A comparative study between the correlation of the *in silico* and *in vitro* hydrolysis of whey proteins: Discovery of potential new antimicrobial peptides

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Abstract

Whey proteins are present in the yellowish liquid remaining after the precipitation of caseins, a procedure used in the cheese manufacture. Throughout the years, whey has become a high value product, not just because of its high biological value and content in essential amino acids, but also because it has been proven to be a precursor of a wide range of bioactive peptides. These bioactive peptides are inactive within the parent molecule but can be released by diverse methods, being the enzymatic hydrolysis the most common method.

This work is thus divided into two main goals. The first one was the release, in silico, of peptides from whey proteins upon hydrolysis with α-chymotrypsin and the characterization of the antimicrobial peptides. The second goal was the production, in vitro, of whey protein hydrolysates that resulted from the hydrolysis of whey also with α chymotrypsin and their characterization in terms of molecular weight, isoelectric point, and antimicrobial potential against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Burkholderia contaminans*.

The *in silico* hydrolysis of whey proteins revealed 13 potential new peptides: α-LA f(80 99), β-LG f(78-98), LF f(19-27), f(85-91), f(102-111), f(113-123), f(129-138), f(291-297), f(443-446), f(594-608), f(701-705), and k-CN f(1-5) and f(83-95). The *in vitro* hydrolysis of whey proteins revealed that at least one of the peptides identified with antimicrobial activity mainly against *S. aureus* appears to correspond to the fragment LF f(443-446) with a molecular weight of 0.83 kDa and a pI of 6.12 identified by Tricine SDS-PAGE and IEF.

Keywords: Whey proteins, hydrolysis, *in silico*, *in vitro*, α-chymotrypsin, antimicrobial peptides

1. Introduction

Milk proteins are the principal source of bioactive peptides encrypted within primary amino acid sequence, which can be released by digestive enzymes (both *in vivo*, during gastrointestinal transit, or *in vitro*), fermentation or ripening during food processing to achieve their specific bioactive roles (Korhonen & Pihlanto 2006; Benkerroum 2010; Chakrabarti *et al.* 2014). Some of these peptides have been shown to possess antihypertensive, antibacterial, antioxidant, immunomodulatory and opioid-like properties (da Costa *et al.* 2007; López-Expósito *et al.* 2007; Pihlanto 2006; Gauthier et al. 2006; Mizuno et al. 2005). Despite the notable success of lactoferricin (LFcin) and kappacin, few studies have addressed the antimicrobial properties of peptides released from dairy proteins by enzymatic hydrolysis (Florisa *et al.* 2003; Clare *et al.* 2003; Kamau *et al.* 2010). In response to the changing mindset of consumers, who demand more products without chemical additives, the research for new antimicrobial peptides (AMPs) to be used as food-grade biopreservatives or as health-promoting food supplements is increasing (Théolier *et al.* 2014). A second driving force for research on AMPs is the increasing resistance to conventional antibiotics. In fact, the majority of AMPs present similar physicochemical properties with conventional antibiotics, including small molecular size and cationic and

amphiphilic properties, that seem to be essential for their activity. Their broad spectrum of action and the low number of observed phenomenon of resistance are advantages over conventional antibiotics, although their mode of action is not fully understood and not always fully studied (Seo *et al.* 2012).

While native proteins such as casein, β-Lactoglobulin (β-LG), α-Lactalbumin (α-LA) and serum albumin (SA) are apparently inactive (Clare & Swaisgood 2000), a few milk proteins, primarily lactoferrin (LF) and lysozyme, appear to have antimicrobial properties. Meanwhile, numerous reports have confirmed the release of AMPs by hydrolysis of the caseins of several animal species (López-Expósito, Minervini, *et al.* 2006; López-Expósito, Gómez-Ruiz, *et al.* 2006). Besides caseins, few works demonstrated the presence of antimicrobial peptides within sequences of whey proteins with the exception of LF. Bellamy *et al.* (1992) were the first to describe the antimicrobial peptide LFcin, released from the N-terminal domain of bovine LF hydrolyzed by pepsin *in vitro* and found later *in vivo* (Kuwata *et al.* 1998). LFcin displays a broad spectrum of activity against bacteria, fungi, viruses and parasites (Bellamy *et al.* 1992) and is now being used in the food processing industry and cosmetics (Tomita *et al.* 2002). In comparison, other whey proteins have so far provided relatively few antimicrobial peptides. Notable works performed by Pellegrini *et al.* (1999) and Pellegrini *et al.* (2001) described negatively charged domains isolated from α-LA and β-LG using trypsin and/or chymotrypsin and active against Gram-positive bacteria. Finally, some whey-derived peptides that have no demonstrable antimicrobial activity *in vitro* stimulate certain immune system functions *in vivo* and appear thus to increase resistance to gastrointestinal pathogens (Gauthier *et al.* 2006).

The potential of bovine whey proteins to yield antimicrobial peptides appears to be under exploited. In this sense, the purpose of this work was to identify and characterize potentially antimicrobial peptides derived from whey proteins by *in silico* and *in vitro* hydrolysis using α-chymotrypsin.

2. Materials and Methods

2.1. Materials and microorganisms

Chymotrypsin (≥40 units/mg), tricine (P≥99%), acrylamide (P≥99%), bis-acrylamide (P=99%), ammonium persulfate (APTS, P>98%), sodium chloride, N, N, N', N'–tetramethyl-ethylenediamine (TEMED), and Tris(hydroxymethyl)aminoethane (P≥99.9%) were purchased from Sigma-Aldrich. Coomassie Brilliant Blue R-250 was purchased from Fluka. Trichloroacetic acid (P>99.5%), β-mercaptoethanol, dodecyl sulfate sodium salt (SDS) were purchased from Merck. Glycerol (86-88% w/w) was purchased from Scharlam. Microbial growth inhibition assays were performed using one Gram-positive (*Staphylococcus aureus* Newman) and three Gram-negatives (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* PA14, and *Burkholderia contaminans* IST408). Bacterial strains were grown in Mueller-Hinton broth at 37 ºC for 16h.

2.2. Precipitation of caseins

For the enzymatic precipitation of caseins, approximately 10 drops of liquid chymosin (purchased from a local pharmacy) were added to 500 mL of pre-heated (37 ºC) pasteurized skimmed milk. The milk was left to clot for approximately 30 minutes. The caseins were filtered and the remaining liquid was centrifuged at 7000 rpm and 4ºC for 15 minutes.

