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Identification and Characterization of Bioactive Peptides with Antimicrobial and Immunoregulating Properties Derived from Bovine Colostrum and Milk

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Declaration:

Hereby I declare that this doctoral thesis, submitted for the doctoral degree at Tallinn University of Technology (Estonia) and Aarhus University (Denmark), is my original investigation and achievement and has not been submitted for any academic degree.

/Mary-Liis Kütt/

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LOODUS- JA TÄPPISTEADUSED B185

Antimikroobsete ja immuunsüsteemi stimuleerivate omadustega bioaktiivsete peptiidide identifitseerimine ja karakteriseerimine lehma piimast ja kolostrumist

MARY-LIIS KÜTT

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Contents

Abstract

Peptides are an important nutritional source of amino acids and display many specific biological functions, such as direct killing of bacteria and modulating the innate immune response. Host defense antimicrobial peptides (AMPs) are produced by various mammalian cells and excreted into bodily fluids including colostrum and milk. The most well characterized AMPs are defensins and cathelicidins. Both peptide classes have a high content of cationic amino acid and amphiphilic regions in their structure. AMPs, if present in colostrum, may be responsible for combating invasive pathogens, preventing infection and stimulating the innate immune response of newborns. AMPs have been detected in both colostrum and milk using both immunoassays and proteomic approaches.

The aim of this doctoral work was: i) to use proteomic tools to identify AMPs in colostrum; ii) to investigate antimicrobial properties of bovine colostrum and a potential role of colostrum as a modulator of an appropriate innate immune response; iii) to gain further insight into the role of peptides as growth promoting substances.

A non-selective proteomic approach (Nanostructure-Assisted Laser Desorption/Ionization - NALDI) was used to identify bioactive peptides from colostrum. The most abundant peptides identified were fragments from caseins. Although the antimicrobial β-casein fragment was detected, even if the analyte was 10^6 fold diluted no host defense AMPs were found in bovine colostrum samples. Furthermore, initial attempts to detect antimicrobial activity in colostrum were unsuccessful. Because the presence of AMPs in colostrum has been previously demonstrated, we presumed that our negative results were due to suppressing effects caused by proteins in the colostrum. Therefore, a model system of bovine colostrum and added piscidin, a fish-derived AMP, was developed to study possible interactions that might preclude straightforward detection of AMPs.

The results showed that colostrum completely abrogated the antimicrobial activity of the added piscidin, which strongly bound to high molecular weight components in colostrum, presumably casein micelles. No apparent binding to IgG or whey proteins was observed and degradation of piscidin in colostrum was found to be negligible. In fact, colostrum even protected piscidin against degradation by additional proteases. The antimicrobial activity of the piscidin in this model system could be recovered using strong denaturants. Colostrum treatment with strong denaturants and fractionation did not display any antimicrobial activity against bacteria and we concluded that colostrum from a healthy cow does not contain host defense AMPs in amounts that inhibit microbial growth.

However, the dialyzed LMW colostral fractions displayed AMP-like activity with respect to the innate immune response using a murine intestinal cell line. Low molecular weight colostral components appeared to interfere with bacterial lipopolysaccharides (LPS). AMPs that bind strongly to LPS have previously been shown to prevent LPS from binding to specific immune cell surface receptors and reduce or dampen the inflammatory response. Thus, specific colostral LMW components may have immune-modulating effects in mammals.

Besides inhibitory effects, small peptides can also promote microbial growth, even in media abundant in free amino acids. Experiments with ^{15}N-labelled yeast hydrolysates showed that both free and peptide-bound amino acids were consumed by *Lactococcus lactis* in parallel. The proportion of amino acids derived from peptides was up to 60 % of the total amino acid consumption. During later stages of fermentation the utilization of peptide-bound amino acids decreased, thus indicating that the more readily assimilated peptides are gradually exhausted from the growth medium. A considerable efflux of many free amino acids occurred during growth.

These findings helped us to identify potential metabolic bottlenecks and this knowledge can be applied to design novel yeast hydrolysates tailored for specific bacterial strains with an optimized content of bioactive peptides that will maximize growth rates and production yields.

This dissertation contributes to our understanding of the effects of various bioactive functions of small peptides, including, antimicrobial effects, immune enhancement, and growth inducing activity. Our results indicate that known host defense AMPs, even when present in colostrum, cannot display antimicrobial activity because of ionic interactions with caseins. A more apparent role of small peptides in colostrum is their ability to modulate the innate immune response of mammals. Finally, bacterial consumption patterns of several amino acids helped us to identify potential metabolic bottlenecks.

Resumé

Peptider er vigtige kilder til aminosyrer, og mange specifikke peptider har interessante biologiske aktiviteter, som f.eks. direkte drab af bakterier og modulering af det innate immunsvar. Antimikrobielle peptider (AMP'er) bliver produceret i forskellige mammale celler og udskilles i kropsvæsker, herunder mælk. De mest velkarakteriserede AMP'er er defensiner og cathelicidiner, som har et højt indhold af kationiske aminosyrer og amfifile strukturer. AMP'er er fundet i råmælk, hvor de kan medvirke i forsvaret mod patogener, forhindre infektioner og stimulere det innate immunforsvar i nyfødte. AMP'er i råmælk og mælk er påvist i immunassays og ved proteomics analyser, men ikke ved direkte antimikrobielle effekter overfor patogener.

