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Unlocking the biological potential of whey proteins through lactic acid bacteria fermentation

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ABSTRACT

Fermenting whey with lactic acid bacteria (LAB) provides a sustainable route to valorize dairy byproducts while generating peptide-rich beverages with health benefits. In this study, a model whey beverage (WM) was fermented for 72 h with twelve LAB strains isolated from raw-milk dairy products. Fermentation resulted in strain-dependent protein hydrolysis and accumulation of low-molecular-weight peptides. Notably, β-lactoglobulin (β-Lg), the major whey allergen, was partially degraded during fermentation, with reductions ranging from 12 % to 33 %, depending on the strain. Among the tested strains, *Lactobacillus delbrueckii* subsp. *delbrueckii* LbL2, despite only moderate overall proteolysis, released the highest levels of peptides <3 kDa (1.05 ± 0.23 mg/mL), typically linked to health-promoting functions. *L. delbrueckii subsp. lactis LbL37* showed the strongest antioxidant capacity, increasing DPPH radical-scavenging activity by approximately 40 % compared to the unfermented control, while *Streptococcus macedonicus* LbL43 exhibited the highest ferric-reducing power. The most pronounced ACE-inhibitory effect was observed for *St. macedonicus* LbL43 (4.07 ± 0.02 mg/mL) and *L. delbrueckii* subsp. *delbrueckii* LbL37 (4.07 ± 0.02 mg/mL). Several fermentates also inhibited pathogenic bacteria, notably *Listeria monocytogenes*. These improvements were not linked to extensive proteolysis but rather to a moderate, selective hydrolysis pattern that may have preserved key bioactive sequences. Altogether, this study demonstrates the potential of autochthonous LAB as a promising strategy to produce whey-based fermented beverages with reduced allergenicity, enhanced health benefits, and environmental sustainability by valorizing dairy by-products.

1. Introduction

Fermentation has been used for centuries as a natural method to enhance the safety, shelf-life, and sensory qualities of food (Ibrahim et al., 2021). More recently, microbial fermentation has gained renewed scientific interest for its ability to transform food matrices into carriers of health-promoting compounds, particularly bioactive peptides (BPs) (Daliri et al., 2018). These peptides, encrypted within native food proteins, are typically released through proteolytic processes and can exert diverse biological activities - including antioxidant, antihypertensive, antimicrobial, antidiabetic, and immunomodulatory effects (Guo et al., 2023; Helal et al., 2023; Vermeirssen et al., 2003).

Among microbial groups, lactic acid bacteria (LAB) have emerged as valuable biocatalysts for the release of BPs, due to their well-characterised proteolytic systems and widespread use in food fermentations. LABs possess cell-envelope proteinases, peptide transporters, and intracellular peptidases that allow them to degrade proteins into peptides and free amino acids, which are essential for their growth. Under specific conditions, peptide accumulation in the fermentation medium can be favoured, particularly when peptide uptake is inefficient or autolysis occurs, enhancing the potential for recovery of extracellular BPs (Raveschot et al., 2020).

Whey, a by-product of cheese production, is rich in high-quality proteins such as β-lactoglobulin, α-lactalbumin, and serum albumin. Despite its nutritional value, whey remains underexploited in the context of functional food development (Olvera-Rosales et al., 2023). Most valorisation strategies have focused on physicochemical treatments (e.g., ultrafiltration), enzymatic hydrolysis, or microbial fermentation for lactose conversion (Di Filippo et al., 2024). However, biological strategies aimed at enhancing the biofunctionality of whey proteins via LAB fermentation remain limited and strain-dependent.

Recent studies have demonstrated that specific LAB strains - such as *Streptococcus thermophilus* and *Lactobacillus helveticus* - can hydrolyse whey proteins and release BPs with notable bioactivities

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Table 1
Bacterial strains and culture conditions used in this study.

Species	Strain	Culture conditions
L. delbrueckii subsp. *delbrueckii*	LbL2	MRS, 37 °C
L. delbrueckii subsp. *delbrueckii*	LbL14	MRS, 37 °C
L. delbrueckii subsp. *delbrueckii*	LbL12	MRS, 37 °C
L. delbrueckii subsp. *lactis*	LbL33	MRS, 37 °C
L. delbrueckii subsp. *lactis*	LbL37	MRS, 37 °C
L. delbrueckii subsp. *delbrueckii*	LbL38	MRS, 37 °C
L. delbrueckii subsp. *delbrueckii*	LbL39	MRS, 37 °C
L. delbrueckii subsp. *bulgaricus*	LbL9	MRS, 37 °C
Enterococcus malodoratus	A2	M17, 37 °C
Lacticaseibacillus paracasei	AF43	MRS, 30 °C
Streptococcus macedonicus	LbL43	MRS, 37 °C
Lactiplantibacillus plantarum	297	MRS, 30 °C

(Mazorra-Manzano et al., 2022; Solieri et al., 2022). Nevertheless, much less is known about the potential of non-starter and non-commercial LAB strains, especially those isolated from traditional raw-milk dairy products. These strains often exhibit unique proteolytic traits and metabolic behaviours, which may favour the production of peptides with specific functional properties.

Within this framework, the present study aimed to assess the ability of twelve LAB strains - isolated from raw-milk cheeses and natural milk cultures - to ferment whey protein concentrate (WPC) and release low-molecular-weight peptides with biological activity. The impact of fermentation was evaluated in terms of protein hydrolysis, peptide profile, and three key bioactivities: antioxidant capacity, ACE-I-inhibitory effect, and antimicrobial action. This work seeks to expand the current knowledge on strain-dependent biopeptide production from whey, with potential applications in sustainable food innovation and functional ingredient development.

2. Material and methods

2.1. Materials

All culture media, including Maximum Recovery Diluent (MRD), de Man Rogosa Sharpe (MRS) broth, M17 broth, and Brain Heart Infusion (BHI) medium, along with lactose, glucose, and bacteriological agar, were procured from Oxoid (Milan, Italy). WPC containing 77 % protein was obtained from BulkTM (Colchester, UK). Chemicals and standards, including acetonitrile, trifluoroacetic acid, bovine serum albumin (BSA), β-lactoglobulin (β-Lg), α-lactalbumin (α-La), caseinomacropeptide (CMP), aprotinin from bovine lung, C-peptide, L-glutathione oxidase, L-serine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), absolute ethanol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), potassium chloride (KCl), hydrochloric acid (HCl), iron(III) chloride ($FeCl_3$), angiotensin I-converting enzyme (ACE-I), hippuryl-L-histidyl-L-leucine (HHL), potassium-phosphate buffer, and sodium chloride, were all purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO, USA).

2.2. Bacterial strains and culture conditions

Twelve bacterial cultures (Table 1) were previously isolated from natural milk cultures and raw-milk Italian cheese, identified by partial 16S rRNA gene amplification (Innocente et al., 2023), and selected for their proteolytic activity (Renoldi et al., 2024). The isolates were stored at −80 °C in MRS or M17 broth supplemented with 30 % glycerol (v/v) for a maximum of 1 year. Cultures were reactivated in 1 mL of appropriate medium, then streaked onto agar plates and incubated for 48 h under anaerobic conditions.

2.3. Whey medium preparation

Whey medium (WM) was prepared by dissolving lactose (50 g/L) and WPC (15 g/L) in distilled water (pH 6.5 ± 0.1) as described by Solieri et al. (2022). The suspension was stirred for 1 h at 4 °C, then centrifuged at 5000×*g* for 10 min at 4 °C. The supernatants were subsequently filtered twice through 0.45 μm and 0.22 μm polystyrene filters.

2.4. Turbidimetric growth curves in whey medium

Overnight cultures were prepared by inoculating a single colony of each strain in 1 mL of MRS or M17. Each overnight culture was centrifuged (13,000×*g* for 5 min at 4 °C), washed twice, and resuspended in 1 mL of MRD. The strains were inoculated into 96-well U-bottomed microplates, with each well containing 10 μL of a 1:100 diluted culture (final concentration approximately 5×10^4 cfu/mL) and 190 μL of WM. Control wells containing inoculated MRS or M17 broth were prepared for each strain. Blank wells containing uninoculated media were also prepared. Microbial growth was monitored for 72 h at 30 °C or 37 °C. The optical density at 630 nm was recorded every 30 min, after shaking for 5 s, using a Sunrise microplate reader (Tecan, Milan, Italy).

