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Antimicrobial potential of chickpea flour hydrolysates against foodborne pathogens

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Abstract*– In the context of higher education it is imperative that students focus on solving real problems during their professional education. The objective of this project was to motivate 7th semester students of Biotechnology Engineering, under a scheme of problem-based learning, to develop an innovative solution to solve a real problem affecting the food industry. The proposed problem was the escalating bacterial resistance and associated risks posed by foodborne pathogens to both food quality and public health. It had created the necessity for natural alternatives to the conventional chemical preservatives employed in the food industry. The research developed assessed the antimicrobial potential of hydrolysates derived from chickpea flour germinated in the presence of Se, Zn or the combination of both. The protein fraction within the flour was extracted by a solubility-based method and digested using pepsin or pancreatin. The resulting hydrolysates were evaluated for their antimicrobial efficacy at concentrations of 5.0, 10.0, and 15.0 mg/mL against pathogenic foodborne bacteria (Salmonella enterica, Staphylococcus aureus, Escherichia coli, and Listeria monocytogenes), employing a resazurin assay. Notably, pancreatin demonstrated the highest hydrolysis efficiency. Pepsin hydrolysates exhibited no discernible antimicrobial activity against the tested bacteria and, in fact, promoted their growth. Conversely, three pancreatin hydrolysates displayed antimicrobial activity against Staphylococcus aureus (S. aureus) at 15.0 mg/mL, while the remaining hydrolysates neither inhibited its growth or that of other bacteria. In conclusion, these identified peptides present a potential alternative to chemical antimicrobial preservatives, in combating S. aureus contamination.

***Keywords*—Selenized peptides; antimicrobial activities; food preservatives; chickpea; higher education**

I. Introduction

In accordance with the FAO, one third of the food produced for human consumption is wasted each year. Consequently, food quality and safety emerged as paramount concerns for the food industry, as microbial contamination possess substantial risk to public health and results in considerable losses [1]. Even though most microbial contamination is caused by non-pathogenic microorganisms, implying alterations on nutritional and sensory characteristics of food products; pathogenic spoilage is responsible for 600,000,000 cases of foodborne disease, 420,000 deaths and 27,000,000 years of life lost, per year [2].

As a first attempt to preserve the food, the industry employs the addition of antimicrobial preservatives as strategy that allows to control the natural spoilage and/or avoid/control contamination by microorganisms. However, many chemical antimicrobials commonly employed in food industry, such as benzoates, sorbates, and propionates, are subject to concentration restrictions due to potential health implications [3]. The excessive use of these preservatives has led to the development of bacterial resistance, encompassing various foodborne pathogenic microorganisms, including *Salmonella spp., Escherichia coli (E. coli), Listeria monocytogenes (L. monocytogenes), and Staphylococcus aureus (S. aureus)* [4]. Therefore, the customers become more vulnerable to foodborne diseases. In addition, the demand for food products with a lower content of chemical preservatives has been increasing [5]. Consequently, this has led to establish a market for natural compounds with antimicrobial capacity.

One of the most popular natural alternatives to conventional preservatives are antimicrobial peptides (AMPs). These compounds are protein residues constituted from 10 to 50 amino acids, with hydrophobic and hydrophilic regions that generate an amphipathic structure, giving them conformational flexibility and allowing penetration into bacterial cells to induce membrane lysis, by the electrostatic interactions [6].

The action mechanism of the AMPs had been described by different model proposals of membrane disruption. In the barrel-stave pore model, the peptides insert perpendicularly into the membrane to form transmembrane pores. Whereas, in the toroidal pore, once reached the threshold, the peptides are incorporated to the membrane forming pores that allow the membrane to curve around it [7]. While, in the carpet model the peptides are adsorbed parallel to the membrane till the threshold reach allows disintegrate the membrane by the formation of micelles and pores [8].

Alternatively, to the membrane disruption mechanism of action, some peptides may have intracellular targets allowing them to interfere on intracellular processes, and eventually cause bacterial death [9]. Furthermore, it is put forward the possibility that AMPs employ multiple and complementary actions to achieve its antimicrobial effect, which helps to evade bacterial resistance development [10].

As well as AMPs, other alternatives as metallic nanoparticles (NPs) had been explored to substitute traditional antimicrobial agents. Among it, selenium NPs (SeNPs) had

shown antimicrobial activities [11] with a minimum inhibitory concentration (MIC) between 17 - 25 µg/mL against pathogenic bacteria [12]. The general antibacterial mechanisms of SeNPs can be summarized as penetration of the cell wall, cell membrane damage and contents leakage, inhibition of the formation of biofilms, and induction of oxidative stress [13].

Meanwhile, zinc NPs (ZnNPs) presented MICs against pathogenic bacteria between 1.0 - 3.5 mg/mL [14,15]. Literature had described the different action mechanisms, being that the direct contact of ZnNPs with cell walls causes destruction of bacterial cell integrity, liberation of antimicrobial ions, reactive oxygen species (ROS) formation [16].

