Antibacterial activity of papain hydrolysed camel whey and its fractions

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ABSTRACT

Camel whey (CW) was hydrolysed with papain from Carica papaya and fractionated by size exclusion chromatography (SEC). The antibacterial activity of the CW, camel whey hydrolysate (CWH) and the obtained SEC-fractions was assessed using the disc-diffusion method. The CWH exhibited significantly higher antibacterial activity than the unhydrolysed CW. SEC-F2 (fraction 2) exhibited the highest antibacterial activity against Staphylococcus aureus, whereas Escherichia coli was the least affected. Transmission electron microscopy (TEM) micrographs showed that the SEC-F2 caused changes in the treated bacterial cells. Additionally, LC/MS analysis was used to characterise the peptides profile of CW, CWH and SEC-F2. Two major peptides (414.05 and 456.06 Da mass) were detected in CWH and SEC-F2, with higher concentration in the latter. This study has demonstrated that hydrolysis of CW with papain generates a wide range of potent antibacterial peptides against selected pathogenic bacteria.

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1. Introduction

Microbial contamination is one of the main problems that may affect the shelf life of food and may also cause consumer illness. Therefore, many chemicals are used as preservatives to increase the safety and shelf life of food products. As a result of the increased awareness of the consumer about the deleterious effects of chemical preservatives and the increasing preference for natural components, researchers have focused on the generation of natural additives that demonstrate antimicrobial significance to be used in the food industry (Osman, Mahgoub, & Sitohy, 2013).

Although whey has been labelled over the years as polluting waste, whey compounds, in particular proteins, exhibit a number of functional, physiological, nutritional and antimicrobial properties. The antimicrobial properties of whey proteins have partially been assigned to lactoferrin, lactoperoxidase, lysozyme and immunoglobulins. Moreover, very potent antimicrobial peptides have been released by the enzymatic hydrolysis of whey proteins of different

species (Atanasova & Ivanova, 2010; Jenssen & Hancock, 2009; Kappeler, Heuberger, Farah, & Puhan, 2004; Osman, Goda, Abdel-Hamid, Badran, & Otte, 2016). Four peptide fragments with bactericidal activity were generated after tryptic hydrolysis of bovine β-LG (Haque & Chand, 2008; Pellegrini, Dettling, Thomas, & Hunziker, 2001). Furthermore, human and bovine lactoferrin hydrolysates have demonstrated a potent inhibition against a number of pathogens (Gobbetti, Minervini, & Rizzello, 2007; Yamauchi, Tomita, Giehl, & Ellison, 1993). Hydrolysis of caprine and ovine lactoferrin by pepsin resulted in hydrolysates with antibacterial properties against Escherichia coli and Micrococcus flavus (Recio & Visser, 2000; Vorland, Ulvatne, Rekdal, & Svendsen, 1999). Whey proteins hydrolysates of buffaloes milk from enzymatic hydrolysis by trypsin, chymotrypsin, pepsin, papain and Proteinase K. showed antibacterial activity against E. coli, Staphylococcus aureus, Streptococcus agalactiae and Streptococcus dysgalactiae isolated from mastitis milk (Meignanalakshmi & Vinoth Kumar, 2013). Peptic hydrolysis of bovine lactoferrin increased the inhibition of E. coli growth (Tomita et al., 2009). Bovine lactoferrin hydrolysates by different proteolytic enzymes exhibited antibacterial activity against Listeria monocytogenes (Ripolles et al., 2015).

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Camel milk is a rich source of proteins with potential antimicrobial activity (Benkerroum, Mekkaoui, Bennani, & Hidane, 2004; Salami et al., 2010). Additionally, camel whey proteins have shown higher antimicrobial activity than whey proteins of other milk sources (El-Agamy, Nawar, Shamsia, Awada, & Haenlein, 2009; El-Hatmi, Girardet, Gaillard, Yahyaoui, & Attia, 2007; Merin et al., 2001; Shamsia, 2009). Few studies have evaluated the antibacterial properties of camel whey proteins hydrolysates. Salami et al. (2010) reported that limited hydrolysis of camel whey proteins by chymotrypsin, proteinase K, trypsin and thermolysin enhanced their antibacterial activities against *E. coli*; however, a limited hydrolysis by either trypsin or chymotrypsin did not enhance their antimicrobial activity.

Most camel whey peptides demonstrating antimicrobial activity, reported in the literature, are released by enzymatic hydrolysis of animal origin. However, proteolytic enzymes from microbial and plant sources have been successfully applied to release peptides from whey proteins exhibiting several biological properties (Korhonen & Pihlanto, 2006). In this study papain, a cysteine protease derived from the papaya plant (*Carica papaya* L), is employed. It has been shown to have a broad proteolytic activity against numerous proteins (Gartika, Sasmita, Satari, Chairulfattah, & Hilmanto, 2014), and has previously been used to hydrolyse milk proteins to produce low phenylalanine milk hydrolysate, antibacterial peptides, antioxidant peptides and ACE-inhibitory peptides (Bezerra et al., 2013; Lee, Kim, Ryu, Shin, & Lim, 2005; Meignanalakshmi & Vinoth Kumar, 2013; Silvestre et al., 2012; Soares et al., 2006). Papain was also used in association with Alcalase to hydrolyse cow whey proteins to reduce its allergenic activity (Wroblewska et al., 2004).

To the best of our knowledge, the antibacterial activity of camel whey hydrolysate produced by papain has not earlier been reported on. Such hydrolysis might release antibacterial peptides and could be a useful candidate as natural food preservative. Therefore, the aim of this work was to investigate the antimicrobial activity of camel whey hydrolysate and its fractions after papain hydrolysis against selected pathogenic and spoilage bacteria, i.e., *S. aureus*, *Bacillus cereus*, *Salmonella typhimurium* and *E. coli*.

