Original article

Process design for the production of peptides from whey protein isolate with targeted antimicrobial functionality

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Summary The antimicrobial potential of peptides from whey proteins has been widely demonstrated. However, it is fundamental to design the best processing conditions able to form peptides with targeted antimicrobial functionalities. This study aimed to investigate the relationship between process parameters (*i.e.* enzyme and hydrolysis time) and antimicrobial activity against *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella enterica* subsp. *enterica*. The *in vitro* hydrolysis was performed with Alcalase 2.4 and Protamex for 30–480 min reaching a hydrolysis degree higher than 90% after 120 min. As hydrolysis time increased peptide molecular weight decreased, and a different molecular weight profile was observed depending on the enzyme. Alcalase 2.4 was more prone to form peptides with molecular weight <0.4 kDa (80%) compared to Protamex (61%) after 480 min. The antimicrobial activity increased as the peptide molecular weight in the mixture decreased. Alcalase-produced more active peptides against *Salmonella* regardless of the hydrolysis time. After 30 min, Protamex was more effective against *Staph. aureus* reducing its viability by 38.61% compared with Alcalase (18.94%). The same trend was maintained at 240 min. A strong linear correlation between the prevalence of peptides having MW < 0.4 kDa and the percentage of inhibition of each target pathogen was found. Results evidence the crucial role of process design to obtain peptides with targeted antimicrobial functionality, which could be potentially exploited to produce new functional ingredients. The latter may open new possibilities in the preservation of foods where the growth of alternative microorganisms could reduce the shelf-life.

Keywords Antimicrobial activity, hydrolysis, processing conditions, whey proteins.

Introduction

Whey proteins are one of the most widely used ingredients among milk derivatives in food being nutritionally complete proteins with a clean taste and versatile functionalities (*e.g.* solubility, viscosity, gelation, aroma retention capacity, emulsifying, and foaming capacity). In addition to their well-known technological properties which broadly govern their behaviour in foods, whey proteins are also known to have antimicrobial activity, mainly attributed to the presence of β-lactoglobulin (β-Lg) (Demers-Mathieu *et al.,* 2013; Osman *et al.,* 2016). The antimicrobial potential of whey protein can be enhanced upon enzymatic hydrolysis. Depending on the hydrolytic enzyme as well as the processing conditions adopted, bioactive peptides with different antimicrobial activities can be formed

(da Cruz *et al.,* 2020). Specific peptides derived from enzymatic hydrolysis of bovine β-Lg and α-lactalbumin (α-La) showed bacteriostatic activity against pathogen strains of *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* (Pihlanto-Leppälä *et al.,* 1999), while peptides obtained by tryptic and chymotryptic hydrolysis of bovine α-La have bactericidal capacity against *Staph. epidermidis* (Pellegrini, 1999). Although the molecular structure-function relationship of bioactive peptides is not well established, it is known that the exerted activities are due to the sequence of the amino acids, hydrophobicity, hydrophilicity, amphipathicity, net charge and spatial configuration (Sun & Udenigwe, 2020). The most widely accepted mechanism is that peptides attach or are adsorbed to bacterial membranes through electrostatic forces leading to the creation of pores, which induces irreversible changes such as cell permeability increase and membrane breakdown (Brandelli *et al.,* 2015).

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Most of these peptides are negatively charged and this might explain their lower effectiveness against Gram-negative bacteria, which have an outer membrane largely composed of negatively charged lipopolysaccharides, repelling anionic peptides (Torcato *et al.*, 2013).

The antimicrobial activity of peptides from whey protein is today in the spotlight not only for its potential application in the treatment of human infections caused by antibiotic-resistant bacteria but also, for food applications (Park *et al.*, 2011). Indeed, these peptides can find application to prevent foodborne diseases and food spoilage as alternatives to or in combination with conventional preservation techniques (Aguilar-Toalá *et al.*, 2020). The exploitation of these potentialities is in line with the ever-worrying incidence of foodborne pathogens, *e.g. Listeria monocytogenes*, *Staph. aureus* and *E. coli*, in foods as well as consumer concerns regarding the use of traditional chemicals and salt acting as antimicrobic agents. The latest EFSA report on zoonosis monitoring activity highlights that, although the presence of various microbial pathogens is common in many food chains, many of these are more often found in specific foods. This is the case of *Salmonella*, which in 2021 was found at the manufacturing level much more frequently in mechanically separated meat (13.3% of the tested samples) than in cheeses, butter, and cream made from raw milk (0.23%) (EFSA, 2021). Consequently, in the food field, there is a constant need for ingredients with targeted antimicrobial action, and from this point of view, the use of whey peptides can be one of the most interesting avenues to pursue (Martín *et al.*, 2022). Another possible application of these peptides could be as antimicrobial agents in edible food packaging films (Aguilar-Toalá *et al.*, 2020). However, to claim a specific activity process optimisation is highly demanded to obtain the targeted antimicrobial activity.

The objective of the present study was to evaluate the effect of enzyme and hydrolysis time on the activity of whey protein hydrolysates (WPHs) against four well-known microbial foodborne targets *i.e.*, *L. monocytogenes*, *Staph. aureus*, *E. coli*, and *Salm. enterica* subsp. *enterica*. To this aim, a commercial whey protein isolate (WPI) was hydrolysed for different time intervals up to 480 min using Alcalase 2.4L and Protamex, that are respectively a serine endopeptidase produced by *B. licheniformis* and a mixture of endo- and exopeptidase from *Bacillus* spp. In addition, the hydrolysis degree, and the mass profile of active peptides as a function of the hydrolysis time were evaluated by liquid chromatography, to find a possible correlation between the inhibitory activity against a specific microbial target and the prevalence of fractions in a definite MW range.

Materials and methods

Materials

Whey protein isolate (WPI) (Davisco Food International, Le Sueur, MN, USA) was obtained from a commercial supplier and characterised for dry matter, total nitrogen, ash, and fat by standard analytical procedures (IDF, 1993; AOAC, 1997). Alcalase® 2.4L, Protamex®, bovine serum albumin (BSA), β-Lactoglobulin (β-Lg), α-Lactalbumin (α-La), aprotinin from bovine lung, L- glutathione oxidised, Lys-Lys-Lys, and L-Serine were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Salm. enterica* subsp. *arizonae* DSM 9386 was obtained from DSMZ (Braunschweig, D), whereas *E. coli* DIAL1411, *L. monocytogenes* DSA198, and *Staph. aureus* DIAL411 were previously isolated (Innocente *et al.*, 2019). Potassium-phosphate buffer, trifluoracetic acid (TFA) and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich. Maximum Recovery Diluent (MRD) and Brain Heart Infusion (BHI) were obtained from Oxoid (Milan, Italy). Deionised water System advantage A10® (Millipore S.A.S, Molsheim, France) was used. All other chemicals used were of analytical reagent grade.

