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Research article

Bioactive protein hydrolysates obtained from amaranth by fermentation with lactic acid bacteria and *Bacillus* species

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ABSTRACT

Protein hydrolysates are a promising source of bioactive peptides. One strategy by which they can be obtained is fermentation. This method uses the proteolytic system of microorganisms to hydrolyze the parental protein. Fermentation is a little-explored method for obtaining protein hydrolysates from amaranth. Different strains of lactic acid bacteria (LAB) and *Bacillus* species isolated from goat milk, broccoli, aguamiel, and amaranth flour were used in this work. First, the total protein degradation (%TPD) of amaranth demonstrated by the strains was determined. The results ranged from 0 to 95.95%, the strains that produced a higher %TPD were selected. These strains were identified by molecular biology and were found to correspond to the genera *Enterococcus, Lactobacillus, Bacillus,* and *Leuconostoc*. Fermentation was carried out with amaranth flour and the selected strains. After this process, water/salt extracts (WSE) containing the released protein hydrolysates were obtained from amaranth doughs. The peptide concentration was measured by the OPA method. The antioxidant, antihypertensive and antimicrobial activity of the WSE was evaluated. In the FRAP test, the best WSE was LR9 with a concentration of 1.99 μMTE/L ± 0.07. In ABTS, 18C6 obtained the highest concentration with 19.18 μMTE/L ± 0.96. In the DPPH test, there was no significant difference. In terms of antihypertensive activity, inhibition percentages ranging from 0 to 80.65% were obtained. Some WSE were found to have antimicrobial properties against *Salmonella enterica* and *Listeria monocytogenes*. Fermentation of amaranth with LAB and *Bacillus* spp. allowed the release of protein hydrolysates with antioxidant, antihypertensive, and antimicrobial activity.

1. Introduction

Currently, several diseases affect people's quality of life. According to the World Health Organization (WHO), the diseases that cause the highest death rates are cardiovascular diseases, cancer, chronic respiratory diseases, and diabetes [1].

One of the factors that can cause these diseases is oxidative damage to lipids, proteins, and nucleic acids, caused because there is no balance between the antioxidants found in the living organism and reactive oxygen species (ROS) [2]. The formation of ROS is an inevitable process that arises as a side effect of respiration [3]. These are neutralized by the body's natural antioxidant defense system or by the antioxidants consumed. However, sometimes an excess of ROS is produced due to various factors, causing the capacity of

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antioxidants to be exceeded and, unfortunately, the generation of fatal diseases [4].

According to WHO, another disease that is impacting today's society is hypertension, as it is the cause of death of 12.8% of the world's population [1]. This condition is defined as the repeated elevation of blood pressure above 140/90 mmHg [5]. Hypertension can develop due to different causes, one of which is dysfunction of the renin-angiotensin-aldosterone system (RAAS) [6]. It has been identified that when the angiotensin-converting enzyme (ACE) hydrolyzes angiotensin I decapeptide, angiotensin II octapeptide is produced, which upon binding to the cell surface receptor AT1R, induces vasoconstriction and inactivates bradykinin, a vasodilator [7].

Another current problem is the growing number of microorganisms that have developed resistance to antibiotics, causing an increase in infections. Which are difficult to treat with current antibiotics [8]. This is originated from the inherent resistance that protects the microorganism, such as the outer membrane in Gram-negative bacteria, or from the acquired resistance that develops due to genetic mutations that confer this characteristic to the microorganism through different mechanisms [9].

In recent years, the importance of protein hydrolysates has increased due to their nutritional properties and bioactive effects [10]. They are also considered a promising source of bioactive peptides [11]. Bioactive peptides are biomolecules derived from specific proteins composed of 2–20 amino acid subunits [12]. However, there are some exceptions, such as Lunasin, a bioactive peptide of 44 amino acids [13]. They have multiple bioactivities such as immunomodulatory, antihypertensive, anticancer, anti-inflammatory, antioxidant, and antimicrobial, among others [14].

Protein hydrolysates containing bioactive peptides have been obtained from animal and vegetable food proteins. In recent years, the trend has been to obtain these molecules from plants because of their social and environmental impact [15].

Amaranth is a pseudocereal that belongs to the foods considered promising for the future, mainly because of its nutritional contribution [16]. It is a good source of lipids, dietary fiber, vitamins, and antioxidants. Compared to other cereals, it has a high protein content [17,18]. Due to its excellent composition, it has an enormous potential to be considered a source of bioactive molecules, for example, protein hydrolysates and bioactive peptides. There are already reports of protein hydrolysates and bioactive peptides obtained from amaranth [11,19,20]. However, the only technique that has been used so far to release them from amaranth parental protein has been enzymatic hydrolysis.

A little-explored technique to hydrolysate amaranth protein is fermentation. This method involves the use of microorganisms to obtain protein hydrolysates and bioactive peptides since the parental protein is broken by the action of the peptidases that the microbes can secrete during fermentation [21,22].

Certain advantages that this method has in comparison with the enzymatic hydrolysis method are that there is a greater diversity of microbial proteases and there are high levels of protease activity since all the microorganism's own proteases act and not just one; it is of lower cost and therefore cost-effective; it gives added value to the substrate used as they demonstrate to improve the nutritional profile of this, reduce the content of antinutrients and improve the organoleptic properties; in addition, it is also considered to be a more eco-friendly method than enzymatic hydrolysis [21,23–25].

The selection of the microorganism is key in this process since it is in charge of generating the proteolytic enzyme; however, it must be given adequate environmental conditions to promote its growth (temperature, time, aw, pH) [26].

Lactic acid bacteria (LAB) are one of the most relevant microorganisms in obtaining protein hydrolysates and bioactive peptides by fermentation because they have a highly specialized proteolytic system consisting of a cell-envelope proteinase that initiates degradation, a transport system, and intracellular peptidases [27]. In addition, they have certain advantages, such as easy adaptation to animal and vegetable substrates, some genres are considered GRAS by the FDA, and are easy to work with [28,29]. It is important to mention that the proteolytic activity of the LAB is characterized by being strain-dependent, this causes a high diversity of proteolytic activities since it even differs among strains of the same species.

Bacillus species can produce a high number of non-specific proteases to enhance hydrolysis [30,31]. In addition, they have high growth performance because they consume low-cost carbon sources and grow in harsh environments with limited or unavailable nutrients [32,33]. Some strains have GRAS status, such as *Bacillus subtilis*, which has generated peptides with diverse biological activity by fermenting soybeans [34]. However, there are other *Bacillus* species that have high proteolytic activity and should be evaluated to demonstrate their ability to generate protein hydrolysates or bioactive peptides. For example, *B. thuringiensis*, *B. megaterium,* and *B. proteolyticus* are species for which there are no reports so far.