2.3. Hydrolysis of whey proteins

2.3.1. *In silico* hydrolysis

The hydrolysis of whey proteins *in silico* was performed using the “Enzyme(s) action” application present on the BIOPEP database (http://www.uwm.edu.pl/biochemia). The option involving a single enzyme was applied for protein hydrolysis with chymotrypsin. Of the peptides released, the ones composed of 5 to 30 amino acid residues were submitted to statistical prediction of antimicrobial activity. The antimicrobial activity of the released peptides was determined with the use of the Prediction Antimicrobial Peptides tool in the CAMP database (Thomas *et al.* 2010). Four multivariate statistical methods were used for prediction: RF (Random Forest), SVM (Support Vector Machines), ANN (Artificial Neural Networks) and DA (Discriminant Analysis). The

process of model development and evaluation has been described in detail by Waghu *et al.* (2014). The prediction results are presented with the relevant scores, excluding ANN, and peptides are classified as AMPs or non-AMPs. In this study, peptides were classified as AMPs if the resulting score was higher than 0.45 and if a positive recognition was obtained for at least two statistical methods. Then, the selected peptides were processed in the APD database to describe their amino acid content and structure and to determine the presence of residues on the same hydrophobic surface of the molecules (Wang *et al.* 2009).

2.3.2. *In vitro* hydrolysis

Hydrolysis of whey proteins was performed according to a Sigma protocol (Cupp-Enyard 2008) as follows: 18 mL of sweet whey and 3.6 mL of chymotrypsin at 2 mg/mL were incubated at 37ºC for 10 minutes. The reaction was stopped by adding 18 mL of 110 mM trichloroacetic acid (TCA). The mixture was then kept at 37ºC for 30 minutes and finally centrifuged at 13,000 rpm for 5 minutes. The soluble total protein concentration was assayed by the BCA method (Smith *et al.* 1985).

2.4. Purification of peptides

The small scale purification was performed using 1 mL adsorbent (SP-Sepharose XL™, GE Healthcare). Each column was washed previously with approximately 10 column volumes (CV) of ultra-pure water. The preparation of the adsorbent for purification consisted in the equilibration of the adsorbent with the working buffer (citrate 20 mM pH 4.5) until the working pH is reached. The soluble sweet whey hydrolysate (SWH) was also in the same pH conditions as the column. 1 mL of sample was loaded into the column, then a washing step of the adsorbent was performed with working buffer (~ 4 CV). The elution step was carried out with the working buffer containing NaCl 150, 250 or 350 mM, collecting 10 fractions of approximately 1 mL each, when the sample was corrected to pH 4.5 with working buffer (diluted SWH). When SWH was corrected to pH 4.5 with NaOH 1M (concentrated SWH), 500 µL were loaded into the column and 5 fractions of 2 mL were collected. In this case the elution step was done with working buffer with 250 mM NaCl and citrate buffer 20 mM pH 6. The adsorbent recovery was performed using 2 CV of NaOH 1M and then washed with ultra-pure water (~5 CV). The adsorbent was stored in distilled water in the fridge, at approximately 4ºC.

2.5. Characterization of peptides

2.5.1. Tricine SDS-PAGE

Analysis of the hydrolysate fractions was performed by Tricine SDS–PAGE according to Schägger & von Jagow (1987). Briefly, Tricine SDS-PAGE was carried out in 16.5%T, 6%C polyacrylamide gels (~4.5 cm), with a "spacer" gel of 10%T, 3%C (~1.2 cm), and a stacking gel of 4%T, 3%C (~0.6cm) on a Mini-PROTEAN® Tetra Cell (Bio-Rad Laboratories). Electrophoresis was performed at room temperature, cooled only by the ambient air, using a voltage stepped procedure: voltage was kept constant (30 V) until the samples completely left the stacking gel and then the voltage was increased 15 V per min for 4 times. Voltage was maintained constant at 90V until the tracking dye reached the bottom of the gel. The polypeptide SDS-PAGE standards molecular weight marker (MW ~1,400 ~26,600 Da; Bio-Rad Laboratories) was used and it is composed of Trisephosphate isomerase (26.625 kDa), Myoglobin (16.950 kDa), α-Lactalbumin (14.437 kDa), Aprotinin (6.512 kDa), Insulin b chain (oxidized, 3.496 kDa), and Bacitracin (1.423 kDa).. Gels were firstly stained with Coomassie Brilliant Blue and, whenever the intensity of the bands was too low, the gels were subsequently stained with silver nitrate. Gels were scanned with the GS-800™ Calibrated Densitometer (Bio-Rad Laboratories) and saved as .jpeg files.

2.5.2. Isoelectric focusing

Isoelectric focusing (IEF) was performed in order to assess the isoelectric points of the peptides present in the hydrolysate fractions. The IEF was performed in a Pharmacia PhastSystem separation module using PhastGel® IEF 3-9 with 50×46×0.45 mm (GE Heatlthcare). Gels were run at 2000 V for 410 Vh (~30 minutes), after a 75 Vh prefocusing step at 200 V for 15 Vh (~10 minutes). The pI markers used were a Broad pI Kit, pH 3-10 (GE Healthcare), composed by, amylglucosidase (pI=3.50); methyl red (pI=3.75); trypsin

inhibitor (pI=4.55); β-lactoglobulin A (pI=5.20); bovine carbonic anhydrase B (pI=5.85); human carbonic anhydrase B (pI=6.55); myoglobin, acidic band (pI=6.85); myoglobin, basic band (pI=7.35); lentil lectin, acidic (pI=8.15); lentil lectin, middle (pI=8.45); lentil lectin, basic (pI=8.65) and trypsinogen (pI=9.30). In addition a sample of myoglobin (from horse skeletal muscle, Sigma Aldrich) was also run in the gel to help with the identification of the pI protein samples. The myoglobin sample was prepared by adding 1 mL of ultra-pure water (Merck Millipore), 300 µL NaCl 1M and 50 µL of Tris-HCl 1.5 M pH 8.5 to 2.5 mg myoglobin. Gels were stained with silver nitrate.