Preparation of WPHs

Whey protein isolate (WPI) was dissolved at 3% (w/w, protein basis) in potassium-phosphate buffer (0.1 M; pH 7), then gently stirred overnight at 4 °C (Venir *et al.*, 2010; Innocente *et al.*, 2011; Segat *et al.*, 2015). The solution was then divided into two batches and subjected to enzymatic *in vitro* hydrolysis with Alcalase 2.4L and Protamex (Ortiz-Chao *et al.*, 2009). The hydrolysis was carried out under constant stirring in a water bath at 50 and 55 °C and an enzyme-to-substrate ratio of 1:10 and 1:5, for Alcalase 2.4L and Protamex, respectively. Hydrolysed samples were collected at 30, 60, 120, 240, 360 and 480 min. Thereafter, the enzymatic reaction was stopped by the addition of 1 M HCl until pH 2.3 ± 0.2. All WPHs obtained were stored at −20 °C and then freeze-dried for further analysis.

Determination of hydrolysis degree

Freeze-dried WPHs and WPI samples were solubilised (3% w/v) in potassium phosphate buffer (0.1 M; pH 7), diluted 1:5 with water, and filtered using a 0.45-μm syringe filter (Lab Logistic Group, Meckenheim, Germany). 30 μL of each sample were injected in an HPLC series Pro Star mod. 230 apparatus (Varian, Walnut Creek, CA) consisting of a Rheodyne mod. 7725 injector, 40 μL loop, and a photodiode array detector series Pro Star mod. 330 (Varian). A PLRP-S

column (150 mm × 4.6 mm, 5 μm, 300 Å; Polymer Laboratories, Church Stretton, UK) was used. The flow rate was 1 mL min^{-1} using eluting solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The gradient of elution, as solvent B proportion, was: 0–8 min, 25–35%; 8–10 min, 35–36%; 10–17 min, 36–38%; 17–23 min, 38–45%; 23–23.5 min, 45–100%; 23.5–25 min 100–25%. After each run the column was equilibrated for 15 min with solvent A. Detection was carried out at 205 nm and samples were analysed in triplicate (De Noni *et al.*, 2007). The degree of hydrolysis (DH) was calculated using eqn (1).

$$\text{DH (\%)} = \left(\frac{A_{\text{tot}}\text{control} - A_{\text{tot}}\text{WPH}}{A_{\text{tot}}\text{control}} \right) \times 100 \quad (1)$$

Where A_{tot} means the sum of the area of the different protein fraction's peaks (α-La, BSA, and β-Lg) of the non-hydrolysed sample (CTRL) and WPH.

Estimation of MW distribution

MW profiles of WPHs were estimated by high-performance gel-filtration chromatography (Liu *et al.*, 2012; Cui *et al.*, 2022). The same HPLC equipment previously described was used with a TSKgel 2000 SWXL 300 mm × 7.8 mm column (Tosoh Bioscience, Griesheim, Germany). A 0.5 mL min^{-1} flow rate was used to deliver the mobile phase consisting of water/acetonitrile/TFA (55/45/0.1, v/v/v). The column temperature was 30 °C, the UV detector was set at 220 nm, and 10 μL of the sample (30 mg mL^{-1}) was injected into the HPLC system. An MW calibration curve was obtained from the elution time of the peak volume from six standards: BSA (66 446 Da), β-Lg (18 400 Da), α-La (14 478 Da), aprotinin (6511 Da), L-glutathione oxidised (612 Da), Lys-Lys-Lys (403 Da) and L-Ser (106 Da). The data analysis was performed using gel permeation chromatography software STAR ver. 5.3 (Varian).

Evaluation of the antimicrobial activity of WPI and WPHs

Salm. enterica subsp. *arizonae* DSM 9386, *E. coli* DIAL1411, *L. monocytogenes* DSA198 and *Staph. aureus* DIAL411 were stored at −80 °C as 30% (v/v) glycerol stock-cultures in BHI. Overnight cultures were prepared, before each experiment, by subculturing 100 μL of stock cultures in 1 mL of BHI at 37 °C for 24 h. Cells were recovered by centrifugation at 13 000 × *g* in a DLAB series D3024 centrifuge (DLAB Scientific Inc., CA, USA) at 4 °C for 5 min, washed three times, and diluted 1:100 in MRD (final concentration about 10^5 CFU mL^{-1}).

The effect of WPI and WPHs on growth parameters was assessed at 37 °C by turbidimetric measurements (Bisson *et al.*, 2021). Growth curves were obtained by adding 10 μL of culture and 190 μL of BHI containing 1% (w/v) of WPI or WPHs in duplicate wells of sterile 96-well microplates during 48 h. Control conditions for each microorganism (CTRL; BHI broth) were also assessed. The optical density at 630 nm was recorded every 30 min, prior to 5 s shaking, using a Sunrise microplate reader (Tecan Italia Srl, Cernusco s. N., Milan, Italy). Growth curve data were fitted using OriginLab 2021 (Northampton, UK) by reparametrised Gompertz equation (Zwietering *et al.*, 1991) as reported in eqn (2):

$$y = A \exp\left\{-\exp\left[\frac{\mu_{\text{max}} \bullet e}{A} (\lambda - t) + 1\right]\right\} \quad (2)$$

Where t is time, y is the log-transformed correct OD value, A (amplitude) is the maximum OD reached value, μ_{max} the maximum specific growth rate (log OD/h) and λ the lag time (h).

The optical density at 630 nm recorded after 24 h of incubation for each duplicate sample was used to calculate the per cent of inhibition as shown in eqn (3):

$$\text{(\%) Inhibition} = \left(\frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}}\right) \times 100 \quad (3)$$

where $\text{OD}_{\text{control}}$ and $\text{OD}_{\text{sample}}$ are the optical density of the CTRL and sample.

Statistical analysis

Data were reported as the mean of at least three measurements carried out on two replicated experiments ($n \geq 3$). One-way analysis of variance (ANOVA) was carried out using R v. 4.1.2 for Windows (The R Foundation for Statistical Computing, Wien, Austria). Bartlett's test was used to check the homogeneity of variance. Tukey's test was used as a *post hoc* test to assess significant differences among means ($P < 0.05$). The relationship between the % of peptides with MW <0.4 and <3 kDa and antimicrobial activity was assessed using the linear correlation coefficient (R^2).

