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ANTIMICROBIAL ACTIVITY OF A PEPTIDE FRACTION FROM LIMA BEANS (<10 KDA) AGAINST PATHOGENIC AND CONTAMINATING BACTERIA, AS WELL AS ITS INCORPORATION INTO BIOACTIVE FILM-FORMING SOLUTIONS

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ABSTRACT: Microbial contamination of food is a constant challenge for the food industry, affecting product quality, safety, and shelf life. This study evaluated the antimicrobial activity of a peptide fraction derived from lima bean **Phaseolus lunatus**) proteins and incorporated into film-forming solutions (FFS) based on flamboyant gum (**Delonix regia**) and lima bean starch, with the aim of developing bioactive materials. Enzymatic hydrolysis of the protein concentrate was performed with the enzyme pepsin, followed by ultrafiltration to obtain a fraction smaller than 10 kDa with a protein concentration of 1.42 mg/mL. The antimicrobial activity of the peptide fraction obtained was evaluated by the minimum inhibitory concentration (MIC) against pathogenic and spoilage bacteria **L. monocytogenes, B. thermosphacta, S. enteritidis, P. aeruginosa, L. lactis, L. acidophilus, S. aureus**, and **E. coli O157:H7**, obtaining values of 1.139, 0.427, 0.996, 0.142, 0.996, 0.996, 1.139, and 1.139 mg/mL, respectively. The peptide fraction had an inhibitory effect on most bacterial strains, both pathogenic and spoilage, relevant to public health and the food industry. Gram-positive (G+) bacteria, such as **B. thermosphacta, L. monocytogenes, L. lactis**, and **L. acidophilus**, were the most susceptible, with inhibition percentages above 50% and even total inhibition in the case of **B. thermosphacta**. In contrast, Gram-negative (G-) bacteria showed lower sensitivity; **P. aeruginosa** showed only 1% inhibition, and **Escherichia coli O157:H7** was not only not inhibited but showed a 33% increase in population.

KEYWORDS: *Phaseolus lunatus, bioactive peptides, antibiotic resistance, enzymatic hydrolysis, food preservation*

Introduction

Various bacterial genera, such as *Bacillus, Escherichia*, *Listeria*, *Salmonella*, and *Staphylococcus*, are commonly associated with both foodborne illnesses and food spoilage. These bacteria can proliferate during production, processing, and storage, highlighting the need for effective control strategies (Mafe et al, 2024).

In response to the limitations of synthetic preservatives and growing concern about antimicrobial resistance, there has been increasing interest in exploring bioactive compounds of natural origin, especially those derived from plant sources (de Olivira et al. 2025). The lima bean (*Phaseolus lunatus*) has been shown to be a good source of peptides with biological activity with proven effects in the prevention and treatment of chronic degenerative diseases, such as diabetes and high blood pressure (Ciau-Solís et al., 2024).

One of the current concerns is antibiotic resistance, which represents a global social and health crisis, where the search for alternatives, including the use of antimicrobial peptides (AMPs), is crucial (Scieuzo et al., 2023). AMPs are the first line of defense against invading pathogens when host cells detect contamination or active infection. They are cationic molecules due to their high lysine and arginine content and are organized in an amphipathic structure. They have hydrophilic and hydrophobic ends, which give them solubility in both aqueous and hydrophobic environments. AMPs bind electrostatically to specific targets on the cell membrane that have a negative surface charge, as they generally have a positive charge, and this interaction is critical in determining a molecule's affinity for the anionic site of the target (Zasloff, 2019).

AMP can be obtained through the hydrolysis of food proteins, which is usually done biologically using enzymes or microbial fermentation. The application of AMP in industry has gained relevance due to its high efficacy against pathogenic microorganisms and contaminant , without the risks associated with antibiotic residues from human and animal use. Today, consumers are more concerned about the indiscriminate use of chemical compounds and are therefore looking for safe and effective alternatives to replace them. For example, the use of AMPs is presented as a promising alternative to food preservatives, antibiotic growth promoters, and phytosanitary agents (Corrêa et al. 2023).

Film-forming polymeric solutions represent innovative drug delivery systems, and their formulation includes the active ingredient, a film-forming agent, a plasticizer, and other excipients such as penetration enhancers or solvents (Milinković, & Đekić 2025). When this liquid formulation is applied, the solvent evaporates, forming a film or coating that regulates the release of the active compound. The resulting coating is usually transparent, which significantly influences the acceptance of the biomaterial. Natural products such as α-mangostin, madecassoside, and asiaticoside have been incorporated into film-forming solutions (Ahmed et al., 2023).

These systems were developed primarily for skin or wound treatments where topical and transdermal drug delivery is possible because a coating is formed upon contact with the target therapeutic region using one or more polymers as a matrix for film formation (Dhimmar et al 2023). This strategy has been adapted for applications in the food industry, where biopolymer-based coatings not only act as barrier layers against gas diffusion, fragrance alterations, water migration, and various volatile exchanges (Sabbah et al., 2019), and are used to produce ecological, smart, and active packaging for food protection and preservation, as they are good carriers of functional ingredients such as antimicrobial peptides and antioxidants to improve durability, functionality, and extend the shelf life of food (De Pilli, 2020).

