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Bioactive peptides released by lactic acid bacteria fermented pistachio beverages

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ABSTRACT

Fruit and vegetable are the raw materials for many popular fermented beverages with potential health benefits. In the present study, a novel pistachio beverage fermented with selected strains of lactic acid bacteria belonging to the species *Leuconostoc pseudomesenteroides* and *Companilactobacillus paralimentarius* was produced, and the extent of proteolysis was evaluated. Physico-chemical and microbiological analyses, together with peptidomics and proteomics, were carried out on the beverage after 24 h of fermentation, using both the non-inoculated and chemically acidified beverage as controls. Amino acid sequence of hundreds of peptides mainly released from 2S albumin, 11S and 7S globulin were characterized. The number and frequency of identified peptides were higher in beverage started with *Leuconostoc pseudomesenteroides*, followed by *Companilactobacillus paralimentarius* and acidic beverage. These results suggest that, in addition to endogenous proteases active at acidic pH, the proteolytic system of LAB directly participated to peptide degradation to some extent. According to the BIOPEP database, a group of 31 peptides were potentially bioactive, and primarily associated with antioxidative properties, ACE and DPP-IV inhibition. The beverage started with *Leuconostoc pseudomesenteroides* showed the highest amount and number of bioactive peptides. This study lays the foundations for the design of novel pistachio-fermented beverage with potential health properties.

1. Introduction

Fermentation is a natural process practiced since ancient times to improve nutritional and sensory properties and food preservation (Ross and Morgan, 2022). Numerous scientific evidences have proven the high nutritional values and health benefits of fermented foods compared to the unfermented matrix, such as reduction blood cholesterol levels, increasing immune defences, preventing carcinogenesis, osteoporosis, diabetes, obesity, allergies, atherosclerosis, and alleviating symptoms in lactose-intolerant subjects (Şanlier et al., 2019).

Food fermentation is deeply embedded in local culture and traditions (Galimberti et al., 2021). Most traditional fermented foods are inducted by microorganisms spontaneously present in the starting raw material, resulting in a product of variable quality (Cuamatzin-García et al., 2022). Nowadays, modern food biotechnologies tend to optimize and standardize the fermentation process by employing selected starter microorganisms with well-defined characteristics, with the aim to improve flavor, safety and achieving high nutritional value and sustainability (Mannaa et al., 2021).

A wide variety of microbes can be responsible for the fermentation process, including lactic acid bacteria (LAB), acetic acid bacteria, yeasts and moulds (Campbell-Platt, 1994). In particular, LAB have been considered the most critical microbial group contributing to the beneficial effects of fermented foods/beverages (Ashaolu & Reale, 2020; de Souza et al., 2023; Kumar et al., 2022). LAB are able to ferment a variety of food substrates, such as milk, meat, fish, cereal, vegetables, and legumes (Ross and Morgan, 2002; Cuamatzin-García et al., 2022; Brown et al., 2017). Proteolytic events that occur during LAB fermentation lead to the release of bioactive peptides with potential health-promoting activities (Chai et al., 2020; Venegas-Ortega et al., 2019). Antioxidant, antihypertensive and antimicrobial peptides are the most remarkable subgroup of bioactive peptides that can be found in fermented foods (Martinez-Villaluenga et al., 2017, pp. 23–47).

Proteomics science has become an indispensable tool to examine

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proteolytic activity of microorganisms, providing insights on the function of fermenting microbiota in food products (Yang et al., 2020). The analysis of the peptidome and the dynamic changes during food fermentation and storage process, have been the subject of research over the years (Martini et al., 2021; Reale et al., 2021). Proteomic and peptidomic studies have been conducted to characterize and quantify the peptides released by proteolysis during LAB fermentation process (Dallas et al., 2015).

Dairy based products are popular fermented foods with beneficial effects on human health, and some of those benefits are related to protein-derived products (Widyastuti et al., 2021). Noteworthy, the consumption of dairy products is limited for a class of consumers suffering of lactose-intolerant, hypercholesterolemic or allergy, as well as for subjects adhering to vegan or vegetarian diets (Jaiswal & Worku, 2022). Vegetable foods/beverages have garnered significant attention as viable substitutes for dairy products (Mäkinen et al., 2016; Sethi et al., 2016). The impact of LAB on the nutritional properties over fermentation process of vegetable matrices has been previously investigated (Holscher, 2017; Kumar et al., 2022; Penha et al., 2020; Rekha & Vijayalakshmi, 2010; Zhao & Shah, 2014). Soybean and almond are the most used seeds to produce plant-based beverages, because of their technological properties. Peanuts, cashew, sesame, and rice are also employed to a lower extent (Harper et al., 2022).

Pistachio seeds are an important source of nutrients, including amino acids, dietary fibre, vitamins, and minerals. Clinical studies associated a diet including pistachios to a reduction in LDL levels and therefore lower risk of cardiovascular diseases and inflammatory bowel syndrome (Holligan, et al., 2014). In addition, the regular consumption of pistachio promotes, glycaemic control, appetite management, and weight control (Dreher, 2012). Pistachios are also a potentially suitable matrix for the fermentation process, as shown in previous studies (Sánchez-Bravo et al., 2020; Di Renzo et al., 2023) and the development of pistachio-based beverages, subject to additional fermentation, could enrich the range of plant-based beverages as an alternative to dairy–based products. In the present study, we used two starter cultures, *Leuconostoc pseudomesenteroides* D4 and *Companilactobacillus paralimentarius* G3, previously selected for their growth ability and acidifying activity in the pistachio matrix and for their impact on the odor profile of the model-beverage (Di Renzo et al., 2023). We performed a detailed evaluation of the proteolytic event occurring in the fermentation process of the pistachio beverage and the peptidome generated by the LAB was characterised using a proteomic approach with a focus on the identification of potentially bioactive peptides.

