this study. This suggests that higher concentrations of FPCs are required to possibly observe a bactericidal effect.

Stability assays revealed that F <1 remained active after incubation at temperatures close to 100°C, characteristic of interest for food applications. However, further studies are needed to evaluate the interaction of F <1 from chia, with food components. In the dairy industry, pasteurization implies heating every particle of milk or milk product, in properly designed and operated equipment to specified temperatures and held continuously during a given period. Batch pasteurization requires heat to 63°C for 30 min. For continuous flow pasteurization, HTST (High Temperature Short Time) or HHST (Higher Heat Shorter Time) could be applied. HTST involves a 72°C – 15 s treatment, while HHST can be applied with any of the following conditions: 89°C - 1 s, 90°C - 0.5 s, 94°C - 0.1 s, 96°C - 0.05 s y 100°C - 0.01 s. Ultra-pasteurization requires processing at or above 138°C, for at least 2 s, either before or after packaging, so as to produce a milk/or milk product, which has an extended shelf-life (FDA 2017). According to results reported in the present study, F <1 is able to preserve activity up to 80°C for 1h, which indicates that F <1 antimicrobial activity could be sustained after a batch pasteurization process or a HTST continuous flow pasteurization. Processing at higher temperatures, such as HHST pasteurization and ultra-pasteurization, could compromise F <1 stability. For fish and fishery products, heat treatments are also applied in order to eliminate most resistant pathogenic bacteria, such as *Clostridium botulinum*. Food and Drug Administration considers a 6D (meaning 6 log reduction) process for *C. botulinum* (type E and nonproteolytic B and F) to be generally suitable for pasteurized seafood products. In

designing a thermal process, a minimum cumulative total lethality of $F_{90°C}$ (i.e., equivalent accumulated time at 90°C = 10 min) is adequate for pasteurized fish and fishery products (FDA 2020). Following this parameter, F <1 could lose or reduce its antibacterial activity as the temperature rises to 100°C in fish or fishery products.

According to the Code of Federal Regulations of the United States (CFR) for egg and egg products should be a "*Salmonella* negative product" with specific time and temperatures for liquid egg products: albumen (56°C – 3.5 min or 55.6°C – 6.2 min); whole egg blends, sugar whole egg and plain yolk (61.1°C – 3.5 min or 60°C – 6.2 min); fortified whole egg and blends (62.2°C – 3.5 min or 61.1°C – 6.2 min); salt whole egg, sugar yolk and salt yolk (63.3°C – 3.5 min or 62.2°C – 6.2 min). For spray-dried albumen, a temperature of 54.4°C is held for 7 days, and for pan-dried albumen, a 51.7°C – 5 days treatment is used (C.F.R. 2012). Therefore, heat treatments for egg and egg products would not affect antibacterial activity of F <1, since temperatures applied are below 80°C.

Juice pasteurization regulated by FDA requires a process with a minimum 5-log reduction of the most resistant microorganism of public health significance identified as the pertinent pathogen under Hazard Analysis and Critical Control Point (HACCP). The target microorganisms are dependent on the juice product and process, including *E. coli* O157:H7, *Salmonella* and *Cryptosporidium parvum* or *C. botulinum* (FDA 2004). Generally, heat treatments for juices and beverages are classified according to the intensity of the process. HTLT (High Temperature Long Time) treatment, with temperatures ≥80°C held for >30 s, is the most commonly used method in the processing of juices and beverages; it can be classified as: pasteurization (<100°C), canning (~100°C), or sterilization (>100°C). HTST thermal pasteurization (≥ 80°C for ≤ 30 s), MTLT (Mild temperature-long time) heat treatment (<80°C for >30 s), MTST (Mid temperature-short time) heat processing (<80°C for <30 s) are also used for juices and beverages (Petruzzi *et al*. 2017). Given these conditions, F <1 could preserve its antibacterial activity in juices and beverages after MTLT and MTST thermal processing. However, in HTLT and HTST treatments the antimicrobial activity could decrease when exceeding a temperature of 80°C.

As temperature, pH is another factor that can have an effect on the antimicrobial activity of AMPs (Hitchner *et al*. 2019). F <1 was sensible to pH due to its activity was reduced when it was exposed to a pH lower than 5 and above 8. The pH range of some common foods are 7.1 - 7.9 for eggs, 6.3 - 8.5 for milk, 5.3 - 5.8 for bakery products, 5.0 - 7.0 for meat, 4.8 - 7.3 for fish, 4.0 - 7.0 for vegetables, 3.3 - 7.1 for fruits and 3.1 - 4.5 for berries (FDA 2012). F <1 was more active at pH 8, consequently, it is possible that it shows a higher protective effect against microorganisms in products with pH values that are closer

to neutrality, as in eggs or milk, and lower antibacterial activity in acid foods, such as berries and fruits.