Results and discussion

Degree of hydrolysis and molecular weight distribution of peptides

The proximate analysis (moisture, protein, ash, and fat content) of commercial WPI used in this study (Table S1) revealed a composition similar to those reported in the literature (Wright *et al.*, 2009; Qi *et al.*, 2017). HPLC chromatogram showed the presence of three main peaks, typically referring to the native BSA, α-La and β-Lg (Sturaro *et al.*, 2016). The

protein samples were composed of β-Lg, α-La and BSA at 75.23, and 2% content of total protein, respectively (Fig. 1a).

Whey protein isolate (WPI) was subjected to *in vitro* enzymatic hydrolysis for increasing times using Alcalase and Protamex enzymes. These enzymes were chosen because they are characterised by excellent performance, with wide specificity, besides being of low cost, which makes their use feasible at the industrial level (Zheng *et al.*, 2008; El Hajj *et al.*, 2023). Preliminary trials were carried out to select the best enzyme-to-substrate ratio allowing to ensure the formation of low molecular weight (MW) peptides. The latter could exert a higher antimicrobial activity and easily diffuse in the microbial cell membrane (Tomita *et al.*, 1991; Najafian & Babji, 2012; Khan *et al.*, 2018). The hydrolysis degree (% DH) as a function of process time was estimated (Fig. 2). Results highlighted different hydrolysis kinetic in the two proteases. Alcalase was revealed to act more rapidly than

Protamex, inducing a higher extent of hydrolysis (97%) just after 30 min in comparison to Protamex (83%), which could be attributed to the broader specificity of Alcalase with respect to Protamex (Osman *et al.*, 2016). The latter reached a hydrolysis degree >95% only after 120 min of reaction.

The HPLC profiles of WPHs obtained using Alcalase (Fig. 1b–d) and Protamex (Fig. 1e–g) showed that the amount of native BSA, α-La and β-Lg was drastically reduced after 30 min of hydrolysis by using both enzymes, in agreement with hydrolysis degree data. In concomitance with native protein peak reduction, a broad signal with overlapping peaks appeared within the first 5 min of hydrolysis. The shape of this multipeak signal varied as a function of the enzyme type and hydrolysis time, suggesting the formation of different peptides depending on the process conditions applied.

This observation was confirmed by studying high-performance gel-filtration chromatography the MW
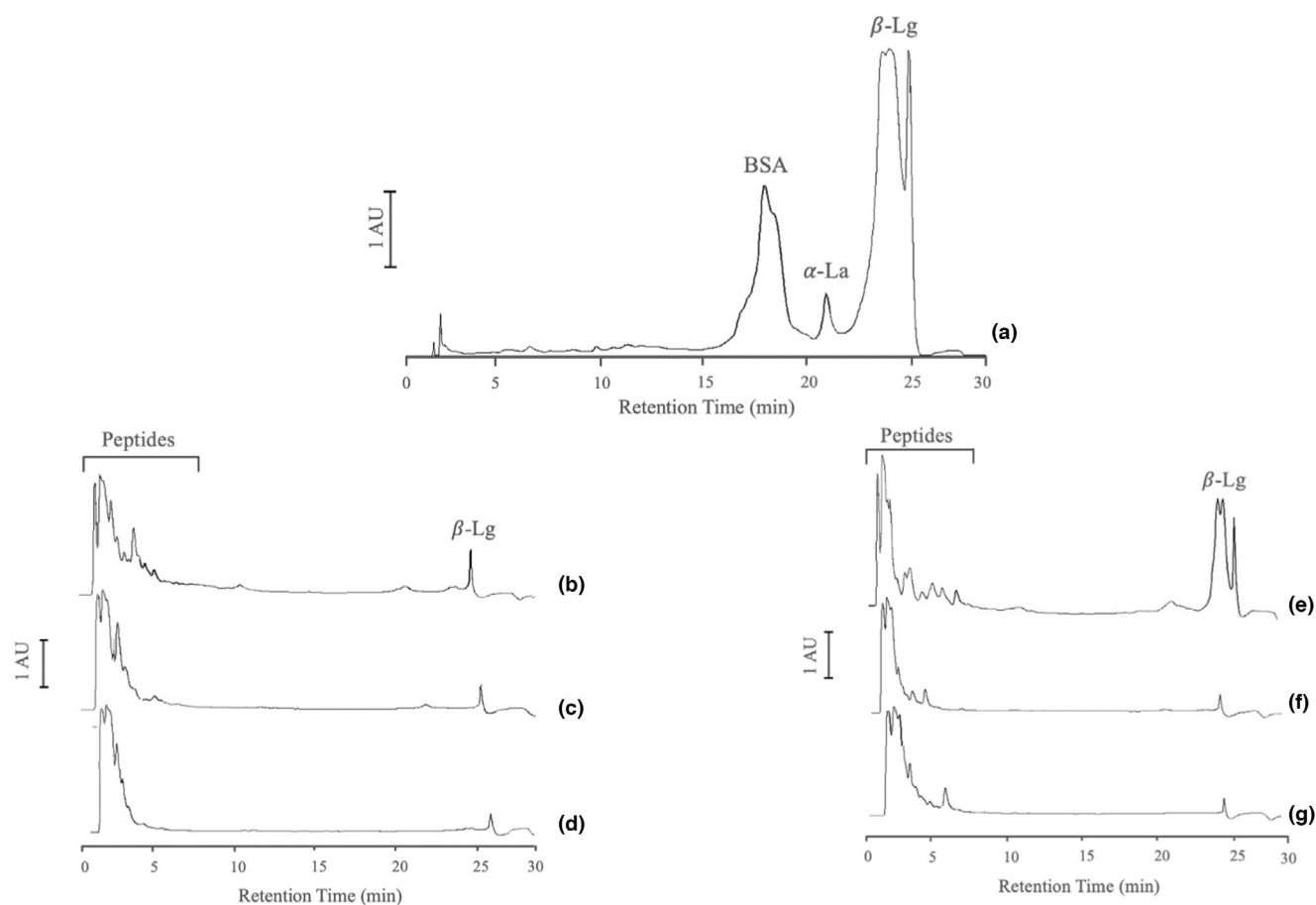
Figure 1 HPLC chromatograms of WPI (a) and WPH hydrolysed by Alcalase for 30 min (b), 240 min (c), 480 min (d), and Protamex for 30 min (e), 240 min (f) and 480 min (g).
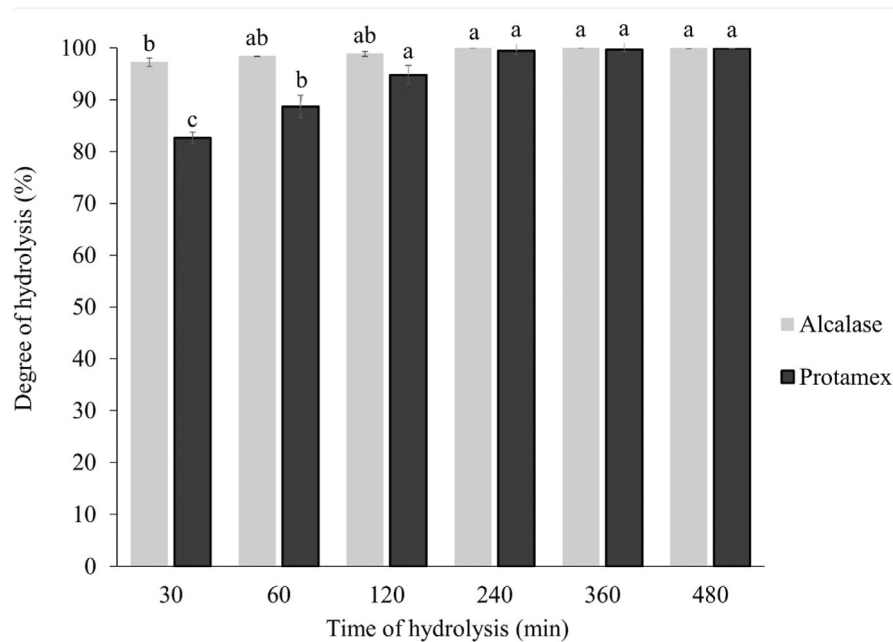
Figure 2 Hydrolysis degree (mean $\pm$ SD) of WPI obtained from the enzymes Alcalase and Protamex as a function of hydrolysis time. Different letters indicate means statistically different ($P < 0.05$).