The versatility of action exhibited by these compounds has led to their integration into innovative delivery technologies, such as active packaging. Active packaging is design to interact with the food product or its surrounding environment, and diminish, restrain, or impede the proliferation of microorganisms that may reside on food surfaces. [17, 18, 19]. In this context, AMPs stand out due to their facile extraction process and widespread presence across various life forms. Notably, plants represent a noteworthy source of AMPs, constituting an abundant group of proteinaceous compounds with robust microbicidal properties against a spectrum of pathogens, including viruses, bacteria, fungi, parasites, and protozoa [20]. Plant-derived AMPs offer a promising avenue for exploration in the development of antimicrobial strategies. In fact, multiple legumes extracted peptides had demonstrated effectiveness as antimicrobial agents for the reduction of bacterial contamination in food. Purified peptides extracted from peas seed (*Pisum sativum*) achieve the inhibition of bacterial growth of *S. aureus* at 75 µg/ml and 100 µg/ml [46]. Whereas soybean peptides had demonstrated antimicrobial activity against *L. monocytogenes* at concentrations above 312.5 µM [47], and Chickpeas (*Cicer arietinum*) peptides exhibited an antibacterial effect against *E. coli, L. monocytogenes, S. aureus and Salmonella typhimurium* at concentrations of 0.13, 1.04, 2.08 and 1.04 mg/mL [48].

Specifically, peptides sourced from plants such as chickpeas emerge as a compelling option, given the relatively high protein content of chickpeas (24.6%). Noteworthy is the fact that countries like Mexico produce substantial quantities of chickpeas, approximately 121,000 tons per year [21], thereby presenting a viable and sustainable peptide source for further research and application in antimicrobial packaging.

Meanwhile, the mineral properties of metallic nanoparticles could be introduced to the AMPs through the increase of proteins with selenium and zinc present in the plant material to enhance its antimicrobial activity. This enhancement could be achieved by germinating the plant material in the presence of these minerals [22].

In this regard, the present study established as objective to assess the antimicrobial potential of hydrolysates derived from germinated chickpea flour in the presence of selenium, zinc, or their combination by resazurin assay.

II. MATERIALS AND METHODS

2.1 Chickpea germination

Kabuli chickpea (*Cicer arietinum L.*) seeds germination was carried out on an industrial scale at "Alimentos Lee," maintaining a controlled environment with a temperature of 19 °C and a relative humidity of 80%. Irrigation was systematically applied every 4 hours with a relation 1:3 w/v under the following treatments: (T1) Water, (T2) 24 mg of Na_2SeO_3 per liter of water, (T3) 480 mg of $ZnSO_4$ per liter of water, (T4) 24 mg of $ZnSeO_3$ per liter of water, and (T5) a combination of 24 mg of $ZnSO_4$ and Na_2SeO_3 per liter of water.

2.2 Chickpea flour production

Sprouted chickpeas were freeze-dried during 72 hours at −50 °C and 0.036 mbar (LABCONCO, Kansas City, MO, USA). Then, dried chickpea seeds were milled with a coffee grinder (KRUPS GX4100, Germany) and sieved through a mesh no. 100.

2.3 Protein extraction

Chickpea flours were defatted by incubating with hexane (1:4 w/v) for 24 hours at 50°C with constant agitation. Subsequently, 20 g of chickpea flour was combined with 200 mL of distilled water, and the pH was adjusted to 8.5 using NaOH 1 M. The samples were incubated for 2 hours at 30°C and 200 rpm, followed by centrifugation at 10,000 g and 4°C for 20 minutes (Sorvall ST1R Plus-MD, ThermoFisher Scientific, Germany). The resulting pellet and supernatant were separated, with the pellet resuspended in distilled water (1:5), incubated, and subjected to centrifugation under the same conditions. The supernatant obtained from the first and second centrifugation rounds was combined, and the pH was adjusted to 4.5, with stirring at the same conditions. The samples were centrifuged again, and the resulting pellet was recovered and subjected to freeze-drying during 96 hours at −50 °C and 0.036 mbar for future use (LABCONCO, Kansas City, MO, USA).

2.4 Enzymatic hydrolysis

The protein extracts were solubilized in milli-Q water (5% w/v) and the pH was adjusted to the optimum value for each reaction (Pepsin: pH 2.2, Pancreatin: pH 7.5) with NaOH 1 M and HCl 1 M (Sigma-Aldrich). Next, the enzyme was added (4% w/w, protein base). The samples were incubated at 37°C for 1 hour. After, the reaction was stopped by submerging the samples in a water bath at 90°C for 5 minutes. Then they were centrifuged for 10 minutes at 10,000g and 4°C. The supernatant was collected, freeze-dried, and stored at 4°C until further use. The hydrolysis degree was calculated as described by [25].

2.5 Quantification of minerals in the flour

For the quantification of Se and Zn present in the flour of the different treatments, the chickpea flour was digested using a microwave digestion system (MARS 6^{TM}, CEM, United States), by adding 10 mL of nitric acid to the fume hood and 500 mg of the sample in the liners. Following the digestion, the

carousel was removed and placed in the hood, and each liner was carefully opened to release the nitric acid fumes. The resulting liquid was then transferred into 25 mL volumetric flasks and graduated with 15 mL of Milli-Q water. After thorough mixing, the liquid was filtrated using Whatman Grade 41 paper filters and subsequently poured into centrifuge tubes. The concentration of minerals, including Se and Zn, was determined using an inductively coupled plasma mass spectrometer (ICP-MS) X Series2 (Thermo Scientific, NC), following the methodology outlined by [50].

2.6 Culture conditions

A colony from each bacterial strain, including *Salmonella enterica, E. coli*, *L. monocytogenes, and S. aureus*, sourced from an established bacterial stock, were selected. Subsequently, each chosen colony was inoculated into 6 mL of Mueller Hinton Broth (MHB) media (Oxoid, United Kingdom). The bacterial cultures were then incubated under standardized conditions at 37°C and 200 rpm for a duration of 24 hours.