2.5. Whey medium fermentation

Overnight cultures were prepared by inoculating a single colony of each strain in 1 mL of MRS or M17. Each overnight culture was washed once in MRD, diluted 1:100, inoculated (1 % v/v) in WM (final concentration approximately 10^4 cfu/mL), and incubated at 30 °C or 37 °C for 72 h. Uninoculated WM served as a control. Bacterial counts of fermentates were performed by serially diluting the samples, spread-plating onto MRS agar for lactobacilli, and M17 agar supplemented with 10 % (v/v) lactose for streptococci and enterococci, and incubating at 30 °C or 37 °C for 48 h under anaerobic conditions. pH of fermentates was measured using a pH meter Basic 20 (Crison, Barcelona, Spain) equipped with a pH electrode previously calibrated with standard solutions at pH 4.01, 7.00, and 9.21.

2.6. Determination of residual sugars

Residual sugars (lactose, glucose, and galactose) from WM and fermentates were quantified following the method proposed by Rossi et al. (2024) with slight modifications. At the end of fermentation, the cell-free supernatant was obtained by centrifugation (5000×*g*, 5 min, 4 °C) and subsequent filtration through 0.22 μm polystyrene filters (Minisart™, Sartorius, Göttingen, Germany). Samples were diluted 1:10 with 25 mmol/L H_2SO_4 and homogenised for 5 min. The resulting suspension was centrifuged at 15,000×*g* for 20 min at 4 °C. Separation of sugars was achieved using an LC-4000 HPLC system (Jasco Europe, Lecco, Italy) equipped with an Aminex HPX-87H ion-exclusion column (300 mm × 7.8 mm × 9 μm; Bio-Rad, Hercules, CA), an AS-4050 autosampler, a 20 μL injection loop, and a column oven set at 65 °C. Chromatographic runs were performed at a flow rate of 0.7 mL/min with 25 mmol/L H_2SO_4 as the mobile phase. Detection of sugars was monitored with an RI-4030 detector (Jasco Europe) maintained at 40 °C. Quantitative analysis was performed by external calibration with standard solutions prepared at concentrations ranging from 0.001 to 100 mg/mL ($R^2 = 0.99$).

2.7. Extent of protein hydrolysis and molecular weight distribution

The extent of protein hydrolysis and the molecular weight (MW) distribution of peptides released during fermentation were estimated using SE-HPLC analysis, as described by Cui et al. (2022) and Innocente et al. (2023). At the end of fermentation, the cell-free supernatant was obtained by centrifugation (5000×*g*, 5 min, 4 °C) and subsequent filtration through 0.22 μm polystyrene filters (Minisart™, Sartorius, Göttingen, Germany) and used for the analysis. A TSKgel 2000 SWXL 300 mm × 7.8 mm column (Tosoh Bioscience, Griesheim, Germany) with a mobile phase consisting of water/acetonitrile/trifluoroacetic acid

(55/45/0.1, v/v/v) was used. The flow rate was 0.5 mL/min, and the column temperature was maintained at 30 °C. A 10-μL sample was injected into the HPLC system (LC-4000, Jasco Europe, Cremella, Italy) equipped with a PDA detector set at 220 nm. BSA (66,463 Da), β-Lg (18, 400 Da), CMP (6800 Da), aprotinin (6511 Da), C-peptide (3183 Da), L-glutathione oxidised (612 Da), and L-Serine (106 Da) were run as standards. Data analysis was performed using the chromatography software ChromNAV2 (Jasco Europe, Cremella, Italy).

The extent of protein hydrolysis (PH, %) was evaluated by measuring the reduction in whey protein area following fermentation. First, the total protein reduction was calculated by comparing the sum of peak areas in the fermentation cell-free supernatant and WM. In addition, the PH (%) was determined for individual whey protein fractions (e.g., BSA, α-La, β-Lg, and CMP) by monitoring the decrease in their specific peak areas. In both cases, PH% was expressed using the formula shown in Equation (1).

$$PH\ (\%) = \frac{(Area\_WM - Area\_F)}{Area\_WM} \times 100 \quad \text{(Equation 1)}$$

Where *Area_WM* and *Area_F* represent the chromatographic peak areas of the unfermented WM and fermentates, respectively.

A calibration curve was then generated by injecting MW standards at concentrations ranging from 0.1 to 5 mg/mL. This curve was first applied to quantify the total protein content of the samples and subsequently used for MW distribution analysis to quantify the amount of peptides below 3 kDa.

2.8. Radical scavenging activity

DPPH radical scavenging activity was determined using the method described by Cui et al. (2022) with some modifications. Briefly, the cell-free supernatant was obtained by centrifugation (5000×*g*, 5 min, 4 °C) and subsequent filtration through 0.22 μm polystyrene filters (Minisart™, Sartorius). 50 μL of each fermentation cell-free supernatant was mixed with 150 μL of fresh DPPH ethanolic solution (0.05 mg/mL) in wells of a U-bottomed 96-well polystyrene microplate (Corning Life Sciences, US). After 20 min of incubation in the dark, the absorbance was read at 517 nm at 25 °C using a Sunrise microplate reader (Tecan s.r.l., Cernusco s. N, Milan, Italy). The radical scavenging activity was computed as reported in Equation 2:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{(Absi - Absf)}{Absi} \times 100 \quad \text{(Equation 2)}$$

where *Absi* and *Absf* are the absorbances at the beginning and the end of the assay, respectively.

Trolox® solutions at increasing concentrations (0, 10, 20, 30, 40, 50, 60 mg/L) were assayed under the same conditions to obtain a calibration curve, and the radical scavenging activity of samples was expressed as Trolox Equivalents on the protein content of each cell-free supernatant (mgTE/g protein).

2.9. Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP) of fermentation supernatants was determined using the method described by Benzie and Strain (1996) with some modifications. FRAP reagent was obtained by mixing KCl/HCl buffer (0.2 M; pH 2.2), 10 mmol/L TPTZ, and a 20 mmol/L FeCl3 solution in a 10:1:1 ratio, while the cell-free supernatant was obtained by centrifugation (5000×*g*, 5 min, 4 °C) and subsequent filtration through 0.22 μm polystyrene filters (Minisart™, Sartorius). 100 μL of the cell-free supernatant and 100 μL of FRAP reagent were placed in U-bottomed microplate wells. The absorbance reading was carried out after 40 min using a Sunrise microplate reader at 593 nm at 37 °C. The FRAP (%) was calculated according to Equation 3:

$$FRAP\ (\%) = \frac{(Absf - Absi)}{Absf} \times 100 \quad \text{(Equation 3)}$$

where *Absi* and *Absf* were the absorbances at the beginning and the end of the assay, respectively. FRAP was expressed as Trolox Equivalents on the protein content of each cell-free supernatant (mgTE/g protein), as described for the DPPH assay.

2.10. ACE-I (angiotensin converting enzyme)-I-inhibitory activity

ACE-I inhibitory activity was measured by HPLC following the method proposed by Cushman and Cheung (1971) with some modifications. Hippuryl-L-histidyl-L-leucine (HHL; 5 mM) and ACE-I (0.1 U/mL) were dissolved in 0.1 mol/L potassium phosphate buffer (pH 8.3) containing 0.3 mol/L NaCl. The cell-free supernatant was obtained by centrifugation (5000×*g*, 5 min, 4 °C) and subsequent filtration through 0.22 μm polystyrene filters (Minisart™, Sartorius). The assay was performed by mixing 120 μL of HHL solution with 30 μL of each fermentation cell-free supernatant or milliQ water as a control. After 10 min of incubation at 37 °C, the reaction was started by adding 30 μL of ACE-I solution. The samples were incubated at 37 °C for 60 min under shaking, and then 150 μL of HCl (1 mol/L) was added to stop the reaction. Samples were filtered through a 0.45 μm nylon syringe filter. Hippuric acid (HA) released by ACE-I was analysed by injecting 6 μL of the sample into a Water Nova-Pak C18 column (4.6 mm × 150 mm, 4 μm) connected to a Jasko HPLC system and detected at 228 nm. Elution was performed at a flow rate of 0.8 mL/min. The mobile phase comprised 0.1 % (v/v) TFA in milliQ water (solvent A) and 0.1 % (v/v) TFA in acetonitrile (solvent B), with a 10 %–60 % B gradient for 10 min, then returned to 10 % B in 2 min and followed by isocratic elution for 3 min. The percentage inhibition was calculated following Equation 4:

$$ACE-I\ inhibitory\ activity\ (\%) = \frac{(Area\_control - Area\_sample)}{Area\_control} \times 100 \quad \text{(Equation 4)}$$

where *Area_sample* is the chromatographic peak area of HA in the presence of the fermentate, and *Area_control* is the area obtained in the absence of the fermentate. The IC_{50} values (mg/mL) were determined by plotting ACE-I inhibition activity (%) as a function of protein concentration, selecting values that fell within the linear range of the inhibition response. IC_{50} represents the concentration of protein in each cell-free supernatant required to achieve 50 % inhibition of ACE-I activity.