Proteases have been constantly introduced in the food industry due to proteolysis is useful in the modification of food protein properties, promoting modifications in solubility, gelation, emulsification, foam formation, allergen reduction, flavor transformation or bioactive peptide release (Tavano 2013). The use of proteases to improve meat tenderness has increased due to the need of the meat industry to give added value to lower-grade meat cuts. Proteases are also used in milk clotting processes in the dairy industry, one of the most important uses of proteases is cheese manufacturing. In the baking industry, wheat grain contains proteins that cause allergic reactions such as gluten, which is also insoluble and expands to form lattice-like structures when it is hydrated. Therefore, gluten should be hydrolyzed to obtain more moldable dough. The use of proteases improves the quality of the dough, enhances its softness and size during the baking process (Fernández-Lucas *et al.*, 2017). In this sense, it is relevant that AMPs used in food systems, are resistant to the action of proteases. In accordance with results reported in the present work, antimicrobial activity of F <1 was not preserved after exposure to proteases such as trypsin and pepsin. Pepsin and chymosin are used as coagulants in cheese production (Karahalil 2020), while trypsin, chymotrypsin, and pepsin have been used for the degradation of gluten in grains (Zhang *et al*., 2018). F <1 could present limitations for application in the above-mentioned processes. AMPs degree of susceptibility is related to the specificity of the enzyme used, trypsin preferentially cleaves cationic residues such as arginine and lysine (Torres *et al*. 2019) and pepsin has specificity towards hydrophobic residues (Tavano *et al*. 2018), both are a prerequisite for AMPs activity (Wang *et al*. 2019). Due to this susceptibility to proteases, different strategies have been addressed to increase its stability to degradation (Jia *et al*. 2019). Some of these include the introduction of unnatural amino acids and the modification of terminal regions by cyclization. The incorporation of D-amino acids has been shown to increase the stability of an AMP analogous to W3R6, in the presence of trypsin for up to 72 h (Y. Li *et al*. 2019). These could be useful tools for the preservation of AMPs' stability in processes including the use of proteases for the transformation of food products.

Since the amino acid sequence has an important contribution to the antimicrobial activity of AMPs, it is highlighted the presence of lysine in KLKKLN that provides a net positive charge (+3). Several studies have correlated cationicity with the antimicrobial activity observed in antimicrobial peptides. The attributed relevance lies mainly in the interaction between the positive charge in PAMs and the negatively charged bacterial surface via electrostatic interactions (Mojsoska and Jenssen 2015). Lysine and arginine (positively charged residues) are the most common amino acids found in cationic AMPs. Their side chains contribute substantially to the first coulombic interactions between peptide and

phospholipids in the membrane. However, lysine presents a lower tendency to be toxic for mammalian cells when compared to arginine, due to the localization of the positive charge density (Pedron *et al*. 2019; Yount *et al*. 2019). The chain group of lysine possesses a more localized positive charge density on its terminal amine, this results in fewer atoms interacting with water molecules. Lesser interactions with water molecules and the biological membrane phospholipid head groups are produced in consequence (Armstrong *et al*. 2016; Li, Vorobyov, and Allen 2013). On the contrary, a delocalized charge density is present in the side chain terminal guanidinium group of arginine. It promotes more interactions with hydrophobic/hydrophilic interfaces, water, and phospholipid head groups, enabling destabilization of completely hydrophobic environments, such as bacterial membrane interior. In this way, the amino group side chain of the lysine residue reacts less with water than the guanidium group from the arginine residue. This is important since solvation is considered a characteristic of amino acid side chain groups that contributes to cytotoxicity (Rice and Wereszczynski 2017). Therefore, lysine-rich AMPs could be better alternatives for applications that implies human consumption. Palman *et al*. (2020) refer to lysine as the most active cationic amino acid residue and to leucine and phenylalanine as the most active hydrophobic amino acid residues. According to Mikut *et al*. (2016), a primary rule for activity is dictated by a balance of positive charge and hydrophobicity. Consistent with this interpretation, KLKKLN contains amino acid residues in their sequence that confer not only a cationic character but hydrophobicity, which is also required for the interaction with bacterial membranes. The KLKKLN chain length is also noticeable because short length AMPs have been shown to have enhanced antimicrobial activities, higher stability and lower toxicity to human cells (Yan *et al*. 2020). Short length AMPs also present ease of synthesis and lower reagent consumption, offering certain advantages from an economic and environmental standpoint (Johnson C.P. Santos *et al*. 2018). Similarity with BP100 and L5K5W suggests that the repetitive KL peptide fragment could be influencing the antimicrobial activity of KLKKNL. BP100 has been demonstrated to effectively inhibit in vitro the growth of the Gram-negative bacteria *Erwinia amylovora*, *Pseudomonas syringae* pv. *syringae*, *Xanthomonas axonopodis* pv. *vesicatoria*, and *Escherichia coli*, as well as *in vivo* the growth of *E. amylovora* (Alves *et al*. 2012). On the other hand, L5K5W is active against *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermis, Micrococcus luteus, E. coli, Shigella dysenteriae, S.* Typhimurium, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa,* inhibiting Gram-positive bacteria to a higher extent (Kang *et al*. 2009). Despite similarity with other AMPs, it should be emphasized that further studies are needed to confirm the antimicrobial activity of KLKKNL.