2.6. Determination of antibacterial activity

Mueller-Hinton broth (MHB) prepared double-strength was added (70 µL) to the round-bottomed wells of a sterile polystyrene 96-well microplate (Greiner Bio One International GmbH). The hydrolysate fractions were all tested (70 µL) without serial dilution. Wells were then inoculated with 70 µL of mid log-phase culture of bacterial strain suitably diluted in MHB. Negative controls were the working and the elution buffers (70 µL), and they were also inoculated. The microplates were then incubated at 37 ºC for 24h. Positive controls were obtained using penicillin-streptomycin solution at a final concentration of 0.03x (prepared from a 100x stock solution, Sigma-Aldrich) added to inoculated wells. This concentration inhibited all tested bacteria. After 24h of incubation microplates were read at 640 nm on a microplate reader (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany). The percentage of inhibition was measured as indicated in the equation below.

$$Inhibition\ (\%) = \left[1 - \left(\frac{OD_{640nm} sample}{OD_{640nm} negative\ control}\right)\right] \times 100$$

Equation 1

3. Results and Discussion

3.1. Bioinformatic analysis

In this study, the enzyme chymotrypsin from the BIOPEP database was used to stimulate the proteolysis of β-LG (UniProtKB P02754), α-LA (UniProtKB P00711), LF (UniProtKB P24627), and also κ-CN (UniProtKB P02668) that can remain in whey after the destruction of casein micelles. The simulation produced hundreds of fragments, but antimicrobial activity predictions were based solely on peptide chains containing 5 to 30 amino acids. In the case of fragments derived from β-LG, only 15 out of 38 fragments were analyzed. For fragments derived from α-LA, 12 out of 30 fragments were analyzed, for LF-derived fragments, only 57 out of 137 and for k-casein only 10 out of 31 peptide fragments were further evaluated. Additionally, the “Search for active fragments” command was used to determine released fragments whose structure corresponded to that of peptides with known antimicrobial activity. Those fragments were not further examined.

The antimicrobial potential of the obtained fragments was evaluated with the use of four statistical models available in the CAMP database. The results were used to manually select peptides where a positive result was reported in at least two algorithms. Overlapping and partially overlapping peptides based on known AMPs from milk proteins, as well as peptides with poor water solubility (as predicted at Innovagen’s Peptide Property Calculator), were excluded. A total of thirteen potentially antimicrobial peptides with scores higher than 0.45, or a positive recognition in the case of ANN, for at least two statistical models (Table 1) were thus selected. An analysis of the selected peptides with the use of APD tools revealed that seven fragments may form amphipathic helices.

Fragment AVAPNHAVVSRSDRAAHVKQVL (Table 1) contains five cationic (one lysine, two arginine and two histidine) and one anionic (aspartic acid) residues, one proline and it may have five residues on the same hydrophobic surface.

Peptide ENGECAQKKIIAEKTKIPAVF has four positively charged (four lysines) and three negatively charged (three glutamic acids) residues, it has nine hydrophobic residues, but only two have been predicted on the same hydrophobic surface. Peptide AVAVVKKGSNF fragment is cationic due to the presence of two lysines with a high hydrophobic ratio of 54%, but only two residues have been predicted on the same hydrophobic surface. In the same way, fragment MMKSF is a cationic peptide due to the presence of one lysine with a high hydrophobic ratio of 60%, but also only two residues have been predicted on the same hydrophobic surface. Peptide

Table 1. Characteristics of potential AMPs from bovine whey proteins released during *in silico* proteolysis and predicted by statistical models available in CAMP database (SVM, RF, ANN, DA).

Sequence	Origin and position	Molecular mass (Da)	pI	Instability index	Aliphatic index	GRAVY index	Boman index (kcal/mol)	Net charge	SVM	RF	ANN	DA
CKDDQNPHSSNICNISCDKF	α-LA f(80-99)	2268.4	5.3	83.31	39	-1.01	3.04	-1	0.354	0.5345	NAMP	0.594
ENGECAQKKIIAEKTKIPAVF	β-LG f(78-98)	2317.7	8.25	19.41	83.81	-0.352	1.33	1	0.176	0.3645	AMP	0.611
AAPRKNVRW	LF f(19-27)	1097.2	12.01	85.56	54.44	-1.233	3.56	3	0.936	0.4885	AMP	0.120
EAGRDPY	LF f(85-91)	806.8	4.37	8.57	14.29	-1.857	3.97	-1	0.848	0.5785	NAMP	0.000
GTKESPQTHY	LF f(102-111)	1147.2	6.75	126.91	0	-1.96	3.03	0.1	0.718	0.625	NAMP	0.007
AVAVVKKGSNF	LF f(113-123)	1119.3	10	-14.91	97.27	0.591	0.13	2	0.943	0.483	AMP	0.912
QGRKSCHTGL	LF f(129-138)	1086.2	9.51	58.17	39	-1.11	2.85	2	0.488	0.2205	AMP	0.007
GKNKSRSF	LF f(291-297)	923	11.17	106.74	0	-1.875	4.44	3	0.124	0.466	AMP	0.190
RPTEGY	LF f(443-446)	721.7	6	58.38	0	2	3.91	0	1.000	0.4985	NAMP	0.025
AVAPNHAVVSRSDRAAHVKQVL	LF f(594-608)	2325.6	10.84	45.77	106.36	0.036	1.73	2.2	0.662	0.319	NAMP	0.461
EACAF	LF f(701-705)	539.6	4	95.88	40	1.08	-0.21	-1	0.988	0.3665	AMP	0.006
MMKSF	k-CN f(1-5)	642.8	8.50	2.24	0.00	0.380	0.25	1	1.000	0.405	NAMP	0.914
AKPAAVRSPAQIL	k-CN f(83-95)	1321.5	11.00	115.48	113.08	0.292	0.63	2	0.527	0.428	AMP	0.193

[a]SVM: Support Vector Machines; [b]RF: Random Forest,; [c]ANN: Artificial Neural Networks; [d]DA: Discriminant Analysis
AMP:Antimicrobial peptide; NAMP: non antimicrobial peptide