The objective of this study was to evaluate the antimicrobial effect of a peptide fraction greater than 10 kDa from *Phaseolus lunatus* obtained by enzymatic hydrolysis followed by ultrafiltration on pathogenic and contaminating bacteria, as well as film-forming solutions prepared with flamboyant gum (*Delonix regia*) and native lima bean starch, thus contributing to the development of sustainable and safe strategies for food preservation.

Materials and Methods

Materials

Baby Lima beans (*Phaseolus lunatus*) were obtained from the local market in the city of Mérida, Mexico, and flamboyant seeds (*Delonix regia*) were collected randomly from whole, dry pods from trees in the city of Mérida, Yucatán, Mexico. These were opened and seeds in good physical condition and larger than 1 cm in length were selected. Ten kilograms of seeds were obtained and stored hermetically at room temperature until processing. The enzyme used for hydrolysis was pepsin (P7125-500G Sigma-Aldrich). The bacterial strains were acquired from the American Type Culture Collection (ATCC): *Listeria monocytogenes*

ATCC 51414, *Brochotrix thermosphacta* ATCC 11509, *Salmonella entertidis* ATCC 13076, *Pseudomona aeruginosa* ATCC 27853, *Lactococcus lactis* ATCC 314 and *Lactobacillus acidophilus* ATCC 11454, *Staphylococcus aureus* ATCC 5083, and *Escherichia coli* O157:H7 (ATCC 43888).

Obtaining peptide fractions and starch from Phaseolus lunatus

The methods used were based on those reported by Ciau-Solís et al. (2024). The Baby Lima bean seeds were cleaned manually to remove any impurities they contained. They were then ground sequentially in a Cemotec 1990 roller mill and a Cyclotec impact mill (Tecator, Sweden). The flour obtained was sieved through a 200 mesh screen to obtain a particle size of 500 µm. The flour was wet fractionated to obtain starch and protein concentrate by preparing a suspension of the flour with distilled water in a 1:6 w/v ratio, adjusting the pH to 11 with a 1M NaOH solution and stirring for one hour.

Enzymatic hydrolysis of the protein concentrate was performed using the enzyme pepsin. The hydrolysis parameters were: substrate concentration 4%, enzyme-substrate ratio 1/10 w/v, temperature 37°C, pH 2 for 10 min in a 2 L glass reactor placed in a Cole-Parmer Poystat® water bath. Stirring was performed with a mechanical stirrer (Caframo RZ-I®) at 300 rpm, using a thermometer and an Orion Star A215® potentiometer electrode ( ). The soluble part of the enzymatic hydrolysate was subjected to ultrafiltration fractionation with a 10 kDa membrane using an Amicon Model 2000 ultrafiltration unit (Millipore, Inc., Marlborough, MA, USA). It was passed through the membrane, collecting the fraction retained by the 10 kDa membrane (F10-UF). The peptide fraction was freeze-dried at -47°C and 13 x 10-3 mbar in a freeze dryer (Labconco Freezone 18 L). The protein content of the fraction obtained by ultrafiltration was determined using the Lowry method (Satpathy et al., 2020). Quantification was performed using a calibration curve prepared from a bovine serum albumin (BSA) solution, finding a concentration of 1.42 mg/mL of protein in the peptide fraction.

Extraction and modification of flamboyant gum

The extraction of native flamboyant gum was performed using an adaptation of the method described by Chel-Guerrero et al. (2022). To extract the endosperm, the seeds were hydrated in a 1:5 (w/v) ratio with distilled water at 70 °C under constant mechanical agitation (Caframo RZ-1) for 12 h. Subsequently, the germ, pericarp, and endosperm were separated by pressure from a smooth roller system. The endosperm was dispersed in distilled water at a ratio of 1:4 v/v and liquefied for 5 min, then heated to 50 °C for 30 min and sieved through a 50 (351 µm) and 100 (149 µm) mesh to separate the fibrous particles of native flamboyant gum. Ethanol (96%) was added in a ratio of 1:2 (v/v) with constant stirring to precipitate the gum, which was dried for 12 h at 60 °C in a convection oven and then ground in a Thomas Wiley machine to obtain a particle size of 173 mm (80 mesh).

Growth of bacterial strains

The bacterial strains were reactivated using specific culture systems for each microorganism: *Brochothrix thermosphacta* was cultured in brain heart infusion broth (BHI-Oxoid®, GA, USA) at 26 °C; *Staphylococcus*

aureus, *Listeria monocytogenes*, and *Salmonella enteritidis* in BHI broth (BHI-Oxoid®, GA, USA) at 37 °C for 18 h; *E. coli* 057:H7 in tryptic soy broth (Oxoid®, GA, USA) at 37 °C °C; *Pseudomonas aeruginosa* ATCC 27853 in nutrient broth at 37 °C; *Lactococcus lactis* ATCC 314 and *Lactobacillus acidophilus* ATCC 11454 in Man-Rogosa-Sharpe (MRS) medium at 37 °C for 24 hours under anaerobic conditions ( ). All bacteria were placed in a Lab-line Imperial III incubator. Once the bacteria were reactivated, their growth kinetics were determined using the McFarland scale (Leber, 2016).