2. Material and methods

2.1. Chemicals

Trichloroacetic acid (TCA), trifluoroacetic acid (TFA), formic acid, acetonitrile, ammonium bicarbonate (AMBIC), cycloheximide, sodium chloride (NaCl), β-mercaptoethanol, Tris-HCl and Urea were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents for electrophoresis analysis were purchased from Bio-Rad (Segrate MI, Italy). Reagent for 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay was provided by Thermo Fischer Scientific (Bremem, Germany).

2.2. Strains and culture conditions

The LAB strains named *Leuconostoc pseudomesenteroides* D4 and *Companilactobacillus paralimentarius* G3 were previously identified and characterized for their tolerance to salinity, sucrose, ethanol, acid, and for proteolytic activity (Reale et al., 2020, 2021). Strains, belonged to the microbial culture collection of the Institute of Food Science - National Research Council (ISA-CNR, Avellino, Italy) were kept as frozen stocks (in 50% glycerol v/v) and routinely propagated in DeMan Rogosa and Sharpe (MRS) medium (Oxoid, Milan, Italy), pH 6.8 for 24 h at 30 °C.

Hereafter in the text, the LAB strains *Leuconostoc pseudomesenteroides* D4 and *Companilactobacillus paralimentarius* G3 will be referred to as D4 and G3, respectively.

2.3. Preparation of fermented pistachio beverage

Pistachio seeds (*cv* Bronte) were provided by a certified Italian producer of the Bronte variety (Aroma Sicilia, Bronte, Italy) produced in 2019 and store at 4 °C. De-hulled pistachio seeds were mixed with water (1:5 w:v) and ground in a colloidal mill (S.A.R. Group – model HOMO-Master 120) for about 5 min with recirculation, at room temperature. The resulting slurry was thermized at 70 °C for 30 min and cooled at 4 °C until the microbial inoculum. The inocula were prepared by subculturing each lactic acid bacteria strain in MRS broth incubated at 28 °C for 16 h, followed by centrifugation at 2000*g* for 10 min at 21 °C, washing of the pellets with sterile physiological solution (0.9% w/v NaCl) and final resuspension in the beverage at a final concentration of ~6 log
CFU/mL beverage. The beverages were fermented for 24 h at 28 °C and then stored at 4 °C for 30 days. In parallel, two control samples were prepared: a beverage without starter (control) and a beverage with 1% of lactic acid:acetic acid in ratio 4:1 v:v (acidified control). All beverage samples were collected after 24 h of incubation and stored at −80 °C until chemical-physical, microbiological and proteomic analysis.

2.4. Determination of pH, acetic/lactic acid and LAB count

The pH value was determined using a BASIC 20 pH-meter (Crison Instruments, Barcelona, Spain) after diluting 1 mL of the beverage sample with 9 mL of distilled water, under magnetic stirring. For microbiological analysis, 1 mL of pistachio beverage was diluted with 9 mL of sterile physiological solution (0.9% w/v NaCl).

Acetic and lactic acid concentrations (expressed as g/L) were quantified using the RIDA®CUBE Assay Kits (Acetic acid RCS4226, D/L-Lactic Acid RCS4240, respectively) according to the manufacturer's instructions (R-Biopharm, Melegnano MI, Italy);.

LAB were counted on MRS (de Man, Rogosa and Sharpe) agar medium supplemented with 4 mg/100 mL of cycloheximide, after incubation at 28 °C for 72 h under anaerobic conditions (Gas Pack AnaeroGen TM, Oxoid, Milan, Italy).

Analyses of pH, LAB count, lactic and acetic acids were carried out in triplicate.

2.5. Degree of hydrolysis in fermented pistachio beverage

Proteolytic activity was determined by measuring the concentration of total primary amino groups (–NH2) by using the TNBS assay as reported by Adler-Nissen (1979). Briefly, 1 mL of freeze-dried pistachio beverage was solubilized in 0.5 M NaCl and 150 mM sodium phosphate at pH 6.8 to a ratio of 1:3 (w:v) and mixed for 30 min at 21 °C. After centrifugation at 12,000 *g*, for 20 min at 4 °C, 250 μL of supernatant was added to 250 μL of buffer borate and 500 μL of TNBS (%). Samples were incubated for 60 min at 37 °C. Reaction was stopped with 1 mL of phosphate buffer. Absorbance of the solution was measured spectrophotometrically at 420 nm using Ultrospec 160 2100 pro, (Amersham Bio-sciences, Uppsala, Sweden). The calibration curve was prepared using leucine (Leu) as standard in a range 0.0–1.0 mmol/L of Leu, and results were expressed as milligrams of Leu/L of beverage. The standard was assayed under reaction condition identical to those utilized for the samples. Experiments were performed in triplicate. TNBS analysis was performed using SYSTAT 13.0 for Windows (Systat Software Inc., Richmond, CA, USA).

2.6. SDS-PAGE analysis

The proteins of pistachio beverage (100 μL) were precipitated by adding an equal volume of acetone/TCA 20% and kept at −20 °C overnight. After centrifugation at 10,000 *g* for 10 min at 21 °C, the supernatant was discarded. Protein pellets were washed with 10 vol (three times) of pre-chilled acetone followed by centrifugation (10,000 *g* for 10 min at 21 °C). Pellets were dissolved in 20 μL of Laemmli buffer (0.125 M Tris−HCl pH 6.8, 5% SDS, 20% glycerol, 0.02% bromophenol blue) containing β-mercaptoethanol and heated in boiling water bath (100 °C) for 5 min. Proteins were separated by SDS-PAGE (Mini-Protean, Bio-Rad, Segrate MI, Italy) on a 12% polyacrylamide precast gels at 100 V. Gels were stained with Coomassie brilliant blue R-250 and destained in methanol:acetic acid:water(1:1:8) solution. The stained gels were imaged using GEL-DOCXR+ (Bio-Rad, Segrate MI, Italy).