The aim of this work is to release bioactive protein hydrolysates from amaranth doughs through the fermentation process using lactic acid bacteria and different *Bacillus* species (*B. thuringiensis*, *B. proteolyticus,* and *B. megaterium*). These protein hydrolysates could contain bioactive peptides, but purification and sequencing techniques are needed in future studies to verify this.

2. Methodology

2.1. Proximal composition of amaranth

Amaranth seeds (*Amaranthus hypochondriacus*) were purchased in Saltillo, Coahuila, Mexico. They were ground and sieved using an 8 × 2 in. mesh until a particle size of 425 microns was achieved to obtain flour. Protein (total nitrogen × 5.7), lipid, ash, and moisture contents were determined according to AACC-approved methods (46-11th, 30-10.01, 08-01, and 44-15th, respectively) [35]. The determination of total fiber was carried out by the methods of AOAC 962.09 [36]. Total carbohydrates were calculated by difference: 100-(protein + lipids + ash + moisture) [15].

2.2. Isolation of lactic acid bacteria and Bacillus species

For this study, 123 strains of LAB and *Bacillus* species were used. The strains from goat milk (43), broccoli (24), and aguamiel (26) were previously isolated. They belong to the Food Department of the Universidad Autonoma de Coahuila (Mexico) collection. New isolates (30) were also made from autochthonous amaranth dough microorganisms. For this, 7 mL of water was added to 3 g of non-defatted amaranth. It was incubated at 37 °C for 48 h. Dilutions (10^{-1}–10^{-5}) were made from 1 g of fermented amaranth dough. They were inoculated on MRS agar and Elliker agar. They were incubated at 37 °C for 24 h. The different colonies were purified and preserved.

2.3. Determination of the amaranth total protein degradation by LAB and Bacillus species

LAB and *Bacillus* spp. strains were reactivated in MRS broth for 18 h at 37 °C, and then an aliquot was transferred to amaranth-maltose broth. This broth was composed of the following (in g/L): maltose 10, glucose 10, defatted amaranth 2, K_2HPO_4 2, sodium acetate 5, $MgSO_4 \cdot 7H_2O$ 0.2, $MnSO_4 \cdot 1H_2O$ 0.05, Tween 80 1 ml, pH 6.2–6.6; as described by Pepe et al. [37], but varying the nitrogen source to amaranth flour only. The strains were incubated at 37 °C for 24 h. Using the Bradford method, total protein degradation (TPD) was determined by taking a control at 0 h and using the equation one:

$$100 - ((TPf/TPi)*100) \quad (1)$$

where TPf is the final total protein of the sample and TPi the initial protein or control. A BSA curve (0–200 mg/L) was used. The percentage of TPD was the indicator of proteolytic activity. The strains with the best TPD were selected according to the statistical analysis performed.

2.4. Molecular identification and phylogenetic analysis of LAB and Bacillus species with the best TPD

DNA extraction was performed by enzymatic lysis of the selected strains with the best TPD. Amplification of 16S rRNA was carried out using the primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1512R (5′-ACG GCT ACC TTG TTA CGA CTT-3′) [38]. PCR products were visualized on 1.5% agarose gel. The amplified products were purified and sequenced. Molecular identification of the isolated strains was carried out by local alignment of the obtained sequences with those reported in the NCBI database using the BLAST algorithm.

For phylogenetic analysis, the 16S rRNA gene sequences were aligned in the MAFFT v. 7.130 program. Alignments were analyzed manually to find conserved regions and to infer a phylogenetic tree. Phylogenies were estimated using the MrBayes program with the best-estimated nucleotide substitution model using the MEGA tool version 7.0.18. Phylogenetic trees were also evaluated using the maximum likelihood method and the Neighbor-joining method in MEGA. All trees were performed with 500 resamples (bootstraps). The topology of the trees obtained by the different methods was compared.

2.5. Amaranth fermentation

LAB strains and *Bacillus* species selected based on the highest % TPD were cultivated into their respective medium for 18 h at 37 °C. Cells were recovered by centrifugation (10,000 × g, 10 min, 4 °C) and washed twice with 50 mM sterile potassium phosphate buffer (pH 7.0). They were resuspended in water to reach a cell density of 8.0 log CFU/mL. Thirty g (30 g) of non-defatted amaranth flour and 70 mL of water containing the cell suspension of each LAB or *Bacillus* (cell density in the dough of: 7.0 log CFU/mL) were used to prepare 100 g of dough. This was mixed and placed in tray-type reactors. The doughs were fermented for 24 h at 37 °C, according to the conditions previously set up by Coda et al. [39]. Uninoculated amaranth doughs at 0 and 24 h were taken as controls.

2.6. Water soluble/salt extracts (WSE)

The extracts were prepared from the fermented dough obtained at the final incubation time, according to the method described by Rizzello et al. [15]. An aliquot of each dough (3.75 g) was taken and diluted with 15 mL of 50 mM Tris-HCl (pH 8.8), held at 4 °C for 1 h with agitation every 15 min. Subsequently, centrifugation was performed at 20,000 g × 20 min, and the supernatants were recovered, containing the water/salt soluble nitrogen fraction, which were stored at −20 °C until analysis.

2.7. Peptide concentration

The peptide concentration of WSE were determined by the o-phthaldialdehyde (OPA) method [40] with some modifications by Muhialdin et al. [41]. Briefly, the OPA solution was prepared by mixing 25 mL of 100 mmol/L sodium tetraborate, 2.5 mL of 20% (wt/wt) SDS, 40 mg of OPA (dissolved in 1 mL of methanol), and 100 μL of β-mercaptoethanol, this was diluted to a final volume of 50 mL with deionized water. To a 96-well microtiter plate, 36 μL of the sample and 270 μL of OPA reagent were added. A standard curve using tryptone (0.25–1.5 mg/mL) was used as a reference. The solution was incubated in the dark for 3 min at room temperature, and the absorbance was measured at 340 nm.

2.8. Antioxidant activity

The antioxidant activity of the WSE was determined by three different methods.