2. Materials and methods

2.1. Materials

Camel milk (3.3% total protein, 3.3% fat), obtained from the local market (Bilbeis City, Sharkia Governorate, Egypt), was heated to 37 °C and immediately skimmed by centrifugation (5000 × *g*, 15 min). The caseins (CNs) were precipitated by addition of 1 M HCl to pH 4.6 and subsequent centrifugation (5860 × *g*, 60 min at 4 °C). The whey was collected, dialysed against 50 mM phosphate buffer, pH 7.8, lyophilised and stored at −20 °C until use. Papain (from *C. papaya* L) was obtained from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.2. Enzymatic hydrolysis of camel whey (CW)

Lyophilised camel whey (CW) was dissolved in 0.1 M Na_2H-PO_4–NaH_2PO_4 buffer pH 6.0 (100 g L^{-1}) and hydrolysed batch-wise by treatment with papain (E/S ratio of 1:200, w/w) at 37 °C and pH 6.0. The hydrolysis was allowed to proceed for 240 min, and the degree of hydrolysis was determined according to previously published method (Adler-Nissen, 1986) after 60, 120 and 240 min. At the end of the hydrolysis, the enzyme was inactivated by heating in a boiling water bath for 15 min. Hydrolysate was clarified by centrifugation at 4000 × *g* for 30 min at 4 °C to remove insoluble substrate fragments, and the supernatant was lyophilised and

frozen at −20 °C until further use. Camel whey hydrolysis was performed in triplicate.

2.3. SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions according to Laemmli (1970). Twenty milligrams of camel whey hydrolysates were mixed with 500 μL of a reducing sample buffer containing 0.3 M Tris-HCl (pH 6.8), 5% SDS, 50% glycerol and 100 mM dithiothreitol, before being heated at 95 °C for 5 min and loading 10 μL of protein into each well of a pre-cast 3−17% acrylamide, 10 × 10 cm, Tris-glycine gel in an AcquaTank mini gel unit. Following separation by electrophoresis at a constant 200 V for 1 h, gels were removed from the electrophoresis unit, fixed, stained for protein (overnight at room temperature) with Coomassie brilliant blue R-250 and destained (O'Regan & Mulvihill, 2009).

2.4. Fractionation of camel whey hydrolysate

Camel whey hydrolysate (CWH) was fractionated by size exclusion chromatography (SEC) using a Sephadex G-25 superfine grade resin (1.6 × 20 cm, Pharmacia™) on an FPLC system (Äkta™, Uppsala, Sweden). A sample containing 40 mg mL^{-1} CWH was injected and eluted with distilled water at a flow rate of 5 mL min^{-1}, and detected at 280 nm. Fractions of 10 mL were collected and lyophilised to evaluate their antibacterial activity. A bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) was used to determine protein concentration of SEC fractions.

2.5. Antibacterial activity

2.5.1. Bacterial strains

Two Gram-positive bacteria, i.e., *S. aureus* (ATCC 25923) and *B. cereus* (ATCC 33018) and two Gram-negative bacteria, *Sal. typhimurium* (ATCC 14028) and *E. coli* (ATCC 25922) were used to evaluate the antibacterial activity of CW, CWH and SEC fractions. The strains were obtained from Cairo University Research Park (CURP), Faculty of Agriculture, Giza, Egypt.

2.5.2. Disc diffusion assay

The antibacterial activity of CW, CWH and SEC-fractions were evaluated by the disc−diffusion assay (Bauer, Kirby, Sherris, & Turck, 1966). Tested samples were dissolved in distilled water at concentration of 10 mg protein mL^{-1} and filtrated through 0.45 μm cellulose acetate membrane filter (ADVANTEC MFS, Inc., Japan). Bacterial strains were grown overnight at their optimum temperature (37 °C for *S. aureus*, *E. coli*, *Sal. typhimurium* and 30 °C for *B. cereus*) in Mueller-Hinton broth medium. After incubation, the cultures were diluted to approximately 6.0 log cfu mL^{-1}. Bacterial suspensions of the tested bacteria (6.0 log cfu mL^{-1}) were spread on Mueller-Hinton agar. The sterile paper disc (5 mm in diameter) was loaded with 15 μL of tested samples and placed on the surface of inoculated Mueller-Hinton agar plates. Plates were stored at 4 °C for 2 h, and then incubated for 24 h under optimal temperature for each strain. Antibacterial activity was evaluated by measuring the diameter of the growth inhibition zones in millimetres. Kanamycin and ampicillin were used as a positive control against Gram-positive and Gram-negative bacteria, respectively.

2.5.3. Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of selected samples was determined as described earlier (Yamamoto, Togawa, Shimosaka, & Okazaki, 2003). Each sample was serially diluted two fold and discs saturated with 15 μL of each dilution were placed

on the inoculated agar medium as mentioned above. The lowest concentration (highest dilution) of the tested samples that showed visible clear zone on Mueller-Hinton agar plates was regarded as the minimal inhibitory concentration.

2.6. Transmission electron microscopy examination

Morphological and ultrastructural changes of bacterial cells upon the treatment with antibacterial active SEC fraction were examined using transmission electron microscopy (TEM) as described by Sitohy, Mahgoub, Osman, El-Masry, and Al-Gaby (2013). Prior to TEM imaging, each bacterial strain was grown in tryptone glucose yeast extract broth supplemented with or without SEC-F2 (at a concentration of $2\times$ MIC) and incubated at their corresponding optimum temperature for 24 h.

2.7. Peptide profile by ultra-high performance liquid chromatography/tandem mass spectrometry (UHPLC/MS)

Peptides were characterised by liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis using an ultra-high performance liquid chromatograph UHPLC+ Ultimate 3000 (Thermo Fisher Scientific, 4000 Roskilde, Denmark) mounted with a C18 column (Aeris peptideXB-C18, 150 × 2.1 mm, 1.7 μm, 100 Å, 40 °C) coupled with a Q Exactive Biotech mass spectrometer (Thermo Fisher Scientific, 4000 Roskilde, Denmark). Buffer A was 0.1% formic acid in water, and buffer B was 0.1% formic acid in 80% acetonitrile. Five microlitres of CW and SEC fraction and 25 μL of CWH were injected. The flow rate was 0.25 mL min^{-1} and the gradient consisted of 100% buffer A for 5 min, followed by a linear increase from 0% to 60% buffer B in 70 min. On-line MS/MS spectra were recorded in the positive mode using first the Full MS method from 200 to 2000 m/z with a resolution of 70,000, AGC target of 3^{e}6, and max IT of 50 ms. The Top 7 spectra from the MS analysis were analysed using the dd-MS2 method, with a resolution of 35,000 and an AGC target of 1^{e}6. Mass spectra of peptides were exported and compared with the camel whey protein from the Swissprot database (http://web.expasy.org) using Proteome Discoverer (v1.4, Thermo Fisher Scientific, Roskilde, Denmark). Sequest HT was the search method used, with a fragment mass tolerance to 0.05 Da and phosphoserine as dynamic modification, otherwise default settings. The confidence of the peptide identifications was validated using the target Fixed Value PSM validator method.