distribution of peptides, which was displayed in Fig. 3. The native proteins β-Lg (16 kDa), α-Lg (14.4 kDa), and BSA (66.4 kDa), associated with the range MW >14 kDa, were detectable in a very small amount after 30 min (<10%) and 60 min (<3%) of hydrolysis performed with Alcalase. As for Protamex, instead, the prevalence of native high-MW proteins was about 26, 15, and 3% after 30, 60, and 120 min of hydrolysis, respectively, confirming the different hydrolysis rates associated with the two enzymes. As for low-MW peptides, Alcalase led to the formation of a higher prevalence of peptides with MW < 0.4 kDa (42%) than Protamex (29%) just after 30 min. By increasing the hydrolysis time, a progressive rise of small peptides was observed for both enzymes in concomitance with the decrease of the protein fragments with intermediate MWs as well as native protein residues.

Complete hydrolysis with no further differences in the relative percentage of peptide fractions was reached after 240 min for both Alcalase and Protamex. However, after 480 min Alcalase was more active in forming small peptides (80%), whereas the maximum number of small peptides obtained with Protamex was 61%. At 480 min, a significantly higher prevalence of peptides with intermediate MWs was present in Protamex-WPH in comparison to Alcalase-treated samples. These results agree with those reported by other authors that highlighted the different behaviour of Alcalase and Protamex. Although both enzymes are endopeptidase with broad specificity to hydrophobic amino acids, Alcalase was

reported to produce a higher level of low molecular weight peptides compared to Protamex as observed in soybean and fish protein (Hoa & Dao, 2017; Da Rocha et al., 2018). Protamex is a blend of peptidases, some of which necessitate the presence of a metal ion to activate the nucleophilic attack. Instead, Alcalase is a serine protease that does not require the presence of metal ions, which may explain its higher hydrolytic activity compared to Protamex (Klompong et al., 2007; Benítez et al., 2008).

Antimicrobial activity of WPHs

Four bacterial strains including two Gram-positive bacteria (*Staph. aureus* and *L. monocytogenes*) and two Gram-negative bacteria (*E. coli* and *Salm. enterica*) were considered to assess the antibacterial activity of WPI and WPHs generated by Alcalase and Protamex. The turbidimetric growth curves of the selected bacteria in the presence of WPI and WPHs are reported in Figure S1 and Figure S2. To compare microbial growth curves, data were modelled with the Gompertz equation to estimate the values of the lag time (λ), the maximum growth rate (μ_{max}, OD/h), and the maximum OD (amplitude). All the data resulted well described by the Gompertz model with $R^2 > 0.9$.

The use of a turbidimetric approach, followed by the modelling of the growth curve and the estimation of the relevant growth parameters, in investigating the effect of AMPs from whey proteins could help clarify
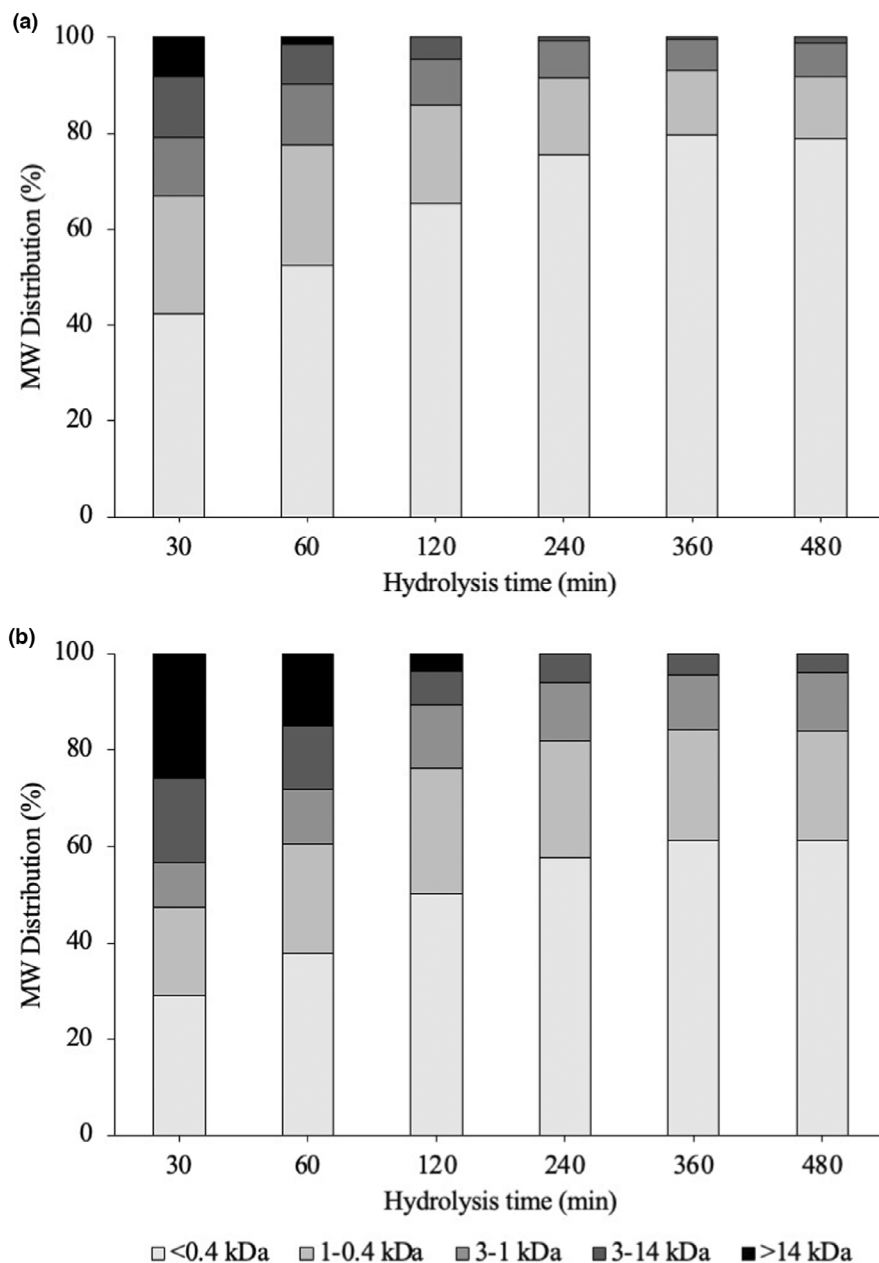
Figure 3 MW distribution of WPHs obtained from Alcalase (a) and Protamex (b) hydrolysis.