Determination of μ_{max} and t_d

The maximum specific growth rate (μ_{max}) and doubling time (t_d) were estimated using two complementary methods. First, the viable counts (CFU/mL) were transformed to ln(N) and the exponential window was identified by searching for sliding windows (3–6 points), selecting the one with the maximum slope with $R^2 \geq 0.95$. A linear regression ln(N) vs. time was fitted to that window, and the slope was reported as μ_{max}. Primary models were then fitted using the modified Gompertz equation and Baranyi--Roberts model (Zwietering et al., 1990) by nonlinear least squares to estimate the parameter λ (lag phase) parameter, which was adopted as the logarithmic phase entry time t_e when the fit was robust, and these data were used to estimate the doubling times (t_d). The models were fitted using the biogrowth package for R (Garre et al., 2023).

Determination of the minimum inhibitory concentration (MIC)

The method described by Kowalska--Krochmal & Dudek-Wicher (2021) was used. Microplates were used in which 100 mL of each bacterial solution to be evaluated was added at a concentration of 1 x^{10^6}CFU/mL. In another well, 100 mL of the broth inoculated individually with the bacteria under study and 100 mL of Nisin® at a concentration of 11 mg/mL (positive control) were added as a second positive control. in another well, 100 mL of the nutrient broth inoculated with the bacteria to be evaluated and 100 mL of Amikacin® at a concentration of 4 mg/mL (control +) were added. For the negative control, 100 mL of the nutrient broth inoculated with the bacteria to be evaluated and 100 mL of sterile distilled water were used. 100 µL of broth inoculated with the bacteria to be evaluated was added to each of the wells for each microorganism. Then, 100 mL of peptide fraction < 1 kDa was added in the following concentrations: 100, 90, 80, 70, 60, 50, 40, 30, 20, and 10%, relative to the initial concentration of 1.42 mg/mL of protein. The microplates were incubated at 37 °C for 24 h. Finally, a 5 mg/mL solution of triphenyltetrazolium chloride was prepared in sterile distilled water, where a red color indicated the presence of viable cells (Moreno et al., 2023). The response variable was the minimum concentration of peptide fractions required to inhibit the bacteria. Three replicates were performed per experiment, and each response variable was measured in triplicate.

Antimicrobial activity of the film-forming solution

The method described by Rosas *et al.* (2021) was used to prepare the film-forming solution (FFS), with some modifications. A 3% (w/v) starch suspension was prepared. The suspension was gelatinized in a Brabender PT-100 viscoamylograph and heated from 30°C to 95°C at an increase of 3°C/min. Flamboyant gum was added to the mixture when it reached 70 °C, the tem-

perature at which starch begins to gelatinize, and was kept at 95 °C for 10 min . It was then allowed to cool to room temperature and stored refrigerated at 4 °C until use.

To determine antimicrobial activity, the method described by Kana & Meimandipour (2017) was used, with some modifications. A peptide solution was prepared at a concentration of 1.4 mg/mL with sterile distilled water, 10 mL of SFP was taken, and 10 mL of the peptide solution was added. The mixture was poured into a beaker and sterilized by UV for 30 min in a CleAir CL-B-262D-04 laminar flow hood. Eight mL of the solution was taken and placed in sterile test tubes at a temperature of 37 °C. One mL of the bacterial solution in exponential phase was added and stirred with a vortex mixer. One mL of the peptide solution at a concentration of 14 mg/mL was added, bringing the concentration at the end of the sample to 1.4 mg/mL. It was homogenized and incubated at 37 °C in a Barnstead MaxQ 7000 water bath for 24 h. The exception was the bacterium *B. thermosphacta*, which was incubated at 24 °C.

After the incubation period, 100 µL of the mixture was taken and serial dilutions were made up to 10^{-5}in Eppendorf tubes containing 900 µL of sterile saline solution. They were homogenized, 20 µL was taken and poured into a Petri dish containing BHI, Nutritive, MRS, and TSA agar, as appropriate for the microorganism. We waited about 20 min until the drop dried and performed the plate spread. They were incubated for 24 h at 37 °C, except for *B. thermosphacta*, which was incubated at 24 °C. After 24 h, the number of colonies was counted ( ) and the percentage of inhibition of the film-forming solution was obtained (Equation 1).

$$\% \; de \; Inhibición = \left(1 - \frac{C.f}{C.i}\right) * 100 \qquad \text{(Ec 1)}$$

Statistical analysis

The results obtained were evaluated using one-way analysis of variance to establish statistically significant differences ($p<0.05$) in the inhibitory effect for each microorganism using Duncan's method of comparison of means using the Statgraphics Centurion Version 19 software package (Statgraphics Technologies, Inc., The Plains, VA, USA) in accordance with the methods of Montgomery, (2020).