Formålet med dette PhD projekt var at opnå øget forståelse for fysiologiske effekter af peptider fra forskellige fødevarematricer – 1) at undersøge antimikrobiel aktivitet i forskellige fraktioner fra råmælk fra køer og yderligere undersøge om råmælk påvirker det innate immunsvar; 2) at anvende proteomics værktøjer til at identificere og karakterisere AMP'er i råmælk; 3) at måle forbrugsmønstre af frie og peptidbundne aminosyrer for en specifik stamme af *Lactococcus lactis* for at opnå større forståelse for peptiders rolle som aminosyrekilder ved overskud af frie aminosyrer.

En non-selektiv proteomics tilgang (Nanostruktur-Assisteret Laser Desorption Inonisering –NALDI) blev anvendt til at identificere små peptider i råmælk. Peptider, der kunne identificeres ved sekventering, var fragmenter fra kaseiner, specielt et fremtrædende antimikrobielle β-kasein fragment, der kunne detekteres selv efter 10^6 ganges fortynding. Der kunne ikke detekteres antimikrobielle peptider. Desuden var mine forsøg på at måle antimikrobiel aktivitet i råmælk og derigennem at identificere AMP'er uden succes.

Derfor udvikledes et modelsystem til at studere mulige interaktioner mellem råmælk og tilsat piscidin, et AMP fra fisk, der kunne forklare vanskelighederne ved direkte påvisning af AMP'er. Mine resultater viste, at råmælk fuldstændigt eliminerede den antimikrobielle aktivitet af tilsat piscidin, og at fluorescens-mærket piscidin binder kraftigt til komponenter i råmælk med høj molekylvægt, formentlig kasein-miceller, uden binding til IgG eller valleproteiner. Da nedbrydning af piscidin i råmælk var ubetydelig, faktisk beskyttede råmælk mod nedbrydning af piscdin efter tilsætning af proteaser, viser mine resultater, at kaseiner er potentielle transportører af AMP'er, som defensiner og cathelicidiner, i råmælk og mælk. Selvom tilsat piscidin kunne genisoleres fra råmælk og kaseiner ved hjælp af denaturerende reagenser, kunne jeg ikke påvise endogene AMP'er i råmælk. Hverken råmælk eller lavmolekylære fraktioner herfra udviste antimikrobiel aktivitet over for bakterier.

Imidlertid viste fraktioner fra råmælk AMP-lignende aktivitet med hensyn til innat immunaktivitet over for en muse tarm celle linie. Lavmolekylære komponenter fra råmælk interfererede tilsyneladende med bakteriel lipopolysakkarid (LPS), som også kunne ses for AMP'er, der binder kraftigt til LPS, en binding der forhindrer LPS i at binde til specifikke receptorer på immuncellernes overflade og derigennem reducerer det inflammatoriske respons.

Små peptider kan også fremme vækst af bakterier. Tilsætning af hydrolysater fra gær til vækstmedier, der allerede indeholder overskud af aminosyrer og nukleotider, forøgede vækstraten af bakterier. Forsøg med ^{15}N-mærkede gær hydrolysater viste, at både frie og peptidbundne aminosyrer blev forbrugt af *Lactococcus lactis* parallelt. Andelen af aminosyrer fra peptider udgjorde op til 60% af det samlede forbrug af aminosyrer. Senere i vækstforløbet reduceredes forbruget af peptid-bundne aminosyrer som tegn på, at de lettere optagne peptider gradvist blev forbrugt i vækstmediet. Der var en betydelig frigivelse af især frie aminosyrer i vækstforløbet.

Denne afhandling bidrager til forståelse for forskellige bioaktive funktioner af små peptider: antimikrobielle effekter, immun-stimulerende og vækst inducerende. Vore resultater indikerer, at AMP'er enten er tilstede i råmælk i meget lave mængder eller er tæt bundne til kaseiner og derfor ikke har en direkte antimikrobiel effekt over for patogener. Modelsystemet med piscidin og råmælk gav indsigt i en mulig rolle for kaseiner som transportører og beskyttere af AMP'er. Den mere oplagte rolle af råmælk vil derfor være at modulere det innate immunsvar hos pattedyr. Endelig hjalp bestemmelse af forbrugsmønstre for flere forskellige aminosyrer os til at identificere begrænsninger i det bakterielle stofskifte. De sidstnævnte resultater vil kunne anvendes til at designe nye gærhydrolysater med optimeret indhold af bioaktive peptider til at opnå forbedrede vækstrater og produktudbytter for bestemte bakteriestammer.