2.7 Antimicrobial assay

The antimicrobial efficacy of the hydrolysates was assessed through resazurin assay. Two distinct hydrolysates, digested either with pepsin or pancreatin, were tested at concentrations of 5.0, 10.0, and 15.0 mg of dry hydrolysate per mL. Each well of the sterile plate was loaded with 50 µL of the test substance, 30 µL of MHB, 10 µL of resazurin solution (1 mg/mL) (Sigma-Aldrich, MO, USA), and 10 µL of bacterial suspension at a concentration of $1x10^4$ cfu/mL. The prepared plates were incubated at 37 °C for a period of 24 hours and subsequently measured at 570 nm using a plate reader (Synergy HT, Biotek). Sterilized water served as the negative control and sodium benzoate at 10 mg/mL as positive control. Furthermore, sodium benzoate was subjected to testing at concentrations of 1.5, 3.0, 5.0, 7.5, and 10.0 mg/mL.

2.8 Statistical analysis

All experiments were performed at least in triplicate and the results were expressed as the mean ± standard deviation. JMP-Software Software was used to perform the ANOVA analysis followed by a post hoc Tukey´s test with a 95% of confidence level.

III. RESULTS AND DISCUSSION

3.1 Enzymatic hydrolysis

The hydrolysis degree (DH) achieved at the digestion of protein extracts by pancreatin, showed a significant increase against pepsin performance (Fig. 1). Specifically, the DH values for pepsin hydrolysates ranged between 13.07% - 16.34%. While the pancreatin hydrolysates went from 32% to 56%. Among these T5 showed a significant DH increase compared to the other treatments. Conversely, T2 exhibited significant lower DH values in comparison with the other treatments.

The significant difference in the DH between the enzymes may be attributed to pancreatin composition, which comprises a mixture of two different enzymes trypsin and chymotrypsin [23]. These enzymes exhibit a preferential cleavage of peptide bonds with specific residues. Trypsin favors the cleavage of bonds containing residues of lysine or arginine. While chymotrypsin targets bonds with basic or aromatic amino acids such as histidine, arginine, lysine, phenylalanine, tyrosine, or tryptophan [24]. In contrast, pepsin is an individual protease with preference for cleavage of peptide bonds containing methionine, phenylalanine, or leucine [25]. The amino acid profile of chickpeas, after 48 hours of germination, presents a predominant concentration of lysine at 13% and leucine at 8.3%, succeeded by phenylalanine at 4.8%, tyrosine at 3.3%, followed by methionine at 1.4%, and tryptophan at 1.2% [26]. It is noteworthy that the germination process, exhibited the potential to reduce the concentration of certain amino acids, as leucine and phenylalanine, and a significant increase was specifically demonstrated in lysine concentration [26,49]. Therefore, the amino acid composition of the protein extracts from the chickpea flours might favored the enzymatic activity of the pancreatin complex. Moreover, this favoring effect could had been intensified by an extended germination time beyond the initially reported 48-hour timeframe.

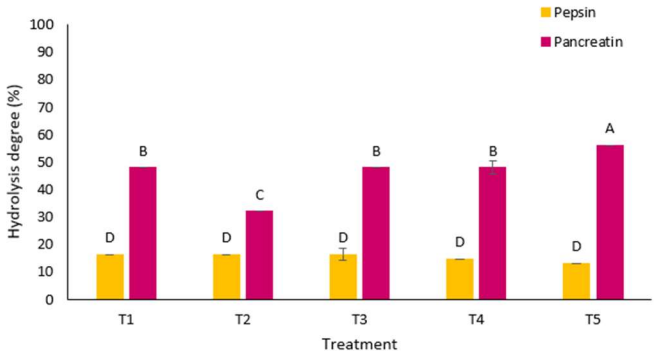


Fig. 1 Hydrolysis degree of sprouted dry chickpea protein in the presence of different minerals: (T1) Water, (T2) Na_2SeO_3, (T3) $ZnSO_4$, (T4) $ZnSeO_3$, (T5) $ZnSO_4$ + Na_2SeO_3. Error bars correspond to standard deviation of two replicates. Data Bars sharing the same letter were not significantly different to the rest of the tested samples ($p < 0.05$).

On the other hand, the significant increase of DH showed by the pancreatin on T5 compared with the other hydrolysates may been a consequence of the presence of zinc sulfate and selenium selenite during chickpea germination. This treatment could potentially induce the binding between the metalloids and the protein [27], conferring greater stability to the protein structure [28, 29] and consequently protect the protein from denaturation during the hydrolysis process, leading to a higher DH than the other treatments.

Meanwhile, the significant decrease in the DH performed by the pancreatin in T2 could be attributed to an enzymatic inhibition of pancreatin activity by the action of the Se-peptide complexes generated during the hydrolysis. Being that previous studies have reported the enzyme inhibitory capacity of

peptides derived from metalloproteins [30, 31], and selenium has been identified as a tool for the inhibition of enzymatic activity in the pancreatic system [32].

3.2 Quantification of minerals in the flour

The quantification of minerals in chickpea flour revealed distinct patterns in the concentrations of Zn and Se across different treatments (Fig. 2). Notably, Zn concentrations surpassed Se concentrations in all treatments, underscoring the prevalence of Zn in the chickpea matrix. T3 exhibited the highest Zn concentration, surpassing other treatments, especially those where selenium was concurrently administered (Fig. 2A). This suggests that the absence of selenium in T3 allowed for a more efficient uptake of zinc, resulting in elevated concentrations. In contrast, the Se concentration in chickpea flour not treated with selenium during germination was notably lower than in treatments where selenium was present. Among the selenium-containing treatments, Na_2SeO_3 emerged as the most effective reactant for incorporating selenium into the chickpea flour, as evidenced by the highest selenium concentrations achieved in treatments where Na_2SeO_3 was added during germination.