AKPAAVRSPAQIL has two cationic amino acid residues (one lysine and one arginine), which makes it a cationic peptide. It also has two prolines, a hydrophobic ratio of 53%, and three residues on the same hydrophobic surface. There are two negatively charged peptides each with possibly two residues on the same hydrophobic surface. Peptide CKDDQNPHSSNICNISCDKF has three negatively charged residues (three aspartic acids) and three positively charged residues (two lysines and one histidine), and peptide EACAF has one negatively charged residue (glutamic acid), but it has four hydrophobic residues (two alanines, one lysine and on phenylalanine) that contribute for a hydrophobic ratio of 80%. The remaining six peptides: AAPRKNVRW, EAGRDPY, GTKESPQTHY, QGRKSCHTGL, GKNKSRSF and RPTEGY – do not have residues on the same hydrophobic surface, according to the APD database. Three of them are cationic, two are neutral and one is anionic. Fragment AAPRKNVRW contains three positively charged residues (two arginines and one lysine) and four hydrophobic residues (two alanines, one valine and one tryptophan) that contribute for a total hydrophobic ratio of 44%. Fragment QGRKSCHTGL contains 3 cationic residues (lysine, histidine and arginine) and two glutamic acids. Fragment GKNKSRSF contains no anionic residues and two cationic residues (lysine and arginine). The hydrophobic ratio for both fragments is relatively low at 20% and 12%, respectively. Fragment GTKESPQTHY contains two cationic residues (one lysine and one histidine) and one anionic residue (glutamic acid), but due to the presence of glutamine, the overall charge is 0.1. Fragment RPTEGY is a neutral peptide with one positively charged residue, but it also contains one proline and one glycine. The latter two peptides have a hydrophobic ratio of 0%. Fragment EAGRDPY contains two negatively charged and one positively charged residue, and also one proline and one glutamic acid typical for anionic peptides. Despite not forming amphipathic structures, these peptides could potentially exhibit antimicrobial activity, since there are known antimicrobial peptides derived from milk proteins that do not form these structures either. Some examples are VLVLDTDYK, YYQQKPVA, IKHQGLPQE and LECIRA. Another important feature of antimicrobial peptides is stability. Molecules with an instability index higher than 40 are considered as unstable, and therefore

they are characterized by lower bioavailability and shorter half-time. In the group of selected peptides (Table 1), 3 were predicted to be stable. Nevertheless, some processing techniques, such as chemical modification or incorporation of synthetic amino acids, could be applied to increase peptide stability.

3.2. Protein hydrolysis and extraction of peptide fractions

The aim of this portion of the study was to extract peptides released from sweet whey hydrolysed with chymotrypsin. The soluble sweet whey hydrolysate to sweet whey ratio was 43% for the 10 minutes of reaction. The total soluble protein concentration was 1.05 mg/mL, compared to the initial sweet whey total protein concentration of 2.45 mg/mL. The purification process, quantified in terms of relative soluble protein concentration in the sample loading, removal of non-attached peptides (washing step) and recovery of the desired peptides/proteins (elution step) in relation to the soluble protein concentration in the sweet whey hydrolysate is shown in Figure 1. Regarding the column used, it can be said that the adsorption was successful, as the percentage of protein in the sample loading was approximately 16±2%, which means that the reaming 84±2% of hydrolysate remained adsorbed in the column. In the washing step, the proteins/peptides that did not adsorb are eluted and this corresponds to a protein concentration of approximately 24±5%. Finally, regarding the elution steps, it is possible to observe that for higher concentrations of salt (250 and 350 mM), this step is more effective, since around 76±1% of the proteins/peptides adsorbed are eluted. In contrast, for the lower salt concentration (150 mM), only approximately 39% of proteins/peptides are eluted (Figure 1A).

For both the experiments with concentrated soluble SWH (Figure 1B) the mean of the load, washing, and elution steps were calculated and yielded the respective values of 23.6±3%, 8.9±4%, and 16.4±7%. Particularly, in the case if the elution step with a salt concentration of 250 mM, the percentage of protein in the sample loading was approximately 21.5%, which means that the remaining 78.5% remained adsorbed in the column, whereas for the elution with citrate buffer 20 mM pH 6, 76.4% remained adsorbed. In terms of the washing steps,

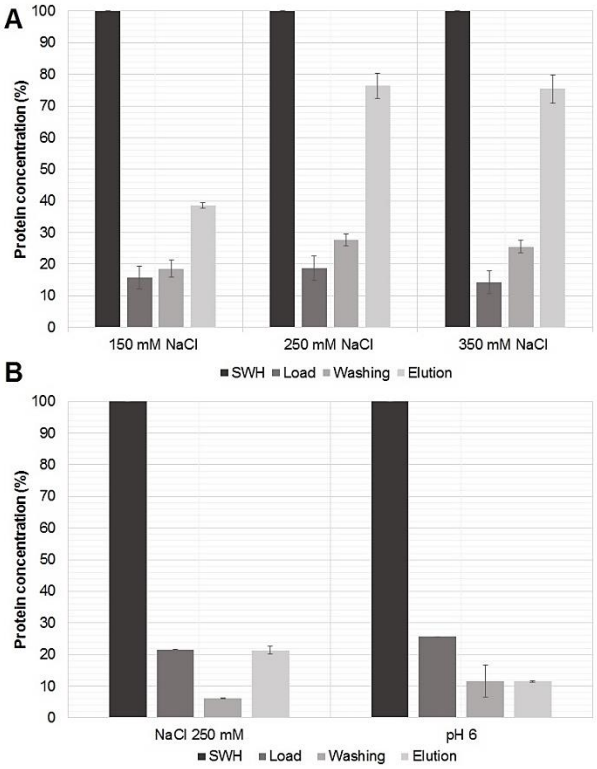

Figure 1. Percentage of soluble protein concentration obtained in each chromatographic step (Load, Washing and Elution) concerning the SP-Sepharose XL™ adsorbent (1 mL) used to fractionate and purify the soluble sweet whey hydrolysate (SWH) corrected at pH 4.5 with A) citrate buffer 20 mM pH 4.5 (diluted SWH) and B) NaOH 1M (concentrated SWH). The initial soluble concentration (100%) was 1.05 mg/mL.

6.1% and 11.7%, respectively, of the non-attached proteins/peptides were eluted with the working buffer. Finally, regarding the elution steps, these represent 21.3% and 11.6% of the soluble total protein for elution with salt and citrate buffer pH 6, respectively, suggesting that not sufficient fractions were collected, since approximately 50% of the proteins are still to be collected.