2.11. Antimicrobial activity

The antimicrobial activity of each fermentation cell-free supernatant was tested against *Staph. aureus*, *S. enterica*, *E. coli*, *L. monocytogenes*, *Enterococcus,* and *P. fluorescens* by turbidimetric growth curves. For each microbial target, pools of three different strains were tested. *Staph. aureus* Di4A_226, *Staph. aureus* Di4A_CAR1-N, *Staph. aureus* DSMZ 2569, *Salmonella enterica* spp. *arizonae* DSMZ 9386, *S. enterica* Di4A_#40, *S. enterica* Di4A_#52, *Escherichia coli* Di4A_8048, *E. coli* Di4A_TOL, *E. coli* Di4A_UD, *L. monocytogenes* Scott A, *L. monocytogenes* Di4A_248, *L. monocytogenes* DSMZ 7644, *Enterococcus faecium* DMSZ 2146, *E. faecium* Di4A_TO, *E. durans* Di4A_173, *Pseudomonas fluorescens* Di4A_LS1, *P. fluorescens* Di4A_LS3, and *P. fluorescens* Di4A_LS22 were stored at −80 °C in BHI added with 30 % (v/v) glycerol for a maximum of 1 year. The overnight culture was prepared for each target strain by inoculating 1 mL of BHI with a loopful of the cryopreserved stock culture, followed by incubation at 37 °C overnight. Cells were recovered by centrifugation at 13,000×*g* for 5 min at 4 °C (Mikro 20 centrifuge; Hettich Italia s.r.l., Milan, Italy) and washed twice with MRD. Microbial pools were created by combining strains belonging to the same genus. The cell-free supernatant was obtained by centrifugation (5000×*g*, 5 min, 4 °C) and subsequent filtration through 0.22 μm polystyrene filters

Table 2
Cell viability (log cfu/mL), pH, and residual sugars (mg/mL) detected in whey medium (WM) and fermentates. Data are expressed as mean ± standard deviation.

Strain	Viability	pH	Lactose	Galactose
WM	–	$6.50^{a} \pm 0.10$	$54.77^{a} \pm 1.19$	n.d.
L. paracasei AF43	$8.10^{a} \pm 0.02$	$4.06^{i} \pm 0.02$	$47.45^{d} \pm 0.05$	n.d.
L. plantarum 297	$7.74^{b} \pm 0.19$	$4.12^{h} \pm 0.03$	$54.01^{a} \pm 0.09$	n.d.
St. macedonicus LbL43	$6.29^{g} \pm 0.02$	$4.18^{g} \pm 0.02$	$50.98^{b} \pm 0.08$	n.d.
L. delbrueckii subsp. *delbrueckii* LbL38	$6.30^{fg} \pm 0.43$	$4.20^{g} \pm 0.02$	$52.56^{ab} \pm 0.03$	n.d.
L. delbrueckii subsp. *bulgaricus* LbL9	$6.60^{f} \pm 0.02$	$4.26^{f} \pm 0.03$	$53.81^{ab} \pm 0.35$	$1.28^{b} \pm 0.01$
L. delbrueckii subsp. *delbrueckii* LbL39	$6.17^{g} \pm 0.18$	$4.30^{ef} \pm 0.02$	$52.31^{ab} \pm 0.06$	$0.42^{f} \pm 0.01$
L. delbrueckii subsp. *lactis* LbL37	$6.92^{d} \pm 0.11$	$4.33^{e} \pm 0.03$	$49.69^{bc} \pm 0.06$	$1.32^{b} \pm 0.07$
L. delbrueckii subsp. *lactis* LbL33	$6.81^{de} \pm 0.05$	$4.36^{de} \pm 0.03$	$53.22^{ab} \pm 0.03$	$1.14^{c} \pm 0.01$
Ent. malodoratus A2	$7.04^{cd} \pm 0.37$	$4.40^{d} \pm 0.02$	$53.14^{ab} \pm 0.04$	n.d.
L. delbrueckii subsp. *delbrueckii* LbL12	$7.23^{c} \pm 0.04$	$4.46^{c} \pm 0.03$	$48.37^{c} \pm 0.05$	$1.60^{a} \pm 0.03$
L. delbrueckii subsp. *delbrueckii* LbL14	$6.74^{e} \pm 0.06$	$4.55^{b} \pm 0.02$	$48.41^{c} \pm 0.05$	$1.03^{d} \pm 0.02$
L. delbrueckii subsp. *delbrueckii* LbL2	$6.81^{de} \pm 0.05$	$4.58^{b} \pm 0.01$	$50.08^{b} \pm 0.04$	$0.81^{e} \pm 0.00$

Statistical differences ($p < 0.05$) within each column are indicated by different letters.
nd. not detected.

(Minisart™, Sartorius). Wells of a U-bottomed 96-well polystyrene microplate were filled with 100 µL of BHI broth 2 × and 100 µL of each cell-free supernatant (final pH 6.8 ± 0.2). Each well was inoculated with 10 µL of each microbial pool (final viability about 5×10^4 cfu/mL). Wells containing inoculated BHI broth were used as a control (BHI), and wells containing non-inoculated medium as a blank. Microbial growth was monitored at 30 °C (for *Pseudomonas* spp.) or 37 °C for 48 h using a Sunrise microplate reader. The optical density at 630 nm (OD_{630}) was recorded every 30 min, preceded by 5 s shaking. Growth curve data were fitted using OriginLab 2021 (Northampton, UK) by the re-parametrised Gompertz equation (Zwietering et al., 1990) as reported in Equation 5:

$$y = A exp\left\{ - exp\left[\frac{\mu_{max} * e}{A}(\lambda - t) + 1\right]\right\} \quad \text{(Equation 5)}$$

where t is time (h); y is response i.e., the log-transformed OD value; A (amplitude) is the upper asymptote; μ_{max} is the maximum specific growth rate (log OD/h); and λ is the lag time (h). The parameters λ, μ_{max}, and A were estimated from the fitted model.

2.12. Statistical analysis

All trials were carried out at least in three biological replicates, and values were expressed as the means ± standard deviation (SD). Statistical analysis was performed using R v 4.3.0 (The R Foundation for Statistical Computing, Vienna, Austria). Bartlett's test was used to assess homogeneity of variance; the difference between means was calculated using a *t*-test for pairwise comparisons and a one-way ANOVA for multiple comparisons, followed by a Tukey's post hoc test to determine statistically significant differences among means ($p < 0.05$). Comparisons were performed between fermentates and the unfermented control (WM), as well as among strains to assess strain-dependent variability.

3. Results and discussion

WM was subjected to 72-h fermentation by 12 different strains of LAB, previously isolated from dairy products and selected for their proteolytic metabolism and ability to grow in the presence of whey proteins. All the strains showed the ability to grow in WM (Fig. S1), with viability values ranging from 6.17 to 8.10 log cfu/mL (Table 2). The growth was associated with a marked acidification of the medium, with pH values decreasing from 6.50 ± 0.10 in WM to 4.06–4.58 in the fermentates.

The extent of acidification varied among strains ($p < 0.05$), reflecting differences in carbohydrate metabolism. The unfermented WM contained lactose (54.77 ± 1.19 mg/mL) as the sole carbohydrate, while glucose and galactose were not detected. After 72 h of fermentation, the extent of lactose consumption varied among strains, with *L. paracasei* AF43 showing the highest utilisation, consistent with its strong acidifying activity. In contrast, *L. plantarum* 297 exhibited limited lactose consumption, in agreement with reports indicating that the efficiency of lactose uptake and β-galactosidase expression in *L. plantarum* is highly strain-dependent and influenced by environmental conditions (Xu et al., 2022). *L. delbrueckii* strains also showed a considerable ability to

Table 3
Extent of protein hydrolysis (PH, %) of different lactic acid bacteria (LAB) strains on total whey protein and specific whey protein fractions, including bovine serum albumin (BSA), β-lactoglobulin (β-Lg), α-lactalbumin (α-La), and caseinomacropeptide (CMP). Data are expressed as mean ± standard deviation.