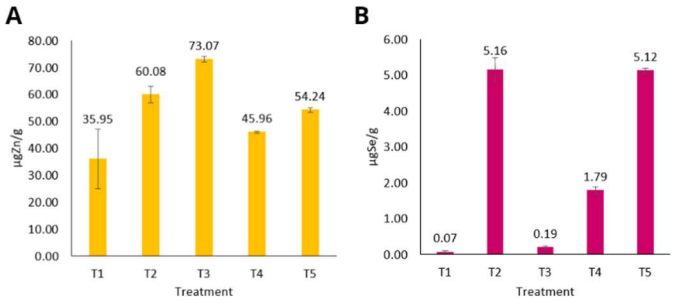


Fig. 2 Mineral concentration on the chickpea flour per gram of chickpea. **(A)** Zn concentration **(B)** Se concentration. (T1) Water, (T2) Na_2SeO_3, (T3) $ZnSO_4$, (T4) $ZnSeO_3$, (T5) $ZnSO_4$ + Na_2SeO_3

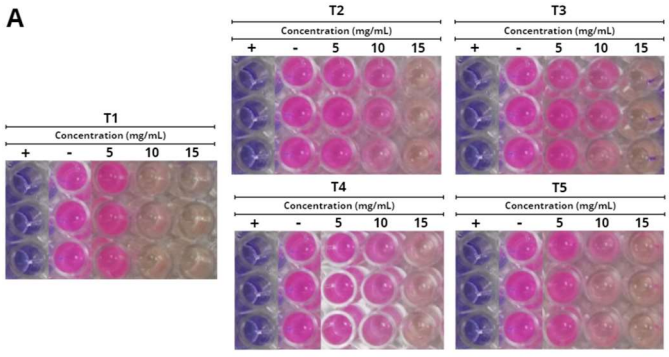
3.2 Antimicrobial assay

The antimicrobial capacity of protein hydrolysates from chickpea flours was assessed through a resazurin assay (fig. 3 – 10). Resazurin is a water-soluble dye subjected to reduction through electron transfer reactions associated with cell respiration, yielding resorufin, a product easily quantifiable through light spectrometry and that induces a color shift from blue to pink [33]. However, resorufin can further undergo reduction to hydroresorufin, a nonfluorescent product resulting in a visible color transition from pink to colorless, ultimately causing a bleaching effect in the assay when culture metabolic activity increases [34, 35].

As observed in fig. 3 – 6, all the pepsin hydrolysates exhibited a tendency to promote bacterial growth with increasing concentration. Despite the decrease in resorufin concentration indicated in section B of each figure exhibited by the absorbance reduction, the color presented in section A demonstrated a bleaching effect by the presence of hydroresorufin, contrary to the absence of such effect in the negative controls of the strains. Particularly in *S. aureus* (Fig. 4), the bleaching effect was also observed in the negative control of the assay. This may mistakenly suggest by the absorbance values (Fig. 4B), that the positive control allows for greater metabolic activity than the hydrolysates. However, a comparison of the visual colour results (Fig. 4A) between samples reveals that although the positive control does not inhibit bacterial growth, it does prevent the bleaching effect observed in the rest of the samples. Contrary to the rest of the bacteria (Fig. 3, 5, 6) where was possible to observe a reduction in resorufin concentration in the positive control compared with the pepsin hydrolysates at 5 mg/mL (where the bleaching effect did not occur), suggesting inhibition or reduction of bacterial metabolic activity.

The bleaching tendency of the samples could be attributed to the absence of antimicrobial peptides in the hydrolysates utilized for the assay, suggesting that the pepsin hydrolysis of proteins extracted from chickpea flour does not yield antimicrobial peptides against the selected bacterial strains and the peptides produced are capable of favor bacterial growth of the tested bacteria. Nevertheless, it remains possible that the hydrolysates may exhibit antimicrobial effects against other strains or types of microorganisms. Furthermore, the observed increase in bacterial growth with rising concentration can be explained by the hydrolysis products serving as a nutrient-rich source for bacteria. Higher concentrations of hydrolysates imply an elevated availability of essential amino acids and peptides, serving as crucial building blocks for bacterial protein synthesis and subsequent growth [36].

Related to the presence of Zn, Se or the combination of them in the hydrolysates resulted from the treatments applied to the tested flours, it is noteworthy that, despite the reported antimicrobial potential of both metalloids against foodborne bacteria [37, 38], the concentration of these elements in the hydrolysates was intended to complement the peptides bioactivity. Then that quantity may not be enough to exhibit any effect over the bacterial growth.



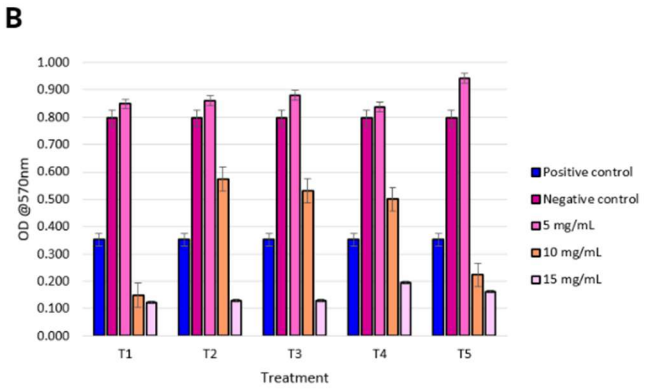


Fig. 3 Resazurin assay in *Salmonella enterica* in the presence of pepsin hydrolysates. **(A)** Visual result, **(B)** Absorbances at 570 nm from each sample: (-) Negative control: with sterile water, (+) Positive control: sodium benzoate 10 mg/mL, (T1) Water, (T2) Na_2SeO_3, (T3) $ZnSO_4$, (T4) $ZnSeO_3$, (T5) $ZnSO_4$ + Na_2SeO_3.