Based on the evidence presented in this work, a sequential pepsin-pancreatin system for 90 min, is considered useful for the releasing of short chain peptides and ascending order of ultrafiltration membranes favored the concentration of peptides less than 1 kDa in F <1. Electrophoretic profile revealed low molecular weight bands confirming the

effectiveness of hydrolysis and fractionation by ultrafiltration. F <1 reported the greatest antibacterial activity against *L. monocytogenes*, which highlights its potential application as an antibacterial agent in food products with a reported incidence of this bacterium, such as those derived from milk and meat products. Stability tests indicate that F <1 could resist heat treatments such as HTST pasteurization of milk, pasteurization of egg and egg products, and MTLT/MTST pasteurization of juices and beverages. F <1 can have an antimicrobial role in foods that have pH values in a range of 5 – 8, being more active in foods with a pH close to neutrality than acidic foods. Antimicrobial activity of F <1 could be restricted in processes involving proteases such as trypsin or pepsin due to its susceptibility to proteolytic degradation. However, new strategies, such as the incorporation of D-amino acids or the cyclization of the terminal regions may be useful alternatives to promote protease resistance and, consequently, their application in the food industry. Additionally, future research is necessary to determine the antimicrobial activity of short-chain peptides from chia peptide fractions, such as KLKKNL.

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## CONCLUSIONES

El GH obtenido (33.79% ± 2.14) se consideró extensivo, siendo el sistema secuencial PP, útil en la generación de péptidos de cadena corta. F <1 registró el mayor contenido de proteína después del hidrolizado, lo que indica que el orden ascendente en el que se utilizaron las membranas de ultrafiltración favoreció la concentración de péptidos menores a 1 kDa en F <1. El perfil electroforético de las fracciones peptídicas de *S. hispanica* L. reveló bandas de bajo peso molecular dentro de los rangos establecidos por las membranas de ultrafiltración, confirmando la efectividad de la hidrólisis y del proceso de separación por peso molecular con membranas de ultrafiltración. F <1 presentó la mayor actividad en las pruebas de actividad antibacteriana *in vitro* a una CMI de 635.47 ± 3.6532 µg/mL contra la bacteria Gram positiva *L. monocytogenes,* lo cual destaca su potencial aplicación como agente antibacteriano en productos que pueden presentar el crecimiento de esta bacteria, como los derivados de la leche y productos cárnicos. Los ensayos de estabilidad indican que F <1 podría resistir tratamientos térmicos como la pasteurización HTST de la leche, la pasteurización del huevo y productos del huevo, así como la pasteurización MTLT y MTST en jugos. F <1 puede tener un papel antimicrobiano en alimentos que presentan valores de pH en un rango de 5-8, siendo más activo en alimentos con pH cercano a la neutralidad, que en alimentos ácidos. La actividad antimicrobiana de F <1 podría verse restringida en procesos que involucren la aplicación de proteasas como tripsina o pepsina debido a su susceptibilidad a la degradación proteolítica. Sin embargo, nuevas estrategias como la incorporación de D-aminoácidos o la ciclización de las regiones terminales pueden ser alternativas útiles para promover la resistencia a proteasas y, por consiguiente, su aplicación en la industria alimentaria. Además, el estudio de péptidos de cadena corta en fracciones peptídicas de chía, como KLKKNL, es necesario para la determinación de su actividad antibacteriana.

## ANEXO

**Cronograma de actividades**

| Actividad | Semestre I | Semestre II | Semestre III | Semestre IV |
|---|---|---|---|---|
| Revisión bibliográfica | ✓ | ✓ | ✓ | ✓ |
| Redacción del protocolo | ✓ | | | |
| Acondicionamiento de la materia prima | ✓ | | | |
| Obtención de la harina desgomada y desgrasada | ✓ | ✓ | | |
| Determinación de humedad | | ✓ | | |
| Hidrólisis enzimática pepsina-pancreatina | | ✓ | | |
| Grado de hidrólisis | | ✓ | | |
| Utrafiltración del hidrolizado | | ✓ | | |
| Determinación del contenido de proteína | | ✓ | | |
| Electroforesis | | ✓ | ✓ | |
| Reactivación de microorganismos | | | ✓ | |
| Antibiogramas | | | ✓ | |
| Ensayos de difusión en disco | | | ✓ | |
| Determinación de la concentración mínima inhibitoria y bactericida | | | | ✓ |
| Efecto de pH, temperatura y proteasas | | | | ✓ |
| Análisis multicriterio | | | | ✓ |
| Redacción del artículo de investigación | | | | ✓ |