Results and discussion

Bacterial growth kinetics

The growth kinetics during reactivation of the bacteria evaluated are shown in Figure 1. With the exception of *L. monocytogenes*, which begins its exponential phase after 2 hours of incubation, the other bacteria showed accelerated growth from time zero, reaching their exponential phase at the start of the test or shortly thereafter, suggesting rapid adaptation to the medium. This behavior can be interpreted as effective activation of the cells after reactivation, which reduced the typical "lag" phase. The bacteria *S. aereus, L. monocytogenes, L. acidophilus*, and *E. coli* O157:H7 (Figure 1a) completed their exponential phase after 6 hours of incubation, while the bacteria *S. enteritidis* and *L. lactis* (Figure 1b) completed it after ~3 and ~4 hours, respectively. On the other hand, *P. aeruginosa* and *B. thermosphacta* bacteria maintained constant growth until 10 h (Figure 1b).

From a methodological perspective aimed at evaluating the efficacy of the *Phaseolus lunatus* peptide fraction, the observed bacterial growth profile is particularly rele-

vant. In this context, the data derived from the growth curves allow us to define a window of intervention for applying the peptide fraction during the active logarithmic phase, when cells tend to be more susceptible, rather than waiting for the stationary phase, when the tolerance of the population may increase (Rodríguez-Rojas & Rolff, 2022). Likewise, in the present study, the rapid entry of most strains into the exponential phase makes it feasible to apply the fraction at the moment of greatest microbial vulnerability, since trials with AMPs have shown that actively growing cells are more sensitive than those in the latency or adaptation phase (Zhang, 2025).

When comparing the species analyzed, *Listeria monocytogenes* showed an approximate delay of 2 h before entering its logarithmic phase, and for this strain, it will be appropriate to schedule antimicrobial treatment to coincide with the onset of cell multiplication. In contrast, *Salmonella enteritidis* and *Lactococcus lactis* took between 2.5 and 3 hours to reach the logarithmic phase, which would imply that intervention should be carried out earlier to maximize contact with the population when it is most vulnerable. In the case of *Pseudomonas aeruginosa* and *Brochothrix thermosphacta*, the prolonged active growth phase observed indicated a different dynamic with a window of vulnerability that could be extended for a longer period for the late application of the peptide fraction. These differences underscore the need to define the timing of treatment individually according to the bacterial strain (Ma et al., 2024), which constitutes an important methodological improvement in studies of antimicrobial coatings or films.

Growth rate (μ_{max}) and doubling time (t_d)

The kinetic parameters obtained indicated clear differences in the growth of microorganisms, reflecting both their physiological characteristics and their adaptive capacity in the culture medium (Table 1). *S. aureus* showed a high maximum growth rate (0.80 h^{-1}), no lag phase, and a relatively short doubling time, indicating rapid adaptation to the medium and greater susceptibility during the first hours of incubation . In contrast, *L. monocytogenes* had a longer doubling time (1.54 h), a lower μ_{max}(0.45 h^{-1}), and a delay of ~2 h before its logarithmic phase, associated with its need for metabolic reprogramming. Therefore, the application of the antimicrobial peptide fraction of *Phaseolus lunatus* should be synchronized in each species to maximize its effect. In the case of *S. aureus*, the rapid entry into the logarithmic phase suggests that exposure should occur immediately after inoculation, maximizing contact with actively dividing cells. In contrast, for *L. monocytogenes,* treatment should be applied a few minutes after the start of incubation, around 2 h. These findings are consistent with recent studies showing that the sensitivity of these bacteria depends on their physiological state and growth phase (Sousa et al., 2024; Medveďová et al., 2019).

Estimates indicated that the pathogenic enterobacteria studied (*E. coli* and *S. enteritidis*) had the shortest doubling times (~26–28 min), which is consistent with studies in enriched media, where typical $\mu_{m\ ax}$ are high and doublings are rapid (Ndraha et al., 2024). These figures imply that the exponential phase is intense and brief for these species; therefore, to maximize the effectiveness of an antimicrobial agent such

as the peptide fraction of *Phaseolus lunatus*, application must coincide with the onset or beginning of the exponential phase to intercept cells when they are actively synthesizing cell wall, membrane, and macromolecules (Rodríguez-Rojas & Rolff, 2022).

Lactic acid bacteria (LAB) and *Brochothrix thermosphacta* had significantly longer doubling times (~69–139 min), with correspondingly lower $\mu_{m\ ax}$ values. This indicates that active growth is more widespread, so the application of the antimicrobial may be less critical at a specific time, but require longer exposures or higher concentrations to achieve the same logarithmic reduction. The growth rate and physiological state modulate susceptibility, and LAB in particular, due to their physiology and membrane, are more resistant to antimicrobial agents (Śliżewska & Chlebicz-Wójcik, 2020).

Pseudomonas aeruginosa showed an intermediate t_d(~46 min) but a sustained growth pattern up to 10 h, suggesting prolonged metabolic activity under the conditions tested. In these cases, the useful window for intervention is prolonged, but challenges also arise, as *P. aeruginosa* has mechanisms of resistance , such as the formation of biofilms and efflux pumps, which can reduce the effectiveness of external agents and promote the formation of protective structures (Illikoud et al., 2019). The same applies to *B. thermosphacta*, whose long $t_d$indicated slower growth.