their mechanism of action on microbial cells. Traditionally, the biological activity of new AMPs is routinely *in vitro* evaluated using standard procedures such as disk diffusion and broth/agar dilution methods, *e.g.*, MIC (Minimum Inhibitory Concentration) (Osman et al., 2016; Yang et al., 2020). However, the endpoint determination of MIC does not show the potential effects of a sub-inhibitory concentration of AMPs. Instead, evaluating the entire time progress of

AMPs' activity would allow a more in-depth reveal of their bioactive effects. Indeed, the shape of the growth curve and the size of relevant growth parameters reflect the main cellular events and biochemical processes that occur because of the interaction of microbial cells with the test AMP. This information is useful to assess whether in a specific environmental condition cell multiplication may reach a certain threshold or if growth occurs in the presence of some AMPs.

Table 1 Effect of 1% WPI and WPHs using Alcalase and Protamex on growth parameters (mean ± SD) of bacterial strains[†]

Strain	Time (min)	λ Alcalase	λ Protamex	μ_{max} Alcalase	μ_{max} Protamex	Amplitude Alcalase	Amplitude Protamex
E. coli	CTRL	1.40 ± 0.12		0.30 ± 0.14		1.60 ± 0.05	
	WPI	1.44 ± 0.14		0.29 ± 0.01		1.59 ± 0.03	
	30	0.83 ± 0.18	1.51 ± 0.22	0.12* ± 0.00	0.22* ± 0.00	1.35* ± 0.01	1.39 ± 0.02
	60	1.15 ± 0.15	1.72 ± 0.17	0.13* ± 0.00	0.22* ± 0.00	1.31* ± 0.11	1.34* ± 0.05
	120	2.46* ± 1.00	1.84 ± 0.11	0.13* ± 0.00	0.21* ± 0.02	1.14* ± 0.16	1.20* ± 0.04
	240	2.28* ± 0.63	1.67 ± 0.55	0.14* ± 0.00	0.14* ± 0.00	1.23* ± 0.18	1.09* ± 0.10
	360	2.54* ± 0.02	1.78 ± 0.03	0.14* ± 0.00	0.17* ± 0.00	1.07* ± 0.01	1.12* ± 0.00
	480	2.42* ± 0.08	1.94 ± 0.05	0.14* ± 0.00	0.14* ± 0.00	1.10* ± 0.00	1.17* ± 0.04
Salm. enterica	CTRL	1.05 ± 0.16		0.29 ± 0.01		1.43 ± 0.05	
	WPI	3.82* ± 0.14		0.33 ± 0.01		1.42 ± 0.03	
	30	2.95* ± 0.14	0.86 ± 0.26	0.09* ± 0.00	0.11* ± 0.00	0.38* ± 0.01	1.28 ± 0.07
	60	3.37* ± 0.19	1.18 ± 0.17	0.08* ± 0.00	0.11* ± 0.00	0.35* ± 0.01	1.09* ± 0.05
	120	3.70* ± 1.41	0.75 ± 0.05	0.07* ± 0.03	0.09* ± 0.01	0.35* ± 0.01	1.16* ± 0.03
	240	4.13* ± 0.04	1.54 ± 0.41	0.09* ± 0.01	0.10* ± 0.01	0.29* ± 0.01	0.92* ± 0.09
	360	4.67* ± 0.17	1.23 ± 0.03	0.07* ± 0.00	0.09* ± 0.00	0.26* ± 0.01	0.92* ± 0.00
	480	3.76* ± 0.53	1.64 ± 0.01	0.07* ± 0.00	0.11* ± 0.00	0.29* ± 0.01	0.87* ± 0.03
Staph. aureus	CTRL	2.66 ± 0.09		0.53 ± 0.03		1.46 ± 0.08	
	WPI	2.79 ± 0.18		0.24 ± 0.02		1.16 ± 0.06*	
	30	3.22 ± 0.37	2.25 ± 0.52	0.31* ± 0.00	0.10* ± 0.00	1.20 ± 0.05	0.93* ± 0.07
	60	3.32 ± 0.37	2.65 ± 0.33	0.14* ± 0.00	0.12* ± 0.01	0.84* ± 0.07	0.63* ± 0.02
	120	3.27 ± 0.15	2.95 ± 0.27	0.06* ± 0.01	0.07* ± 0.02	0.48* ± 0.06	0.47* ± 0.08
	240	3.39 ± 1.22	2.85 ± 0.77	0.07* ± 0.00	0.06* ± 0.01	0.54* ± 0.17	0.53* ± 0.17
	360	3.12 ± 0.38	2.99 ± 0.76	0.05* ± 0.00	0.02* ± 0.00	0.36* ± 0.00	0.28* ± 0.03
	480	3.43 ± 0.30	2.79 ± 0.32	0.07* ± 0.00	0.03* ± 0.01	0.40* ± 0.01	0.38* ± 0.03
L. monocytogenes	CTRL	4.19 ± 0.41		0.36 ± 0.04		1.23 ± 0.08	
	WPI	2.03 ± 0.20		0.16 ± 0.04		1.04* ± 0.05	
	30	3.82 ± 0.53	3.62 ± 0.12	0.03* ± 0.00	0.05* ± 0.00	0.56* ± 0.02	0.62 ± 0.01
	60	3.88 ± 0.49	3.85 ± 1.18	0.03* ± 0.00	0.03* ± 0.01	0.45* ± 0.10	0.39* ± 0.10
	120	NG[‡]	4.30 ± 2.25	NG	0.02* ± 0.00	NG	0.29* ± 0.00
	240	NG	4.01 ± 0.60	NG	0.01* ± 0.00	NG	0.25* ± 0.05
	360	NG	NG	NG	NG	NG	NG
	480	NG	NG	NG	NG	NG	NG