2.7. LC–MS/MS analysis

Lyophilized pistachio beverage (1 mL) was suspended into 1 mL of urea 7 M Tris buffer saline (TBS) (50 mM Tris-HCl 150 mM NaCl) at pH 8 and incubated overnight at 4 °C under constant magnetic stirring. Sample was centrifuged at 10,000 *g* for 20 min at 21 °C, and the supernatant was collected. Proteins (MW > 7 kDa) and peptide (MW < 7 kDa) were fractionated by size exclusion chromatography (*SEC*), using an Econo-pack 10-DG pre-packed desalting column (Bio-Rad, Segrate MI, Italy), equilibrated, and eluted with 25 mM ammonium bicarbonate. The effluent was monitored by UV absorbance at 280 nm (Ultrospec 160 2100 pro, Amersham Biosciences, Uppsala, Sweden). The peptide-containing fractions were desalted using Sep-Pak C18 pre-packed cartridges (Waters, Milford, MA, USA), according to the manufacture's instruction and lyophilized. Peptide fraction was analyzed by LC–MS/MS using a Q Exactive Orbitrap mass spectrometer, equipped with a nano-electrospray ion source, and coupled online with an UltiMate 3000 RSLC nano system (Thermo Fischer Scientific, Bremem, Germany). Sample was suspended in 0.1% (v/v) TFA solution and transferred to polypropylene vials and nearly 1 μg was loaded through a 5 mm long, 300 mm i.d. pre-column and separated by an EASY-Spray™ PepMap C18 column (15 cm × 75 mm i.d.), 3 mm particles, 100 Å pore size (Thermo Fischer Scientific, Bremem, Germany). The separation was carried out with a linear gradient from 4% to 50% of 0.1% formic acid (v/v) in 80% acetonitrile (eluent B), over 50 min at a flow rate of 300 nL/min, after 5 min equilibration at 4% B. Eluent A was 0.1% formic acid (v/v) in Milli-Q water. The mass spectrometer was operated in data-dependent mode, and all MS1 spectra were acquired in the positive ionization mode in the mass scanning range 250–1600 *m/z*. Normalized collision energy was set to 27. The top-10 most intense precursor ions were selected for fragmentation in MS/MS mode, with 10 s of dynamic exclusion. A resolving power up to 70,000 full widths at half maximum (FWHM), an automatic gain control (AGC) target of 10^6 ions and a maximum ion injection time (IT) of 120 ms were set as standard values to generate precursor spectra. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM. Spectra were elaborated using the Xcalibur Software version 3.1 (Thermo Fischer Scientific, Bremem, Germany).

2.8. Processing of LC-MS/MS data

Raw files were processed the Andromeda search engine of the MaxQuant open-source bioinformatic suite (https://maxquant.org). The searches were taxonomically restricted to the *Pistacia vera* databases (Taxon ID 55513) downloaded from UniprotKB. The search conditions included unspecific cleavage and no static modification. In both cases, methionine oxidation and pyroglutamic acid at N-terminus glutamine were selected as variable modifications. The mass tolerance value was 10 ppm for the precursor ion and 0.08 Da for MS/MS fragments. Peptide Spectrum Matches (PSMs) were filtered using the target decoy database approach with an e-value of 0.01 peptide-level false discovery rate (FDR), corresponding to a 99% confidence score. The average intensity of peptides was calculated based on the triplicate determination of the ion counts.

2.9. Bioinformatics analysis

Principal component analysis (PCA) was performed to show differential in mass peptide distribution among the fermented pistachio beverages using Perseus bioinformatics tools. Significant differences between means were determined by using one-way ANOVA followed by post hoc Bonferroni multiple comparison tests; a p-value <0.05 indicated statistical significance.

Potential bioactive peptides, released during fermentation, were in silico searched using as reference all peptides listed in the BIOPEP (http://www.uwm.edu.pl/biochemia) database (Iwaniak et al., 2016).

2.10. Statistical analysis

The statistical analysis was performed using the statistical software GraphPad Prism version 9.0 (GRAPH PAD software Inc, California, USA). Experiments were performed in triplicate and results were expressed as the mean ± standard deviation (SD). Significant differences ($P < 0.05$) were determined by analysis of one-way ANOVA, followed by Dunnett's test for multiple comparisons.

3. Results

3.1. Beverage preparation and characterization

Fig. 1 summarizes the analytical workflow applied to determine the enzymatic proteolysis in the inoculated, acidified and non-inoculated Pistachio beverages. All beverages were sampled at 24 h of fermentation for subsequent microbiology and physico-chemical and proteomics analyses.

3.1.1. Microbiology and physico-chemical analysis

The pistachio-based beverage before inoculum had a pH of 6.45. In Table 1 are reported the results related to the pH, organic acid, microbial count and TNBS in the pistachio-based beverage after 24 h of fermentation.

Both the strain starters (D4 and G3) were able to acidify the pistachio beverage. In fact, after 24 h of fermentation, the pH of LAB-fermented beverages decreased to 3.88 ± 0.08 (D4) and 3.91 ± 0.04 (G3), consistent with the production of lactic acid values, which ranged between 9.12 ± 0.03 (D4) and 7.87 ± 0.08 g/L (G3). The D4 and G3 starter cultures also induced production of acetic acid with a concentration of 0.43 ± 0.03 and 0.14 ± 0.04 g/L respectively. The low pH values of LAB-fermented beverages were comparable to the chemically acidified beverage, which had a value of about 3.95, which remained unchanged over the time. Similar, the pH and concentration of acetic acid and lactic acids of control beverage, did not change significantly over the 24 h incubation time.

As expected, the LAB counts of pistachio beverage at time 0 h were lower than 1.0 log CFU/mL, because of heat treatment of the beverage and LAB count was also negligible (1.0 log CFU/g) over the fermentation of control and acidified control beverages.