2.8.1. ABTS test

The radical cation 2,20-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS+; Sigma-Aldrich) was used to measure the anti-radical capacity. For this, 7 mM/L ABTS was mixed with 2.45 mM/L potassium persulfate (Sigma-Aldrich). Subsequently, this was incubated for 16 h in the dark and at room temperature. It was diluted to an absorbance of 0.7 ± 0.02. To each well of a 96-well plate, 95 μL of the diluted $ABTS^{+}$ solution and 5 μL of the WSE were added. It was incubated for 1 min at room temperature. The absorbance was measured at 754 nm. Antioxidant capacity was expressed in micromoles of Trolox equivalents per liter (μM TE/L). Based on the Trolox calibration curve constructed [42].

2.8.2. DPPH test

The antiradical activity was measured using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH; Sigma-Aldrich). For this, the DPPH radical was prepared at 60 μM with methanol. In a 96-well plate, 7 μL of each WSE and 193 μL of the DPPH solution were added to each well. This was incubated for 30 min in the dark at room temperature. Its absorbance was measured at 520 nm. The results are expressed as μM TE/L. Based on the Trolox calibration curve constructed [42].

2.8.3. FRAP test

Ferric reducing antioxidant power (FRAP) solution was prepared. For this, acetate buffer (0.3 M, pH 3.6), $FeCl_3$ (20 mM in 40 mM HCl) and 2,4,6-Tris(2-pyridyl)-s-triazine (10 mM in 40 mM HCl) were mixed in a 10:1:1 ratio. In a 96-well plate, 10 μL of the WSE and 290 μL of the FRAP solution were added to each well. This was incubated for 15 min in the dark at room temperature. The results were expressed in μM TE/L. Based on the Trolox calibration curve constructed [42].

2.9. Antihypertensive activity

The antihypertensive activity of WSE were determined by a colorimetric method using the ACE kit-WST (Dojindo, Japan) according to the protocol described by the manufacturer. The absorbance was read at 450 nm. The results are expressed as % inhibition of ACE activity using the equation two:

$$\text{ACE inhibitory activity (inhibition rate \%)} = [(A_{\text{blank 1}} - A_{\text{sample}})/(A_{\text{blank 1}} - A_{\text{blank 2}})] \times 100 \quad (2)$$

where:

$A_{\text{blank 1}}$ is the absorbance of the positive control (no ACE inhibition)
$A_{\text{blank 2}}$ is the absorbance of the reagent blank
A_{sample} is the absorbance of the sample

2.10. Antimicrobial activity

The antimicrobial activity of WSE were determined according to the method described by Avaiyarasi et al. [43] and modified by Venegas-Ortega et al. [44]. Two pathogenic strains were used: *Salmonella enterica* and *Listeria monocytogenes*. These were inoculated on trypticase soy agar at a density of 8.0 log CFU/mL. Once the agar was solidified, 9 mm holes were punched with a sterilized punch. To each well, 50 μL of each WSE was added. The plates were incubated at 37 °C for 18 h. To calculate the antimicrobial activity, the clear zones formed around the wells were measured. The results were expressed in arbitrary units (AU) according to the equation three.

$$\text{AU} = \text{Inhibition area (mm}^2\text{)} - \text{Well area (mm}^2\text{)/Volume sample (mL)} = \text{mm}^2\text{/mL} \quad (3)$$

2.11. Electrophoresis

WSE were analyzed by Tricine SDS-PAGE according to Haider et al. [45], using a 4% stacking gel and a 15% resolving gel. WSE were mixed with loading buffer (1:1 ratio) and heat denatured at 70 °C for 10 min. Electrophoresis was performed with a vertical electrophoresis chamber, "Mini-Protean Tetra Cell II (Bio-Rad Laboratories)," at a constant voltage of 80 V for stacking gel and resolving gel. Protein bands were stained with silver stain. Molecular weight was determined using the Page-Ruler® (Thermo Scientific™) unstained low-range protein ladder (3.4–100 kD).

2.12. Statistical analysis

Data were subjected to one-way ANOVA; pair-comparison of treatment means was achieved by Tukey's procedure at $P < 0.05$, using the statistical software Statistica (Statistica7.0 per Windows).

3. Results and discussions

3.1. Proximal composition of amaranth

The proximal composition of the amaranth flour used in this study coincides with several values reported in the literature, as shown in Table 1. It differs only in the case of crude fiber since the value obtained is lower than that reported. This is because its content depends on the variety of amaranth used and the environmental conditions in which it has developed [48].

3.2. Determination of the amaranth total protein degradation by LAB and Bacillus species

TPD of 123 previously isolated LAB and *Bacillus* species (goat milk, aguamiel, broccoli, amaranth) was evaluated as shown in Table 2. These values are within the range found in reports such as that of Valerio et al. [49]. However, she obtained a maximum TPD of 73.7% ± 0.7. And in this work, a maximum TPD of 95.95 ± 5.73 was obtained. These differences are because Valerio et al. [49] use *Weissella cibaria* and *Lactiplantibacillus plantarum* to ferment amaranth. In this work the %TPD of several and different bacteria were evaluated. Although some of the strains evaluated are also *L. plantarum*, it should be noted that the proteolytic system of LAB is strain-dependent, so differences in protein degradation can be obtained even when dealing with the same strain species.

The degradation of the native protein of cereals and pseudocereals during sourdough fermentation is influenced by the pseudocereal enzymes, which release oligopeptides (primary proteolysis), and by the metabolic activity of microbial peptidases and proteinases, which release small peptides and free amino acids (secondary proteolysis) [50]. As mentioned above, the proteolytic and peptidase activity of LAB is strain-dependent [51]. In the case of *Bacillus,* it has been identified in several reports that protease activity is also strain-dependent [34]. It is, therefore, necessary to select the best strains that can degrade amaranth protein and thus produce various protein hydrolysates which could contain bioactive peptides.

The LS15, 4A4, AE13, 9C4, 11C4, AE7, 18C6, 3A2, 2M2, LS19, and LR9 strains proved to be those with the highest percentage of TPD. Therefore, when degrading the amaranth protein found in the amaranth-maltose broth, it was determined that these strains have enzymes capable of breaking down these macromolecules and thus hydrolyzing the protein. However, further analysis is needed to identify these enzymes and determine if there are common proteases among the strains or if they are different.

Some strains obtained a %TPD of 0. This is due to different factors, such as genetic variations of each species and the environmental conditions in which they grow, since these factors affect the modulation of the expression of proteolytic genes [52]. Also, the source of isolation of the bacteria influences, as it can be observed that all the strains isolated from amaranth were able to degrade amaranth protein. However, some of the strains isolated from other sources fail to degrade amaranth protein.

3.3. Molecular identification and phylogenetic analysis of LAB and Bacillus species with the best TPD

The molecular identification of the 11 selected strains with the best TPD was performed by 16S rRNA sequencing. According to the BLAST database, strain identification is shown in Table 3.