Strains	Extent of protein hydrolysis (%)				
	Total	BSA	β-Lg	α-La	CMP
L. paracasei AF43	$21.84^{f} \pm 2.11$	$58.43^{b} \pm 4.77$	$13.66^{bc} \pm 5.38$	$21.24^{bd} \pm 2.27$	$61.02^{ac} \pm 8.01$
L. plantarum 297	$24.53^{f} \pm 2.29$	$92.49^{a} \pm 1.04$	$2.44^{cd} \pm 0.42$	$11.95^{d} \pm 4.69$	$46.55^{c} \pm 8.29$
St. macedonicus LbL43	$25.72^{f} \pm 0.20$	$91.8^{a} \pm 4.36$	$0.80^{d} \pm 0.13$	$12.92^{d} \pm 0.29$	$52.29^{ac} \pm 3.42$
L. delbrueckii subsp. *delbrueckii* LbL38	$42.56^{bc} \pm 1.57$	$89.04^{a} \pm 4.04$	$27.72^{ab} \pm 2.37$	$32.2^{bc} \pm 4.18$	$59.96^{ac} \pm 3.48$
L. delbrueckii subsp. *bulgaricus* LbL9	$36.22^{d} \pm 3.39$	$85.13^{a} \pm 7.4$	$18.72^{abc} \pm 8.1$	$30.24^{cd} \pm 8.9$	$65.02^{ac} \pm 5.02$
L. delbrueckii subsp. *delbrueckii* LbL39	$46.13^{ab} \pm 1.31$	$93.29^{a} \pm 1.51$	$30.31^{ab} \pm 2.57$	$36.13^{bc} \pm 4.97$	$60.31^{ac} \pm 4.03$
L. delbrueckii subsp. *lactis* LbL37	$46.04^{ab} \pm 4.12$	$99.12^{a} \pm 0.06$	$28.3^{ab} \pm 5.67$	$39.87^{a} \pm 8.15$	$66.17^{bc} \pm 2.15$
L. delbrueckii subsp. *lactis* LbL33	$47.67^{a} \pm 0.32$	$94.35^{a} \pm 2.28$	$33.2^{a} \pm 1.30$	$36.78^{bc} \pm 4.66$	$60.25^{ac} \pm 4.36$
Ent. malodoratus A2	$31.25^{e} \pm 1.34$	$95.17^{a} \pm 0.87$	$12.55^{bc} \pm 6.99$	$26.43^{cd} \pm 0.01$	$86.11^{a} \pm 6.06$
L. delbrueckii subsp. *delbrueckii* LbL12	$40.85^{c} \pm 1.89$	$87.06^{a} \pm 1.88$	$28.21^{ab} \pm 5.78$	$30.79^{abc} \pm 2.6$	$64.27^{ac} \pm 6.01$
L. delbrueckii subsp. *delbrueckii* LbL14	$41.24^{c} \pm 0.44$	$85.67^{a} \pm 0.81$	$30.49^{ab} \pm 2.26$	$30.78^{abc} \pm 0.54$	$67.74^{ab} \pm 3.93$
L. delbrueckii subsp. *delbrueckii* LbL2	$32.02^{de} \pm 1.28$	$84.64^{a} \pm 11.52$	$12.04^{bc} \pm 5.19$	$30.64^{abc} \pm 4.04$	$15.42^{c} \pm 3.26$

Statistical differences ($p < 0.05$) within each column are indicated by different letters.

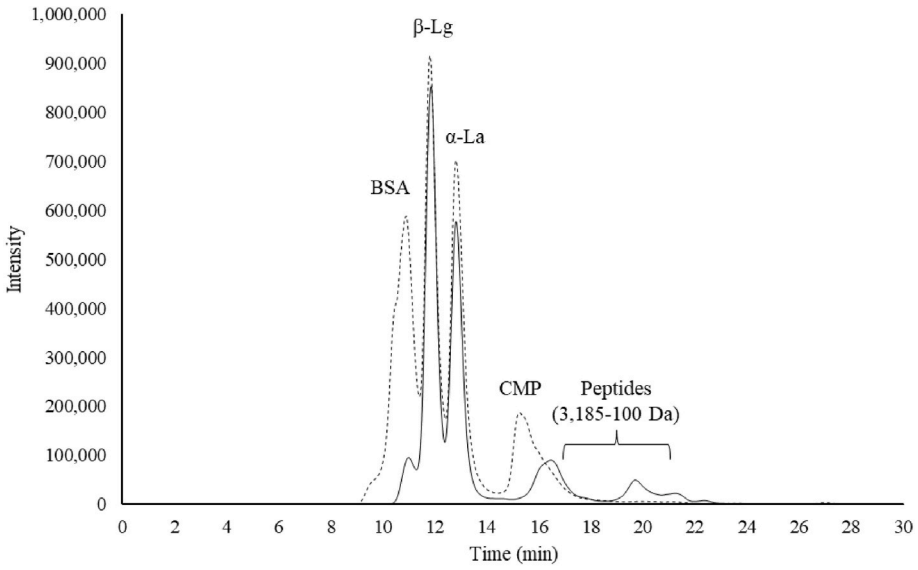

Fig. 1. SE-HPLC chromatograms of whey medium (WM; dotted line) and *Lactobacillus delbrueckii subsp. delbrueckii* LbL39 fermentate (continuous line). Peaks correspond to bovine serum albumin (BSA), β-lactoglobulin (β-Lg), α-lactalbumin (α-La), and caseinomacropeptide (CMP).

metabolise lactose. These species are generally lactose-positive, possessing both lactose transport systems and β-galactosidase enzymes that facilitate lactose hydrolysis (Bintsis, 2018; Papadimitriou et al., 2014). However, in *L. delbrueckii* fermentations, the higher lactose consumption did not correspond to a more substantial acidification, probably because part of the galactose obtained from lactose hydrolysis was not metabolised but rather accumulated in the medium. Some *L. delbrueckii* subspecies preferentially export galactose instead of channelling it into glycolysis (Iskandar et al., 2019). By contrast, glucose was not detected in any fermentate, indicating its rapid uptake and complete metabolism. Consequently, although these strains efficiently degraded lactose, incomplete galactose metabolism limited overall acid production, contributing to the observed strain-dependent variability in fermentation performance.

The unfermented WM contained an initial total protein concentration of 9.72 ± 1.71 mg/mL. This value is consistent with concentrations typically reported for whey-based fermentation systems and is suitable to sustain bacterial growth and proteolytic activity (Dineshbhai et al., 2022; Solieri et al., 2022). After fermentation, the extent of proteolysis varied significantly among the tested strains, indicating that strains were able to hydrolyse whey protein differently (Table 3). PH (%) ranged from approximately 21.84 %–47.7 %, with *L. delbrueckii* subsp. *lactis* LbL33 showing the highest total proteolytic activity, followed by *L. delbrueckii* subsp. *delbrueckii* LbL39 (46.13 ± 1.31 %) and *L. delbrueckii* subsp. *lactis* LbL37 (46.04 ± 4.12 %). In contrast, *L. paracasei* AF43 and *L. plantarum* 297 exhibited the lowest total reductions (21.84 ± 2.11 % and 24.53 ± 2.29 %, respectively), highlighting a much weaker proteolytic effect.

Fig. 1 shows a representative chromatogram of unfermented WM and a fermentate, illustrating the decrease of major whey proteins and the concurrent increase in low-molecular-weight peptide fractions after fermentation. Identifying which fractions are most susceptible to proteolysis is important, not only because they represent valuable sources of BPs, but also because certain proteins, such as β-Lg and α-La, are major milk allergens whose degradation can enhance the hypoallergenic properties of whey-based products (Pescuma et al., 2012). In this study, BSA was the most degraded protein across all strains, with reduction values often exceeding 85 %, suggesting that BSA is particularly susceptible to LAB proteases. The most efficient BSA hydrolysis was observed with *L. delbrueckii* subsp. *lactis* LbL37, with a reduction value almost reaching 100 %. By contrast, β-Lg and α-La were generally more resistant to proteolysis. The hydrolysis of β-Lg remained below 20 % for most strains, except for LbL33, which reached a maximum reduction of 33.2 ± 1.3 %. Similarly, α-La degradation was generally low, ranging from 11.95 ± 4.69 % (*L. plantarum* 297) to 39.87 ± 8.15 % (*L. delbrueckii* subsp. *lactis* LbL37). Although LAB proteolytic systems can act on all whey protein fractions, they typically show a preference for caseins (Solieri et al., 2022). Consistently, CMP appeared to be moderately hydrolysed, with most strains achieving reductions between 45 % and 67 %. Notably, *Ent. malodoratus* A2 showed the highest reduction of this fraction (86.11 ± 6.06 %), despite a moderate total proteolytic activity (31.25 ± 1.34 %), suggesting a possible specificity of its proteolytic system toward CMP. Overall, *L. delbrueckii* subsp. *lactis* strains displayed the most extensive and balanced proteolytic activities, efficiently degrading multiple whey protein fractions and potentially contributing both to improved digestibility and reduced allergenicity.