Furthermore, the pistachio matrix proved to be a suitable substrate for the growth of the strains. In fact, the cultures, inoculated at time zero with a value of about 6.0 log CFU/g, increased considerably reaching, after 24 h of fermentation, values of 9.30 ± 0.13 and 8.50 ± 0.15 log CFU/g for D4 and G3, respectively.

The proteolytic activity of LAB was determined by measuring the content of free amino groups in fermented pistachio beverages by TNBS assay. As shown in Fig. 2, the release of free amino groups was significantly higher in the beverages started by LAB G3 (1.38 ± 0.09 mg/L)

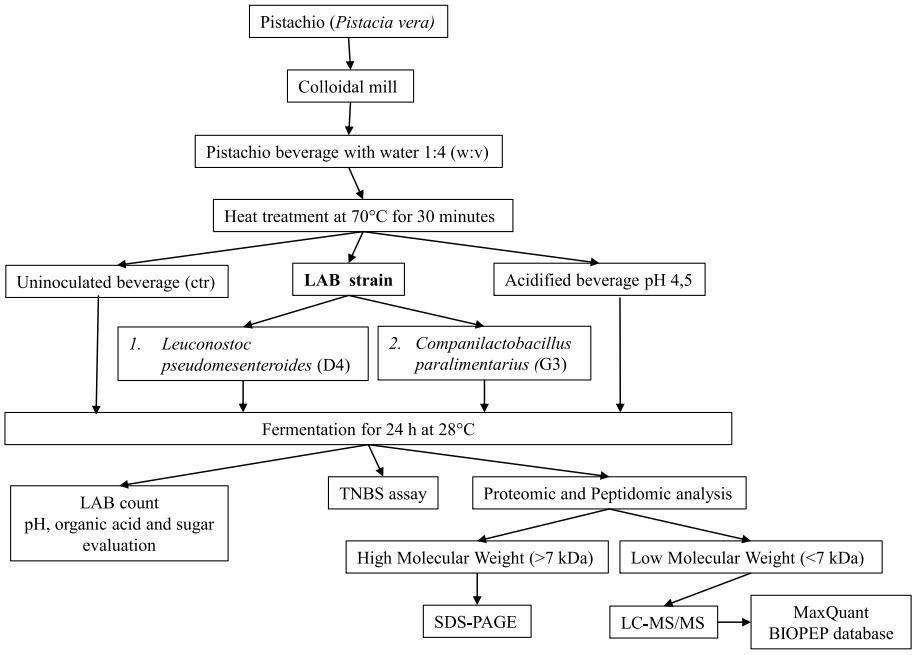

Fig. 1. Experimental workflow illustrating the technological and analytical strategy applied in this study for the assessment of pistachio fermented-beverages.

Table 1
Evaluation of pH, microbial count, acidification and TNBS value in pistachio-based fermented beverages. Data are expressed as the mean ± SD of three experiments.

Beverage sample	pH	MRS log *CFU/mL*	Lactic acid *g/L*	Acetic acid *g/L*	TNBS *mg Leu eq./L*
Control (without microbial starter)	6.39 ± 0.01	<1.00	not detected	not detected	1.06 ± 0.08
Beverage chemically acidified	3.95 ± 0.02	<1.00	4.91 ± 0.04	0.76 ± 0.06	1.20 ± 0.04
Beverage fermented with D4	3.88 ± 0.08	9.30 ± 0.13	9.12 ± 0.03	0.43 ± 0.03	1.33 ± 0.10
Beverage fermented with G3	3.91 ± 0.04	8.50 ± 0.15	7.87 ± 0.08	0.14 ± 0.04	1.38 ± 0.09

and D4 (1.38 ± 0.10 mg/L) *versus* the control sample (1.06 ± 0.08 mg/L). Proteolysis degree over the 24 h was also observed for acidified beverages with a content of free-amino group of 1.20 ± 0.039 mg/L.

3.1.2. Protein and peptide fraction analysis

As part of the analytical strategy outlined in Fig. 1, the proteomic analysis of sample beverages relied on the fractionation of protein fraction (MW > 7 kDa) from peptide fraction (MW < 7 kDa) by SE-chromatography. The protein fraction (MW > 7 kDa) was investigated by SDS-PAGE (Fig. 3). The electrophoretic patterns of control pistachio beverage ranged from 5 to 75 kDa corresponding to 2S albumin (9–15 kDa), 11S acid globulin subunit (20–30 kDa) and 11S basic globulin subunit (17–20 kDa), as previously reported (Di Stasio et al., 2022). No marked differences were observed in the electrophoretic profiles of control and acidified beverage samples. Conversely, LAB affected the SDS-PAGE profile due to their proteolytic activity. As a result of the fermentation process, LAB G3 and D4 beverage samples showed significant degradation of MW protein fractions 30–40 kDa which is accompanied by an increase in small polypeptides with molecular weight <20 kDa.

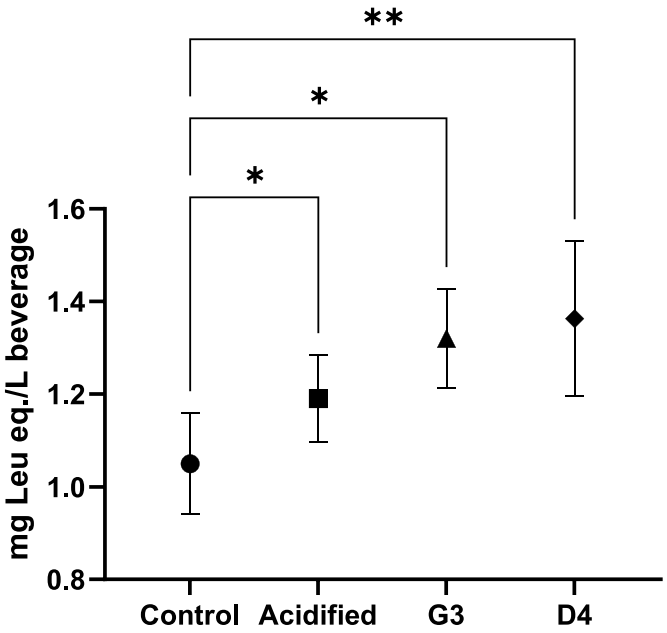

Fig. 2. Degree of hydrolysis of pistachio-fermented beverages expressed as milligrams of Leucine/L of beverage. Each value is mean ± SD (n ≥ 3), and different superscripts (*; **) are significantly different at p < 0.05 and p < 0,01 respectively by Dunnett's multiple comparisons test.