Kokkuvõte

Peptiidid on olulised aminohapete allikad erinevatele organismidele, lisaks on neil veel bakterite kasvu inhibeeriv, aga ka indutseeriv ning immuunsüsteemi tugevdav toime. Antimikroobseid peptiide ekspresseeritakse paljude imetajate rakkudes, kust nad omakorda satuvad ringlusega organismi erinevatesse kudedesse. Nii jõuavad antimikroobsed peptiidid imetajate rinnapiima, kus nende funktsiooniks on võidelda organismi tungivate patogeensete bakteritega, hoida ära infektsiooni tekkimist ning lisaks stimuleerida vastsündinu immuunsüsteemi. Hetkel on kõige paremini ja täpsemini kirjeldatud kaks suurt antimikroobsete peptiidide rühma – defensiinid ja katelitsidiinid, millel on hulgaliselt aluselisi, positiivselt laetud aminohappeid ja amfifiilset struktuuri. Ternespiimast (kolostrumist) on antimikroobseid peptiide leitud nii immunoloogiliste kui ka proteoomika meetoditega, aga üllatuslikult puuduvad uuringud, mis otseselt tõestaks selle olulise imetajate sekreedi patogeensete bakterite kasvu inhibeerivat toimet.

Käesoleva uurimuse eesmärgiks oli välja selgitada ternespiimas leiduvate peptiidide bioaktiivne toime, kontsentreerudes otsestele või kaudsetele antimikroobsetele omadustele ning lisaks uurida ternespiima rolli immuunsüsteemi tugevdamisel. Selleks arendasime ja katsetasime erinevaid, nii biotestidel kui ka proteoomikal põhinevaid metoodikaid. Lisaks arendasime välja metoodika uurimaks peptiidide transporti ja ainevahetust bakterirakkudes, kasutades ^{15}N-märgist ja massspektromeetriat.

Proteoomika meetoditest kasutasime esmakordselt madalmolekulaarsete peptiidide detekteerimiseks ternespiimast nanostruktureeritud laserdesorptsioon-ionisatsiooni (NALDI). See võimaldas meil identifitseerida β-kaseiini antimikroobse fragmendi, kuid kahjuks mitte ühtegi defensiini ega katelitsidiini.

Ternespiima bakterite kasvu inhibeerivat toimet ei õnnestunud demonstreerida ka otsestes bioaktiivsuse testides. Kuna aga kirjanduse andmed viitasid selgelt defensiini ja katelitsidiini olemasolule ternespiimas, kontrollisime bioloogilise testi toimimist kaladest pärit antimikroobse peptiidiga piscidin, millel on väga sarnane molekulaarne struktuur eelmainitud bioaktiivsete peptiididega. Testid näitasid, et kui piscidini lahus inhibeeris nii Gram positiivsete kui ka negatiivsete bakterite kasvu, siis kolostrumi lisamisega see efekt kadus. Edasised katsed demonstreerisid veenvalt, et piscidin seondub tugevalt ternespiimas leiduvate kaseiinidega. Veelgi enam, kolostrum kaitses seotud piscidini proteolüüsi eest. See viitab kaseiinide võimele siduda ja transportida madalmolekulaarseid, kaasa arvatud antimikroobseid komponente imetaja seedetraktis. Siiski, hoolimata asjaolust, et me olime võimelised vabastama seotud piscidini kaseiinidest guanidiinhüdrokloriidi toimel, ei suutnud me ternespiimast samu võtteid kasutades vabastada ega ka identifitseerida ühtegi teist antimikroobset peptiidi.

Edasi jätkasime katsetega tõestamaks lehma ternespiima immuunsüsteemi aktiveerivat toimet imetaja seedetrakti rakkudes. Katsete käigus tõestasime, et kolostrumi madalmolekulaarne komponent seondus bakteriaalse lipopolüsahhariididega läbi elektrostaatilise interaktsiooni, nagu seda teeb ka enamik antimikroobseid peptiide. Selline seondumine takistab lipopolüsahhariididel koerakkude seintele kinnitumast ning seondumist spetsiifiliste rakupinna retseptoritega ning hoiab ära edasise põletikulise reaktsiooni tekke. Seega, ternespiimal võib olla imetaja immuunsüsteemi aktiveeriv ja tugevdav toime.

Lisaks antimikroobsele efektile uurisime peptiidide võimet stimuleerida rakkude kasvu. Töötasime välja mudelsüsteemi, kus peptiidide allikana kasutasime ^{15}N märgistatud pärmiekstrakti. Töötasime välja metoodika LC/MS-ile, et määrata peptiid-seotud (^{15}N rikastatud) ja vabade aminohapete tarbimist *Lactococcus lactis*es.

Tulemused näitasid, et *L. lactis* tarbis nii vabu kui ka pärmi hüdrolüsaadist saadud aminohappeid paralleelselt. Kui katse alguses tarbis bakter

umbes 60% ulatuses aminohappeid peptiidsel kujul, siis fermentatsiooni lõppedes oli märgata rohkem vabade aminohapete tarbimist, kuna paremini omastatavad peptiidid lõppesid toitekeskkonnast otsa.