Table 4
Peptide concentration (mg/mL) in the <3 kDa molecular weight (MW) in whey medium (WM) and the fermentates. Values are reported as mean ± standard deviation.

Strain	Peptide <3 kDa (mg/mL)
WM	0.18^{d} ± 0.04
L. paracasei AF43	0.39^{cd} ± 0.01
L. plantarum 297	0.47^{bd} ± 0.04
St. macedonicus LbL43	0.49^{bd} ± 0.06
L. delbrueckii subsp. *delbrueckii* LbL38	0.56^{bc} ± 0.08
L. delbrueckii subsp. *bulgaricus* LbL9	0.58^{bc} ± 0.02
L. delbrueckii subsp. *delbrueckii* LbL39	0.60^{bc} ± 0.05
L. delbrueckii subsp. *lactis* LbL37	0.64^{bc} ± 0.01
L. delbrueckii subsp. *lactis* LbL33	0.62^{bc} ± 0.06
Ent. malodoratus A2	0.66^{bc} ± 0.13
L. delbrueckii subsp. *delbrueckii* LbL12	0.71^{ab} ± 0.05
L. delbrueckii subsp. *delbrueckii* LbL14	0.78^{ab} ± 0.01
L. delbrueckii subsp. *delbrueckii* LbL2	1.05^{a} ± 0.23

Statistical difference ($p < 0.05$) is indicated by different letters.

To further assess the impact of fermentation on whey proteins, peptide fractions in the MW range <3 kDa were quantified (Table 4). Importantly, bioactivities are generally reported to be highest for peptides with MW < 0.8 kDa (*i.e.*, <7 amino acids), and still significant for those with MW between 0.8 and 3 kDa. For this reason, the fraction of peptides with MW < 3 kDa is particularly noteworthy, while those with extended chains are typically the least effective (Capriotti et al., 2015). All strains tested released peptides in the <3 kDa range, indicating

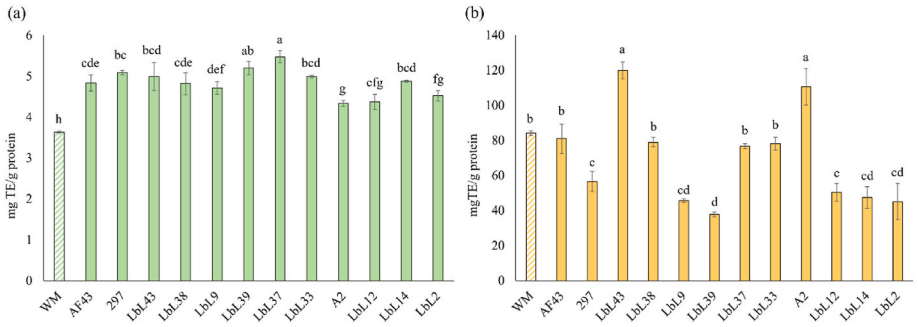

Fig. 2. Antioxidant activity of whey medium (WM) and fermentates, expressed as mg TE/g protein, determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (a) and the ferric reducing antioxidant power (FRAP) assay (b). Different letters indicate statistically significant differences ($p < 0.05$).

proteolysis and extracellular accumulation.

L. delbrueckii subsp. *bulgaricus* and subsp. *lactis* are widely recognised for their ability to hydrolyse whey proteins and release peptides (Chourasia et al., 2024; Hebert et al., 2008; Pescuma et al., 2008). Despite the highest reduction of proteins shown in Table 3, *L. delbrueckii* subsp. *lactis* LbL33 did not generate the highest concentration of low-MW peptides, possibly due to cellular uptake of the released peptides to meet physiological needs (Ter et al., 2024). In contrast, several strains of *L. delbrueckii* subsp. *delbrueckii* (e.g., LbL2, LbL12, and LbL14) showed a marked ability to accumulate small peptides (<3 kDa), with *L. delbrueckii* subsp. *delbrueckii* LbL2 reaching 1.05 ± 0.23 mg/mL, significantly higher than all other strains ($p < 0.05$). To the best of our knowledge, no previous studies have confirmed peptide release by this subspecies. In all cases, however, the unfermented WM contained significantly lower amounts of peptides compared to the fermentates. Interestingly, *L. delbrueckii* subsp. *delbrueckii* LbL2 exhibited a slightly lower overall protein reduction (32.02 %) compared to LbL33, yet was the most effective in generating peptides in the <3 kDa range. According to Table 3, proteolysis in this strain was primarily directed toward BSA, and to a lesser extent, α-La, while β-Lg and CMP were only marginally affected. These findings suggest that *L. delbrueckii* subsp. *delbrueckii* LbL2 promotes a controlled and selective proteolysis, favouring the accumulation of short peptides.

Conversely, species such as *St. macedonicus*, *L. plantarum,* and *L. paracasei* are known to be poorly proteolytic (Pescuma et al., 2013; Savijoki et al., 2006; Çetin et al., 2024). In fact, they produced comparatively lower peptide concentrations.

Peptide yields from fermentation were considerably lower than those obtainable through enzymatic hydrolysis, which can release over 90 % of peptides in just a few hours (Huang et al., 2010; Innocente et al., 2023). However, fermentation remains a promising approach because it allows the production of a wide range of qualitatively diverse peptides and enables *in situ* biofortification. In addition, fermentation offers a cost-effective and sustainable alternative to enzymatic hydrolysis, as it relies on the intrinsic proteolytic systems of microorganisms rather than expensive commercial enzymes (Tonini et al., 2024; Ulug et al., 2021).

The accumulation of low-molecular-weight peptides provided the experimental basis for investigating the biological properties of the fermentates, as described in the following sections.

The antioxidant activity is relevant both in the food industry, where various substances are prone to oxidation with a negative impact on food products, and physiologically, as the oxidation of cellular components triggers ageing and cellular degeneration (Tonolo et al., 2024; Zhu et al., 2024). Several mechanisms contribute to the antioxidant capacity of food matrices (Marazza et al., 2012). Therefore, the antioxidant activity of the cell-free fermentation supernatants was assessed in terms of radical scavenging activity (DPPH) and ferric-reducing antioxidant power (FRAP) (Fig. 2).

WM exhibited a certain radical scavenging activity (3.63 ± 0.03 mg TE/g protein) and ferric-reducing ability (84.13 ± 1.3 mg TE/g protein), highlighting the antioxidant properties of whey proteins. These findings are consistent with those of other authors (Di Filippo et al., 2024; Lin et al., 2012). The antioxidant activity of native whey proteins is primarily attributed to the high concentration of hydrophobic and aromatic amino acids, which can stabilize radicals through proton or electron donation (Halavach et al., 2024). In addition, reducing sugars added to WM (Table 2) may contribute to its high ferric-reducing capacity, as these compounds are known to enhance FRAP values (Liu et al., 2014). It cannot be excluded that other antioxidant molecules naturally present in milk (e.g., vitamins and minerals) and that may remain in the WPC also contribute to the overall antioxidant activity (Khan et al., 2019).