The LS15, AE13, 9C4, LS19, and LR9 strains were identified as belonging to the *Enterococcus* genus, which has been isolated from dairy foods, water, soil, and plants, although they naturally inhabit the gastrointestinal tract of humans and warm-blooded animals [53,54]. This study isolated them from goat milk, broccoli, and amaranth flour. It has been demonstrated by different reports that this genus has proteolytic activity since it can produce bioactive protein hydrolysates and even bioactive peptides [55–57]. However, there are few reports where *Enterococcus* strains are mentioned as opportunistic pathogens, causing nosocomial and urinary tract infections or endocarditis; therefore, they are not considered GRAS [58]. Because of this, safety testing of *Enterococcus* strains is necessary.

The 11C4 strain isolated from broccoli was identified as *Lactiplatibacillus plantarum*. This genus is auxotrophic for several amino acids; therefore, it uses its proteolytic system to hydrolyze proteins and obtain the amino acids necessary for its growth [59]. This makes it a genus with high proteolytic capacity. Currently, there are several reports where *L. plantarum* is used to hydrolyze the proteins of certain substrates and obtain peptides with different bioactivities [60–62].

Two strains, AE7 and 18C6, were identified as *Leuconostoc mesenteroides*. This LAB genus is commonly found in cereals, milk, vegetables, or meat [63]. For example, in this work, it was isolated from broccoli and amaranth flour. *L. mesenteroides* is a strain of biotechnological importance because it produces metabolites of interest (exopolysaccharides, vitamins, ethanol, among others) [64–66]. However, it has been reported that it has a poor proteolytic system [67]. On the other hand, *L. mesenteroides* is auxotrophic for certain amino acids, so it must hydrolyze proteins to grow and therefore has proteolytic enzymes. According to Ruiz et al. [68], this

Table 1
Proximate analysis of amaranth flour.

	Present work (*Amaranthus hypochondriacus*)	Rodríguez et al. [46] (*Amaranthus mantegazzianus*)	Bojórquez-Velázquez et al. [47] (*Amaranthus hypochondriacus cv* Nutrisol)
Fat (%)	7.08 ± 0.2	7.3 ± 0.2	6.9 ± 0.1
Protein (%)	15.3 ± 0.4	15.3 ± 0.6	15.8 ± 0.1
Ash (%)	2.90 ± 0.1	2.4 ± 0	3.5 ± 0.2
Crude fiber (%)	1.05 ± 0.1	-	2.4 ± 0
Moisture (%)	8.61 ± 0.4	8.2 ± 0.2	-
Carbohydrates (%)	65.06 ± 0.9	69 ± 3	71.4 ± 0.1

Table 2
Amaranth total protein degradation (TPD) by the action of LAB isolated from different sources.

Goat milk strains	% TPD	Aguamiel strains	% TPD	Broccoli strains	% TPD	Amaranth strains	% TPD
LS15	95.95 ± 5.73*	4A4	93.89 ± 3.83*	AE13	93.88 ± 4.79*	9C4	92.41 ± 1.30*
LS19	86.56 ± 1.99*	3A2	88.38 ± 2.35*	AE7	90.84 ± 12.93*	11C4	92.41 ± 1.30*
LR9	86.49 ± 9.56*	2M2	86.72 ± 10.16*	AE8	84.41 ± 3.83	18C6	90.04 ± 2.09*
LS23	79.92 ± 2.28	2A9	84.60 ± 6.48	AE6	81.70 ± 6.70	14C4	85.89 ± 2.09
LS18	76.70 ± 2.85	2M12	84.60 ± 2.6	AE9	75.27 ± 11.96	1I4	85.30 ± 2.93
LT1	76.50 ± 10.53	3M9	71.31 ± 5.83	AE10	72.90 ± 3.83	5C2	82.93 ± 3.77
LS7	75.70 ± 1.99	2M1	70.85 ± 1.29	AE2	66.81 ± 3.83	2I6	81.74 ± 2.09
LP12	75.69 ± 0.86	4A3-1	68.16 ± 5.74	A3	65.44 ± 4.29	18C6-1	79.66 ± 12.58
LP10	73.08 ± 0.57	2M9	66.73 ± 0.65	A11	62.69 ± 2.74	2I4	77.88 ± 1.68
LS21	71.63 ± 9.55	2M3	65.99 ± 0.39	A15	62.68 ± 9.77	1I2	77.59 ± 1.26
LS13	70.95 ± 4.78	3M2	64.90 ± 0.65	A41	62.13 ± 9.77	2IC6	72.99 ± 0.98
LS1	68.06 ± 6.54	2A3-1	52.55 ± 0.86	AE15	58.00 ± 0.95	I2	70.78 ± 4.19
LR24	67.65 ± 0.28	2A13	51.95 ± 3.44	A7	56.60 ± 14.50	I0	68.83 ± 0.98
LS20	67.58 ± 9.55	2M6	49.78 ± 7.78	A10	55.50 ± 1.96	20C6	65.36 ± 5.88
LR33	66.90 ± 6.69	4A8	49.77 ± 6.48	AE12	54.95 ± 11.97	24C8	41.43 ± 7.13
LR6	64.90 ± 15.3	2A4-1	49.51 ± 0.0	A12	54.66 ± 4.69	3C2	28.60 ± 4.91
LR15	64.19 ± 2.86	3M1	47.1 ± 18.9	A2	53.56 ± 0.78	12C4	21.32 ± 3.44
LP6	63.03 ± 3.98	3M7	46.47 ± 4.3	A8	53.28 ± 0.40	2I0	20.28 ± 0.98
SA1	62.84 ± 2.86	2M10	45.19 ± 1.29	AE5	51.36 ± 1.91	15C4	19.58 ± 0.98
LR21	62.02 ± 7.11	3A9	31.46 ± 1.56	AE3	51.23 ± 0.95	2I2	19.24 ± 7.35
LS22	60.81 ± 11.46	2A1-1	24.57 ± 5.83	A14	47.48 ± 9.38	2I8	6.75 ± 3.43
LS12	55.41 ± 1.91	3A5	23.35 ± 4.3	A1	45.83 ± 7.04	25C10	4.67 ± 0.43
LR18	49.95 ± 8.82	3M8	19.75 ± 1.44	A13	45.55 ± 5.08	6C2	0
LO	49.55 ± 11.66	2M7	0	AE11	34.31 ± 0.0	1I10	0
LP4	48.74 ± 4.84	3A3	0	A5	34.22 ± 7.81		
LS14	45.27 ± 0.95	2MII	0	AE1	32.30 ± 16.03		
LS9	43.92 ± 10.51	2M5	0	AE14	30.59 ± 3.34		
LR3	42.58 ± 10.51			AE4	8.92 ± 2.39		
LS4	37.17 ± 6.69			A43	7.1 ± 7.5		
LR30	32.44 ± 11.47						
LS3	31.64 ± 1.14						
LS10	31.09 ± 1.91						
LS11	29.73 ± 5.73						
LS5	28.38 ± 3.83						
LS17	28.02 ± 6.26						
LP8	26.01 ± 7.97						
LS16	24.20 ± 7.68						
LP2	23.19 ± 3.99						
LS2	18.56 ± 1.42						
LR12	13.73 ± 3.13						
LS8	10.31 ± 0.57						
LS6	9.91 ± 0.84						
LR22	0						

* Bacteria with the best %TPD according to the statistical analysis performed.