Minimum inhibitory concentration of the peptide fraction of P. lunatus

The presence of wells without red coloration in the microdilution plates (Figures 2 and 3) clearly indicates the inhibition of bacterial viability by the <10 kDa peptide fraction of *Phaseolus lunatus*, through the reduction of the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) indicator, evidencing metabolic activity when coloration is present. This color change is consistent with the use of validated tetrazolium-based assays to evaluate antimicrobial activity in bacteria, as described by Xu et al. (2023). The results indicated that for *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus*, color elimination, as an indicator of the absence of viability detected, occurred at a concentration of 1,139 mg/mL, while for *Lactobacillus acidophilus*, *Lactococcus lactis*, and *Salmonella enteritidis,* inhibition occurred at 0.996 mg/mL. For *Brochothrix thermosphacta,* the effect was observed at 0.427 mg/mL, and for *Pseudomonas aeruginosa*, a lower value (0.142 mg/mL), which could be due to cell density or peptide release, suggesting that the peptide fraction may require a higher concentration or improved release techniques to overcome intrinsic resistance mechanisms.

This variability in inhibitory concentration between species suggests a combined influence of bacterial physiological factors such as membrane structure, growth rate, density, and peptide availability. In particular, the lower efficacy against Gram-negative bacteria is consistent with the findings of Raheem & Straus (2019) on antimicrobial peptides (AMPs), which indicated that the lipopolysaccharide (LPS)-rich outer membrane acts as a barrier to the penetration and action of the active agent. Likewise, the high effectiveness against *B. thermosphacta* (100% inhibition) and other Gram-positive bacteria confirms that this type of peptide has greater access and efficacy when the structural barrier is lower or contains fewer defense components.

In active coating applications, these findings imply that the formulation must consider both the actual concentration of available peptide and the growth phase and type of target bacteria. Strains that show adaptation delays or slow growth may require longer exposure times or adjustments in peptide release to ensure effectiveness. The review by Li et al. (2024) on AMPs indicated that combining peptides with controlled-release matrices or nanofillers can improve availability and efficacy against G-bacteria.

The <10 kDa peptide fraction extracted from *Phaseolus lunatus* showed differentiated antimicrobial activity against pathogenic and spoilage bacteria (Table 2). The minimum inhibitory concentrations (MIC) found for spoilage bacteria (0.142-0.427 mg/mL) and pathogenic bacteria (0.996-1.139 mg/mL), with particular inhibition of *Pseudomonas aeruginosa* (0.142 mg/mL), indicated a relatively broad spectrum of activity and outstanding efficacy. This pattern is consistent with recent studies showing that plant-derived antimicrobial peptides (AMPs) can exhibit considerable activity against both G+ and G- bacteria. Chen et al. (2025) demonstrated that a plant peptide called NCBP1 was effective against multiple Gram+ and Gram- strains with low MIC values.

The greater susceptibility of Gram+ bacteria observed in this study is consistent with what has been described in the literature: plant-derived peptides tend to have lower inhibitory concentrations against species such as *Staphylococcus aureus* and *Listeria monocytogenes* compared to Gram- bacteria. This can be attributed to structural differences in the cell envelope, as Gram- bacteria have an outer membrane rich in lipopolysaccharides that acts as a permeability barrier, restricting the penetration of antimicrobial molecules (Zhang, 2025). Some of these bacteria are capable of preventing the binding of peptides to the outer membrane, reducing the net negative charge of this layer by modifying the lipid A of the LPS or by reducing the fluidity of the outer membrane. This causes an increase in the number of hydrophobic interactions between the acyl ends of lipid A, reducing its negative charge, the attraction of peptides to the cell surface, and limiting interaction with cationic peptides. In contrast, G+ bacteria lack this outer membrane, allowing peptides to access the cytoplasmic membrane more easily and exert their lethal effect more efficiently (Hickson et al., 2025).

The absence of inhibitory activity of biopeptides derived from food sources against the strains evaluated in this study was evidenced by Parvez et al. (2024), who found that the specificity of the antimicrobial spectrum is dependent on the biological source, amino acid sequence, net charge, and three-dimensional structure. In particular, peptides rich in basic amino acids tend to have greater activity against Gram-, while those rich in hydrophobic residues favor efficacy against G+. In this sense, peptides from *Phaseolus lunatus*, possibly with high contents of basic residues/cysteine and disulfide bridges, could explain their greater activity on certain bacteria such as *P. aeruginosa*.

Antimicrobial activity of the film-forming solution

The antimicrobial activity of the film-forming solution incorporating the <10 kDa peptide fraction of *Phaseolus lunatus* is shown in Table 3. Significant differences ($p < 0.05$) were found, with differentiated antimicrobial performance observed for the inhibition of the bacteria evaluated.

In particular, the *Brochothrix thermosphacta* strain was completely inhibited (100% reduction in viability) after treatment, suggesting very high efficacy under film application conditions. Likewise, species such as *Listeria monocytogenes* (63% inhibition), *Lactococcus lactis* (53%), and *Lactobacillus acidophilus* (72%) also showed significant reductions, positioning this peptide fraction as promising for Gram-positive bacteria or food spoilage bacteria. In contrast, the *Escherichia coli* O157:H7 strain showed a 33% increase, indicating that SFP was not only ineffective against this Gram-negative bacterium, but that its performance was counterproductive. This effect was possibly due to the contribution of nutrients by the peptides through the action of proteases produced as defense mechanisms by the microorganism, thus promoting its growth.