In all samples, fermentation increased the radical scavenging activity ($p < 0.05$), which ranged from 4.34 ± 0.07 mg TE/g protein to 5.48 ± 0.15 mg TE/g protein. *L. delbrueckii* subsp. *lactis* LbL37 showed the best result, increasing antioxidant activity by approximately 40 % as measured by the DPPH assay. In the FRAP assay, fermentation induced a significant increase in antioxidant activity in only two samples. The most vigorous ferric-reducing activity was recorded for *St. macedonicus* LbL43 (119.96 ± 4.80 mg TE/g protein). *Ent. malodoratus* A2 also displayed a high reducing capacity (110.58 ± 10.32 mg TE/g protein), while several *L. delbrueckii* strains and *L. plantarum* 297 exhibited substantially lower activity. The generally lower FRAP values of many fermentates compared to WM may, at least in part, be explained by the consumption of reducing sugars during fermentation. Notably, *St. macedonicus* LbL43 and *Ent. malodoratus* A2 were able to sustain high levels of ferric-reducing activity despite this reduction.

Considering that in WM the nitrogen fraction represented the major component of the dry weight, except for lactose (which decreased during fermentation), the increase in antioxidant activity could be associated with the accumulation of low-molecular-weight peptides resulting from proteolytic activity, which are known to contribute to antioxidant activity (Shirkhan et al., 2023). However, *L. delbrueckii* subsp. *lactis* LbL37, *St. macedonicus* LbL43, and *Ent. malodoratus* A2 did not release the highest overall peptide quantities (Table 4). In contrast, other strains produced considerably higher amounts of small peptides but did not achieve the same antioxidant capacity. This finding suggests that antioxidant capacity is not merely a function of peptide abundance. Instead, it highlights the importance of peptide sequence and composition, since residues such as tyrosine, tryptophan, methionine, cysteine, histidine, and lysine are particularly effective in stabilising radicals (Di Filippo et al., 2025). Moreover, the structural features of peptides, including their hydrophobicity and basic nature, are likely to play a decisive role in determining their antioxidant potential (Ren et al., 2008). Thus, these strains may have generated peptide fractions with a more favourable composition for antioxidant activity, despite their lower overall release of peptides with a molecular weight of less than 3 kDa. In particular, *Ent. malodoratus* A2 showed the highest CMP degradation (Table 3), suggesting that extensive CMP hydrolysis may contribute to sustaining antioxidant potential. Based on its amino acid composition, CMP represents a promising precursor for the generation of antioxidant peptides,

Table 5
Angiotensin-converting enzyme I (ACE-I) inhibitory activity of whey medium (WM) and fermentates, expressed as half-maximal inhibitory concentration (IC_{50}, mg/mL).

Sample	IC_{50} (mg/mL)
WM	$5.96^{a} \pm 0.01$
L. paracasei AF43	$4.74^{d} \pm 0.01$
L. plantarum 297	$4.91^{c} \pm 0.00$
St. macedonicus LbL43	$4.06^{k} \pm 0.02$
L. delbrueckii subsp. *delbrueckii* LbL38	$4.46^{h} \pm 0.01$
L. delbrueckii subsp. *bulgaricus* LbL9	$4.63^{e} \pm 0.02$
L. delbrueckii subsp. *delbrueckii* LbL39	$4.59^{f} \pm 0.02$
L. delbrueckii subsp. *lactis* LbL37	$4.07^{k} \pm 0.02$
L. delbrueckii subsp. *lactis* LbL33	$4.19^{j} \pm 0.01$
Ent. malodoratus A2	$4.33^{i} \pm 0.01$
L. delbrueckii subsp. *delbrueckii* LbL12	$4.33^{i} \pm 0.00$
L. delbrueckii subsp. *delbrueckii* LbL14	$4.98^{b} \pm 0.01$
L. delbrueckii subsp. *delbrueckii* LbL2	$4.55^{g} \pm 0.02$

Statistical differences ($p < 0.05$) are indicated by different letters.

as it can be hydrolysed into smaller peptide fractions enriched in residues such as glutamate and aspartate, which enhance metal ion chelation, especially with pro-oxidant ions like Fe^{2+} and Cu^{+} (Karimidastjerd & Gulsunoglu-Konuskan, 2023).

In addition to low-molecular-weight peptides, other antioxidant compounds, such as glutathione, exopolysaccharides, or metal-chelating compounds, might also be generated during fermentation. To better elucidate the mechanisms underlying the observed antioxidant activity, it would be essential to characterise the peptide fractions in detail, thereby allowing the identification of specific peptide sequences responsible for the bioactivity and clarifying whether their structure-activity relationships are consistent with previously reported antioxidant motifs.

The effect of fermentation on the ACE-I-inhibitory activity of whey proteins was also evaluated (Table 5). ACE-I is an exopeptidase that catalyses the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor. Excessive activity of ACE-I causes an increase in the production of angiotensin II and, therefore, an increase in blood pressure (Olvera-Rosales et al., 2023). Some BPs can bind the active site of ACE-I with higher affinity than angiotensin I. By binding ACE-I, BPs prevent angiotensin I from being hydrolysed into angiotensin II, modulating hypertension (Tondo et al., 2020). In this study, ACE-I-inhibitory activity was expressed as IC50, determined by linear regression of protein concentration in each fermentate versus ACE-I inhibition.

WM exhibited an IC_{50} of 5.96 ± 0.01 mg/mL, and fermentation significantly enhanced its ACE-I-inhibitory activity. *St. macedonicus* LbL43 (4.07 ± 0.02 mg/mL) and *L. delbrueckii* subsp. *delbrueckii* LbL37 (4.07 ± 0.02 mg/mL) showed the highest ACE-I-inhibitory activity. These strains were not among the most proteolytically active strains, suggesting that their effectiveness derives from the release of peptides with particularly favourable sequences rather than from extensive hydrolysis. Consistently, several studies have shown that moderate degrees of hydrolysis are often more effective than extensive ones in generating bioactive properties (Chen et al., 2012; Di Filippo et al., 2024; Guo et al., 2009). Previous literature has shown that ACE-I-inhibitory activity strongly depends on the amino acid sequence at the C-terminal region, with hydrophobic residues such as proline, isoleucine, tryptophan, phenylalanine, and tyrosine conferring strong inhibitory potential (Li et al., 2004).

Interestingly, these strains also enhanced antioxidant activity, reinforcing the idea that fermentation can simultaneously improve multiple bioactivities, thus representing an effective biotechnological tool for valorizing whey proteins. In particular, *L. delbrueckii* subsp. *lactis* LbL37 exhibited the most widespread proteolytic activity across all whey protein fractions (Table 3). This broad-spectrum proteolysis likely contributed to the release of a diverse pool of peptides with both ACE-inhibitory and antioxidant potential, emphasising the role of comprehensive protein hydrolysis in enhancing the functional properties of whey-derived fermentates.

Table 6
Effect of the unfermented whey medium (WM) on growth parameters of *L. monocytogenes*, *Staph. aureus*, *Enterococcus* spp., *E. coli*, *Salmonella* spp., and *P. fluorescens*. Data are expressed as mean ± standard deviation.

	λ (h)	μ_{max} (log OD/h)	A (OD)
L. monocytogenes			
BHI	$7.11^{a} \pm 0.11$	$0.31^{b} \pm 0.01$	$1.05^{a} \pm 0.01$
WM	$3.47^{b} \pm 0.01$	$0.45^{a} \pm 0.04$	$1.20^{a} \pm 0.06$
Staph. aureus			
BHI	$5.32^{a} \pm 0.50$	$0.52^{a} \pm 0.16$	$1.50^{a} \pm 0.03$
WM	$3.52^{b} \pm 0.08$	$0.43^{a} \pm 0.02$	$1.15^{b} \pm 0.02$
Enterococcus spp.			
BHI	$4.84^{a} \pm 0.05$	$0.85^{a} \pm 0.05$	$1.43^{a} \pm 0.01$
WM	$3.69^{b} \pm 0.00$	$0.64^{b} \pm 0.01$	$1.21^{b} \pm 0.02$
E. coli			
BHI	$2.54^{a} \pm 0.15$	$0.39^{a} \pm 0.02$	$1.47^{a} \pm 0.01$
WM	$2.62^{a} \pm 0.08$	$0.35^{a} \pm 0.01$	$1.33^{b} \pm 0.01$
S. enterica			
BHI	$2.74^{b} \pm 0.17$	$0.24^{b} \pm 0.02$	$1.23^{a} \pm 0.02$
WM	$3.51^{a} \pm 0.02$	$0.44^{a} \pm 0.02$	$1.12^{b} \pm 0.01$
P. fluorescens			
BHI	$4.67^{a} \pm 0.02$	$0.29^{a} \pm 0.00$	$1.54^{a} \pm 0.01$
WM	$4.64^{a} \pm 0.03$	$0.29^{a} \pm 0.01$	$1.27^{b} \pm 0.02$

μmax is the maximum specific growth rate; λ is the lag time; A is the upper asymptote.
Statistical differences ($p < 0.05$) from the control (BHI) are indicated by different letters.