2.8. Statistical analysis

Results are expressed as the mean ± standard deviation (SD) of three independent experiments and each analysis was done in triplicate. Data were analysed by one-way analysis of variance (ANOVA), followed by assessment of differences by Tukey's post-hoc test. All statistical calculations were performed using SPSS version 16.0 (SPSS Inc., Chicago, Release 16.0.0, 2007). Results were considered statistically significant at p-value < 0.05.

3. Results and discussion

3.1. Camel whey hydrolysate preparation and fractionation

The extent of protein degradation by papain was estimated by assessing the degree of hydrolysis (DH) and SDS-PAGE analysis. The hydrolysate obtained after 240 min degradation had the highest DH (27%) in comparison with those obtained after 60 and 120 min (11% and 16%, respectively). Different degrees of hydrolysis (DH) of milk proteins could be found in the literature due to the variation of E:S ratio, type of protein and amino acid sequence of this protein. For

bovine whey proteins hydrolysis, DH was found to be 15.9% (Wroblewska & Troszynska, 2005) after two-step hydrolysis with Alcalase and papain and 9.22% (Awaiwanont, Tantituvanont, Suwakul, & Meksawan, 2015) by papain at E:S 1:100 for 5 h. Papain hydrolysis of goat casein had DH of 28.5% using a 1:150 E:S ratio for 5 h (Bezerra et al., 2013). Also, hydrolysis of bovine casein by papain for 5 h and a 1:100 E:S ratio resulted in 13.8% DH (Zhao, Wu, & Li, 2010). Kumar, Chatli, Singh, Mehta, and Kumar (2016) reported 15% DH of camel milk casein hydrolysed by papain for 4 h. Comparing with the %DH of the above results, the %DH in our study reflects the papain broad specificity (hydrophobic-(Arg or Lys)-X except Val) on camel whey proteins.

The molecular masses of CW proteins were estimated by comparing them with the standard marker proteins with molecular masses between 245 and 5 kDa. The electrophoretic pattern of CW showed three fractions corresponding to lactoferrin (LF), serum albumin (SA) and α-lactabumin (α-La) (Fig. 1, lane 1). Similar results were observed by Salami et al. (2008), Saliha, Dalila, Chahra, Saliha, and Abderrahmane (2013) and Tagliazucchi, Shamsia, and Conte (2016). All whey protein fractions were completely hydrolysed within 60 min by papain with the exception of α-La (Fig. 1, lane 2). These results are in accordance with those of Salami et al. (2008) who found that camel α-La was slowly hydrolysed by trypsin but it was more extensively hydrolysed by chymotrypsin. Tagliazucchi et al. (2016) also found that camel α-La was more resistant to digestion by gastric juice for 120 min, whereas addition of the intestinal fluid resulted in complete hydrolysis of camel α-La after 5 min. Two new bands were appeared in 60 and 120 min CW hydrolysates with mass between 20 and 17 kDa that may be liberated from LF or SA (Fig. 1, lanes 2, 3).

The electrophoretic pattern of CWH after 240 min hydrolysis by papain showed that all whey protein fractions were completely hydrolysed (Fig. 1, lane 4), which is in agreement with the highest DH. Therefore, CWH after 240 min hydrolysis was fractionated by SEC. The fractionation profile of CWH showed two major peaks
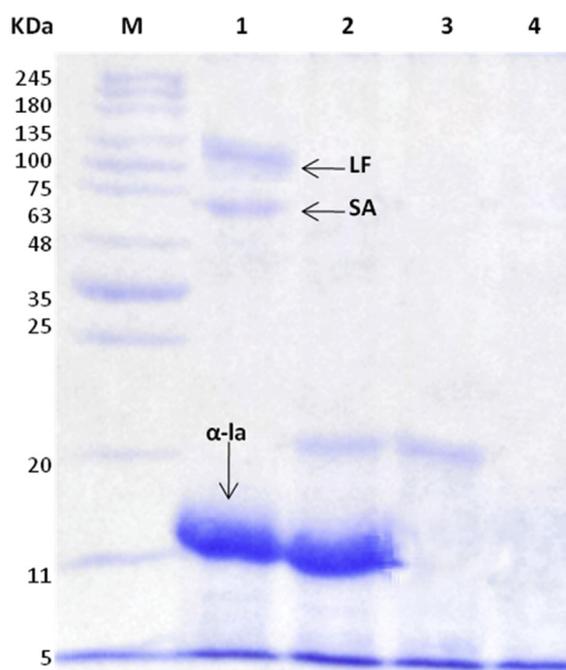

Fig. 1. SDS-PAGE (representative of three independent experiments) of camel whey hydrolysates produced using papain at different times: M, protein marker; lane 1, unhydrolysed camel whey; lanes 2, 3 and 4, camel whey after 60, 120 and 240 min of hydrolysis, respectively. LF, lactoferrin; SA, serum albumin; α-La, α-lactalbumin.
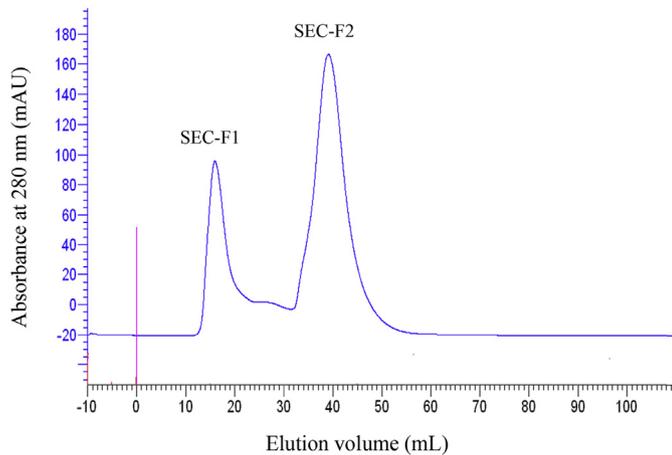
Fig. 2. Size exclusion chromatography (SEC) profile (representative of three independent experiments) of camel whey hydrolysate (CWH) on Sephadex G-25 column. SEC-F1 and SEC-F2: SEC fractions No. 1 and No. 2, respectively.

(SEC-F1 and SEC-F2) at 280 nm (Fig. 2). Peaks were collected, lyophilised and assessed for their antibacterial activity.