Similarly, *Staphylococcus aureus* and *Salmonella enteritidis* showed only 17% inhibition, and *Pseudomonas aeruginosa* barely 1%. These results suggest that the efficacy of the peptide film is clearly modulated by the bacterial species, its physiology (G+ vs. G-) and probably the film's ability to interact with or release the active agent (Rizzetto et al., 2023). This differential behavior is consistent with recent reviews on active films and antimicrobial coatings ( ), which point out that the incorporation of natural antimicrobial agents into films and coatings such as polymer matrices, biopolymers, and bioactive compounds can vary widely depending on the target strain, the release matrix, cell density, and application environment (Fadiji et al., 2023). In particular, it is well documented that antimicrobial systems are more effective against G+ bacteria, due to the absence of an outer membrane, and present challenges against G-, where the outer membrane rich in lipopolysaccharides and porin proteins acts as a barrier to the access and action of the antimicrobial agent (Zhang, 2025). Therefore, the poor effect against *E. coli* O157:H7 and *P. aeruginosa* may be related to this bacterial structure, along with the possible reduction in peptide diffusion through the film, insufficient release, or inactivation upon contact with the application surface.

From the point of view of food applications, the findings indicated that the *P. lunatus* peptide fraction integrated into SFP clearly has greater potential for controlling spoilage bacteria and improving the shelf life of perishable foods than for direct pathogen control. To optimize its effectiveness, it will be necessary to investigate modifications to SFP, such as the addition of plasticizers, controlled-release agents, nanocarriers, or nanofillers (Ribeiro et al., 2021), as well as increasing the concentration of the peptide to overcome the outer membrane barrier of the target bacteria. In addition, it is suggested to evaluate the release of the peptide, its stability in the SFP matrix, and its adhesion to the food substrate, as well as possible interference with the sensory or physical properties of the coating, as indicated by recent analyses of active biofilms (Jabbar et al., 2023).

Conclusions

The <10 kDa peptide fraction obtained from exhibited selective and promising antimicrobial properties against several foodborne pathogenic and spoilage microorganisms. The strong inhibition observed in Gram-positive bacteria is consistent with their structural characteristics, specifically the absence of an outer lipopolysaccharide

membrane. Notably, the complete inhibition of *Brochothrix thermosphacta* and reductions greater than 50% for *Listeria monocytogenes*, *Lactococcus lactis*, and *Lactobacillus* acidophilus highlight the potential of this fraction as an antimicrobial component in food preservation strategies. The integration of microbial growth kinetics provided mechanistic insight into why some species responded more effectively than others. *S. aureus* showed rapid exponential growth ($\mu max = 0.80\ h^{-1}$; td ~ 0.87 h), indicating high vulnerability during early metabolic activation. In contrast, *L. monocytogenes* showed a longer adaptation phase (~2 h), suggesting that maximum susceptibility occurs later during incubation. These species-dependent kinetic behaviors underscore the importance of timing antimicrobial delivery in active packaging systems, ensuring that peptide exposure coincides with the physiological state most conducive to inactivation. In contrast, Gram-negative bacteria showed limited inhibition and, in the case of *E. coli* O157:H7, an unexpected increase in population. These findings support the potential of using *Phaseolus lunatus* peptide fractions as a source of natural antimicrobial peptides, with possible applications in the formulation of food preservatives and in the design of biotechnological strategies for microbial control. Future work should focus on improving peptide stability, diffusion, and controlled release within polymeric matrices, as well as validating performance under real food conditions.