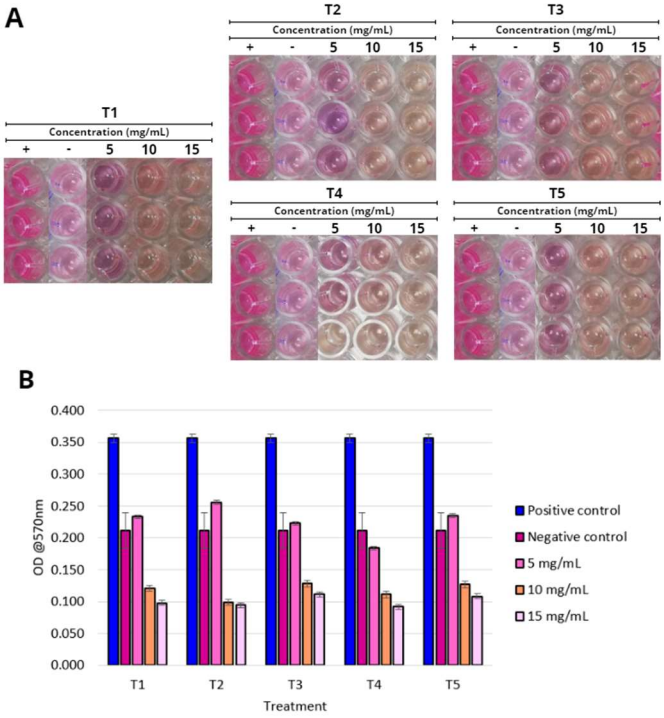


Fig. 4 Resazurin assay in *S. aureus* in the presence of pepsin hydrolysates. **(A)** Visual result, **(B)** Absorbances at 570 nm from each sample: (-) Negative control: with sterile water, (+) Positive control: Sodium benzoate 10 mg/mL, (T1) Water, (T2) Na_2SeO_3, (T3) $ZnSO_4$, (T4) $ZnSeO_3$, (T5) $ZnSO_4$ + Na_2SeO_3.

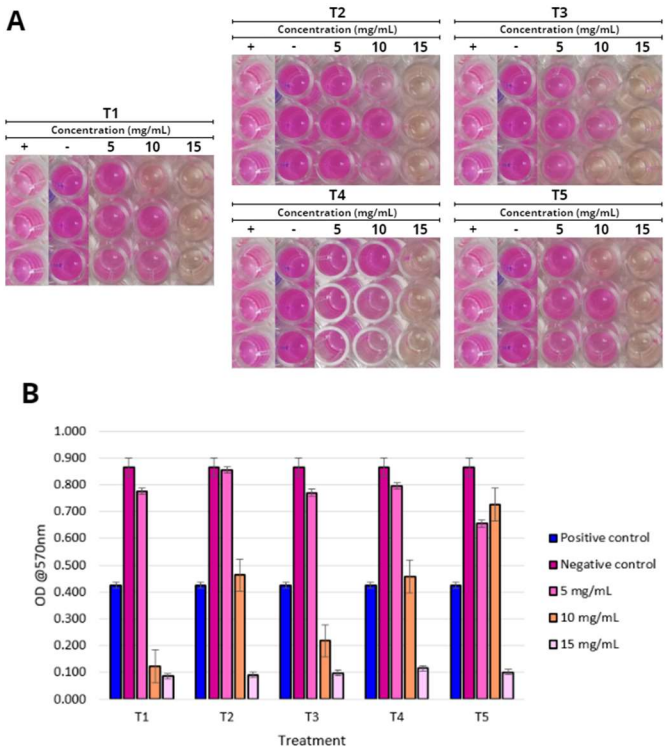


Fig. 5 Resazurin assay in *E, coli* in the presence of pepsin hydrolysates. **(A)** Visual result, **(B)** Absorbances at 570 nm from each sample: (-) Negative control: with sterile water, (+) Positive control: Sodium benzoate 10 mg/mL, (T1) Water, (T2) Na_2SeO_3, (T3) $ZnSO_4$, (T4) $ZnSeO_3$, (T5) $ZnSO_4$ + Na_2SeO_3.