3.2. Antibacterial activity

Antibacterial activity of CW, CWH and SEC fractions against four pathogenic and toxigenic bacteria was evaluated by disc-diffusion method. CW showed antibacterial activity only against *S. aureus*, which may be due to the known antibacterial factors such as lysozyme, lactoferrin, and immunoglobulins in CW (Benkerroum et al., 2004; Salami et al., 2010). Papain enzymatic hydrolysis significantly ($P < 0.05$) enhanced the antibacterial activity of CW (Table 1). This result might be attributed to the cleavage of antimicrobial peptides by the action of papain. Similar results were reported by Salami et al. (2010) where the enzymatic hydrolysis enhanced the antibacterial activity of camel whey. CWH inhibited all tested bacteria to different extents. The highest antibacterial activity of CWH was observed against *S. aureus* with an inhibition zone of 49.3 ± 1.2 mm while, the lowest activity was observed against *B. cereus* with 12.0 ± 1.0 mm inhibition zone diameter, which may be due to the ability of *Bacillus* spp. to produce various soluble extracellular proteases that may inactivate the antibacterial peptides in CWH (Roubos-van den Hil, Dalmas, Nout, & Abee, 2010). Papain hydrolysates of buffalo whey proteins have demonstrated antibacterial activity against *E. coli* and *S. aureus*, with an inhibition zone diameter of 14.5 and 15.4 mm at a concentration of 2 mg mL^{-1} (Meignanalakshmi & Vinoth Kumar, 2013).

Fractionation of CWH by size exclusion chromatography resulted in two fractions SEC F1 and SEC F2. With regard to the antibacterial activities of the obtained fractions, SEC-F1 exhibited no antibacterial activity against *B. cereus*, *E. coli* and *Sal. typhimurium*.

This may be due to the absence of the factors responsible for the inhibition of such bacteria in consequence of the fractionation. In contrast, SEC-F1 exhibited antibacterial activity against *S. aureus*. Nevertheless, it was observed that this antibacterial activity was significantly lower than that of CWH. This could be probably due to either the synergistic effect between the peptides released after the papain hydrolysis and whey proteins or a high concentration of peptides responsible for the inhibitory activity, enhancing the antibacterial activity of CWH against *S. aureus*.

Contrary to SEC-F1, SEC-F2 exhibited a significant ($P < 0.05$) and strong antibacterial activity against *S. aureus*, *B. cereus*, *E. coli* and *Sal. typhimurium* (Table 1). Furthermore, these results illustrate no significant difference between the inhibitory effect of CWH and SEC-F2 against *Sal. typhimurium*, *E. coli* and *B. cereus*, leading to a conclusion that the inhibitory efficiency of camel whey hydrolysate is attributed mainly to SEC-F2 as the most antibacterial peptides were eluted. The strong antibacterial activity of SEC-F2 may be due to the presence of low mass peptides. Since SEC-F2 elutes last, most probably that it contains relatively low mass peptides. Generally, most of the demonstrated antibacterial peptides are short peptides (2–20 amino acids). It is believed that the low mass peptides had higher antibacterial activity than the high mass peptides. Similar results have also been observed by Salami et al. (2010) who reported that permeate from ultrafiltrated (3 kDa cut off) camel whey protein hydrolysates had the highest antibacterial activity against *E. coli*. The antibacterial activity of SEC-F2 could be due to its net charge or hydrophobic properties. Most of the antibacterial peptides are positively charged, thus they bind electrostatically to the negatively charged components located on the bacterial cell wall, potentially leading to destruction of the cell wall (Gobbetti, Minervini, & Rizzello, 2004; Hancock & Rozek, 2002; Jenssen, Hamill, & Hancock, 2006). Also, peptide hydrophobicity plays an important role in the disturbance of the bacterial cell wall and membrane.

The potent antibacterial effect of SEC-F2 was confirmed by its lower MIC values compared with those of CWH. *S. aureus* and *Sal. typhimurium* were the most affected bacteria with the lowest MIC values of 0.01 and 0.09 mg mL^{-1}, respectively (Table 2). The MIC values obtained in this study are considerably lower than that reported by Jrad et al. (2014) for peptic and pancreatic hydrolysates of

Table 2
Minimum inhibitory concentration (MIC) of camel whey hydrolysate (CWH) and size exclusion chromatography fraction 2 (SEC-F2) against susceptible bacteria.[a]

Strains	MIC (mg mL^{-1})	
	CWH	SEC-F2
Sal. typhimurium	0.91	0.09
E. coli	1.00	0.39
B. cereus	0.91	0.09
S. aureus	0.09	0.01

[a] MIC values are the mean of three independent experiments; no variation in MIC values was detected between experiments.

Table 1
Antibacterial activity of camel whey, camel whey hydrolysate and size exclusion chromatography fractions (SEC-F1 and SEC-F2) against tested bacteria.[a]

Samples	Inhibition zone diameter (mm)			
	Sal. typhimurium	*E. coli*	*B. cereus*	*S. aureus*
Positive reference	10.0[b] ± 0.0	14.7[b] ± 0.6	19.3[a] ± 1.2	17.3[c] ± 0.6
Camel whey	NI	NI	NI	18.3[c] ± 1.5
Camel whey hydrolysate	23.3[a] ± 0.6	34.0[a] ± 1.7	12.0[b] ± 1.0	49.3[a] ± 1.2
SEC-F1	NI	NI	NI	16.3[c] ± 1.2
SEC-F2	23.3[a] ± 0.6	35.7[a] ± 0.6	12.0[b] ± 1.7	43.0[b] ± 1.7

[a] The positive reference standards used were ampicillin (10 μg disc^{-1}) for Gram-negative and Kanamycin (30 μg disc^{-1}) for Gram-positive bacteria. Values are means ± SD of three measurements of three independent experiments ($n = 9$); different superscript letters within a column mark significantly different values ($P < 0.05$). NI, no inhibition.

camel milk and colostrum proteins demonstrating antibacterial activity against *Listeria innocua* and *E. coli* at a concentration of 10 mg mL^{-1}. Moreover, the minimum inhibitory concentration of buffalo whey hydrolysate by papain was found to be 100 µg mL^{-1} against *E. coli*, 150 µg mL^{-1} against *S. aureus,* 100 µg mL^{-1} against *Str. agalactiae* and 100 µg mL^{-1} against *Str. dysgalactiae* (Meignanalakshmi & Vinoth Kumar, 2013). Compared with the previous results of Salami et al. (2010), Meignanalakshmi and Vinoth Kumar (2013) and Jrad et al. (2014), the SEC-F2 obtained demonstrates high antibacterial efficiency at low concentrations. Therefore, the hydrolysis of CW with papain seems to be an easy and promising tool to produce natural and significantly strong

antibacterial agent. For this reason, SEC-F2 was subjected to further characterisation as can be found in the following section.