Nonetheless, these preliminary results show a fairly good capacity of these adsorbent, at pH 4.5, to adsorb and purify SWH. Furthermore, in order to better understand the adsorption capacity of peptides to the negatively charged SP-column, an isolation step prior to the purification, for example nanofiltration, could be eventually assessed, since in the whey hydrolysate remained proteins that were not hydrolysed by α-chymotrypsin (results not shown). Even so, the isolated fractions of the different purification strategies were analyzed for their antimicrobial activity.

Table 2. Summary of the percentage of inhibition of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Burkholderia contaminans* (*B. contaminans*) and*Staphylococcus aureus* (*S. aureus*) after a period of incubation of 24 hours, at 37ºC with fractions collected from the SP Sepharose XLTM 1 mL adsorbent with different elution strategies and using sweet whey hydrolysate (SWH) corrected to pH 4.5 either with citrate buffer 20 mM pH 4.5 or with NaOH 1M.

			Inhibition after 24 hours(%)			
	Elution strategy	**Fraction**	***E. coli***	***P. aeruginosa***	***B. contaminans***	***S. aureus***
Diluted SWH (corrected to pH 4.5 with citrate buffer 20 mM pH 4.5)	[NaCl] 150 mM	Load 1	4.8	-	13.7	24.4
		Load 2	4.56	9.2	10.2	-
		Wash 1	5.7	-	13.3	-
		Wash 2	9.4	-	7.8	20.0
		Wash 3	14.7	1.0	11.6	3.5
		Wash 4	19.1	-	2.6	5.8
	[NaCl] 250 mM	Elution 1	16.2	-	13.8	-
		Elution 2	-	-	13.0	-
		Elution 8	4.7	-	-	16.7
	[NaCl] 350 mM	Load 2	6.7	-	-	21.5
		Wash 1	5.2	12.4	4.1	26.5
		Elution 4	-	18.4	7.2	-
		Elution 5	-	18.6	-	-
Concentrated SWH (corrected to pH 4.5 with NaOH 1M)	[NaCl] 250 mM	Wash 1	6.5	-	-	41.7
		Wash 2	5.6	-	-	32.9
		Elution 3	-	-	-	15.9
	pH 6	Wash 1	6.9	-	-	33.5
		Wash 2	4.3	-	-	30.3

3.3. Antimicrobial activity of the peptide fractions

The inhibitory activities of all the fractions were evaluated and some fractions showed a considerable satisfactory capacity of inhibiting some of the bacterial strains tested after 24 hours (Table 2). When studying natural possible antibiotics, such as peptides derived from whey proteins, the results appear to be promising. In particular, when concentrated SWH is used, the fractions of non-adsorbed peptides appear to have an inhibitory effect mainly on *S. aureus,* with a mean value of 38±6% for washed fraction 1, and 32±2% for washed fraction 2. In the other hand, the elution strategy with pH 6 did not showed any fraction with potential inhibitory effect, whereas in the elution strategy with NaCl 250mM, eluted fraction 3 exhibited an inhibitory capacity of approximately 16%.

When comparing the results for the diluted SWH, *P. aeruginosa* seems to be the strain less affected by the antimicrobial effect of the fractions, followed by *E. coli*, and *B. contaminans*. More so, independently of the elution strategies, some peptides in the loaded and washed fractions exhibited potential inhibitory effects. This is the case of loaded fraction 2 and washed fraction 1, which yielded 21.5% and 26.5% inhibition against *S. aureus*, respectively. Also for *S. aureus*, loaded fraction 1 and washed fraction 2 respectively showed inhibitory capacity in the order of 24.4% and 20%. Regarding *B. contaminans*, the antimicrobial effect was around 12-13% for loaded fraction 1, and washed fractions 1 and 3. Finally, the washed fractions 3 and 4 showed an inhibitory capacity of 14.7% and 19.1%, respectively, against *E. coli*. Regarding the different elution strategies applied when using diluted SWH, the most effective were the ones using 250mM and 350mM of NaCl salt. For instance, when a salt concentration of 350mM is used, eluted fractions 4 and 5 show approximately 18.5% of inhibition against *P. aeruginosa*. Moreover, when using a salt concentration of 250 mM, eluted fraction 1 showed an inhibitory capacity of 16.2% and 13.8% against *E.coli* and *B. contaminans*, respectively. Eluted fraction 2 also acted against *B. contaminans*, revealing an inhibitory effect of approximately 13%. Finally, eluted fraction 8 showed only an inhibitory capacity of 16.7% against *S. aureus*.

Regardless of the elution strategy or the effect of diluted or concentrated SWH, the results summarized in Table 2 show that peptides have a greater inhibitory capacity towards *S. aureus*, the only gram-positive bacteria tested. This could be due to the absence of an outer membrane, when in comparison with gram-negative bacteria, which may difficult the protection of gram-positive bacteria against the environment.

The soluble protein concentration found in the tested fractions was in the order of 20 to 157 µg/mL. Although copper-ion based assays, such BCA, are the best option for detecting peptides, due to its detection limits, problems may arise with very small peptides or very dilute solutions. Therefore, the concentration measured may not correspond to the real peptide concentration. Still, the results are encouraging, especially in the case of *S. aureus*, a recognized important pathogen in patients with liver diseases (Kang *et al.* 2010) and that chronically infects the airways of patients with cystic fibrosis (Besier *et al.* 2007).

3.4. Characterization of antimicrobial peptides

3.4.1. Molecular weight

In order to assess the molecular weight of the peptides present in the different antimicrobial fractions, the gels were always stained with silver nitrate after staining with Coomassie dye. This suggests that the majority of the peptides in the fractions collected were at least at concentrations below 100 µg/mL, the detection limit for Coomassie blue staining (Schägger 2006). Moreover, if peptides are visible with silver staining this suggests that they have a concentration at least higher than 1 µg/mL (Schägger 2006). A summary of the molecular weights is given in Table 2. In a first analysis, Lanes 1 and 2 from Gel 3 (Figure 2), corresponding to the loaded fraction 2 and washed fraction 1 from the chromatographic separation of diluted SWH, appear to have a single peptide with an approximate molecular weight of 6.51 kDa. The same happens with Lanes 4 and 7 from Gel 4 (Figure 3), which single band matches to a peptide with a molecular weight of around 800 and 400 Da, respectively. In Gel 1, Lanes 1, 2, and 3 (Figure 2), corresponding to the washed fractions 1 and 2, and eluted fraction 3 from the chromatographic separation of concentrated SWH and eluted with a salt concentration of 250mM, show that a mixture of peptides is present, with molecular weights ranging from 25 to 6 kDa, approximately.