Another interesting activity of fermentates is related to their antimicrobial effect. The ability of LAB strains to release compounds with antimicrobial activity may play a crucial role in the treatment of human infections caused by antibiotic-resistant bacteria, as well as in food safety and preservation (Hernández Figueroa et al., 2024). Antimicrobial activity was tested against six pathogenic and spoilage bacteria of interest in the food field: three Gram-positive (*Enterococcus* spp., *L. monocytogenes*, and *Staph. aureus*) and three Gram-negative bacteria (*E. coli*, *Salmonella* spp., and *P. fluorescens*). Antimicrobial activity was assessed using a turbidimetric approach, as the presence of an antimicrobial substance can alter a microorganism's growth kinetics by extending the lag phase, decreasing the maximum growth rate, or reducing the maximum cell concentration reached during the stationary phase (Innocente et al., 2023). To simplify comparison among growth curves, data were modelled with the Gompertz equation, which allows for estimating the values of the lag time (λ; h), the maximum specific growth rate (μ_{max}; log OD/h), and the amplitude (A; OD). For each genus, target strains were pooled to consider microbial variability in nature. Considering the bioactivities of whey protein against bacteria (Innocente et al., 2023), first of all, the growth of strains in WM was assessed and compared to BHI as a control medium. Table 6 describes the effect of the unfermented WM on the growth parameters of the different microbial targets. The effect of WM on bacterial growth was strain-dependent, showing both antimicrobial and growth-stimulatory properties compared to the control medium (BHI). For *L. monocytogenes* and *Staph. aureus*, WM significantly reduced the lag phase (λ) ($p < 0.05$), indicating enhanced adaptation and earlier growth onset. In *L. monocytogenes*, WM also led to a significantly higher μ_{max}, suggesting a stimulatory effect on the growth rate, although the final cell density (A) remained comparable to that in BHI. Instead, in *Staph. aureus* WM reduced the final OD, indicating a limited overall biomass yield and an inhibitory action of WM. Similarly, *Salmonella* spp. cultured in the presence of WM showed a significantly higher μ_{max} and longer lag phase, suggesting delayed but accelerated exponential growth, even though the final OD was lower. In contrast, *Enterococcus* spp., *E. coli*, and *P. fluorescens* exhibited a significant reduction in final OD (A) in WM, consistent with a moderate inhibitory effect. Additionally, in *Enterococcus* spp., μ_{max} was significantly reduced, and although the lag phase

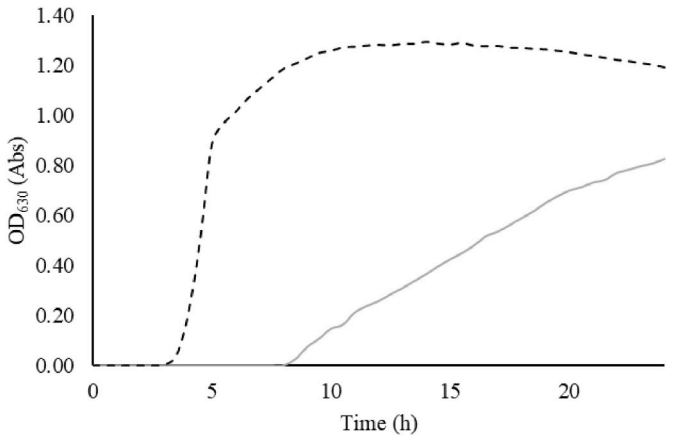

Fig. 3. Turbidimetric growth curves of *Listeria monocytogenes* in the presence of a fermentate (continuous line) or the whey medium (WM; dotted line), expressed as optical density at 630 nm (OD_{630}) over time.

was shortened, the overall growth was suppressed. These findings indicate that unfermented WM may promote growth in certain pathogens by shortening the lag phase or enhancing the growth rate, while inhibiting others. This dual effect may be due to factors such as nutrient availability (e.g., lactose) or the presence of intrinsic antimicrobial compounds in whey (Innocente et al., 2023).

Fermentation significantly improved the antimicrobial properties of WM. Interestingly, *L. monocytogenes* was the most affected. As an example, Fig. 3 illustrates the growth curves of *L. monocytogenes* in the presence of the unfermented WM (dotted line) and the fermentate AF43 by L. *paracasei* (continuous line). As shown, the growth of this pathogen was markedly delayed and reduced in the presence of the fermentate compared to WM.

Despite *L. monocytogenes* was stimulated in growth by the presence of WM (Table 6), its lag phase was prolonged and μ_{max} and A decreased ($p < 0.05$) in the presence of all the fermentates, indicating that fermentation of whey protein significantly impaired *L. monocytogenes* growth kinetics (Table 7). The effects of the fermentates on the other target microorganisms varied depending on both the specific target and the fermentate tested. For *Staph. aureus*, the lag phase increased and μ_{max} decreased in the presence of fermentates. However, the final OD was higher than that observed in WM, suggesting that while fermentation enhanced antimicrobial activity, it did not fully limit growth. For *E. coli* and *S. enterica* (Table 8), the lag phase was shortened, likely due to residual lactose providing initial nourishment, yet both experienced reduced μ_{max} compared to the WM and reached a final OD similar to the BHI control, indicating initial stimulation followed by slowed exponential growth. *Enterococcus* spp. and, to a lesser extent, *P. fluorescens* exhibited prolonged lag phases and reduced μ_{max}, suggesting partial inhibition. Interestingly, while WM promoted *Enterococcus* growth relative to BHI, fermentation reversed this trend. *Pseudomonas* maintained a higher final OD, implying resilience despite slower kinetics. A notable finding was the strong antimicrobial activity of the fermentate from *Ent. malodoratus* A2, which inhibited all pathogens studied.

Table 7
Effect of the fermentates on growth parameters of Gram-positive microbial targets. Data are expressed as mean ± standard deviation.

Sample	*L. monocytogenes*			*Staph. aureus*			*Enterococcus* spp.		
	λ	μ_{max}	Amplitude	λ	μ_{max}	Amplitude	λ	μ_{max}	Amplitude
WM	3.47 ± 0.01	0.45 ± 0.04	1.20 ± 0.06	3.52 ± 0.08	0.43 ± 0.02	1.15 ± 0.02	3.69 ± 0.00	0.64 ± 0.01	1.21 ± 0.02
AF43	6.95* ± 1.02	0.05* ± 0.01	0.75* ± 0.09	5.44* ± 0.15	0.23* ± 0.01	1.27* ± 0.01	4.00* ± 0.06	0.22* ± 0.03	1.03 ± 0.16
297	7.25* ± 0.08	0.25* ± 0.00	0.94* ± 0.02	5.46* ± 0.01	0.24* ± 0.01	1.30* ± 0.03	3.90* ± 0.02	0.21* ± 0.00	1.24 ± 0.09
LbL43	6.74* ± 0.07	0.05* ± 0.00	0.74* ± 0.04	5.51* ± 0.08	0.22* ± 0.01	1.30* ± 0.04	4.11* ± 0.10	0.30* ± 0.01	1.30 ± 0.07
LbL38	7.14* ± 0.95	0.06* ± 0.01	0.83* ± 0.03	5.59* ± 0.10	0.23* ± 0.00	1.26* ± 0.03	4.13 ± 0.23	0.28* ± 0.06	1.19 ± 0.03
LbL9	6.82* ± 0.34	0.05* ± 0.00	0.76* ± 0.01	5.95* ± 0.15	0.22* ± 0.03	1.28 ± 0.04	4.19* ± 0.02	0.27* ± 0.01	1.27 ± 0.08
LbL39	7.31* ± 0.58	0.05* ± 0.01	0.69* ± 0.06	5.53* ± 0.08	0.22* ± 0.01	1.25* ± 0.00	4.19* ± 0.09	0.28* ± 0.02	1.15 ± 0.02
LbL37	7.28* ± 0.10	0.06* ± 0.00	0.78* ± 0.03	5.49* ± 0.01	0.24* ± 0.02	1.33* ± 0.01	4.22* ± 0.01	0.30* ± 0.03	1.19 ± 0.04
LbL33	5.99* ± 0.49	0.06* ± 0.00	0.95 ± 0.17	5.46* ± 0.02	0.25* ± 0.01	1.30* ± 0.02	4.02* ± 0.09	0.28* ± 0.00	1.23 ± 0.08
A2	9.99* ± 0.67	0.04* ± 0.00	0.49* ± 0.01	6.26* ± 0.10	0.15* ± 0.03	1.21 ± 0.08	4.82* ± 0.01	0.19* ± 0.02	1.08 ± 0.06
LbL12	7.12* ± 0.42	0.06* ± 0.01	0.92 ± 0.07	5.59* ± 0.13	0.22* ± 0.01	1.35* ± 0.00	4.23* ± 0.14	0.29* ± 0.04	1.29 ± 0.04
LbL14	7.89* ± 0.29	0.05* ± 0.00	0.76* ± 0.02	5.59* ± 0.06	0.19* ± 0.01	1.28* ± 0.02	4.62* ± 0.08	0.21* ± 0.01	1.20 ± 0.10
LbL2	6.12* ± 0.03	0.06* ± 0.00	0.92 ± 0.13	5.55* ± 0.06	0.24* ± 0.01	1.30* ± 0.00	4.12* ± 0.08	0.27* ± 0.02	1.24 ± 0.03