3.3. Peptide profile

The peptide profiles of the camel whey, CWH, and the SEC-F2 samples were assessed by running the three samples across a C18 column. Intact whey proteins eluted around 47 min in the camel milk whey sample (Fig. 3a) with some degradation peptides. After hydrolysis, these were partially degraded into peptides eluting between 5 and 45 min as well as two major peaks eluting at approximately 17 and 24 min (Fig. 3b) indicating a fairly low
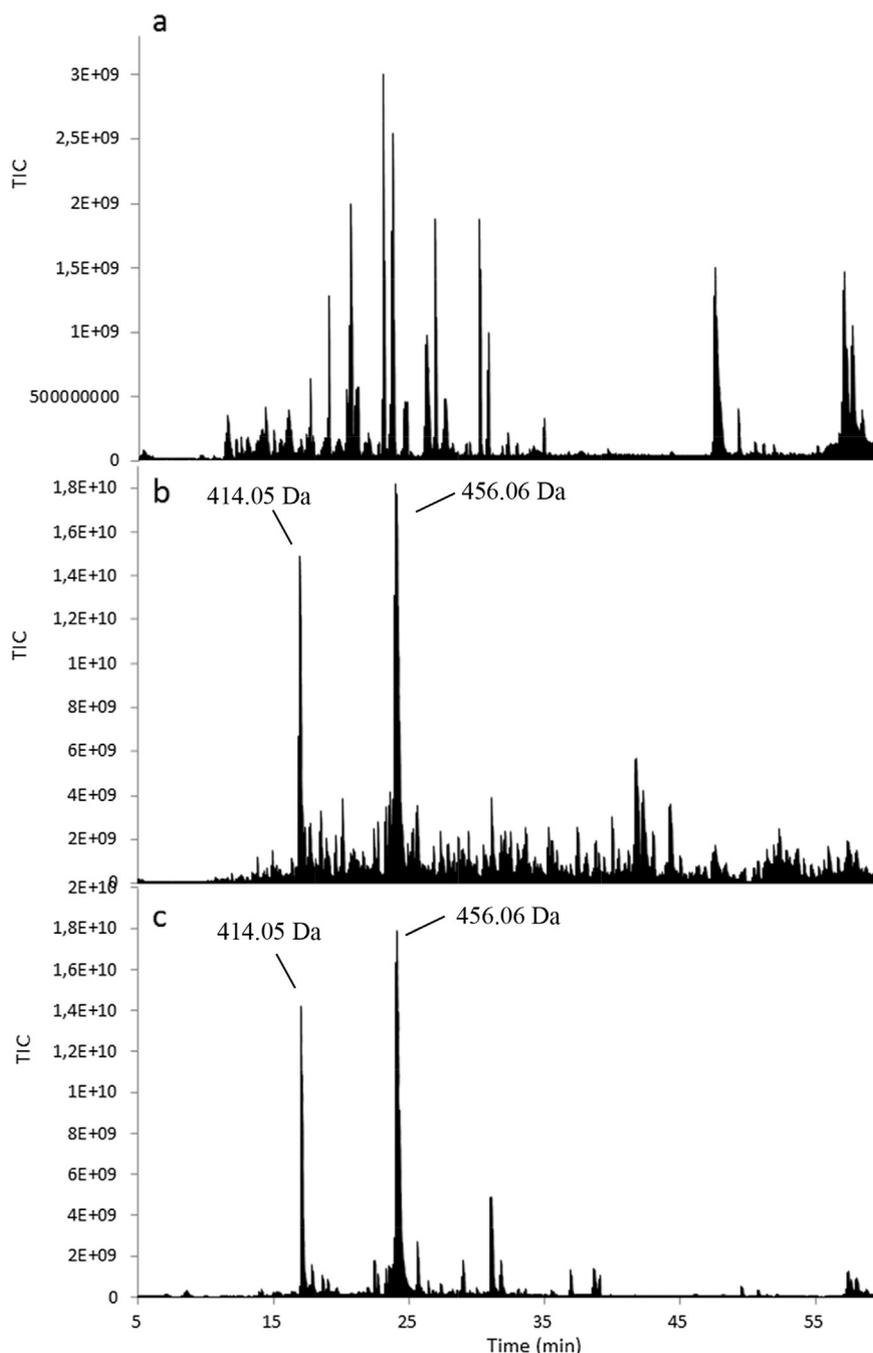

Fig. 3. Chromatographic profiles (representative of three independent experiments) of camel whey (a), camel whey hydrolysate (b), and size exclusion chromatography fraction No. 2 (c).

hydrophobicity. The same two peaks appeared again in SEC-F2 and in higher concentration than CWH (Fig. 3c). The major peaks detected in the CWH and the SEC-F2 contained peptides with masses of 414.05 Da and 456.06 Da. Unfortunately it was not possible to identify the peptides, either with database search or with de novo sequencing and further analysis is needed.

3.4. Transmission electron microscopy

Different studies have revealed that the characteristics of antimicrobial peptides determine their antimicrobial activity and mechanism of action. These characteristics include peptide charge (cationic or anionic), size, amino acid composition, structural conformation, hydrophobicity, and amphipathicity (Guilhelmelli et al., 2013; Jenssen et al., 2006).

The untreated and treated bacterial cells (E. coli, Sal. typhimurium, B. cereus and S. aureus) with SEC-F2 were examined using transmission electron microscopy to assess the mode of action of SEC-F2 as antibacterial agent against these bacteria. TEM images (Fig. 4) of untreated S. aureus, Sal. typhimurium and both lengthwise and transverse sections of untreated E. coli and B. cereus confirmed that the cells had normal morphological and structural features. The cell components including cell wall, cell membranes and nuclear region were distinct. In addition, formation of cleavage furrow was observed in the cells of S. aureus (Fig. 4). In this stage of binary fission, the FtsZ proteins form a ring around the periphery of the midpoint between the two chromosomes. In the cells of E. coli, formation of septum was obvious (Fig. 4). Generally, in the binary fission the FtsZ ring directs the formation of septum between the nucleoids and extending gradually from the periphery toward the cell centre (Madigan, Martinko, Stahl, & Clark, 2012).