Antud töö annab ülevaate peptiidide funktsionaalsusest ternespiimas, seda nii bakterite kasvu inhibeerimise ning stimuleerimise kui ka immuunsüsteemi tugevdamise aspektidest. Meie tulemused näitasid, et antimikroobseid peptiide esineb lehma ternespiimas kas väga minimaalses koguses või on nad tugevalt kaseiinidega seotud ning seega ei saa nad vähemalt enne nende vabastamist toimida. Teisalt näitasid katsed piscidiniga, et kaseiinide roll on olla antimikroobsete peptiidide sidujaks ja kandjaks, kaitstes neid imetaja seedetraktis proteolüütiliste ensüümide eest. Tähtsam kui ternespiima otsene antimikroobne aktiivsus on pigem tema immuunsüsteemi toetav ja aktiveeriv toime. Väikeste peptiidide toime uurimine mikroorganismidele lubab oletada, et ka ternepiimas võivad need bioaktiivsed komponendid pigem stimuleerida kui inhibeerida mikroobide kasvu.

List of publications

The following publications form the basis of this dissertation and are referred to in this text with Roman numerals. Each is reproduced in the appendices with the permission of the publisher.

I. Kütt M-L, Malbe M, Stagsted J (2011). Nanostructure-Assisted Laser Desorption/Ionization (NALDI) for analysis of peptides in milk and colostrum. Agronomy Research, 9, 415 - 420.

II. Kütt M-L., Stagsted J (2014) Caseins from bovine colostrum and milk strongly bind piscidin-1, an antimicrobial peptide from fish. International Journal of Biological Macromolecules, 70, 364 - 372.

III. Kevvai K, Kütt M-L, Nisamedtinov I, Paalme T (2014). Utilization of ^{15}N-labelled yeast hydrolysate in *Lactococcus lactis IL1403* culture indicates co-consumption of peptide-bound and free amino acids with simultaneous efflux of free amino acids. Antonie van Leeuwenhoek, 105(3), 511 - 522.

Author's Contribution to the Publications

I. The author designed and performed the experiments, analyzed the data and wrote the manuscript together with J. Stagsted.
II. The author designed and performed the experiments, developed the mass-spectrometry methods, analyzed the data and wrote the manuscript together with J. Stagsted.
III. The author developed the method for, and performed, all the amino acid analyses, analyzed this data and participated in the process of writing the manuscript.

List of presentations

I. Kütt M-L Effect of colostrum low molecular weight fractions on molecular weight fractions on NF-κB stimulated luciferase expression in mIC_{C12} cells in absence or presence of LPS. Poster presentation at seminar for PhD student from Department of Food Science in Aarhus University, 29-30 September, 2014, Sandbjerg, Denmark

II. Kütt M-L, Paalme T, Stagsted J Caseins from bovine colostrum strongly bind piscidin-1. Oral presentation at 5th scientific conference of TÜ and TTÜ PhD School "Functional materials and technology", 4-5 March, 2014, Tartu, Estonia

III. Kütt M-L, Stagsted J Bovine colostrum strongly binds piscidin-1, an antimicrobial peptide from fish mucus. Oral presentation at seminar for PhD student from Department of Food Science in Aarhus University, 30 September, 2013, Sandbjerg, Denmark

IV. Kütt M-L, Antimicrobial Test: Radial Plate Diffusion Assay. Poster presentation at 4th scientific conference of TÜ and TTÜ PhD School "Functional materials and technology", 7-8 March, 2013, Tallinn, Estonia

V. Kütt M-L, Identification and characterization of bioactive peptides with antimicrobial and immunoregulating properties derived from bovine colostrum and milk. Oral presentation at winter school conference of TÜ and TTÜ PhD School "Functional materials and technology", 6 December, 2012, Rakvere, Estonia

VI. Kütt M-L, Paalme T, Stagsted J Antimicrobial peptides and comparison of MALDI and NALDI for detecting low molecular weight components. Poster presentation at 3rd scientific conference of TÜ and TTÜ PhD School "Functional materials and technology", 29 February-1 March, 2012, Tartu, Estonia

VII. Kütt M-L, Malbe L, Stagsted J Nanostructure-Assisted Laser Desorption/Ionization (NALDI) for analysis of peptides in milk and colostrum. Poster presentation at FLAVOURE conference about Quality and Safety of Food and Feed and Agricultural Risks, 25-28 October, 2011, Tallinn, Estonia

VIII. Kütt M-L., Kodis I, Malbe M, Stagsted, J Identification of Antimicrobial and Host-Defense Peptides from Monkfish Waste. Poster presentation at the 5th Baltic Conference on Food Science and Technology „FoodBalt 2010“, 29-30 October, 2010, Tallinn, Estonia.

Additional publications

I. Akk E, Lõiveke H, Edesi L, Kütt M-L, Lauringson E, Kastanje V (2013) Formation of the abundance of microfungi on the barley grain grown as pure and mixed crops in Central and North Estonia. Estonian Journal of Ecology, 62, 265 - 275.

II. Kütt M-L., Lõiveke H, Tanner R (2010) Detection of alternariol in Estonian grain samples. Agronomy Research, 8, 317 - 322.