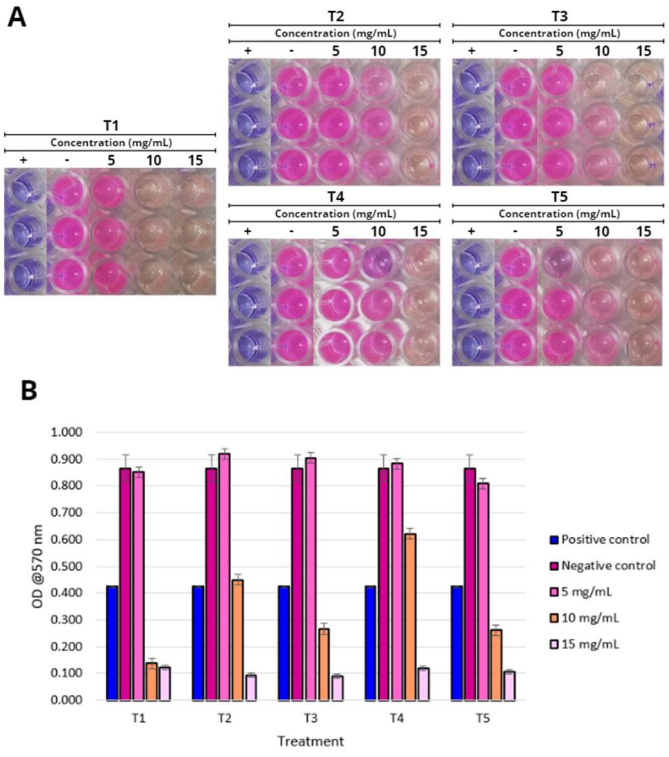


Fig. 6 Resazurin assay in *L. monocytogenes* in the presence of pepsin hydrolysates. **(A)** Visual result, **(B)** Absorbances at 570 nm from each sample: (-) Negative control: with sterile water, (+) Positive control: Sodium benzoate

10 mg/mL, (T1) Water, (T2) Na_2SeO_3, (T3) $ZnSO_4$, (T4) $ZnSeO_3$, (T5) $ZnSO_4$ + Na_2SeO_3.

On the other hand, the hydrolysates resulting from the enzymatic action of pancreatin exhibit similar trends to pepsin hydrolysates, showing an increase in bacterial growth with higher concentrations of hydrolysates and lower inhibition efficiency compared to the positive control (Fig. 7-10).

However, T3 hydrolysate displayed an exception against *Salmonella enterica*, wherein the metabolic activity of the culture exhibited a slight reduction at 15 mg/mL of the hydrolysate (Fig. 7B). While this reduction may not be sufficient to declare antibacterial activity, further analysis at higher concentrations is warranted, given that previous studies have reported antibacterial effects of peptide-Zn complexes against foodborne bacteria, as *Salmonella enterica* [39, 40]. It is essential to note that analysis in terms of resorufin is feasible due to the absence of the bleaching effect.

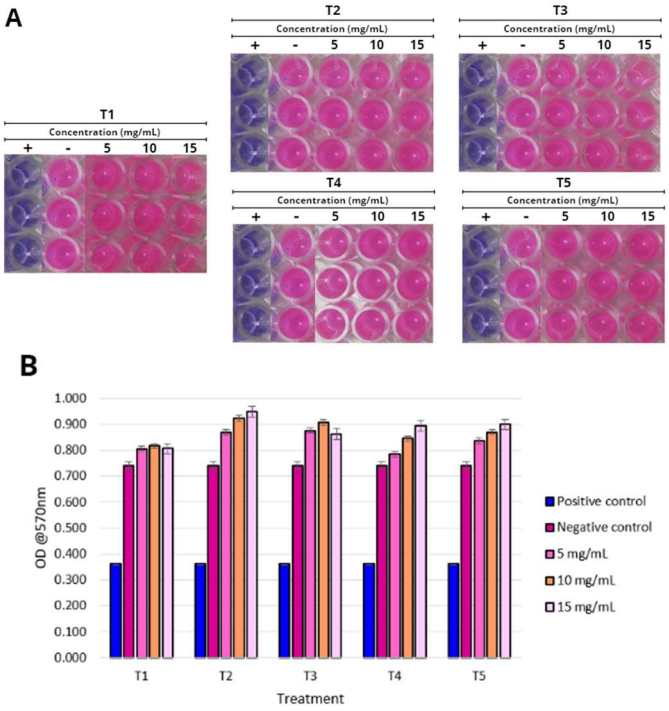


Fig. 7 Resazurin assay in *Salmonella enterica* in the presence of pancreatin hydrolysates. **(A)** Visual result, **(B)** Absorbances at 570 nm from each sample: (-) Negative control: with sterile water, (+) Positive control: Sodium benzoate 10 mg/mL, (T1) Water, (T2) Na_2SeO_3, (T3) $ZnSO_4$, (T4) $ZnSeO_3$, (T5) $ZnSO_4$ + Na_2SeO_3.

Furthermore, in the *S. aureus* strain (Fig. 8), inhibition of bacterial metabolic activity was observed at 15 mg/mL of T2 hydrolysate, and the same concentration of T4 and T5 showed a reduction in the metabolic activity of the culture, indicated by the prevention of the bleaching effect observed in the negative control and other concentrations. Despite these observations, none of the treatments exhibited sufficient similarity in absorbance or color to be declared a Minimum Inhibitory Concentration (MIC), or was as effective as the positive control to prevent the increase in metabollic activity of the culture. Nevertheless, it is noteworthy to mention that the three treatments containing selenium, while the other two lacking bioactivity, also lack selenium.