Strong ultrastructural alterations were observed in SEC-F2 treated E. coli (Fig. 4). These alterations could be summarised as loss of cell integrity and complete cell lysis, allowing the leakage of cytoplasm and its components including DNA fibres, which were observed to be not affected. These actions suggest that the inhibition of cell wall synthesis and destruction of cell membrane can be considered as the main mechanisms by which the SEC-F2 exerts its antibacterial activity against E. coli. These intense alterations in treated E. coli cells may show the bactericidal effect of SEC-F2 against E. coli. Our finding is compatible with the results obtained by Pelegrini, Del Sarto, Silva, Franco, and Grossi-De-Sa (2011) and Nawrot et al. (2014), who reported that some of the antimicrobial peptides act through formation of membrane pores, resulting in ions and metabolite leakage, depolarisation, interruption of the respiratory processes and finally cell death.

TEM examination also revealed that the cells of Sal. typhimurium were less affected than E. coli cells (Fig. 4). Only cell distortion and partial cell wall degradation were representative actions of SEC-F2 as antibacterial agent against Sal. typhimurium. This may be attributable to weak adsorption of proteins to the cell surface compared with that to E. coli.

TEM micrographs illustrate that the antibacterial effect of SEC-F2 against B. cereus extended from cell distortion to cell lysis (Fig. 4).

Interestingly, the formation of vacuoles or blebs in some treated B. cereus cells was observed, while other cells were completely lysed. Similar significant alterations (numerous blebs and lysis of bacterial cells) have earlier also been reported in B. cereus cells treated with a bioactive fraction of peptide extract of a mixture of four Australian plants; namely Backhousia citriodora, Terminalia ferdinandiana, Citrus australasica and Lophopyrum ponticum (Shami, Philip, & Muniandy, 2013).

S. aureus cells were less affected by SEC-F2 as an antibacterial agent (Fig. 4) with an effect varying between weak cell lysis in some treated cells and no visual effect in other cells.

Antimicrobial peptides have been applied to inactivate prokaryotic cells by targeting a number of metabolic processes at extracellular, plasma membrane, and/or intracellular sites. The majority of the natural cationic antimicrobial peptides are 10−50 amino acids in length, range from 2 to 9 kDa, and contain a high amount of hydrophobic amino acids (Yount & Yeaman, 2013).
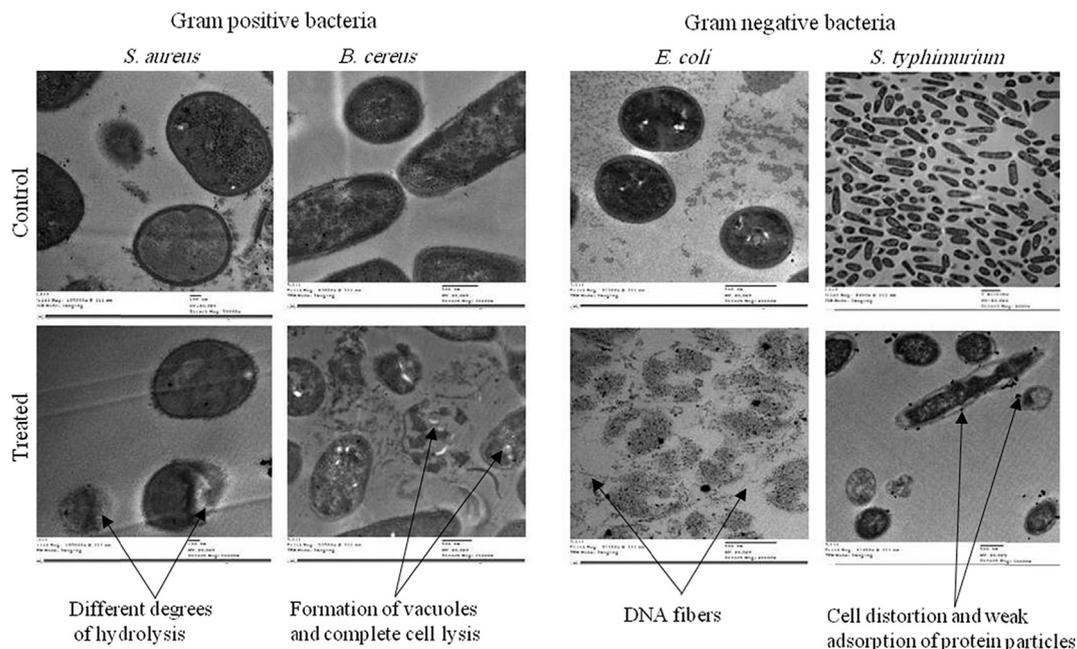

Fig. 4. Electron micrographs of ultrathin sections of control and treated Gram-positive bacteria (Staphylococcus aureus and Bacillus cereus) and Gram-negative bacteria (Salmonella typhimurium and Escherichia coli) as affected by 2× MIC of size exclusion chromatography fraction No. 2 (SEC-F2). Transmission electron microscopy analysis was performed on the third replicate of this work.

Therefore, the adsorption of antimicrobial peptides to the cell surface could be considered as the first step in the interaction between the peptides in the SEC-F2 and bacterial cell. This interaction occurs between the cationic peptide and negatively charged components present in the bacterial cell wall, such as phosphate groups within the outer membrane of Gram-negative bacteria or lipoteichoic acids present on the surface of Gram-positive bacteria. The ability of cationic antibacterial peptides to associate with membranes is a definitive feature of their antibacterial mode of action (Hancock & Rozek, 2002; Varkey, Singh, & Nagarai, 2006). In Gram-negative bacteria this adsorption is followed by insertion of the peptides into the outer membrane structure stimulated by hydrophobic interaction (Jenssen et al., 2006); the antibacterial peptides then cause destruction and permeabilisation of the cytoplasmic membrane.