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Abbreviations

ABC	ATP binding cassette
ACE	Angiotensin I-converting enzyme
ACN	Acetonitrile
AMPs	Antimicrobial peptides
BCAA	Branched-chain amino acid
BSA	Bovine serum albumin
DMEM	Dulbecco's Modified Eagle's medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ESI	Electro spray ionization
FGF	Fibroblast growth factor
FPLC	Fast protein liquid chromatography
GMP	Glycomacropeptide
GndHCl	Guanidine hydrochloride
HIV	Human immunodeficiency virus
HMW	High molecular weight
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGF-II	Insulin-like growth factor 2
IL	Interleukin
LAP	Lingual antimicrobial peptide
LB	Luria Broth
LC-MS/MS	Liquid chromatography coupled with tandem mass-spectrometry
LIT	Linear ion trap
LMW	Low molecular weight
LPS	Lipopolysaccharide
m/z	mass-to charge
MALDI	Matrix Assisted Laser Desorption/Ionization
MFGM	Milk fat globule membrane
MIC	Minimum inhibitory concentration
MS	Mass-spectrometry
NALDI	Nanostructure-Assisted Laser Desorption/Ionization

NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate buffered saline
RT-PCR	Reverse transcription polymerase chain reaction
SCC	Somatic cell count
SEC	Size exclusion chromatography
TAP	Tracheal antimicrobial peptide
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOF	Time of flight

THESIS

1. Introduction

Besides possessing nutritional value, peptides have several important functions in various organisms: antibacterial, antifungal, antiparasite, antiviral, antitumor, antioxidative, antihypertensive, anti-inflammatory, immunoregulating, cell growth inducing, hormone signaling and health activities (Fig. 1). Peptides with antimicrobial activity provide defense against invasive pathogens and have excellent properties to kill many common antibiotic-resistant bacterial strains (Noga et al. 2009; Tomasinsig et al. 2010; Wang et al. 2011). Antimicrobial peptides (AMPs) are generally considered an important part of the innate (non-adaptive) immune defense system.

Antimicrobial peptides are encoded by AMP genes and produced ribosomally, mainly as prepropeptides. Host-defense AMPs are produced in specialized immune-competent cells by all living organisms, including bacteria, fungi, plants, invertebrates, and vertebrates. Roughly 2400 peptide sequences with antimicrobial activity have been described to date (Wang et al. 2009). In addition, some bioactive peptides are encoded within protein sequences with no obvious antimicrobial or other specific function. These peptides are either released during food processing, enzymatic degradation, or gastrointestinal digestion (Korhonen & Pihlanto 2006; Phelan et al. 2009). Some peptides with antibiotic properties, such as vancomycin and surfactin, are produced by bacteria and fungi using non-ribosomal peptide synthetases (Wiesner & Vilcinskas 2010; Marahiel 2009).

AMPs are relatively small molecules that typically possess a net positive charge and rapidly bind to bacterial membranes, form pores, and kill bacteria (Boman 2003; Cormican et al. 2008; Conlon & Sonnevend 2010). Host defense AMPs work to enhance host organism immune systems (Hancock & Sahl 2006; Steinstraesser et al. 2011).

They are able to stimulate the expression of cytokines, which, in turn, control the subsequent immune response (Garofalo 2010).

Furthermore, some AMPs play an anti-inflammatory role by blocking the inflammatory response and septic shock in host organisms (Zhang & Falla 2010).

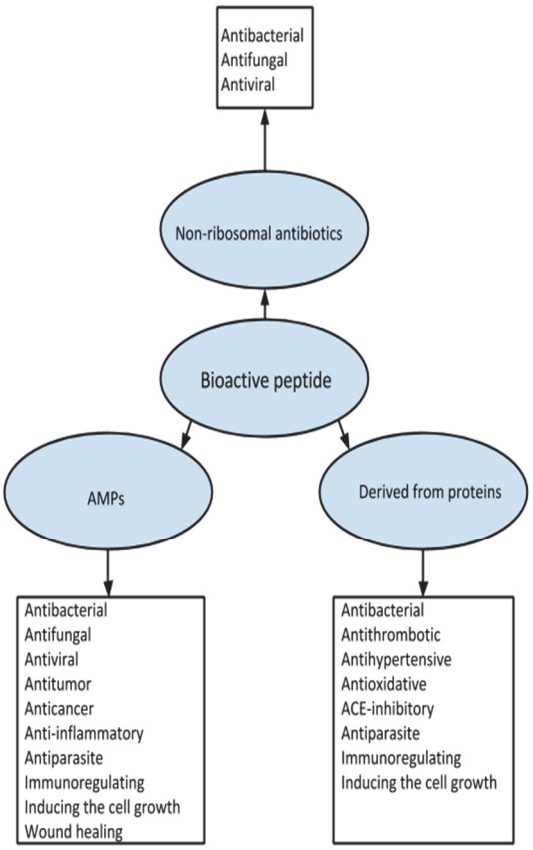

Figure 1. Different types of bioactive peptides and their activities.

Other AMPs are effective agents against various fungi and commensal yeasts, which could turn infectious after the attenuation of the immune systems in several plants and animals (Ng 2004). Some fungi also produce antifungal peptides to combat other competing fungi (Gun Lee et al. 1999).

Many bacteria have adopted the same strategy, such as *Lactococcus lactis,* which produces an antibacterial peptide nisin that is able to kill other Gram positive bacteria (Reddy et al. 2004).