Table 3
Molecular identification of the strains with the best proteolytic activity.

Isolation source	Strain	Species	Percentage of identity	Length	Access
Goat milk	LS15	*Enterococcus faecium*	99.8%	821	NR_113904.1
	LS19	*Enterococcus durans*	100%	971	NR_043403.1
	LR9	*Enterococcus faecium*	94.51%	441	NR_104560.1
Broccoli	9C4	*Enterococcus faecium*	100%	631	NR_113904.1
	11C4	*Lactiplatibacillus plantarum*	100%	851	NR_115605.1
	18C6	*Leuconostoc mesenteroides*	100%	741	NR_157602.1
Aguamiel	4A4	*Bacillus thuringiensis*	100%	761	NR_157602.1
	3A2	*Bacillus proteolyticus*	100%	766	NR_157735.1
	2M2	*Bacillus megaterium*	100%	891	NR_112636.1
Amaranth	AE13	*Enterococcus casseliflavus*	99.89%	931	NR_036922.1
	AE7	*Leuconostoc mesenteroides*	100%	801	NR_114742.1

strain does show proteolytic activity. Still, so far, only one report has been found where it is used to obtain peptides with antihypertensive activity [69].

On the other hand, three strains (4A4, 3A2, and 2M2) were identified to belong to the *Bacillus* genus. These microorganisms have a high capacity to hydrolyze proteins [70], and this genus is considered a good producer of bioactive peptides [71]. There are already

reports of different *Bacillus* strains that generate protein hydrolysates and peptides with various bioactivities [72,73]. But so far, there are no reports of bioactive peptides using *Bacillus proteolyticus, Bacillus megaterium,* and *Bacillus thuringiensis* strains.

The phylogenetic tree performed under Kimura's 2-parameter model revealed four clades (Figure 1). In the first clade are the LAB belonging to the *Enterococcus* genus. It can be observed that *E. faecium*-9C4 and *E. faecium*-LR9 are highly related, even though they were isolated from broccoli and goat milk, respectively. In the second clade are strains belonging to the *Bacillus* genus, all isolated from aguamiel. In the third clade, there is only the *Lactiplantibacillus plantarum* strain isolated from broccoli since it belongs to a different genus. And finally, the fourth clade corresponds to the *L. mesenteroides*-18C6 and *L. mesenteroides*-AE7 strains isolated from broccoli and amaranth, respectively.

The clustering pattern in the phylogenetic tree was associated with the isolated bacterial genus. Clade distribution does not correlate with %TPD and thus with proteolytic activity. Strains belonging to the same clade differ in %TPD. This may be because cell envelope proteinases show high diversity and intraspecific heterogeneity [74].

3.4. Peptide concentration

Peptide concentrations ranging from 1.738 to 1.525 mg/mL DW were obtained from the WSE evaluated (Table 4).

There is a variation in peptide concentration of the different WSE. This is because, in some cases, different LAB and *Bacillus* species were used to obtain the WSE. The proteolytic enzymes of each LAB species are highly variable at the inter- and intraspecific level, mainly for the genes coding for the cell-envelope proteinases (CEP) that are responsible for hydrolyzing the protein [75]. In the case of *Bacillus,* it is known to contain nonspecific proteolytic enzymes that enhance the hydrolytic action [76]. Although its proteolytic system has not yet been studied, it is recognized that the proteolytic activity varies because of the used species [34].

However, the same LAB species (*Enterococcus faecium*) was used to produce WSE LS15, LR9, and 9C4, and the concentrations obtained are different. This is because, in addition to the species, other factors influence the proteolytic activity of LAB, such as the isolation source and the environmental conditions to which they are subjected [75]. The LS15 and LR9 strains were isolated from goat milk, where the number of free amino acids and nitrogenous components is insufficient, so LAB must break down proteins by proteolytic enzymes to meet their needs [77]. On the other hand, the 9C4 strain was isolated from broccoli. In this plant source, a higher amount of free amino acids is found, so breaking down the proteins is unnecessary. The difference in peptide concentration between LS15 and LR9 WSE may be because the LS15 strain has been better adapted to the environmental conditions of fermentation.