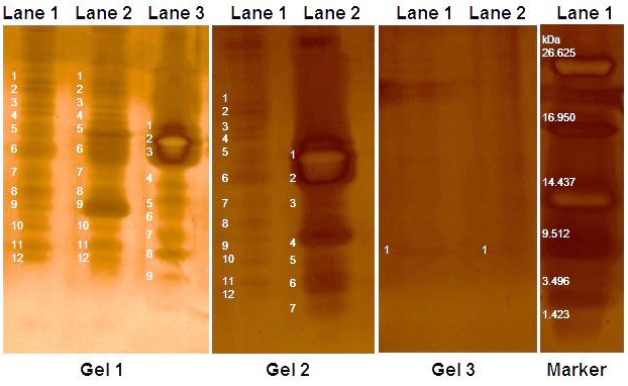

Figure 2. Tricine SDS-PAGE of washed fractions 1 and 2, and eluted fraction 3 from concentrated sweet whey hydrolysate eluted with NaCl 250 mM (Gel 1, Lanes 1, 2 and 3, respectively); washed fractions 1 and 2 from concentrated sweet whey hydrolysate eluted with citrate buffer 20 mM pH 6 (Gel 2, Lanes 1 and 2, respectively; and loaded fraction 1 and washed fraction 1 from diluted sweet whey hydrolysate eluted with NaCl 350 mM (Gel 3, Lanes 1 and 2, respectively). The marker (Lane 1) represents the polypeptide SDS-PAGE molecular weight. All the lanes are at the same scale.

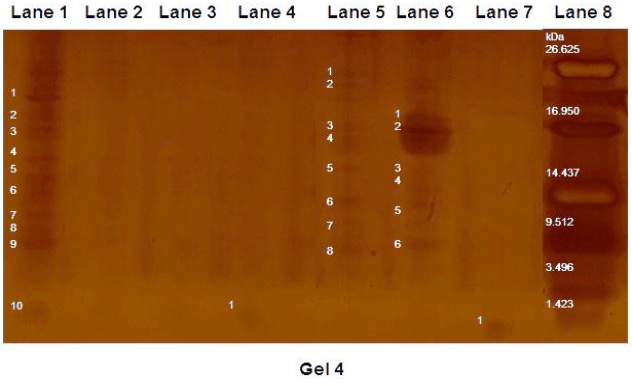

Figure 3. Tricine SDS-PAGE of washed fractions 1 to 4 from diluted sweet whey hydrolysate eluted with NaCl 150 mM (Lanes 1 to 4, respectively), and the eluted fractions 1, 2, and 8 from diluted sweet whey hydrolysate eluted with NaCl 250 mM (Lanes 5, 6, and 7, respectively. Lane 8 represents the polypeptide SDS-PAGE molecular weight. All the lanes are at the same scale.

The same happens to Lanes 1 and 2 from Gel 2 (Figure 2) that correspond respectively to the washed fractions 1 and 2 from concentrated SWH; and Lanes 1, 5, and 6 from Gel 4 (Figure 3) that correspond to washed fractions 1 to 4, and eluted fractions 1 and 2 from diluted SWH eluted with a salt concentration of 250 mM. In this section, it is important to stress that a more sophisticated technique should be applied in parallel, such as reverse phase HPLC (RP-HPLC), in order to identify how many different fragments were present in the fractions that exhibited antimicrobial potential. Furthermore, with this

Table 3. Summary of the predicted molecular weight of the peptides encountered in the chromatograpgic fractions that showed antimicrobial activity.

	Predicted Molecular Weight (kDa)											
	Gel 1			Gel 2		Gel 3		Gel 4				
Lane / Strip	1	2	3	1	2	1	2	1	4	5	6	7
1	25.4	25.4	20.9	24.5	19.4	6.5	6.5	22.2	0.8	15.7	21.4	0.4
2	24.6	24.6	19.6	23.6	17.6			20.2		12.6	20.0	
3	23.3	23.3	18.3	22.2	15.3			17.7		7.8	14.4	
4	22.2	22.2	17.2	21.3	12.0			16.2			12.6	
5	20.0	20.0	15.3	19.9	10.0			14.4			8.3	
6	18.8	18.8	14.0	17.6	7.0			12.1				
7	16.6	16.6	10.6	15.3	5.3			10.8				
8	14.7	14.7	8.6	13.9				8.9				
9	13.2	13.2	6.5	12.0				7.1				
10	11.2	11.2		10.0				0.8				
11	9.7	9.7		8.3								
12	8.4	8.4		6.7								

technique it is important to have an internal standard in order to better quantify the peptides concentration.

Comparing this results with the ones predicted by the bioinformatic analysis, the molecular weights calculated herein are bigger that the ones predicted, in general. Nonetheless, the bioinformatic analysis showed potential antimicrobial peptides with sizes of 0.807 kDa (EAGRDPY), 0.923 kDa (GKNKSRSF) and 0.722 kDa (RPTEGY) and the analysis of the tricine SDS-PAGE showed a peptide (or mixture) with a molecular weight around 0.8 kDa, present in the washed fraction 1 from the separation of diluted SWH. Other predicted peptide by the bioinformatic analysis had a molecular weight of 0.539 kDa (EACAF), comparable with the 0.4 kDa peptide (eluted fraction 8 following elution of diluted SWH with NaCl 250 mM) obtained by tricine SDS PAGE.

3.4.2. Isoelectric point

The assessment of the isoelectric point (IEP) of the peptides present in the separate antimicrobial fractions was performed by isoelectric focusing. The gels were stained with silver nitrate, which the average sensitivity limit of this technique is estimated to be 1 to 5 ng protein per band for IEF (Heukeshoven & Dernick 1985). As mentioned before, the peptides concentrations may be lower than the detection limit of this method and thus some bands were not visible. Therefore, Figure 4 highlights the fractions which bands were visible. As visible in Figure 4, the IEP for the majority of the peptides is around 3.75 and 4.55, with the exception of the washed fraction 1 from diluted SWH (Lane 6), with a pI approximately of 6.12. Moreover, the existence of more than two bands per lane also suggests a mixture of peptides in the chromatographic fractions, as expected from the previous results.