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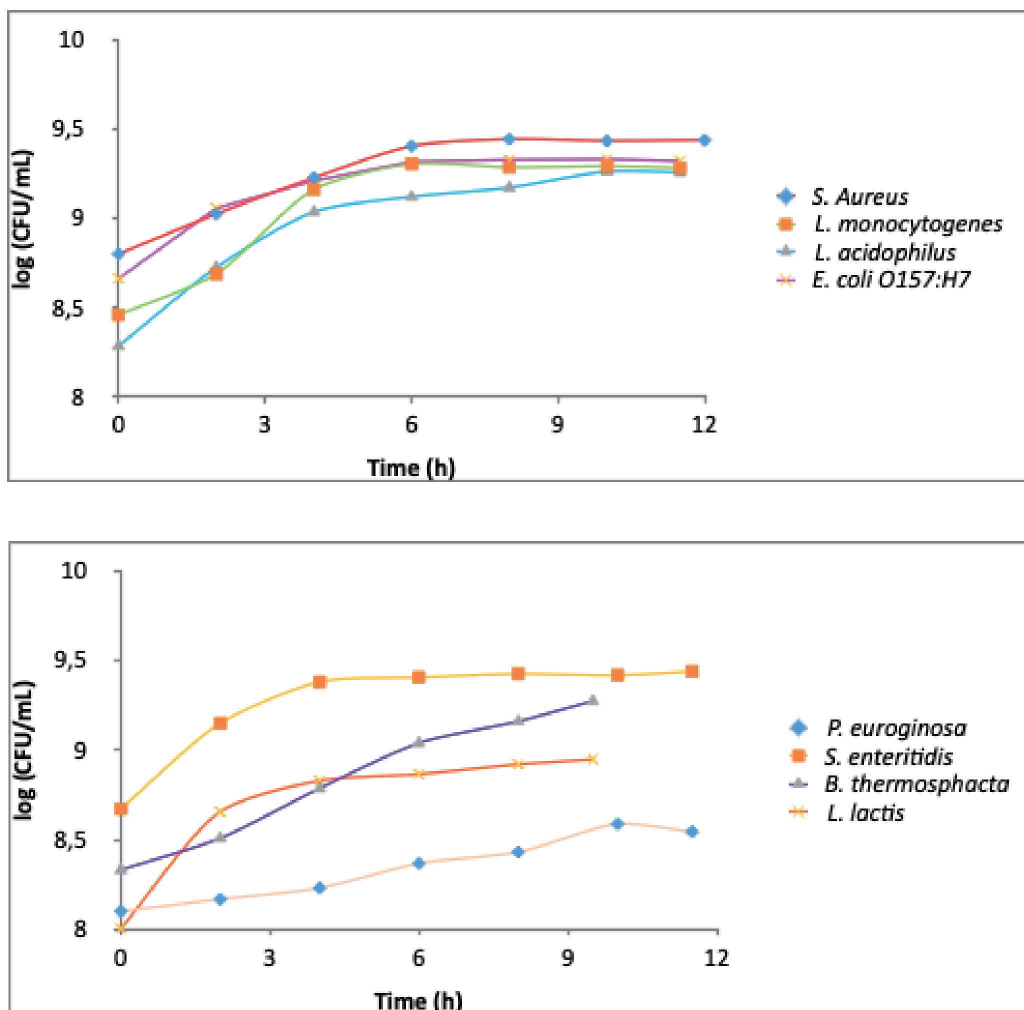


Figure1 . Growth kinetics of pathogenic and spoilage bacteria. a) *S. aureus, L. monocytogenes, L. acidophilus*, and *E. coli* O157:H7 ; b) *P. auroginosa, S. enteritidis, B. thermosphacta*, and *L. lactis*

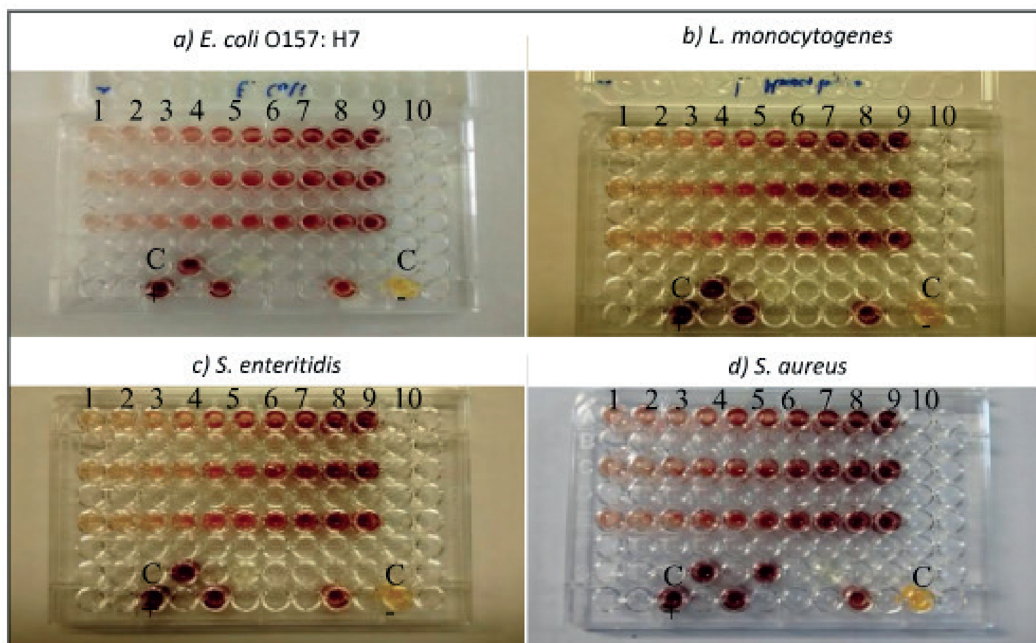
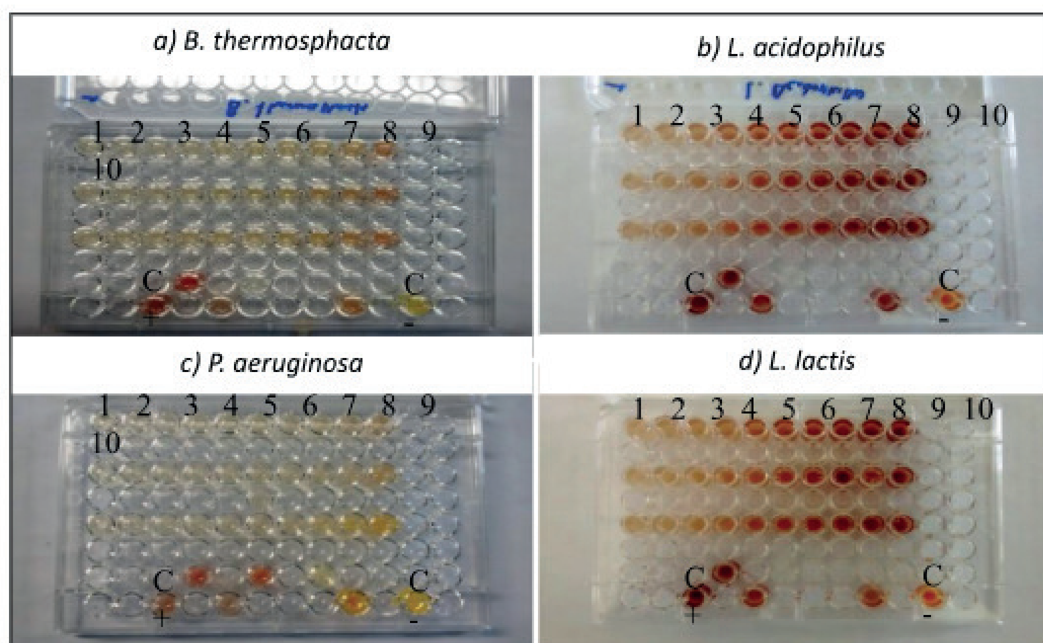