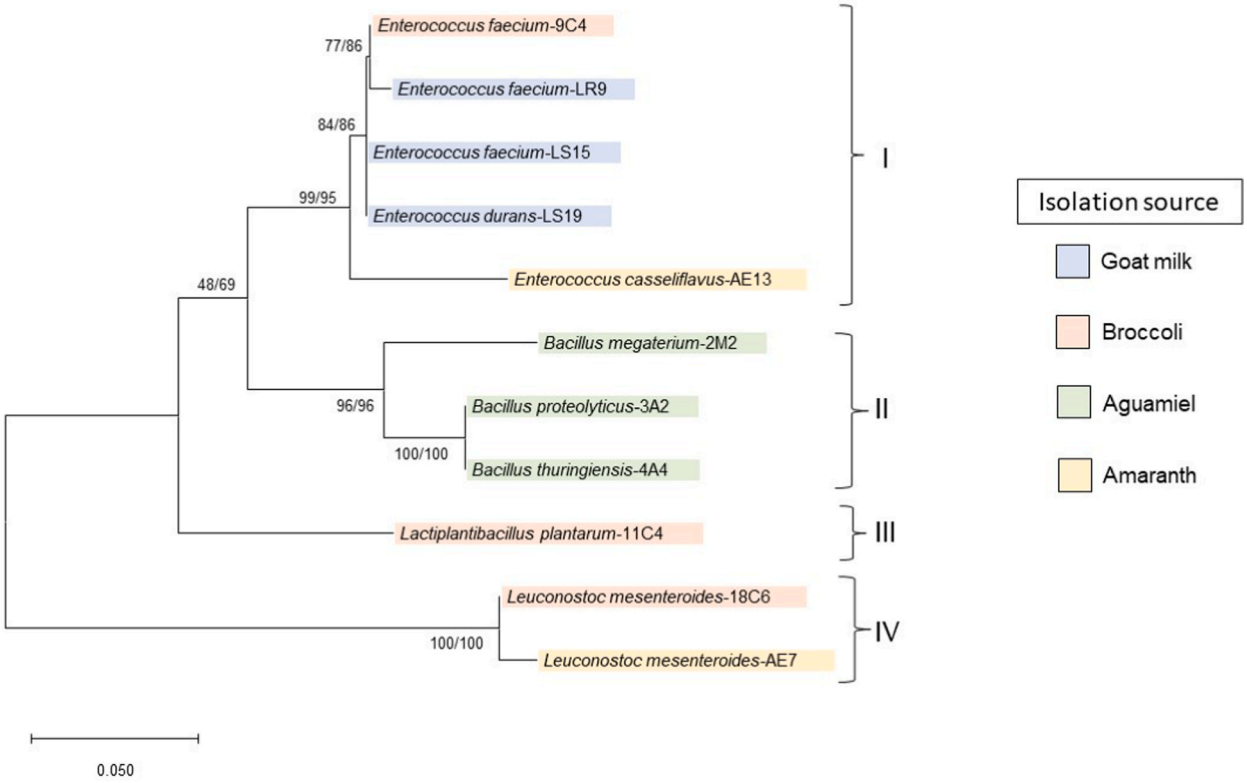

Figure 1. Phylogenetic tree of the isolates that showed the best %TPD. The phylogenetic tree was deduced by neighbor-joining and maximum likelihood based on the deduced amino acid sequences. Numbers above the branches represent bootstrap confidence values (%) based on 500 replicates (ML/NJ). Isolation source is indicated in different color. Clade I: *Enterococcus*, Clade II: *Bacillus*, Clade III: *Lactiplantibacillus plantarum*, Clade IV: *Leuconostoc mesenteroides*.

Table 4
Peptide concentration of the WSE obtained.[a]

Isolation source	Strain	Species	Peptide concentration (mg/mL DW)
Goat milk	LS15	*Enterococcus faecium*	1.74 ± 0.027^{a}
	LS19	*Enterococcus durans*	1.60 ± 0.008^{cde}
	LR9	*Enterococcus faecium*	1.61 ± 0.006^{cde}
Broccoli	9C4	*Enterococcus faecium*	1.63 ± 0.017^{bcd}
	11C4	*Lactiplatibacillus plantarum*	1.64 ± 0.015^{bc}
	18C6	*Leuconostoc mesenteroides*	1.59 ± 0.003^{de}
Aguamiel	4A4	*Bacillus thuringiensis*	1.65 ± 0.006^{b}
	3A2	*Bacillus proteolyticus*	1.62 ± 0.003^{bcde}
	2M2	*Bacillus megaterium*	1.61 ± 0.004^{bcde}
Amaranth	AE13	*Enterococcus casseliflavus*	1.62 ± 0.003^{bcde}
	AE7	*Leuconostoc mesenteroides*	1.59 ± 0.007^{e}

[a] Within the same samples, values with different letters (a, b, c, d, and e) are significantly different ($p < 0.05$).

With the LS15 WSE, it was obtained the highest peptide concentration. *E. faecium* is known to play a positive role in dairy processing due to its proteolytic activity. However, so far, there are no reports on the CEP that it produces.

The strains that with a higher %TPD also showed a higher concentration of peptides in the WSE. However, strains such as LS15 showed a %TPD of 95.95% and only produced 1.75 mg/mL of peptides. This is because it is possible that a higher percentage of protein hydrolysates are being produced than peptides. Also, the Bradford technique was used to determine the %TPD. On the other hand, the OPA method was used to determine the peptide concentration. These techniques have different principles as well as different sensitivities. Amaranth fermentation is a strategy to obtain new peptide sequences with potential bioactivities. But so far, it has not been considered an effective method to obtain a higher production of peptides. On the other hand, enzymatic hydrolysis of amaranth using alcalase shows higher values and is more reproducible [78].

The semi-solid fermentation used in this work generated peptide concentrations similar to those reported by Ayala-Niño et al. [79], who used submerged fermentation to release amaranth peptides. These authors evaluated the peptide concentration generated by *Lacticaseibacillus casei* and *Streptococcus thermophilus* in amaranth (1.945 mg/mL NH:- and 1.645 mg/mL NH:- respectively) by the trinitrobenzene sulfonic acid method (TNBSA). But they obtained a higher peptide concentration (4.245 mg/mL NH:-) when performing the combined culture compared to the results obtained in this work.

However, several advantages have been identified in semi-solid fermentation compared to submerged fermentation. For example, amaranth flour is used as a substrate, which provides an ecological approach since, it valorizes the fermented amaranth dough that can have potential applications [80]. There are also lower sterilization costs, and the volume of water is reduced [81].

Although the protein content of amaranth is considerably high (15.3%), a high peptide concentration was not obtained because the fermentation was not carried out under optimized parameters to obtain this response variable. Since the objective of the work was to produce bioactive protein hydrolysates, it is important to optimize the different fermentation parameters to obtain a higher peptide concentration; however, enzymatic hydrolysis with alcalase is a better strategy if the focus is to obtain more amaranth peptides.

3.5. Antioxidant activity

The antioxidant activity of the 11 WSE in which amaranth peptides were present was evaluated using three assays: DPPH, ABTS, and FRAP. The results are summarized in Table 5.

DPPH and ABTS are antioxidant tests based on the transfer of electrons or hydrogen atoms. However, it is more common for them to use electron transfer due to the solvents used (methanol and ethanol) and the fact that this mechanism is carried out faster than hydrogen atom transfer. FRAP bases its mechanism only on electron transfer [82].