The LC-MS/MS analysis of SEC-enriched peptide fraction, provided direct evidence of LAB-induced proteolytic activity after 24 h of fermentation. Peptide monitoring through LC-MS/MS, showed that the number and intensity of peptides arising from hydrolysis during 24 h of fermentation differed according to the G3 and D4 bacterial strain used to ferment the beverages with respect to control and acidified beverages (Fig. 4A). The detailed list of identified peptides in each beverage sample is shown in Supplementary Table 1. A total of 494 endogenous peptides were identified in the control (uninoculated) beverage. The chemical acidified beverage and those fermented with G4 and D4, had a number

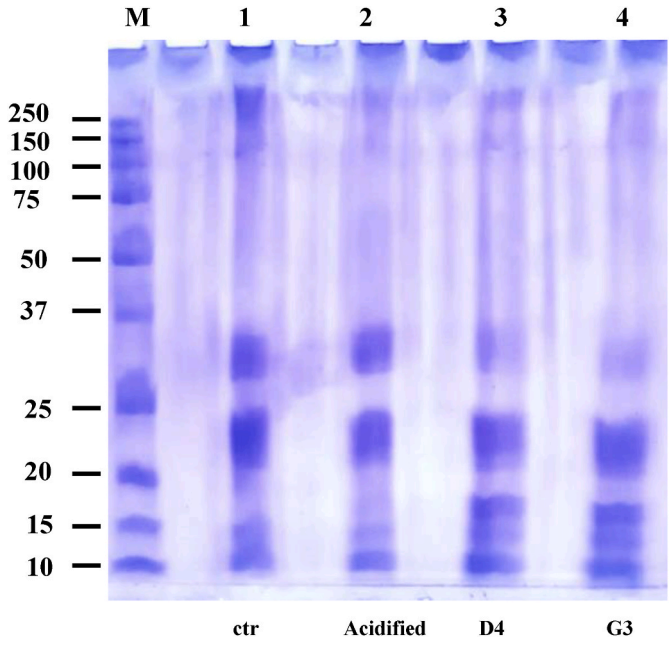

Fig. 3. SDS-PAGE analysis of protein extract from pistachio beverages treated in different ways under reducing conditions. Lane M, Molecular marker (kDa); lane 1, protein of pistachio beverage (control); lane 2, proteins of acidified pistachio beverage; lane 3, proteins of fermented with D4 strain pistachio beverage; lane 4, proteins of fermented with G3 strain pistachio beverage.

of peptides to 1065, 1178 and 1258 respectively. This result was in line with the increased amount of free-NH2 group as determined by TNBS assay. The Venn diagram in Fig. 4B illustrates the peptides found in chemical acidified beverage and those fermented with LAB. There were 661 coexisting peptides present in acidified and started samples. The microbial activity of the LAB determined the complexity of peptides, as 208 and 231 peptides specific peptides were found in the G3 and D4 samples, respectively. A total of 30 and 205 peptides in acidic beverages overlapped with G3 and D4, respectively, while 279 peptides overlapped between G3 and D4.

To examine the distinction between the peptide profiles of beverage samples, a principal component analysis (PCA) was performed considering the abundance of the identified peptides as loadings. As shown in Fig. 4C, the PCA analysis revealed that the replicates in each beverage sample were well clustered. The beverages inoculated with LAB and acidified beverages were significantly different from control beverage. The control sample clustered on its own, while G3 and D4 beverage samples clustered closely and distinctly from acidified sample.

Similarly, differences in peptide profiles were observed by comparison of the masses (Da) of the peptides identified by LC-MS/MS, as shown in Fig. 4D. The height of the bar (counts) indicates the ion abundance of peptides in that particular mass range. The LFQ proteomic analysis revealed an increased number of peptides ranging 1000–4500 Da in the samples fermented with LAB compared with both control samples. The mass ranges overlap between G3 and D4 fermented beverages but do not align perfectly with control and acidified control beverages. The number of counts of G3-and D4-fermented beverages was higher compared to unstarted samples.

3.1.3. *Bioactive amino acid sequences*

The peptides resulting from proteolysis were processed by BIOPEP database (Supplementary Table S2), that includes information about known bioactive peptides, in order to associate potential bioactivities to their sequences (Table 2). Database search was restricted to bioactive sequences longer or equal to 4, in order to increase the confidence of identification. A group of 31 bioactive peptides, mostly belonging to 11S globulin, was identified. These peptides were mainly associated with