4. Conclusion

Hydrolysis of camel whey by papain significantly ($P < 0.05$) enhanced antibacterial activity against tested bacteria. Fractionation of the papain hydrolysate by size exclusion chromatography (SEC) resulted in two major fractions (SEC-F1 and SEC-F2). SEC-F2 contains the low molecular mass peptides and exhibited significantly ($P < 0.05$) stronger antibacterial activity than SEC-F1against tested bacterial strains. The potent antibacterial effect of SEC-F2 was confirmed by its low MIC value. Two major peptides were detected in CWH and SEC-F2 by UHPLC/MS, with higher concentration in the latter, having masses of 414.05 Da and 456.06 Da. TEM examination showed a strong ultrastructural alterations in SEC-F2 treated *E. coli*, whereas, *S. aureus* cells were less affected by SEC-F2 as an antibacterial agent.

The results of the present study are extremely encouraging for the future utilisation of the camel whey hydrolysates and the size exclusion chromatography derived fractions as sustenance additives or antibacterial lead drug applicants.

Acknowledgements

References

Adler-Nissen, J. (1986). *Enzymic hydrolysis of food proteins*. Barking, Essex, UK: Elsevier Applied Science Publishers.

Atanasova, J., & Ivanova, I. (2010). Antibacterial peptides from goat and sheep milk proteins. *Biotechnology and Biotechnological Equipment, 24*, 1799–1803.

Awaiwanont, V., Tantituvanont, A., Suwakul, W., & Meksawan, K. (2015). Scavenging activity of whey protein hydrolysates in HaCaT cells. *Chiang Mai Journal of Science, 42*, 907–917.

Bauer, A. W., Kirby, W. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology, 45*, 493–496.

Benkerroum, N., Mekkaoui, M., Bennani, N., & Hidane, K. (2004). Antimicrobial activity of camel's milk against pathogenic strains of *Escherichia coli* and Listeria monocytogenes. *International Journal of Dairy Technology, 57*, 39–43.

Bezerra, V. S., Campos, J. F., Silva, R. A. d., Porto, T. S., Filho, J. L. d. L., & Porto, A. L. F. (2013). Biotechnological richness of the northeastern semi-arid region: antioxidant activity of casein hydrolysates from Moxotó goat milk (*Capra hircus* Linnaeus, 1758) obtained by papain action. *Food Science and Technology (Campinas), 33*, 513–520.

El-Agamy, E. I., Nawar, M., Shamsia, S. M., Awada, S., & Haenlein, F. W. (2009). Are camel milk proteins convenient to the nutrition of cow milk allergic children? *Small Ruminant Research, 82*, 1–6.

El-Hatmi, H., Girardet, J., Gaillard, J., Yahyaoui, H. M., & Attia, H. (2007). Characterisation of whey proteins of camel (*Camelus dromedarius*) milk and colostrum. *Small Ruminant Research, 70*, 267–271.

Gartika, M., Sasmita, S. I., Satari, H. M., Chairulfattah, A., & Hilmanto, D. (2014). Antibacterial activity of papain against *Streptococcus mutans* ATCC 25175. *International Journal of Development Research, 4*, 2075–2077.

Gobbetti, M., Minervini, F., & Rizzello, C. G. (2004). Angiotensin I-converting enzyme-inhibitory and antimicrobial bioactive peptides. *International Journal Dairy Technology, 57*, 173–188.

Gobbetti, M., Minervini, F., & Rizzello, C. G. (2007). Bioactive peptides in dairy products. In Y. H. Hui (Ed.), *Hand book of food products manufacturing* (pp. 489–517). Oxford, UK: John Wiley & Sons, Inc.

Guilhelmelli, F., Vilela, N., Albuquerque, P., Derengowski, L., Silva-Pereira, L., & Kyaw, C. (2013). Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance. *Frontiers in Microbiology, 4*, 353.

Hancock, R. E. W., & Rozek, A. (2002). Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiology Letters, 206*, 143–149.

Haque, E., & Chand, R. (2008). Antihypertensive and antimicrobial bioactive peptides from milk proteins. *European Food Research Technology, 227*, 7–15.

Jenssen, H., Hamill, P., & Hancock, R. E. W. (2006). Peptide antimicrobial agents. *Clinical Microbiology Reviews, 19*, 491–511.

Jenssen, H., & Hancock, R. E. W. (2009). Antimicrobial properties of lactoferrin. *Biochimie, 91*, 19–29.

Jrad, Z., El Hatmi, H., Adt, I., Girardet, J.-M., CakirKiefer, C., Jardin, J., et al. (2014). Effect of digestive enzymes on antimicrobial, radical scavenging and angiotensin I-converting enzyme inhibitory activities of camel colostrum and milk proteins. *Dairy Science and Technology, 94*, 205–224.

Kappeler, S. R., Heuberger, C., Farah, Z., & Puhan, Z. (2004). Expression of the peptidoglycan recognition protein, PGRP, in the lactating mammary gland. *Journal of Dairy Science, 87*, 2660–2668.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: production and functionality. *International Dairy Journal, 16*, 945–960.

Kumar, D., Chatli, M. K., Singh, R., Mehta, N., & Kumar, P. (2016). Enzymatic hydrolysis of camel milk casein and its antioxidant properties. *Dairy Science and Technology, 96*, 391–404.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Lee, K. J., Kim, S. B., Ryu, J. S., Shin, H. S., & Lim, J. W. (2005). Separation and purification of angiotensin converting enzyme inhibitory peptides derived from goat's milk casein hydrolysates. *Asian-Australasian Journal of Animal Sciences, 18*, 741–746.

Madigan, M. T., Martinko, J. M., Stahl, D. A., & Clark, D. P. (2012). Cell structure and function in bacteria and archaea. In *Brock biology of microorganisms* (13th ed., pp. 49–63). San Francisco, CA, USA: Pearson Education.

Meignanalakshmi, S., & Vinoth Kumar, S. (2013). Antibacterial activity of papain hydrolysates of buffalo milk whey protein against mastitis pathogens. *International Journal of Pharma and Bio Sciences, 4*, 1133–1138.

Merin, U., Bernstein, S., Bloch-Damti, A., Yagil, R., van Creveld, C., Lindner, P., et al. (2001). Comparative study of milk serum proteins in camel (*Camelus dromedarius*) and bovine colostrums. *Livestock Production Science, 67*, 297–301.

Nawrot, R., Barylski, J., Nowicki, G., Broniarczyk, J., Buchwald, W., & Goździcka-Józefiak, A. (2014). Plant antimicrobial peptides. *Folia Microbiologica, 59*, 181–196.

Osman, A., Goda, H. A., Abdel-Hamid, M., Badran, S. M., & Otte, J. (2016). Antibacterial peptides generated by Alcalase hydrolysis of goat whey. *LWT – Food Science and Technology, 65*, 480–486.