Table 5
Antioxidant activity of WSE obtained from amaranth fermentation.[a]

WSE	FRAP (μMTE/L)	DPPH (μMTE/L)	ABTS (μMTE/L)
LS15	1.53 ± 0.03^{b}	211.47 ± 18.86	13.96 ± 1.34^{de}
4A4	0.76 ± 0.01^{e}	220.80 ± 4.00	5.10 ± 0.00^{g}
AE13	0.45 ± 0.01^{f}	219.47 ± 18.87	0.48 ± 0.42^{hi}
9C4	1.22 ± 0.05^{c}	224.80 ± 32.00	16.76 ± 1.22^{bc}
11C4	0.91 ± 0.03^{de}	212.80 ± 20.00	0.40 ± 0.02^{i}
AE7	0.16 ± 0.01^{f}	168.80 ± 0.00	0.94 ± 0.00^{hi}
18C6	0.75 ± 0.26^{e}	219.47 ± 18.86	19.18 ± 0.96^{a}
3A2	0.33 ± 0.02^{f}	219.47 ± 13.60	3.02 ± 1.56^{gh}
2M2	0.29 ± 0.03^{f}	216.80 ± 39.19	2.10 ± 0.00^{hi}
LS19	1.36 ± 0.04^{bc}	206.13 ± 15.08	12.22 ± 0.20^{e}
LR9	1.99 ± 0.07^{a}	228.80 ± 36.00	9.52 ± 1.62^{f}
Control 0	0.32 ± 0.02^{f}	198.13 ± 7.54	0.28 ± 0.10^{i}
Control 24	1.19 ± 0.03^{cd}	211.47 ± 37.14	16.44 ± 0.98^{cd}

[a] Within the same samples, values with different letters (a, b, c, d, e, f, g, h, and i) are significantly different ($p < 0.05$).

According to the statistical analysis, the DPPH test showed no significant difference between the WSE evaluated. However, most WSE showed a higher antioxidant activity concentration than the control at 0 h, except for WSE AE7. This may be because this WSE does not contain acidic, hydrophobic, and aromatic amino acid residues that are essential to demonstrate antioxidant activity [83].

WSE 18C6 had the best antioxidant activity in the ABTS test compared to the other extracts. This result may be because this extract may contain aromatic and hydrophobic amino acid sequences, which can donate electrons or hydrogen atoms to capture free radicals [84,85]. The concentrations obtained in this work by the ABTS method are lower than those achieved by the WSE reported by Rizzello et al. [15] (ABTS: 15-291 μMTE/L). This is because these authors ferment quinoa, with LAB isolated from quinoa. Therefore, the source of the WSE is another important factor in their bioactivity.

In the FRAP test, the LR9 extract showed the best antioxidant activity. The same results were not obtained as in the ABTS test because the FRAP test is limited to detecting only water-soluble antioxidants besides basing its mechanism only on electron transfer. The concentrations obtained are lower compared to ABTS, which is caused by the fact that FRAP is performed at an acidic pH, which causes the redox potential to increase and the values to decrease [86]. The antioxidant activity concentrations obtained by the FRAP method in this work are lower than those obtained by Ayala-niño et al. [79] but higher by ABTS (FRAP: 225.6–381.3 μMTE/L and ABTS: 0.103–0.194 μMTE/L). These authors evaluated amaranth peptides generated by fermentation with *Lacticaseibacillus casei* and *Streptococcus thermophilus*, so this difference is due to the strains used, confirming that for the generation of bioactive protein hydrolysates the type of fermentation, protein source and microorganism used are important.

3.6. Antihypertensive activity

The WSE obtained after fermentation were shown to have antihypertensive activity, as shown in Figure 2.

The 18C6, 9C4, AE13, LS19, LR9, 4A4, LS15, 3A2, and 2M2 samples showed the highest ACE-I inhibition percentage. Sánchez-López et al. [87] after fermenting amaranth with LAB, obtained protein hydrolysates with ACE-I inhibition percentages between 10.2 and 45.8. The results obtained in this work are higher than those reported by these authors. This is because these authors perform a liquid fermentation with amaranth protein and use *Lactobacillus helveticus* and *Lactiplatibacillus plantarum* strains.

Bioactive peptides achieve this bioactivity by binding to the active site of the enzyme, either competitively or non-competitively. Peptides with this bioactivity are characterized by having a small size [88]. Therefore, the WSE produced could contain small peptides that must be confirmed by analyzing their sequence.

Although 11C4 and AE7 are shown to generate protein hydrolysates of low molecular weights (Figure 3), the antihypertensive activity is also influenced by the amino acid sequence and composition [89]. Therefore, these WSE may have amino-acidic characteristics that do not allow them to demonstrate such bioactivity.

Controls at 0 h and 24 h show inhibition of ACE-I in lower percentages. In the case of the unfermented control (0 h), this is because amaranth may have this intrinsic property, according to some reports [90]. On the other hand, in spontaneous fermentation (control at 24 h), the various microorganisms that were able to grow under these conditions may promote the release of protein hydrolysates by using amaranth as a nitrogen source. It can be affirmed that amaranth contains autochthonous microorganisms because, in this work, 30 different strains were isolated. Also, in the electrophoresis carried out later, the presence of bands of different molecular weights were observed (Figures 3 and 4) which were not observed in control at 0 h. This indicates the degradation of the amaranth protein. The protein hydrolysates released during spontaneous fermentation may contain peptides that can inhibit ACE-I since a higher percentage of inhibition is observed compared to the control at 0 h. However, the control at 24 h obtained a lower percentage of ACE-I inhibition

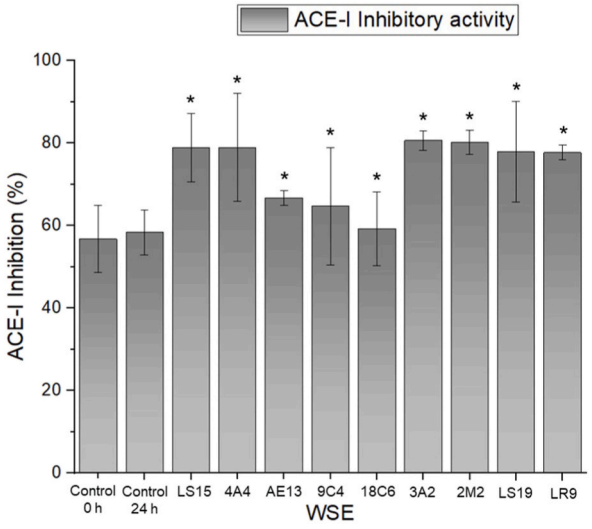

Figure 2. Antihypertensive activity of WSE obtained from amaranth fermentation. The symbol "*" highlights the WSE with better ACE-I inhibitory activity. There is no significant difference between these samples.