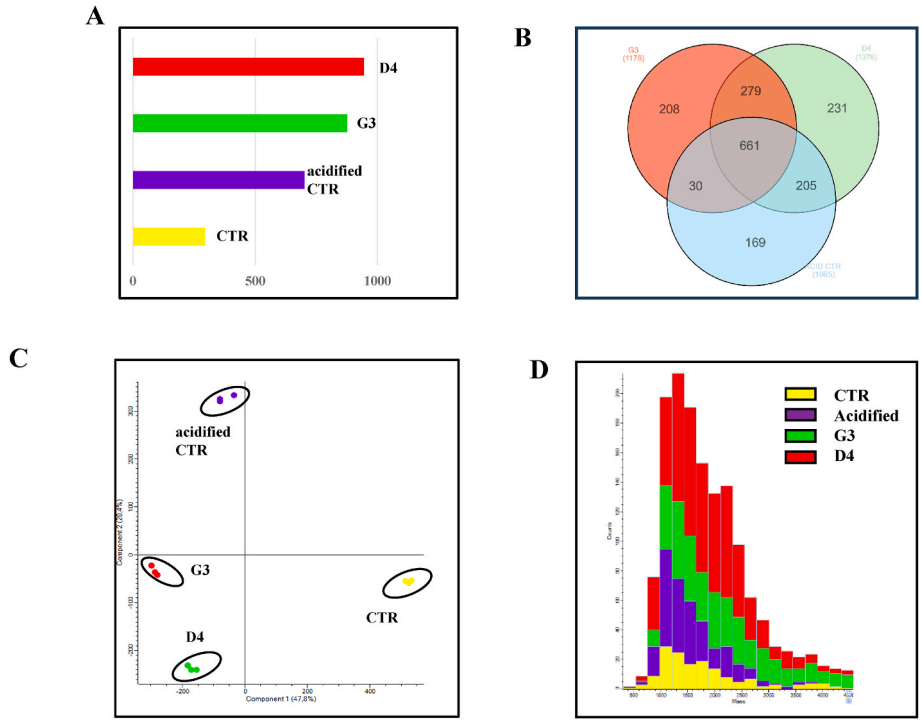

Fig. 4. Peptidomics analysis of beverage samples. A full list of identified peptides is shown in Supplementary Table S1. **Panel A**: number of peptides derived from hydrolysis of pistachio proteins in G3-, D4-, acidified- and control beverages. **Panel B**: Venn diagrams of the number of peptides affected by proteolysis in G3-, D4-LAB strains and acidified control beverages. **Panel C:** Principal Component Analysis (PCA) scores plots of peptidome of G3-, D4-, acidified and control beverages; the contribution of the PC1 was 47,8%, while PC2 contributed for 20.4% of the total variance (when combined, PC1 and PC2 constituted 69,2% of the total variance). **Panel D**: mass distribution of the identified peptides of G3-, D4-, acidified and control beverages.

Table 2
List of potential bioactive peptides identified in pistachio beverages by LC-MS/MS and BIOPEP database. A detailed list of bioactive peptide search is shown in Supplementary Table S2.

ACE inhibithor	Bioactive peptide: GVLY (ID:9325)[a]							
	Length	**Sequence**	**Protein family classification**	**Mass**	**Control**	**Acidified**	**D4**	**G3**
	8	SVEKGVLY	11S globulin Pis v 2.0201	893,48583	ND	ND	1E+09	ND
	9	SVEKGVLYQ	11S globulin Pis v 2.0201	1021,5444	ND	3,13E+08	ND	ND
	10	SVEKGVLYQN	11S globulin Pis v 2.0201	1135.5873	ND	4,57E+08	1E+09	5E+08
	10	VEKGVLYQNA	11S globulin Pis v 2.0201	1119,5924	ND	2,16E+08	7E+08	2E+08
ACE inhibithor/Renin inhibitor	**Bioactive peptide: RALP (ID: 9468–9469)** [a]							
	Length	**Sequence**	**Protein family classification**	**Mass**	**Control**	**Acidified**	**D4**	**G3**
	7	RALPLDV	11S globulin Pis v 2.0101	782,46504	ND	ND	3E+08	ND
	8	FRALPLDV	11S globulin Pis v 2.0101	929,53345	2E+07	4,05E+08	9E+08	1E+08
	9	RVSVFRALP	11S globulin Pis v 2.0101	1043,624	ND	1,86E+08	9E+07	9E+07
	9	VFRALPLDV	11S globulin Pis v 2.0101	1028,6019	ND	13510005	ND	ND
	9	FRALPLDVI	11S globulin Pis v 2.0101	1042,6175	ND	26302331	6E+07	ND
	9	VSVFRALPL	11S globulin Pis v 2.0101	1000,607	ND	ND	2E+07	ND
	10	RVSVFRALPL	11S globulin Pis v 2.0101	1156,7081	ND	48889332	ND	1E+08
	10	FRALPLDVIK	11S globulin Pis v 2.0101	1170,7125	ND	2,41E+08	6E+08	ND
	10	RALPLDVIKN	11S globulin Pis v 2.0101	1137,687	ND	1,34E+08	ND	ND
ACE inhibitors/Antioxidative/Hypotensive peptide	**Bioactive peptide: LPILR (ID: 9708–9716–8661)** [a]							
	Length	**Sequence**	**Protein family classification**	**Mass**	**Control**	**Acidified**	**D4**	**G3**
	6	LPILRF	11S globulin Pis v 2.0201	757,48505	ND	ND	2E+08	ND
	8	NLPILRF	11S globulin Pis v 2.0201	871,52797	3E+07	ND	ND	ND
	7	LNLPILR	11S globulin Pis v 2.0201	837,54362	1E+07	ND	2E+08	3E+07
	8	LNLPILRF	11S globulin Pis v 2.0201	984,61204	1E+08	ND	8E+08	2E+08
	8	ALNLPILR	11S globulin Pis v 2.0201	908,58074	1E+07	ND	4E+08	7E+07
	8	NLPILRFL	11S globulin Pis v 2.0201	984,61204	2E+07	ND	ND	ND
	9	ALNLPILRF	11S globulin Pis v 2.0201	1055,6492	5E+07	2,45E+08	2E+09	4E+08
	9	LNLPILRFL	11S globulin Pis v 2.0201	1097,6961	3E+07	ND	ND	ND
	10	NLPILRFLQL	11S globulin Pis v 2.0201	1225,7547	ND	ND	2E+07	2E+07
	10	NALNLPILRF	11S globulin Pis v 2.0201	1169,6921	1E+07	ND	ND	4E+06
	10	ALNLPILRFL	11S globulin Pis v 2.0201	1168,7332	1E+07	ND	ND	ND
ACE inhibitors/Antioxidative/Renin inhibitor	**Bioactive peptide: LPAGV (ID: 9998–9997–9999)** [a]							
	Length	**Sequence**	**Protein family classification**	**Mass**	**Control**	**Acidified**	**D4**	**G3**
	8	LPAGVAHW	11S globulin Pis v 2.0101	849,44972	ND	3,62E+08	ND	ND
	9	ALPAGVAHW	11S globulin Pis v 2.0101	920,48684	ND	ND	3E+08	ND
	9	VIALPAGVA	11S globulin Pis v 2.0101	809,50109	ND	11999267	ND	ND
	10	IALPAGVAHW	11S globulin Pis v 2.0101	1033,5709	ND	2,73E+09	3E+09	4E+08
	10	VIALPAGVAH	11S globulin Pis v 2.0101	946,56	ND	ND	4E+08	ND
DPP-IV inhibitor	**Bioactive peptide: ILAP (ID:8647)** [a]							
	Length	**Sequence**	**Protein family classification**	**Mass**	**Control**	**Acidified**	**D4**	**G3**
	8	AILAPHWN	11S globulin Pis v 2.0101	920,48684	ND	ND	3E+08	3E+07
	9	RDAILAPHW	11S globulin Pis v 2.0101	1077,572	ND	98612007	5E+07	ND