Some AMPs are thought to impede the spread of Human Immunodeficiency Virus (HIV) and herpes (Zasloff 2002). Furthermore, studies show that AMPs could prevent the transmission of HIV from mother to infant during breast feeding (Bosire et al. 2007; Kuhn et al. 2007). Besides their use in virus therapy, AMPs have also been implicated in the control of cancer. When these peptides are not toxic to healthy eukaryotic cells, but interact electrostatically with bacteria and cancer cells, they could be used to develop new anticancer drugs (Hoskin & Ramamoorthy 2008). Finally, AMPs also function as growth promoting factors and participate in wound healing, thereby contributing to the integrity and health of the organism (Yeung et al. 2011).

Milk and particularly colostrum are considered sources of useful AMPs that are beneficial by specifically defending the mammalian organism from infection. Colostrum peptides have been suggested to provide antimicrobial defense against invasive pathogens and support both the mother and newborn immune system. The most well characterized AMPs in both the human and bovine genomes are defensins and cathelicidins. Both families are comprised of small AMPs that range between 20-40 amino acids with cationic and amphiphilic structures (Wiesner & Vilcinskas 2010). Besides AMPs, milk and colostrum are rich in small peptides derived from proteins that inhibit the angiotensin I-converting enzyme (ACE) (Korhonen & Pihlanto 2006; Korhonen 2010) and provide antioxidative and growth promoting effects that accelerate both growth and healing of the organism (Korhonen & Pihlanto 2006; Erdmann et al. 2008; Godhia & Patel 2013).

The aim of this work is to investigate the presence of various AMPs in colostrum and characterize their potential role as antimicrobial and immune system enhancing agents.

2. Literature review

2.1. Milk and colostrum

Milk is a complex bio-fluid synthesized in the mammary gland and provides an ample supply of nutrients and protective factors (Table 1). Colostrum is a term for the early milk produced a few days before and after delivering the baby and is rich in proteins compared with milk (Fig. 2). These include lactoferrin, immunoglobulins (Ig) and growth factors (Playford et al. 2000; Korhonen 2010; Godhia & Patel 2013). The protein concentration in bovine colostrum can be 149 mg/ml and is much higher than the 37 mg/ml typical of human colostrum (Table 1).

Table 1. Nutritional composition of human and bovine colostrum and milk.

Nutritional component	**Colostrum, (mg/ml)**		**Milk, (mg/ml)**		**Reference**
	Human	**Bovine**	**Human**	**Bovine**	
Protein	37.0[1]	149[1]	12.0[2]	32.0[3]	[1](Godhia & Patel 2013) [2](Ballard & Morrow 2014) [3](Haug et al. 2007)
Casein	3.0[4]	Low concentration[5]	3.2-5.6[4]	28.0[6]	[4](Kunz & Lönnerdal 1990) [5](Christiansen 2010) [6](Morris, G 2002)
Immunoglobulin	0.43-17.35[7]	20-150[8]	0.04-1.0[7]	0.5-1.0[8]	[7](Stelwagen et al. 2009) [8](Korhonen & Pihlanto 2006)
β-lactoglobulin	Not detected[9]	8.0[10]	Not found[9]	3.3[10]	[9](Korhonen 2009) [10](Korhonen 2010)
α-lactalbumin	3.6-5.0[11]	3.0[10]	1.03-1.57[12]	1.2[10]	[11](Montagne et al. 1999) [12](Lönnerdal & Glazier 1985)
Lactoferrin	7.0[13]	1.5[13]	1.0[13]	0.1[8]	[13](Brock 1980)
Lactose	53.0[1]	26.0[1]	78.0[2]	53.0[3]	
Fat	29.0[1]	67.0[1]	36.0[2]	33.0[3]	

Senda et al. (2011) found that the concentration of caseins and immunoglobulins drastically decrease in bovine colostrum 48 hours postpartum, while the concentration of both α-lactalbumin and β-lactoglobulin increase (Senda et al. 2011).

Later, colostrum turns into mature milk that is more dilute and contains less protein mass and more sugars than colostrum (Table 1). For example, human milks contains 87 % water, roughly 4% fat, and 9% other compounds such as lactose, proteins, vitamins, and minerals (Zimecki & Kruzel 2007).

2.1.1. Major proteins in milk and colostrum

The milk and colostrum of most mammals contains many casein micelles, which act to fluidize proteins and solubilize both calcium and phosphate (Farrell et al. 2002). Milk and colostrum contain α_{S1}-casein, α_{S2}-casein, β-casein, and κ-casein and the molecular weights of these proteins range between 20-26 kDa. Caseins are phosphorylated on several serine residues in the Golgi complex to promote interaction with calcium: α_{S1}- casein has eight, β-casein has five, and κ-casein one phosphoserine residue (Kumosinski et al. 1991; Kumosinski et al. 1993a; Kumosinski et al. 1993b). These phosphate groups bind calcium and form large molecular micelles to encapsulate and deliver Ca^{2+} (Farrell et al. 2002). κ-casein is responsible for stabilizing casein micelles and causes micelle coagulation after hydrolysis by rennet (Creamer et al. 1998). In addition, casein micelles are capable of binding antioxidants of plant origin through both hydrophobic and hydrophilic interactions (Bourassa et al. 2013). Moreover, electrostatic binding to casein provides protection against enzymatic degradation for some important small molecules, including insulin (Zhang et al. 2008) and piscidin (Kütt & Stagsted 2014).