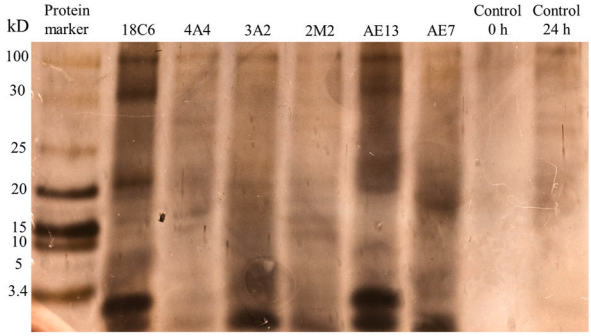

Figure 3. Tricine SDS-PAGE of the WSE 18C6, 4A4, 3A2, 2M2, AE7, and AE13 obtained after fermentation. Controls at 0 h and 24 h are also observed. The full figure is presented as supplementary material.

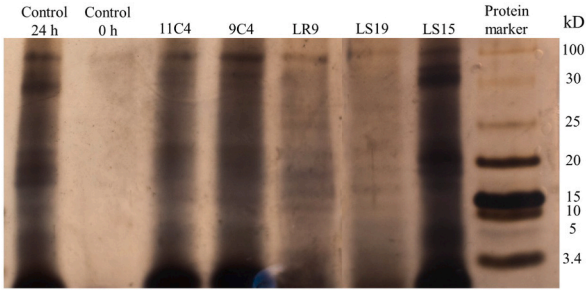

Figure 4. Tricine SDS-PAGE of the WSE 11C4, 9C4, LR9, LS19, and LS15 obtained after fermentation. Controls at 0 h and 24 h are also observed. The full figure is presented as supplementary material.

compared to the WSE evaluated since they use specific LAB strains in appropriate parameters.

3.7. Antimicrobial activity

Some WSE were shown to have antimicrobial activity vs. *Salmonella enterica* and *Listeria monocytogenes,* as shown in Table 6. The 9C4, LR9, and 18C6 extracts showed the best antimicrobial activity vs. *S. enterica*. On the other hand, when evaluating the activity vs. *L. monocytogenes*, the best WSE were 11C4, 18C6, LS19, and 9C4.

Some of the extracts are positive for both *S. enterica* and *L. monocytogenes*. Still, it is observed that more WSE have antimicrobial activity against *L. monocytogenes,* and even the AU is higher. The cause of this is that *S. enterica* is Gram-negative bacterium and *L. monocytogenes* is Gram-positive. This makes *S. enterica* more resistant because it has an outer membrane with lipopolysaccharide molecules attached [91].

Not all extracts had antimicrobial activity, and this is because the protein hydrolysates or peptides must have a sequence of cationic (such as arginine and lysine) and hydrophobic amino acids. This way, they can permeabilize the membrane, target the cell wall and intracellular wall and induce apoptosis [92].

The results obtained are superior to those reported by Banihashemi et al. [93], who evaluated peptides obtained from Koopeh cheese through fermentation with LAB. Since he obtained inhibition halos between 0 and 10.5 mm for *L. monocytogenes* and 0–10.2 mm for *S. enterica*, in contrast, the present work produced inhibition halos between 13 and 25 mm for *L. monocytogenes* and 13–29 mm for *S. enterica*. This may be because, in this work, we used specific LAB and *Bacillus* strains that were shown to have proteolytic activity in amaranth sourdoughs, as compared to Banihashemi et al. [93] which uses the natural LAB that become predominant during the ripening process of Koopeh cheese.

3.8. Electrophoresis

Different bands of molecular weights between 100 kD and 3.4 kD are visualized, as shown in Figures 3 and 4.

In the control at 0 h, only a band of 100 kD is observed since this control was not subjected to the fermentation process. Therefore, there was no protein degradation since the presence of low molecular weight proteins was not observed. On the other hand, in control at 24 h, bands of different molecular weights were visualized, indicating that there was a spontaneous fermentation with the autochthonous microorganisms of the amaranth flour.

Different banding patterns are observed in each WSE because they are produced from different strains, which differ in their proteolytic system (strain-dependent), through which they can release protein hydrolysates of different molecular weights. However,

Table 6
Antimicrobial activity of WSE obtained from amaranth fermentation.[a]

WSE	AU vs *Salmonella enterica* (mm^2/mL)	AU vs *Listeria monocytogenes* (mm^2/mL)
LS15	12.08 ± 5.13^{b}	13.82 ± 2.67^{cd}
4A4	–	–
AE13	–	29.95 ± 20.14^{cd}
9C4	112.82 ± 10.27^{a}	102.14 ± 4.84^{a}
11C4	–	79.17 ± 4.44^{a}
AE7	–	70.02 ± 4.25^{ab}
18C6	128.60 ± 27.72^{a}	95.43 ± 8.21^{a}
3A2	–	–
2M2	–	–
LS19	30.93 ± 25.63^{b}	95.50 ± 10.05^{a}
LR9	113.94 ± 27.33^{a}	76.17 ± 7.52^{ab}
Control 0 h	–	–
Control 24 h	15.71 ± 0.0^{b}	48.90 ± 27.12^{bc}

[a] Within the same samples, values with different letters (a, b, c, and d) are significantly different ($p < 0.05$).

future studies are needed to determine the sequence and molecular mass of the protein hydrolysates. Although low molecular weight bands (3.4 kD) are observed in some WSE, these cannot be considered peptides until purification and sequencing methodologies are performed.

4. Conclusions

Fermentation of amaranth with LAB and *Bacillus* species (*B. thuringiensis*, *B. proteolyticus,* and *B. megaterium*) proved to be a method capable of degrading the protein of this pseudocereal. Protein hydrolysates with important bioactivities, such as antioxidant, antihypertensive, and antimicrobial activities can be obtained by this method. However, this process is strain-dependent.

In this method, it is essential to use microorganisms with high proteolytic activity to obtain protein hydrolysates that can contain peptides with amino acid sequences with suitable characteristics to demonstrate high bioactivities. Based on this, the strains used in this work were selected since these strains showed a high protein degradation in the tests performed.

Enterococcus faecium-LR9 and *Leuconostoc mesenteroides*-18C6 are strains that have not been widely used to obtain protein hydrolysates or even bioactive peptides. However, demonstrated in this work was that they produce protein hydrolysates with interesting bioactivities not previously reported.

The protein hydrolysates obtained were shown to have multiple bioactivities and could therefore be used in functional foods or nutraceuticals as an alternative to prevent various diseases.

Declarations

Author contribution statement

Dora Elisa Cruz-Casas: Conceived and designed the experiments; Performed the experiments; Wrote the paper.
Cristóbal N. Aguilar, Juan A. Ascacio-Valdés, Mónica L. Chávez-González: Analyzed and interpreted the data.
Raúl Rodríguez-Herrera: Contributed reagents, materials, analysis tools or data.
Adriana C. Flores-Gallegos: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e13491.

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