Asterisks indicate a significant difference ($P < 0.05$) with the control (CTRL).

[†]λ is the lag time (h), μ_{max} is the maximum specific growth rate (log optical density/h) and amplitude is the upper asymptote.

[‡]NG: no growth within 48 h.

Interestingly, Alcalase-induced hydrolysis carried out for 120 min produced a WPH that strongly affected *L. monocytogenes* growth, which was suppressed within 48 h (Figure S1). Similarly, Protamex-generated WPHs after 360 and 480 min of hydrolysis inhibited *L. monocytogenes* growth (Table 1 and Table 2). To the best of our knowledge, this is the first study that highlights the possibility of obtaining AMPs active against *L. monocytogenes* starting from whey protein isolates. Considering the high tolerance of this microbial species to stressful conditions, frequently in a biofilm state, as well as its high fatality rate in fragile and immunocompromised populations (Bucur *et al.*, 2018; Marino *et al.*, 2018), these data appear particularly interesting in the perspective of improving food safety without resorting to thermal technologies or antimicrobials.

When growth occurred, the typical sigmoidal curve was obtained with a clear antimicrobial effect for many of the kinetics in presence of WPHs. The estimated kinetic parameters of the microbial growth curves in presence of 1% of WPI and WPHs obtained from Alcalase and Protamex at increasing hydrolysis times were reported in Table 1. A slight antimicrobial action of WPI was observed only against the Gram-positive *Staph. aureus* and *L. monocytogenes*, whose growth kinetics showed a minor reduction in μ_{max} and amplitude values. It might be due to the presence of β-Lg, which showed to be inhibitory against the Gram-positives *Staph. aureus* and *Streptococcus uberis* (Chaneton *et al.*, 2011).

Generally, WPHs caused an antimicrobial effect against all the microbial targets, and bioactivity increased with hydrolysis time, showing that hydrolysis

Table 2 Antimicrobial activity expressed as % inhibition (mean ± SD) of WPHs against different bacteria strains

| Strain	Time (min)	Alcalase	Protamex
E. coli	WPI	<10[c]	
	30	20.98[b] ± 0.38	18.34[b] ± 1.17
	60	26.75[b] ± 5.61	20.71[ab] ± 3.35
	120	31.07[ab] ± 1.59	28.99[a] ± 1.80
	240	32.54[ab] ± 5.98	35.68[a] ± 6.49
	360	34.97[a] ± 0.54	33.73[a] ± 0.08
	480	33.79[a] ± 2.18	30.77[a] ± 2.26
Salm. enterica	WPI	<10[d]	
	30	73.85[c] ± 0.83	16.60[d] ± 3.56
	60	76.92[b] ± 1.02	28.75[bd] ± 2.96
	120	77.97[b] ± 0.60	23.95[cb] ± 0.00
	240	81.05[a] ± 0.14	41.18[abc] ± 6.19
	360	83.01[a] ± 1.20	39.89[a] ± 0.18
	480	81.04[a] ± 0.18	43.14[ab] ± 2.17
Staph. aureus	WPI	23.20[d] ± 2.29	
	30	18.94[d] ± 1.89	38.61[c] ± 1.82
	60	44.54[c] ± 3.27	58.41[bc] ± 6.95
	120	65.67[b] ± 4.29	72.08[b] ± 5.97
	240	61.71[ab] ± 12.88	65.02[b] ± 12.56
	360	75.78[a] ± 0.19	83.63[ab] ± 1.96
	480	73.33[a] ± 0.65	76.37[a] ± 2.01
L. monocytogenes	WPI	18.89[d] ± 2.52	
	30	57.79[c] ± 0.10	53.60[c] ± 0.11
	60	63.83[bc] ± 0.11	69.78[b] ± 8.89
	120	84.13[ab] ± 0.04	76.95[ab] ± 0.56
	240	>95[a]	80.17[ab] ± 2.85
	360	>95[a]	>95[a]
	480	>95[a]	>95[a]

[a-d]For each microorganism, means followed by different letters indicate a significant difference ($P < 0.05$) among samples (columns).

of whey proteins can reveal hidden antimicrobial peptides (AMPs). AMPs may act by inhibiting DNA, RNA and protein biosynthesis or by establishing an electrostatic bonding between the peptide and bacterial membrane, which disturbs its permeability (Zaslaoff, 2002; Aguilar-Toalá *et al.*, 2020). The antimicrobial activity of peptides is linked to their structure, which determines charge, amphipathic properties and flexibility. These key factors influence contact with the microbial cell and subsequent action. It has been shown that the amphipathicity of peptides, determined by the formation of a secondary structure that segregates polar and apolar amino acid residues, can favour the formation of pores and the internalisation of the peptide through the plasma membrane, which is consequently damaged in its functionality (Mihajlovic & Lazaridis, 2012). Furthermore, high conformational flexibility, associated with the ability to assume an α-helix conformation, facilitates the ability to penetrate through the cell membrane and consequently increases the bactericidal activity (Amos *et al.*, 2016). The lag phase, which represents the adaptation to the growth media and conditions for the microorganisms, was lengthened in the presence of the WPHs obtained by Alcalase, but only for Gram-negative bacteria. The different activity of the hydrolysates against the Gram-negative and Gram-positive bacteria tested in this study could refer to the interaction with the outside of the cell, which is very different from a structural point of view in the two microbial groups. The cell wall of Gram-positives and the outer membrane of Gram-negatives are enriched with different amounts of negatively charged lipid molecules, lipoteichoic acids (in Gram-positives), and lipopolysaccharide (in Gram-negatives), which results in a different interaction with positively charged peptides (Yang *et al.*, 2020). Furthermore, the different cell wall structures could also account for the different sensitivity to peptides in Gram-positive and -negative bacteria. It has been hypothesized that peptidoglycan, a component of the cell wall undoubtedly more abundant in Gram-positives, could act as a sponge by attracting the peptides through the wall, which in turn destabilises the inner membrane (Malanovic & Lohner, 2016). These observations induce us to hypothesise different mechanisms of action linked to the microbial species. This activity could be exploitable given extending the shelf-life of foods, for example, refrigerated ones, where the growth of Gram-negative psychrotrophic microorganisms often occurs even at low storage temperatures (Hahne *et al.*, 2019). On the contrary, the exponential and stationary phases were modified by the presence of WPHs. Maximum growth rate (μ_{max}) and amplitude, which are the expression of the minimum generation time during growth and the total amount of microbial biomass produced, were significantly reduced by WPHs in all the tested microorganisms, regardless of the type of enzyme and the hydrolysis time.