Figure 2. Minimum inhibitory concentration of the <10 kDa peptide fraction of *P. lunatus* against pathogenic bacteria: a) *E. coli* O157:H7, b) *L. monocytogenes,* c) *S. enteritidis,* d) *S. aureus.* The wells marked with the numbers 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 correspond to concentrations of 100, 90, 80, 70, 60, 50, 40, 30, 20, and 10%, respectively, relative to the initial protein content (1.42 mg/mL).



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Microorganism	Maximum growth rate $\mu_{m\ ax}$ (h^{-1})	Doubling time t_d (h)
Staphylococcus aureus	0.80 ± 0.20^{bc}	0.866 ± 0.22^{d}
Listeria monocytogenes	0.45 ± 0.15^{de}	1.540 ± 0.13^{b}
Lactobacillus acidophilus (ATCC 11454)	0.30 ± 0.10^{e}	2.310 ± 0.270^{a}
Escherichia coli O157:H7	1.60 ± 0.25^{a}	0.433 ± 0.07^{e}
Salmonella enteritidis	1.50 ± 0.20^{a}	0.462 ± 0.09^{e}
Pseudomonas aerugino-sa ATCC 27853	0.90 ± 0.20^{bc}	0.770 ± 0.15^{d}
Brochothrix thermosphacta	0.30 ± 0.15^{e}	2.310 ± 0.16^{a}
Lactococcus lactis ATCC 314	0.60 ± 0.15^{cd}	1.155 ± 0.19^{c}

Table 1. Kinetic growth parameters μ_m and t_d for pathogenic and spoilage bacteria.

$^{a-e}$Different letters in the same column indicate statistically significant differences ($p<0.05$)

Microorganism	MIC (mg/mL)
Brochothrix thermosphacta	0.427^{c}
Lactobacillus acidophilus	0.996^{b}
Lactococcus lactis	0.996^{b}
Pseudomonas aeruginosa	0.142^{d}
Listeria monocytogenes	1.139^{a}
Escherichia coli O157:H7	1.139^{a}
Salmonella enteritidis	0.996^{b}
Staphylococcus aureus	$1,139^{a}$

Table 2. Minimum inhibitory concentration (MIC) of < kDa peptide fraction of *P. lunatus* against spoilage bacteria and pathogenic .

$^{a-e}$Different letters indicate statistically significant differences ($p<0.05$).

Microorganism	Initial concentration (CFU/mL)	Final concentration (CFU/mL)	% Inhibition
Staphylococcus aureus ATCC 5083	$2x10^7$	$3.3x10^6$	17^{d}
Salmonella enteritidis ATCC 13076	$1x10^7$	$1.7x10^6$	17^{d}
Escherichia coli O157:H7	$3x10^6$	$3.9x10^6$	-33^{f}
Listeria monocytogenes ATCC 51414	$1x10^7$	$6.3x10^6$	$63^{b,c}$
Brochothrix thermosphacta ATCC 11509	$3x10^6$	0	100^{a}
Pseudomonas aeruginosa ATCC 27853	$1x10^5$	$1x10^3$	1^{e}
Lactococcus lactis ATCC 314	$1x10^7$	$5.3x10^6$	53^{c}
Lactobacillus acidophilus ATCC 11454	$1x10^7$	$7.2x10^6$	72^{b}

Table 3. Inhibition of the film-forming solution incorporated with the < kDa peptide fraction of *Phaseolus lunatus*

$^{a-f}$ Different letters indicate statistically significant differences ($p<0.05$)