Osman, A., Mahgoub, S., & Sitohy, M. (2013). Preservative action of 11S (glycinin) and 7S (β-conglycinin) soy globulin on bovine raw milk stored either at 4 or 25°C. *Journal of Dairy Research, 80*, 174–183.

O'Regan, J., & Mulvihill, D. M. (2009). Preparation, characterization and selected functional properties of sodium caseinate-maltodextrin conjugates. *Food Chemistry, 115*, 1257–1267.

Pelegrini, P. B., Del Sarto, R. P., Silva, O. N., Franco, O. L., & Grossi-De-Sa, M. F. (2011). Antibacterial peptides from plants: what they are and how they probably work. *Biochemistry Research International, 2011*. Article ID 250349.

Pellegrini, A., Dettling, C., Thomas, U., & Hunziker, P. (2001). Isolation and characterization of four bactericidal domains in the β-lactoglobulin. *Biochimica et Biophysica Acta, 1526*, 131–140.

Recio, I., & Visser, S. (2000). Antibacterial and binding characteristics of bovine, ovine and caprine lactoferrins: a comparative study. *International Dairy Journal, 10*, 597–605.

Ripolles, D., Harouna, S., Parron, J. A., Cavlo, M., Perez, M. D., Carraminna, J. J., et al. (2015). Antibacterial activity of bovine milk lactoferrin and its hydrolysate prepared with pepsin, chymosin and microbial rennet against foodborne pathogen, Listeria monocytogenes. *International Dairy Journal, 45*, 15–22.

Roubos-van den Hil, P. J., Dalmas, E., Nout, M. J., & Abee, T. (2010). Soya bean tempe extracts show antibacterial activity against *Bacillus cereus* cells and spores. *Journal of Applied Microbiology, 109*, 137–145.

Salami, M., Moosavi-Movahedi, A. A., Ehsani, M. R., Yousefi, R., Haertlé, T., Chobert, J. M., et al. (2010). Improvement of the antimicrobial and antioxidant activities of camel and bovine whey proteins by limited proteolysis. *Journal of Agricultural and Food Chemistry, 58*, 3297–3302.

Salami, M., Yousefi, R., Eshani, M. R., Dalgalarrondo, M., Chobert, J. M., Haertle, T., et al. (2008). Kinetic characterisation of hydrolysis of camel and bovine milk proteins by pancreatic enzymes. *International Dairy Journal, 18*, 1097–1102.

Saliha, S. A. Z., Dalila, A., Chahra, S., Saliha, B. H., & Abderrahmane, M. (2013). Separation and characterization of major milk proteins from Algerian dromedary (*Camelus dromedarius*). *Emirates Journal of Food and Agriculture, 4*, 283–290.

Shami, A., Philip, K., & Muniandy, S. (2013). Synergy of antibacterial and antioxidant activities from crude extracts and peptides of selected plant mixture. *BMC Complementary and Alternative Medicine, 13*, 360–370.

Shamsia, S. M. (2009). Nutritional and therapeutic properties of camel and human milks. *International Journal of Genetics and Molecular Biology, 1*, 52–58.

Silvestre, M. P. C., Silva, M. R., Silva, V. D. M., Souza, M. W. S., Lopes, C. O., Jr., & Afonso, W. O. (2012). Analysis of whey protein hydrolysates: peptide profile and ACE inhibitory activity. *Brazilian Journal of Pharmaceutical Sciences, 48*, 747–757.

Sitohy, M., Mahgoub, S., Osman, A., El-Masry, R., & Al-Gaby, A. (2013). Extent and mode of action of cationic legume proteins against *Listeria monocytogenes* and *Salmonella enteritidis*. *Probiotics and Antimicrobial Proteins, 5*, 195–205.

Soares, R. D., Biasutti, E. A., Capobiango, M., Vieira, C. R., Silva, V. D., Morais, H. A., et al. (2006). Preparation of enzymatic skim milk hydrolysates with low phenylalanine content. *Acta Farmacéutica Bonaerense, 25*, 325–332.

Tagliazucchi, D., Shamsia, S., & Conte, A. (2016). Release of angiotensin converting enzyme-inhibitory peptides during in vitro gastro-intestinal digestion of camel milk. *International Dairy Journal, 56*, 119–128.

Tomita, M., Wakabayashi, H., Shin, K., Yamauchi, K., Yaeshima, T., & Iwatsuki, K. (2009). Twenty five years of research on bovine lactoferrin applications. *Biochimie, 91*, 52–57.

Varkey, J., Singh, S., & Nagarai, R. (2006). Antibacterial activity of linear peptides spanning the carboxy-terminal β-sheet domain of arthropod defensins. *Peptides, 27*, 2614–2623.

Vorland, L. H., Ulvatne, H., Rekdal, Ø., & Svendsen, J. S. (1999). Initial binding sites of antimicrobial peptides in *Staphylococcus aureus* and *E. coli*. *Scandinavian Journal Infectious Diseases, 31*, 467–473.

Wroblewska, B., Karamac, M., Amarowicz, R., Szymkiewicz, A., Troszynska, A., & Kubicka, E. (2004). Immunoreactive properties of peptide fractions of cow whey milk proteins after enzymatic hydrolysis. *International Journal of Food Science and Technology, 39*, 839–850.

Wroblewska, B., & Troszynska, A. (2005). Enzymatic hydrolysis of cow's whey milk proteins in the aspect of their utilization for the production of hypoallergenic formulas. *Polish Journal of Food and Nutrition Sciences, 14*, 349–357.

Yamamoto, Y., Togawa, Y., Shimosaka, M., & Okazaki, M. (2003). Purification and characterization of a novel bacteriocin produced by *Enterococcus faecalis* strain RJ-11. *Applied and Environmental Microbiology, 69*, 5746–5753.

Yamauchi, K., Tomita, M., Giehl, T. J., & Ellison, R. T. (1993). Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infection and Immunity, 61*, 719–728.

Yount, N. Y., & Yeaman, M. R. (2013). Peptide antimicrobials: cell wall as a bacterial target. *Annals of the New York Academy of Sciences, 1277*, 127–138.

Zhao, X. H., Wu, D., & Li, T. J. (2010). Preparation and radical scavenging activity of papain-catalysed casein plasteins. *Dairy Science and Technology, 90*, 521–535.