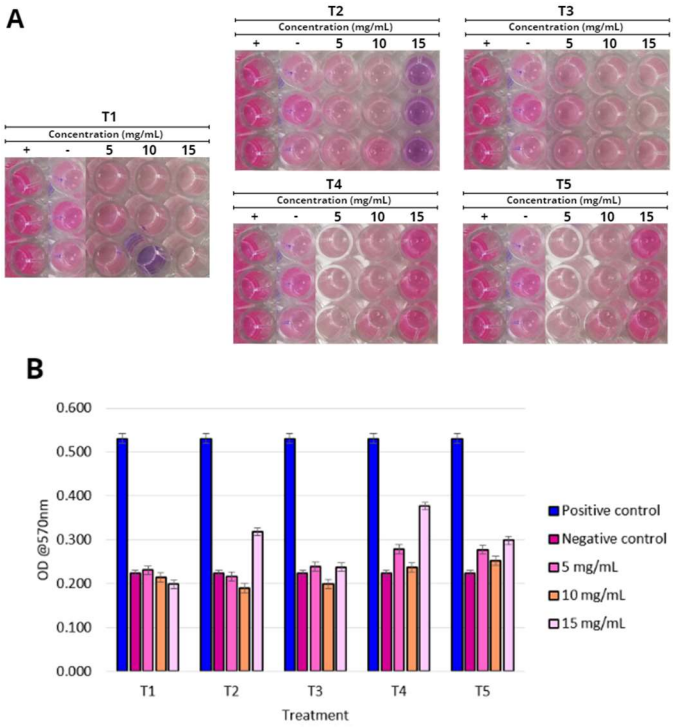


Fig. 8 Resazurin assay in *S. aureus* in the presence of pancreatin hydrolysates. **(A)** Visual result, **(B)** Absorbances at 570 nm from each sample: (-) Negative control: with sterile water, (+) Positive control: Sodium benzoate 10 mg/mL, (T1) Water, (T2) Na_2SeO_3, (T3) $ZnSO_4$, (T4) $ZnSeO_3$, (T5) $ZnSO_4$ + Na_2SeO_3.

As mentioned previously, evidence illustrates that selenoproteins, in the form of Se-peptide complexes, exhibit antimicrobial efficacy against foodborne pathogens. Consequiently, it is of particular interest that T4 and T5 exhibited only a reduction in bacterial metabolic activity. These treatments involved the addition of zinc, alongside selenium, during chickpea germination. Likewise, the only treatment supplemented with only Zn showed no reduction in metabolic activity. It is plausible that *S. aureus* may not be as sensitive to Zn-peptide complexes as it is to Se-peptide complexes, or it may require a higher concentration of hydrolysate for inhibition. This suggestion is supported by previous studies reporting a greater antimicrobial efficiency of SeNPs compared to ZnNPs [41]. Therefore, the lower antimicrobial efficiency of T4 and T5 compared to T2 could be attributed to an insufficient concentration of metallopeptides, as their activity has been shown to be dose-dependent [42].

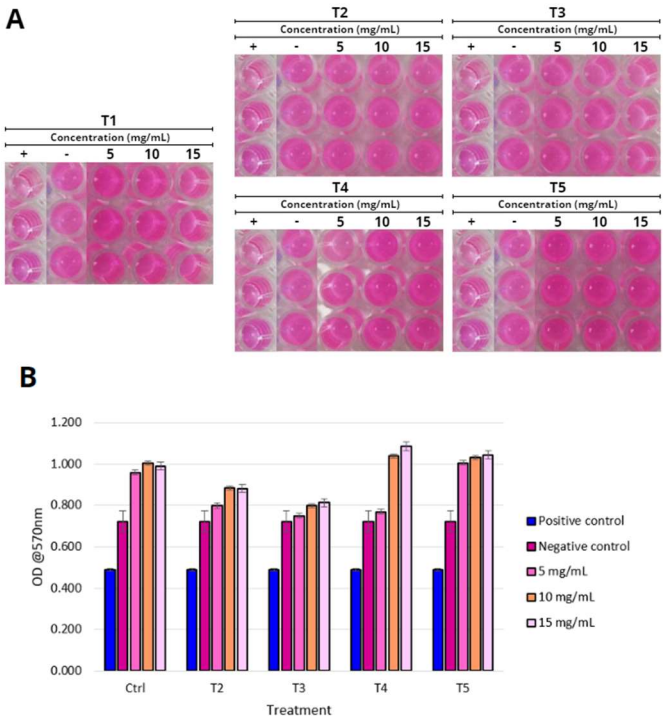


Fig. 9 Resazurin assay in *E. coli* in the presence of pancreatin hydrolysates. **(A)** Visual result, **(B)** Absorbances at 570 nm from each sample: (-) Negative control: with sterile water, (+) Positive control: Sodium benzoate 10 mg/mL, (T1) Water, (T2) Na_2SeO_3, (T3) $ZnSO_4$, (T4) $ZnSeO_3$, (T5) $ZnSO_4$ + Na_2SeO_3.

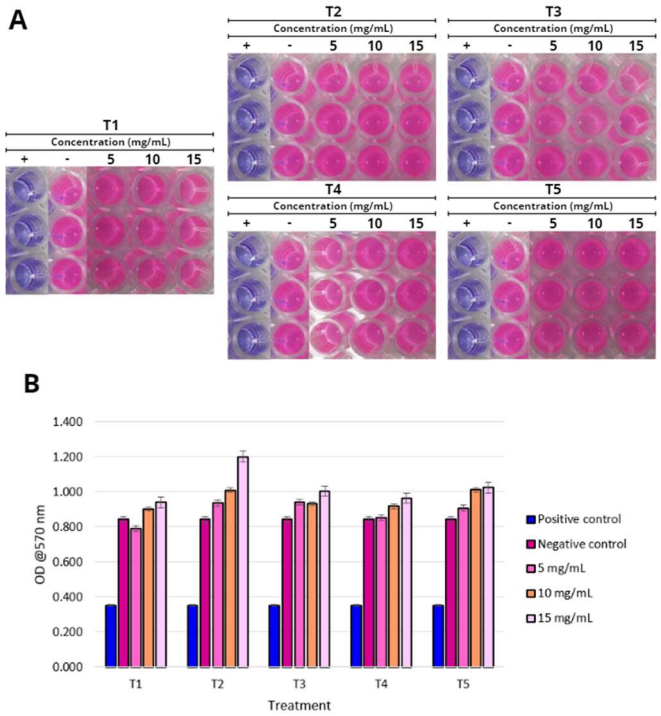


Fig. 10 Resazurin assay in *L. monocytogenes* in the presence of pancreatin hydrolysates. **(A)** Visual result, **(B)** Absorbances at 570 nm from each sample: (-) Negative control: with sterile water, (+) Positive control: Sodium benzoate 10 mg/mL, (T1) Water, (T2) Na_2SeO_3, (T3) $ZnSO_4$, (T4) $ZnSeO_3$, (T5) $ZnSO_4$ + Na_2SeO_3.