[a] ID number of bioactive sequence annotated in BIOPEP database.

Angiotensin-Converting Enzyme (ACE) inhibition, antioxidative properties, dipeptidyl peptidase IV (DPP-IV) inhibition properties. In detail, samples G3, D4, acidified control and control (un-inoculated) contained 13, 20, 15 and 10 bioactive peptides, respectively (Table 2). The peptides harbouring sequences GVLY (BIOPEP ID 9325, ACE inhibitor), RALP (BIOPEP ID 9468–9469, ACE inhibitor and Renin inhibitor), LPILR (BIOPEP ID 9708-9716-8661, ACE inhibitor, antioxidant and hypotensive peptide), LPAGV (BIOPEP ID 9998-9997-9999, ACE inhibitor, antioxidant and renin inhibitor) and ILAP (BIOPEP ID 8647, DPP-IV inhibitor) were identified as the most representative in the pool of peptides generated during pistachio beverage fermentation. Fig. 5 focuses on the abundance of each fragment found in the different beverages. Fermented beverage D4 showed the highest number of identified peptides, the highest hydrolysis degree and notably, the larger abundance of bioactive precursor peptides. In general, the endogenous enzymatic activity under acidic conditions shows a large distribution of low abundant peptides characterised by "ragged ends", while the enzymatic activity of D4-fermented beverage shows some characteristic dominant sequences. For example, the sequence VEK before the bioactive sequence GVLY is well preserved, while the N or F residues are always present respectively at the N- and C-terminus the sequence LPILR. Finally, the fragments containing the bioactive sequence RALP with high amount have characteristic dominating sequences (FRALPLDVIK, FRALPLDV, RALPLDV and FRALPL).

4. Discussion

In this study, we applied proteomics analysis to investigate the release of peptides from pistachio proteins by LAB fermentation of pistachio-based beverage. Certain LAB have a complex proteolytic system that includes proteases to hydrolyze food proteins, transport systems to incorporate these peptides, and peptidase to metabolize peptides into amino acids and nitrogen essential for survival (Savijoki et al., 2006). However, it is documented in different food system that acidification induced by LAB clearly trigger proteolysis through activation of flour endogenous proteases (Gänzle et al., 2008). Previously studies on

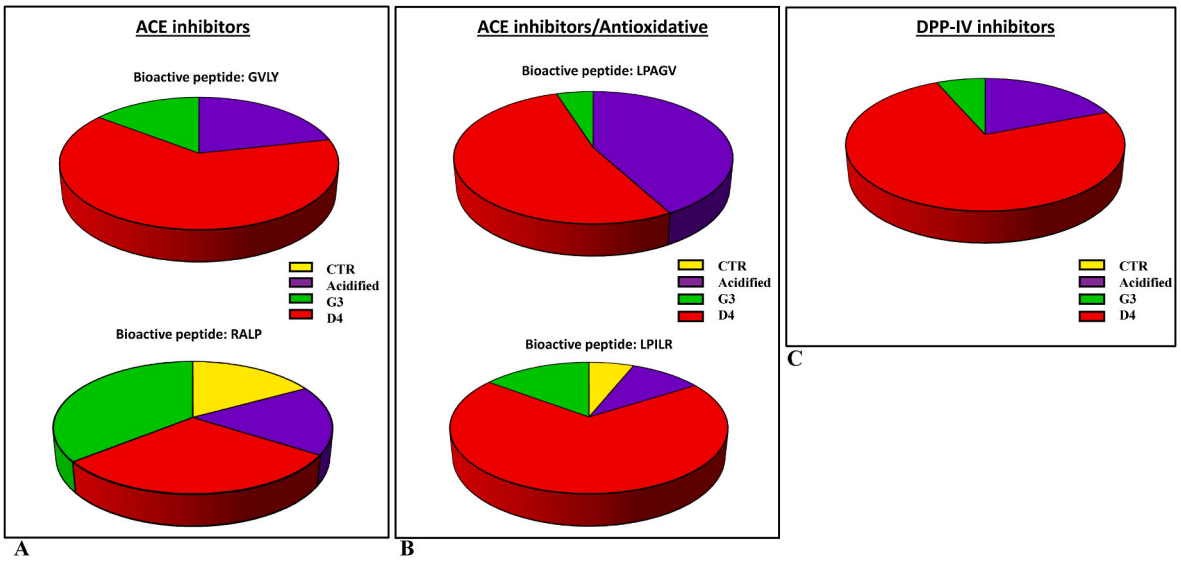

Fig. 5. Graphical representation of the relative abundance of precursor peptides (from 3 up to 10 amino acid residues) of **A**) the angiotensin-converting enzyme (ACE) inhibitor sequence RALP (BIOPEP ID 9468) and GVLY (BIOPEP ID 9325) **B**) the ACE inhibitor (BIOPEP ID 9708) and antioxidative (BIOPEP ID 9716) sequence LPILR and the ACE inhibitor (BIOPEP ID 9998) and antioxidative (BIOPEP ID 9997) sequence LPAGV and **C**) the dipeptidyl peptidase IV inhibitor (BIOPEP ID8647) sequence ILAP.

cereals, for example, have described a comparable protein degradation between chemically acidified matrix and LAB started fermented matrix (Gänzle, 2014; Thiele et al., 2002).