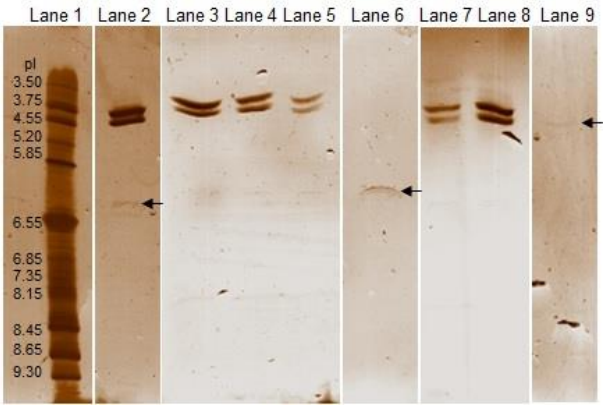

Figure 4. Isoelectric focusing of washed fraction 1 from concentrated SWH (Lane 2), eluted fractions 3, 4 and 5 from concentrated SWH eluted with NaCl 250 mM (Lanes 3, 4, and 5, respectively), washed fraction 1 from diluted SWH (Lane 6), loaded fractions 1 from concentrated SWH (Lanes 7 and 8, respectively), and eluted fraction 2 with NaCl 250 mM from diluted SWH (Lane 9). Lane 1 represents the Broad pI Kit, pH 3-10 (GE Healthcare).

Comparing the results obtained herein with the ones predicted by the bioinformatic analysis, some of the IEP are in agreement with the ones predicted. For example, the peptide EAGRDPY has a theoretical pI of 4.37, peptide RPTEGY a pI of 6 and the peptide EACAF had a predicted pI of 4. This may suggest that one or more of these predicted antimicrobial peptides is present in one of the mixture of peptides in the fractions selected.

4. Conclusions and Future Perspectives

The main conclusions from this work are that, in fact, the employment of α-chymotrypsin as the protease for the hydrolysis of whey proteins originates a complex mixture of peptides and small proteins with potential antimicrobial activity. Also, that this mixture of peptides is more effective against the *S. aureus* bacterial strain, when compared to the *E.coli*, *P. aeruginosa* and *B. contaminans* strains. Nonetheless, as demonstrated by the tricine SDS-PAGE and IEF techniques, the peptides seem to be in very low concentration, when compared to the BCA results. Comparing the results obtained with the ones predicted by the bioinformatic analysis, it appears to be at least one peptide identified, RPTEGY. The bioinformatic analysis predicted a molecular weight of 0.722 Da and a pI of 6 for this peptide, whereas by tricine SDS-PAGE and IEF the molecular weight is around 0.830 Da and the pI 6.12, respectively. This peptide corresponds to the fragment f(443-446) derived from lactoferrin.

In general, the results obtained are considered positive, since it was demonstrated that low concentrated peptides derived from whey proteins have the potential to act as antimicrobial aids. Moreover, it was also possible to correlate the results obtained with a bioinformatic analysis with an *in vitro* approach. Nonetheless, further work is necessary, mainly in terms of concentrating the peptides, to better study their capability of inhibiting the studied bacterial strains or other ones of interest, for instance in the food industry, such as *Salmonella* or *Listeria* . Also, the concentration of peptides would help with their identification by the techniques employed herein. Moreover, as already mentioned, techniques like RP-HPLC would facilitate the quantification of peptides and also it would be a comparable technique in terms of identifying the presence of a single peptide or mixture of peptides in the collected fractions. Another reason to use concentrated peptides is to perform this study on a preparative scale, with higher volumes of sweet whey.

The results presented herein contribute to the transformation of whey from a waste material to a valuable dairy stream for use in the agro-food, biotechnology, medical and related markets. Whey is currently a major by-product of the modern cheese and casein production industries and represents an important source of environmental pollution due to its large volume, production rate and high organic matter content (Silvestre *et al.* 2012) and therefore it would be interesting to have a partnership between academia and a cheese producing company, with the main goal of researching the potential of whey in generating peptides with bioactive activities, in this case antimicrobial. Indeed, a growing body of evidence exists that whey peptides exhibit physiological activities on specific components of the immune system and, consequently, many new products with bioactive peptides have reached the market (Gauthier *et al.* 2006). Moreover, several studies have suggested that milk protein-derived bioactive peptides may be used as preventive/prophylactic agents to alleviate symptoms of various diseases in humans. Although various drugs exist to cure or slow down the progress of specific diseases in humans, their side-effects may sometimes overshadow their benefits. In this context, food protein-derived peptides, in particular milk protein-derived bioactive peptides have potential as natural alternatives to drugs for disease management (Nongonierma & FitzGerald 2015). However, in order to fully verify the potential of whey peptides in immunomodulation, conclusions from *in vitro* models need to be constantly corroborated with physiological data obtained *in vivo* (Roufik *et al.* 2006). Additionally, for a candidate peptide to be labeled as bioactive, its resistance to gastrointestinal conditions must be determined in advance. The exact mechanisms by which whey peptides exert their bioactivities upon reaching the intestine need further elucidation. For instance, there is a need to assess whether their effect is mediated directly in the gut lumen or through receptors on the intestinal cell wall. Therefore, *in vivo* studies are essential not only to validate the potential bioactive peptides, but also to confirm whether they will require

protection from gastrointestinal enzymes when orally administered (Meisel & Schlimme 1996). Concluding, future research should focus on novel hydrolysis pathways for breakdown of whey proteins and peptides, brought about by unusual proteases aimed at releasing unique amino acid sequences. These may include enzymes from the native microbiota of dairy products or from plant rennets (Lamas *et al.* 2001). Additionally, molecular studies concerning the mechanisms by which bioactive peptides exert their activities are to be undertaken (Wada & Lönnerdal 2014). In the specific case of antimicrobial peptides, although neither their effectiveness as food preservatives nor their safety for use as food additives has been sufficiently demonstrated, they offer the advantage of being derivable from generally recognized as safe (GRAS) substances, such as whey. Consequently, their use in medicine and in the food industry should meet with less consumer resistance and easier regulatory approval, on the basis of their natural occurrence (Théolier *et al.* 2013).

5. References

Bellamy, W. et al., 1992. Identification of the bactericidal domain of lactoferrin. *Biochimica et Biophysica Acta*, 1121(1-2), pp.130–136.