3.3 Sodium benzoate MIC

The MIC of sodium benzoate was determined through resazurin assay (Fig. 11). The MIC was found to be 10 mg/mL for *Salmonella enterica* and *L. monocytogenes*, while *S. aureus* and *E. coli* exhibited the necessity of higher concentrations. This suggests an increasing bacterial resistance, aligning with previous studies that reported an MIC range of 1.5–12.5 mg/mL for foodborne bacteria [43, 44]. Moreover, it is of particular interest that, for *S. aureus* the MIC for sodium benzoate appears remarkably similar to the T2 hydrolysate. The last presented antimicrobial activity at 15 mg/mL, causing color change to purple, while sodium benzoate presented visible antimicrobial activity from 3 to 15 mg/mL but maintained the color change to pink, which demonstrates a major concentration of resorufin [45] than in the T2 hydrolysate test. Consequently, the latter emerges as a viable alternative to chemical antimicrobial preservatives in averting *S. aureus* contamination.

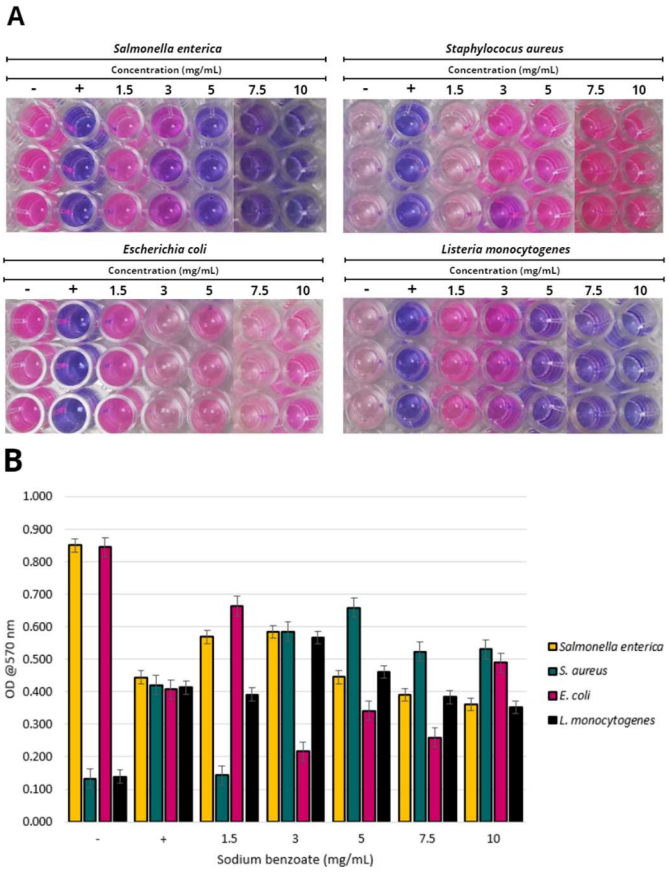


Fig. 11 Rezasurin assay in the presence of sodium benzoate at different concentrations. **(A)** Visual result, **(B)** Absorbances at 570 nm from each sample: (-) Negative control with sterile water, (+) Positive control with 25 µM of penicillin and 25 µM of streptomycin, (T1) Water, (T2) Na_2SeO_3, (T3) $ZnSO_4$, (T4) $ZnSeO_3$, (T5) $ZnSO_4$ + Na_2SeO_3.

IV. CONCLUSION

In conclusion, pepsin hydrolysates demonstrated no discernible antimicrobial activity against common foodborne bacterial strains. However, pancreatin hydrolysates presented antibacterial efficacy against *S. aureus* at 15 mg/mL, particularly when pre-treated with Na_2SeO_3, $ZnSeO_3$, or a combination of $ZnSO_4$ and Na_2SeO_3. Notably, sodium selenite-treated hydrolysates exhibited the highest effectiveness, although further studies are warranted to determine the MIC for each hydrolysate at higher concentrations. Furthermore, pancreatin resulted as the most effective enzyme, exhibiting heightened hydrolytic efficiency when complemented by the presence of zinc or a combination of zinc and selenium in proteins. By contrast, the efficiency was reduced when selenium was introduced in proteins independently.

The gathered information provides a strong basis for considering these treated pancreatin hydrolysates as potential alternatives to chemical preservatives to prevent *S. aureus* contamination, while mitigating concerns related to bacterial resistance. The promising antimicrobial properties of these alternatives underscore their potential application in the food industry, promoting food safety without compromising long-term efficacy.

With respect to the learning strategy, this model based on real problems constitutes an educational innovation not only because of the cognitive and execution skills developed in the process, but also because of the commitment generated in the student with the development of the project and their own learning, knowing that they are attending to a real need and that they could become a potential entrepreneur.

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