μ_{max} is the maximum specific growth rate; λ is the lag time; A is the upper asymptote.
Statistical differences ($p < 0.05$) from the WM are indicated by an asterisk (*).

Table 8
Effect the fermentates on growth parameters of Gram-negative microbial targets. Data are expressed as mean ± standard deviation.

Strain	*E. coli*			*S. enterica*			*P. fluorescens*		
	λ	μ_{max}	Amplitude	λ	μ_{max}	Amplitude	λ	μ_{max}	Amplitude
WM	2.62 ± 0.08	0.35 ± 0.01	1.33 ± 0.01	3.51 ± 0.02	0.44 ± 0.02	1.12 ± 0.01	4.64 ± 0.03	0.29 ± 0.01	1.27 ± 0.02
AF43	1.34* ± 0.23	0.20* ± 0.01	1.40* ± 0.01	1.87* ± 0.06	0.12* ± 0.00	1.34* ± 0.01	4.57 ± 0.08	0.18* ± 0.00	1.45* ± 0.03
297	1.24* ± 0.18	0.18* ± 0.00	1.35* ± 0.00	1.49* ± 0.04	0.11* ± 0.00	1.34* ± 0.00	4.38 ± 0.09	0.17* ± 0.00	1.45* ± 0.00
LbL43	1.58* ± 0.02	0.21* ± 0.01	1.37 ± 0.02	1.46* ± 0.10	0.11* ± 0.00	1.31* ± 0.02	4.91* ± 0.06	0.18* ± 0.01	1.45* ± 0.02
LbL38	1.82* ± 0.01	0.21* ± 0.01	1.35 ± 0.03	1.60* ± 0.05	0.11* ± 0.00	1.27* ± 0.02	5.01 ± 0.14	0.18* ± 0.00	1.39* ± 0.02
LbL9	1.58* ± 0.15	0.21* ± 0.01	1.39 ± 0.03	1.51* ± 0.04	0.11* ± 0.00	1.28* ± 0.03	4.94 ± 0.13	0.19* ± 0.00	1.45 ± 0.05
LbL39	1.66* ± 0.04	0.21* ± 0.00	1.34 ± 0.02	1.39* ± 0.25	0.11* ± 0.01	1.25* ± 0.03	5.03* ± 0.06	0.18* ± 0.00	1.42* ± 0.02
LbL37	1.67* ± 0.08	0.22* ± 0.00	1.38* ± 0.01	1.39* ± 0.05	0.11* ± 0.00	1.31* ± 0.02	4.69 ± 0.06	0.18* ± 0.00	1.44* ± 0.01
LbL33	1.68* ± 0.04	0.22* ± 0.01	1.40* ± 0.01	2.21* ± 0.12	0.15* ± 0.01	1.37* ± 0.01	4.56 ± 0.00	0.19* ± 0.00	1.46* ± 0.01
A2	3.43 ± 0.44	0.16 ± 0.00	1.21* ± 0.04	4.44 ± 0.31	0.11* ± 0.01	1.04* ± 0.01	7.76* ± 0.14	0.18* ± 0.01	1.24* ± 0.06
LbL12	1.62* ± 0.16	0.20* ± 0.00	1.38* ± 0.01	1.72* ± 0.33	0.11* ± 0.00	1.30* ± 0.01	5.03* ± 0.03	0.18* ± 0.00	1.45* ± 0.01
LbL14	1.62* ± 0.23	0.18* ± 0.02	1.34 ± 0.03	2.34* ± 0.07	0.12* ± 0.00	1.27* ± 0.00	5.14 ± 0.16	0.17* ± 0.01	1.40* ± 0.02
LbL2	2.03 ± 0.40	0.24* ± 0.04	1.40 ± 0.03	1.60* ± 0.16	0.12* ± 0.01	1.34* ± 0.04	4.65 ± 0.02	0.18* ± 0.00	1.46* ± 0.02

μ_{max} is the maximum specific growth rate; λ is the lag time; A is the upper asymptote.
Statistical differences ($p < 0.05$) from the WM are indicated by an asterisk (*).

Enterococcus spp., although not considered GRAS nor granted QPS status due to its potential as an opportunistic pathogen and its ability to transmit antimicrobial resistance, is nonetheless common in dairy products and, in some cases, valued for its technological contribution. However, its use requires rigorous case-by-case evaluation to ensure safety (Ogier & Serror, 2008). The *L. delbrueckii* subsp. *delbrueckii* LbL14 fermentate also exhibited substantial inhibitory effects, particularly against Gram-positive targets. The observed antimicrobial effect, presumably attributable to the release of peptides into the medium, highlights the effectiveness of fermentative hydrolysis. The pH of the culture medium prepared for the antimicrobial assay after the addition of fermentates was approximately 6.8, which excludes an inhibition effect due to acidity. A further potential explanation for the antimicrobial activity observed in this study may be the production of bacteriocins in the fermentation medium (Russo et al., 2017). Other metabolites typically associated with LAB, such as exopolysaccharides and hydrogen peroxide, may have also contributed to the observed inhibition (Abdalla et al., 2021; Vieco-Saiz et al., 2019); nonetheless, the increase in peptide prevalence following fermentation strongly supports the hypothesis that peptides are the primary drivers of antimicrobial activity. The inhibitory effect of BPs arises from their ability to hinder nucleic acid and protein synthesis or to interact with bacterial membranes (Elsaadany et al., 2024). BPs bind to the bacterial membrane via electrostatic interactions, deforming it and increasing its permeability. The formation of pores across the membrane leads to the passage of molecules inside the cell, which interfere with typical metabolic processes of microbes, resulting in reduced cell growth and cell lysis (Olvera-Rosales et al., 2023). Overall, Gram-positive bacteria were more affected than Gram-negative ones. This discrepancy may be due to structural differences in cell envelopes: Gram-negative bacteria possess an outer membrane that may impede BPs from reaching their target sites (Torcato et al., 2013).

4. Conclusions

This study provides novel insights into the ability of non-starter LAB strains, isolated from raw-milk cheeses and natural milk cultures, to improve the bioactivities of whey protein through controlled fermentation. The observed strain-specific differences in peptide release and functional enhancement underscore the importance of selecting appropriate microbial candidates not solely based on proteolytic intensity, but on their capacity to generate fermentates with targeted low-molecular-weight peptide profiles. Notably, *L. delbrueckii* subsp. *lactis* LbL37 and *St. macedonicus* LbL43 stood out by contributing to all three bioactivities: antioxidant, ACE-I-inhibitory, and antimicrobial. Thus, they can be promising candidates for the development of a multifunctional whey-based fermented beverage with combined nutritional and bioprotective value. The findings of this study pave the way for tailored biotechnological strategies aimed at the sustainable valorisation of dairy side streams and the incorporation of fermentation-derived biopeptides into functional beverages.

Future research should focus on the identification and characterisation of the specific molecules responsible for the observed bioactivities, the elucidation of their mechanisms of action, and *in vivo* validation of their effects, including their interaction with the gut microbiota and implications for human health.

CRediT authorship contribution statement

Anna Rossi: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Nadia Innocente:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Conceptualization. **Giulia Di Filippo:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Niccolò Renoldi:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Marilena Marino:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2025.108136.

Data availability

Data will be made available on request.

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