The most abundant class of proteins in colostrum and milk are whey proteins. During normal lactation, the proportion of whey proteins within total protein in human milk varies from 80% in early lactation to 50% in late lactation (Lönnerdal 2003).

In bovine colostrum, the proportion of whey proteins and caseins is 60-80% at first and decreases to ~20% in mature milk (Korhonen 2010). The major bioactive proteins in whey are immunoglobulins, β-lactoglobulin, α-lactalbumin, lactoferrin, lactoperoxidase, lysozyme, and growth factors (Ibeagha-awemu et al. 2010; Korhonen 2010).

The most abundant immunoglobulin in bovine colostrum and milk is IgG_1, while IgA is the most abundant immune protein in human milk (Stelwagen et al. 2009). Still, bovine colostrum also contains IgG_2, IgM, and IgA (Stelwagen et al. 2009). Immunoglobulins are acquired intact from both colostrum and milk and play a critical role in providing passive immunity to mammalian newborns (Korhonen et al. 2000; Stelwagen et al. 2009). During late lactation, immunoglobulins are partially replaced by α-lactalbumin (16 kDa) and β-lactoglobulin (19 kDa) (Levieux & Ollier 1999; Senda et al. 2011). β-lactoglobulin constitutes between 5 to 10% of all proteins in bovine milk (Table 1) and half of all proteins found in whey. It functions as a carrier for vitamins, lipids, and peptides, and provides antioxidative, immune system enhancing, anticarcinogenic, and antibacterial properties (Korhonen 2009; Korhonen 2010). α-lactalbumin functions within the lactose synthase protein complex in mammary glands, and is an important factor for lactose production and a valuable source of essential amino acids for newborn mammals. Moreover, peptide fragments from α-lactalbumin hydrolysis display ACE-inhibitory, antimicrobial, and anticarcinogenic activity (Chatterton et al. 2006).

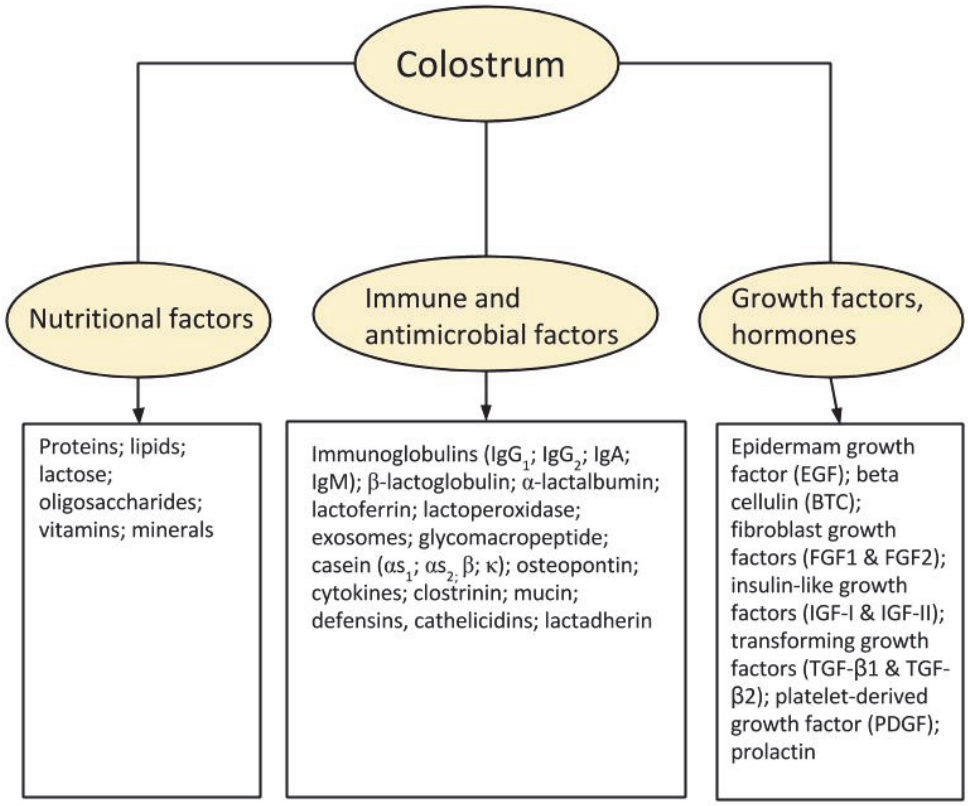

Figure 2. The main components of bovine colostrum.