These results were confirmed by the values of % of bacterial inhibition (*i.e.* the ratio between OD of the CTRL and OD of the sample after 24 h of growth), which progressively increased with hydrolysis time for both enzymes (Table 2). In accordance with previous data, *L. monocytogenes* was fully inhibited by 120 min-Alcalase and 360 min-Protamex hydrolysates, whereas *E. coli* was the less sensitive microorganism, showing an inhibition <37% for all hydrolysates. It is well known that the antimicrobial activity of peptides is higher in the case of Gram-positive than Gram-negative bacteria, due to the presence of the outer membrane in the latter, which can limit the reaching of the cell membrane by the peptides (Torcato *et al.*, 2013). The reduced antimicrobial activity against Gram-negative bacteria observed in this study could be overcome using higher concentrations of peptides. Indeed, it has been observed that the bioactivity of peptides of different origins can be concentration-dependent (Chen *et al.*, 2002; Abdel-Hamid *et al.*, 2020).
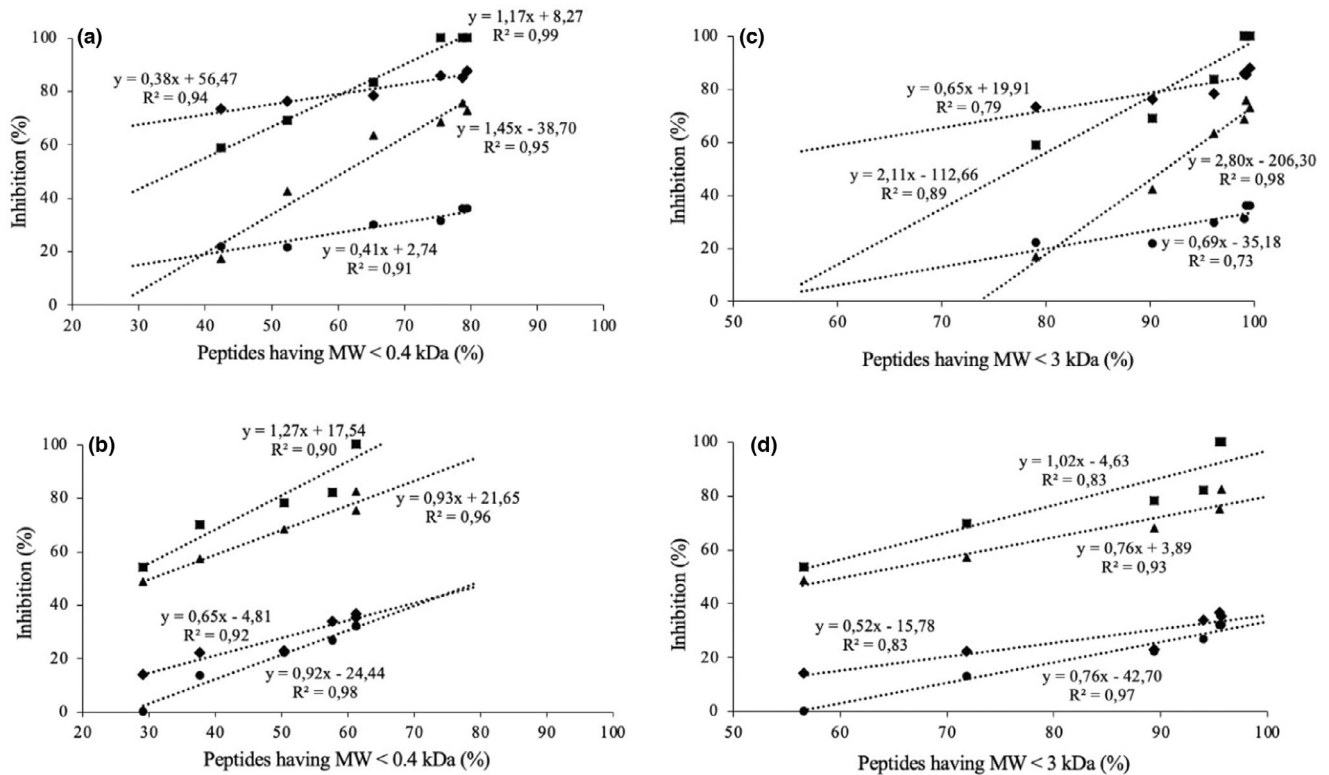
Figure 4 Relation between the % of peptides with MW <0.4 and <3 kDa contained in WPHs obtained by Alcalase (a, c) and Protamex (b, d) at different hydrolysis times and the inhibition of WPHs (%) against *E. coli* (●), *Salmonella* (◆), *Staph. aureus* (▲), and *L. monocytogenes* (■). Line derived from linear regression.

Considering *Salm. enterica*, a remarkable antimicrobial activity was already obtained with 30-min Alcalase-hydrolysates, while Protamex hydrolysates were less active. Lastly, both Alcalase and Protamex WPHs caused a significant inhibition of *Staph. aureus* only for hydrolysis times higher than 60 min. These results strongly suggest that by carefully choosing the type of enzyme, the hydrolysis process (in terms of time), and the concentration of use, it might be possible to obtain a targeted activity against specific pathogens, which may be relevant in specific food fields. For example, the presence of *Staph. aureus* is frequent in catering and the dairy sector, due to the presence of this potential pathogen on the mucous membranes of food handlers, as well as the spread of mastitis disease among dairy animals (Hatakka *et al.,* 2000; Zayda *et al.,* 2020). On the other hand, *L. monocytogenes* poses a particularly frequent threat to smoked fishery products (Chen *et al.,* 2022).