For this reason, in this study, in order to establish the role of endogenous pistachio proteases and LAB proteases, a control acidified beverage was analyzed in parallel. According to our findings, the peptides accumulated in the fermented beverage resulted from both the activity of endogenous pistachio proteases activated at low pH and the activity of LAB proteases. Undoubtedly, the acidification of the medium, caused the activation of the endogenous proteases, as demonstrated by the results of the proteolytic data. It should also be noted that the heat treatment at 70 °C carried out on the drink, was useful in reducing adventitious microorganisms (in fact, the charges were <1 log CFU/mL after treatment), but not adequate in denaturing the endogenous proteases that were still active. Furthermore, although LAB G3 and D4 acidified the beverage to similar pH values, the proteolytic patterns differed from each other and both from the acidified control. The number and frequency of identified peptides were higher in the beverage fermented with D4, followed by G3 and chemically acidified beverage. These results suggest that, in addition to endogenous protease active at acidic pH, the proteolytic system of LAB directly participated in peptide degradation to some extent, in line with previous findings (Reale et al., 2021; Spiecher & Nierle, 1988). After 24 h of LAB fermentation, 2S albumin, 7S and 11S globulins were the main pistachio proteins almost completely hydrolyzed. These proteins are storage proteins involved in seeds allergy. Proteolysis has denatured the 2S albumin, 7S and 11S globulins, thereby reducing their IgE-binding capacity and, potentially, their allergenicity (Gänzle, 2014).

Another important result is that fermentation leads to the production of peptides with potential bioactive properties. Overall, the peptides present in the fermented beverage varied significantly, with D4 containing the most peptides, followed by G3. This could be attributed to variations in enzymatic specificity between the different strains. In fact, LAB cell-envelope proteinases vary in substrate specificity, domain composition and anchoring mechanism, factors that may influence the production of protein hydrolysate (Savijoki et al., 2006). Using a predictive informatics tool based on the BIOPEP database, the peptides identified in fermented beverages showed a high frequency of ACE inhibitory, antioxidant and DPP-IV inhibitors sequences. In general, bioactive peptides contain mainly 3–20 amino acid units, but in some cases the size is larger (Shahidi & Zhong, 2008), and can be considered as components of functional foods which may exert regulatory activities in the human organism, irrespective of their nutritive functions (Gobbetti et al., 2007). Sample D4 had the highest content of ACE inhibitory peptides with antioxidant and DPP-IV inhibitor activity compared to G3. Interest in ACE-inhibitory peptides has grown as it has been shown that they can be competitive substrates for the inhibition of angiotensin I-converting enzyme, which plays a key role in the regulation of blood pressure (Iwaniak et al., 2014). Previous studies have report the identification of ACE inhibitory peptides from fermented plant food (Ambigaipalan et al., 2015; Guang & Phillips, 2009; Ramlal et al., 2022). Similarly, biologically active peptides with potential antioxidant activity have been isolated from plant fermented food matrices. (Rizzello et al., 2016; García et al., 2013; Babini et al., 2017). The antioxidant potential of bioactive peptides is based on their capacity to transfer hydrogen or electrons, preventing oxidative stress associated with numerous degenerative diseases like cancer and atherosclerosis (Chakrabarti and Jahandideh, 2014; Coda et al., 2012). Although the antioxidant properties of fermented foods have mostly been attributed to the presence of phenolic compounds (Chakrabarti and Jahandideh, 2014), the release of antioxidant peptides during fermentation may significantly contribute to the bioactivity of the final product (García et al., 2013). Interest in peptides with DPP-IV inhibitor activity has been discovered in various fermented foods. Dipeptidyl peptidase-IV inhibitor is a peptide that can increase insulin secretion and, therefore, decrease blood glycaemia by preventing incretins inactivation (Zhang et al., 2022).

5. Conclusions

In conclusion, this research has shed light on the potential of fermented-pistachio beverage with probiotic, nutritional and health benefits. A variety of peptides with different sequences and lengths are generated in beverage samples as a consequence of proteolysis induced by LAB. Some of the released peptides own potentially bioactivity like inhibition of ACE, DPP-IV and antioxidant properties. Results also showed that protein degradation is mainly affected by endogenous protease naturally occurred in pistachio seed, but LAB proteases affecting the degradation of proteins during fermentation also increase the concentration and the patterns of peptides. However, to fully harness

benefits of pistachio based-beverage for consumers, further studies are necessary to demonstrate the effectiveness of the health positive activities suggested by the present in silico data. Synthesis of the selected predicted peptides followed by *in vitro* and *in vivo* evaluation may allow to confirm the peptide bioactivity. At the same time, stimulated gastrointestinal conditions and cell culture mimicking the intestinal absorptive environment is needed to fully understand the bioaccessibility and bioavailability of these fermentation-derived peptides.

CRediT authorship contribution statement

Serena Marulo: Writing – original draft, Formal analysis, Data curation. **Salvatore De Caro:** Formal analysis, Data curation, Conceptualization. **Chiara Nitride:** Formal analysis, Data curation, Conceptualization. **Tiziana Di Renzo:** Data curation, Conceptualization. **Luigia Di Stasio:** Writing – review & editing, Visualization, Data curation. **Pasquale Ferranti:** Writing – original draft, Data curation, Conceptualization. **Anna Reale:** Writing – review & editing, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Gianfranco Mamone:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of Competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.103988.

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