Benkerroum, N., 2010. Antimicrobial peptides generated from milk proteins: a survey and prospects for application in the food industry. A review. *International Journal of Dairy Technology*, 63, pp.320–338.

Besier, S. et al., 2007. Prevalence and Clinical Significance of Staphylococcus aureus Small-Colony Variants in Cystic Fibrosis Lung Disease. *Journal of Clinical Microbiology*, 45(1), pp.168-172.

Chakrabarti, S., Jahandideh, F. & Wu, J., 2014. Food-Derived Bioactive Peptides on Inflammation and Oxidative Stress. *BioMed Research International*, 2014, pp.1–11.

Clare, D.A., Catignani, G.L. & Swaisgood, H.E., 2003. Biodefense properties of milk: the role of antimicrobial proteins and peptides. *Current Pharmaceutical Design*, 9(16), pp.1239–1255.

Clare, D.A. & Swaisgood, H.E., 2000. Bioactive Milk Peptides : A Prospectus. *Journal of Dairy Science*, 83(6), pp.1187–1195.

da Costa, E.L., Gontijo, J.A. da R. & Netto, F.M., 2007. Effect of heat and enzymatic treatment on the antihypertensive activity of whey protein hydrolysates. *International Dairy Federation*, 17, pp.632–640.

Cupp-Enyard, C., 2008. Sigma's Non-specific Protease Activity Assay - Casein as a Substrate. *Journal of Visualized Experiments*, 19(899), pp.4–5.

Florisa, R. et al., 2003. Antibacterial and antiviral effects of milk proteins and derivatives thereof. *Current Pharmaceutical Design*, 9(16), pp.1257–1275.

Gauthier, S.F., Pouliot, Y. & Saint-Sauveur, D., 2006. Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. *International Dairy Journal*, 16(11), pp.1315–1323.

Heukeshoven, J. & Dernick, R., 1985. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis*, 6(3), pp.103–112.

Kamau, S.M. et al., 2010. Functional Significance of Bioactive Peptides Derived from Milk Proteins. *Food Reviews International*, 26, pp.386–401.

Kang, C. et al., 2010. Clinical significance of Staphylococcus aureus infection in patients with chronic liver diseases. *Liver International*, 30(9), pp.1333–1338.

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

Kuwata, H. et al., 1998. Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1429(1), pp.129–141.

Lamas, E.M. et al., 2001. Hydrolysis of whey proteins by proteases extracted from Cynara cardunculus and immobilized onto highly activated supports. *Enzyme and Microbial Technology*, 28(7-8), pp.642–652.

López-Expósito, I. et al., 2007. Casein hydrolysates as a source of antimicrobial, antioxidant and antihypertensive peptides. *Le Lait*, 87(4-5), pp.241–249.

López-Expósito, I., Minervini, F., et al., 2006. Identification of Antibacterial Peptides from Bovine κ-Casein. *Journal of Food Protection*, 69, pp.2992-2997.

López-Expósito, I., Gómez-Ruiz, J.Á., et al., 2006. Identification of antibacterial peptides from ovine αs2-casein. *International Dairy Journal*, 16(9), pp.1072–1080.

Meisel, H. & Schlimme, E., 1996. Bioactive peptides derived from milk proteins : Ingredients for functional foods? *Kieler Milchwirtschaftliche Forschungsberichte*, 48(4), pp.343–357.

Mizuno, S. et al., 2005. Antihypertensive effect of casein hydrolysate in a placebo-controlled study in subjects with high-normal blood pressure and mild hypertension. *The British journal of nutrition*, 94(1), pp.84–91.

Nongonierma, A.B. & FitzGerald, R.J., 2015. The scientific evidence for the role of milk protein-derived bioactive peptides in humans: A Review. *Journal of Functional Foods*, 17, pp.640–656.

Pellegrini, A. et al., 2001. Isolation and characterization of four bactericidal domains in the bovine β-lactoglobulin. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1526(2), pp.131–140.

Pellegrini, A. et al., 1999. Isolation and identification of three bactericidal domains in the bovine α-lactalbumin molecule. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1426(3), pp.439-448.

Pihlanto, A., 2006. Antioxidative peptides derived from milk proteins. *International Dairy Journal*, 16(11), pp.1306–1314.

Roufik, S., Gauthier, S.F. & Turgeon, S.L., 2006. In vitro digestibility of bioactive peptides derived from bovine β-lactoglobulin. *International Dairy Journal*, 16(4), pp.294–302.

Schägger, H., 2006. Tricine-SDS-PAGE. *Nature Protocols*, 1(1), pp.16–22.

Schägger, H. & von Jagow, G., 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical biochemistry*, 166(2), pp.368-379.

Seo, M.-D. et al., 2012. Antimicrobial Peptides for Therapeutic Applications: A Review. *Molecules*, 17(10), pp.12276–12286.

Silvestre, M.P.C. et al., 2012. Analysis of whey protein hydrolysates: Peptide profile and ACE inhibitory activity. *Brazilian Journal of Pharmaceutical Sciences*, 48, pp.747–757.

Smith, P.K. et al., 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, 150(1), pp.76–85.

Théolier, J. et al., 2014. Antimicrobial Peptides of Dairy Proteins : From Fundamental to Applications. *Food Reviews International*, 30, pp.134–154.

Théolier, J. et al., 2013. Isolation and identification of antimicrobial peptides derived by peptic cleavage of whey protein isolate. *Journal of Functional Foods*, 5(2), pp.706–714.

Thomas, S. et al., 2010. CAMP: A useful resource for research on antimicrobial peptides. *Nucleic Acids Research*, 38, pp.774–780.

Tomita, M. et al., 2002. Bovine lactoferrin and lactoferricin derived from milk: production and applications. *Biochemistry and Cell Biology*, 80, pp.109–112.

Wada, Y. & Lönnerdal, B., 2014. Bioactive peptides derived from human milk proteins--mechanisms of action. *The Journal of Nutritional Biochemistry*, 25(5), pp.503–514. [Accessed September 25, 2014].

Waghu, F.H. et al., 2014. CAMP: Collection of sequences and structures of antimicrobial peptides. *Nucleic Acids Research*, 42, pp.1154–1158.

Wang, G., Li, X. & Wang, Z., 2009. APD2: The updated antimicrobial peptide database and its application in peptide design. *Nucleic Acids Research*, 37(SUPPL. 1), pp.933–937.