Overall, Alcalase hydrolysates were generally more active than that generated with Protamex, except for some WPHs obtained at the highest hydrolysis times. Despite this, even a limited antimicrobial activity in Protamex-WPHs can be a feature that should not be underestimated, especially when coupled with other mild strategies that can be exploited for reducing microbial counts in foods. Reported results open new opportunities in the shelf-life extension being WPHs potentially applied in different food applications ranging from meat and fish products, moving to pulses and grains, to beverages (Sinha *et al.,* 2007; Sarker, 2022). The strategies most applied in the food context to reduce microbiological risk are based on harsh technologies or on the use of antimicrobial additives, which however can be invasive towards the nutritional and sensory characteristics of the products. Furthermore, they do not satisfy the demand of the modern consumer, who is increasingly turning towards minimally treated products. The biotechnological alternative that most resembles that of this study is represented by bacteriocins, antimicrobial peptides of which nisin is the best example of commercial application (Wu *et al.,* 2023). Nisin is a bacteriocin produced by *Lactococcus lactis*, and which has a rather broad antimicrobial spectrum against Gram-positive bacteria. Unlike what was observed in this study, Gram-negative bacteria are not sensitive to nisin, which in fact finds application only against *L. monocytogenes* in

meat, dairy, and fish products (Pawar *et al.*, 2000; Abdollahzadeh *et al.*, 2014; Fu *et al.*, 2018b). Furthermore, the high cost of production, linked to the growth media and low yields, and purification strongly limits its application on an industrial level (Garsa *et al.*, 2014).

To the best of our knowledge, this is the first study documenting the antimicrobial activity of peptides obtained using the Protamex enzyme. Protamex possesses other properties which make it interesting in the prospect of using it with whey proteins. First, it has a great antiallergenic capacity and can reduce the residual antigenicity of ß-Lg by 8 times. Furthermore, it has proved to be very effective as a debittering agent, which is relevant to the sensory characteristics of food products (Torkova *et al.*, 2016; Fu *et al.*, 2018a).

Correlation between MW of WPHs and inhibitory activity

Combining data on antimicrobial activity and MW distribution of WPHs, it can be inferred that small peptides exerted the highest antimicrobial activity. Although their antimicrobial mechanism has not been completely understood, it has been reported that low MW peptides easily access target sites on the bacterial surface (Korhonen & Pihlanto, 2006). Peptides can also translocate into the bacterial cell, *via* a 'Trojan-horse' like mechanism, interfering with a series of processes and metabolic functions of the bacterial cell (Kapil & Sharma, 2021). A hydrolysed fraction derived from goat milk caseins with low MW (<3 kDa) showed higher antimicrobial activity than that exhibited by the 3–5 or 5–10 kDa fractions (Esmaeilpour *et al.*, 2016). Similarly, peptides from fermented milk (MW <3 kDa) exhibited higher, or at least equal, antimicrobial activity than the 3–10 kDa peptide fraction against diverse bacterial strains (Aguilar-Toalá *et al.*, 2020).

Based on these considerations, for each hydrolysis time, the percentages of peptides with MW <0.4 kDa and <3 kDa were calculated and correlated with the percentage of inhibition for each microorganism (Fig. 4). In all cases, a linear correlation was observed between the inhibition activity and the percentage of the low MW fractions (both <0.4 and <3 kDa). However, observing the coefficients of determination (R^2), the linear model was able to better describe microbial inactivation when considering the lowest MW peptides (<0.4 kDa; Fig. 4a, b). The R^2 values were indeed always higher than 0.9 for both enzymes. The estimated slope values (m) of the regression were higher for Gram-positive bacteria, confirming their higher susceptibility to the presence of peptides in comparison to Gram-negative ones, and the critical role of the structure of the bacterial membrane in determining the antimicrobial activity of peptides (Torcato

et al., 2013). The high correlation between the presence of peptides in a certain MW range and the expected antimicrobial activity against a specific target can be particularly useful in setting up a hydrolysis process aimed at the production of bioactive peptides that can be used as new functional ingredients. Such an approach proved successful in the correlation between the degree of hydrolysis of whey proteins with cardoon extract and the antioxidant and ACE-inhibitory activity (Tavares *et al.*, 2011), and could also be very useful in the prediction of the antimicrobial activity.

Conclusion

In this study, it was shown that the progressive hydrolysis of WPI by Alcalase and Protamex enzyme resulted in increased antimicrobial activity against important microorganisms that endanger food safety all over the world. *L. monocytogenes*, characterised by a certain ability to resist environmental stresses in food, proved to be the most sensitive microorganism to WPH, which offers a new and interesting perspective on the reduction of incidence of listeriosis. As for other pathogens under study, a careful selection of enzyme and hydrolysis conditions, as well as concentration-in-use, might help in developing new bioactive peptides effective against specific microbial targets. A strong linear correlation was found between peptides having MW <0.4 kDa and inhibitory activity, which could be helpful in the prediction of the efficacy of a hydrolysis process on whey proteins in terms of the production of new functional ingredients targeted against specific pathogens. Despite more in-depth studies being needed to confirm the maintenance of the antimicrobial activity in complex food matrices, the acquired results appear particularly interesting for practical applications opening different possibilities in the use of WPHs at different concentrations depending on the intended use, the cost associated to the process, and the impact on food quality attributes.

Author contributions

Nadia Innocente: Conceptualization (equal); project administration (equal); resources (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Sonia Calligaris:** Conceptualization

(equal); resources (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Giulia Di Filippo:** Formal analysis (equal); investigation (equal); validation (equal); visualization (equal); writing – original draft (equal). **Sofia Melchior:** Validation (equal); visualization (equal); writing – review and editing (equal). **Marilena Marino:** Conceptualization (equal); project administration (equal); resources (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Maria Cristina Nicoli:** Conceptualization (equal); resources (equal); writing – review and editing (equal).

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical guidelines

Ethics approval was not required for this research.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Chemical composition (mean $\pm$ SD) of WPI.

Figure S1. Turbidimetric growth curves of (a) *E. coli*, (b) *Salm. enterica*, (c) *S. aureus* and (d) *L. monocytogenes*. CTRL ($\bullet$), WPI ($\times$) and WPH hydrolysed by Alcalase for 30 (■), 60 (+), 120 (▲), 240 ($*$), 360 ($\blacklozenge$) and 480 min (−).

Figure S2. Turbidimetric growth curves of (a) *E. coli*, (b) *Salm. enterica*, (c) *S. aureus* and (d) *L. monocytogenes*. CTRL ($\bullet$), WPI ($\times$) and WPH hydrolysed by Protamex for 30 (■), 60 (+), 120 (▲), 240 ($*$), 360 ($\blacklozenge$) and 480 min (−).