The main antimicrobial protein within colostrum and milk is lactoferrin, which is a glycoprotein with a molecular weight of ~80 kDa (Brock 1980; Saito 2009; Korhonen 2010). Both human and bovine lactoferrin is encoded by the LTF gene and the protein is stored in secondary granules of neutrophils. Degranulation releases this bioactive protein into blood or colostrum and milk, depending on the location of the neutrophils (Brock 1980; Adlerova et al. 2008). In human colostrum, the lactoferrin concentration is seven times higher than in bovine colostrum (Table 1). The main function of lactoferrin is to bind and facilitate absorption of ferric ions in newborn mammals (Brock 1980; González-Chávez et al. 2009).

Furthermore, lactoferrin possess antibacterial, antiviral, anti-inflammatory, antioxidative, and antiparasite activity (Adlerova et al. 2008; González-Chávez et al. 2009; Park 2009; Korhonen 2010). These functionalities are all facilitated by the tight binding or sequestering of iron, which is essential for bacterial metabolism, which explains the observed antibacterial effect (Fig. 3) (Zimecki & Kruzel 2007).

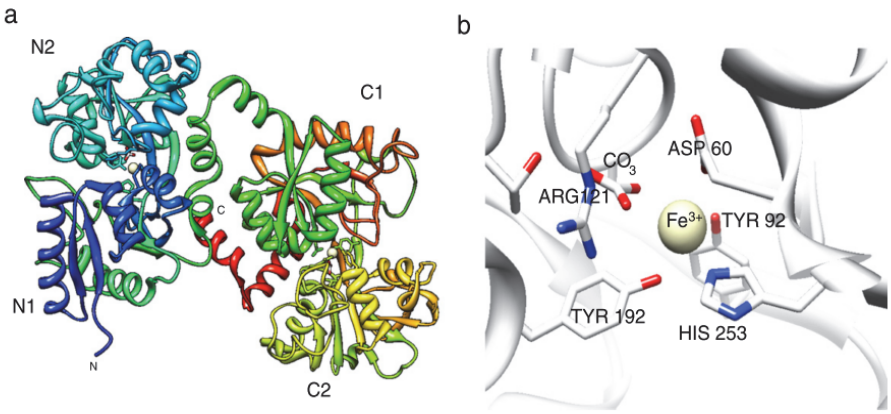

Figure 3. A predicted structure of lactoferrin. a) Lactoferrin polypeptide contains two homologous globular domains, termed the N (amino)-and C (carboxy)-lobes. These both contain two subdomains, N1, N2 and C1, C2. The N-lobe contains one iron binding site and the C-lobe another (indicated with small white globes). The small c illustrates the C-terminus and small N the N-terminus and rainbow coloring indicates the transition from the C- to the N- terminus. b) Iron-binding pocket in the N-lobe of lactoferrin. The iron interacts with one aspartic acid, one histidine, and two tyrosine residues; the arginine residue is responsible for binding a carbonate ion (adopted from (García-Montoya et al. 2012)).

There are several small peptides that result from the enzymatic degradation of lactoferrin that display strong antimicrobial activity. For example, lactoferricin B (FKCRRWQWRMKKLGAPSITCVRRAF) is generated from the lactoferrin$^{36\text{-}60}$ sequence after pepsin digestion. Lactoferricin B has antifungal and antibacterial activity and induces bacterial membrane perturbation (Wakabayashi et al. 1999).

Lactoferrampin (WKLLSKAQEKFGKNKSR) is a bioactive peptide derived from the N1-domain of lactoferrin$^{268-284}$ and has antibacterial and antifungal activity. The antimicrobial activity of this peptide is due to the positively charged amino acids near the C-terminal end, whereas the N-terminal part forms the helical structure (van der Kraan et al. 2005)

Another antimicrobial protein is lactoperoxidase (69 kDa), which forms 1% of whey proteins and is the most abundant enzyme in bovine milk (Smolenski et al. 2007; Saito 2009; Alonso-Fauste et al. 2012). The main function of lactoperoxidase in milk is to catalyze the oxidation of thiocyanate by hydrogen peroxide (Saito 2009) to produce a strong antimicrobial agent hypothiocyanite (Fonteh et al. 2002).

Cytokines are polypeptides expressed in the human and bovine mammary gland that have pro- and anti-inflammatory properties (Compton et al. 2009; Garofalo 2010). Cytokines, such as interleukins (IL) and the tumor necrosis factor (TNF), tend to bind with cellular factors and contribute to both developmental and immune system functions (Garofalo 2010). Furthermore, osteopontin (~31 kDa), the major matrix protein in bones that is also found in milk, has cytokine-like properties (Reinholt et al. 1990) that are primarily responsible for both the maintenance and health of bones and teeth, but also accelerates wound healing (Sodek et al. 2000). In addition, a mixture of proline-rich polypeptides (500-3000 Da), termed clostrinin, can be derived from colostrum. Clostrinin peptides are either specifically secreted or arise from the degradation of proteins. Clostrinin peptides are responsible for leukocyte proliferation and induce expression of cytokines (Kruzel et al. 2001). The antiviral activity of colostrum is provided by mucins, which are high molecular weight glycoproteins that are secreted into milk and transferred into the immunologically naïve neonatal gut to suppress the invasion of rotavirus causing diarrhea (Dowbenko et al. 1993; Newburg & Walker 2007). Mucin-lipid aggregates impede the virus through a fat globule-mucin conglomerate, which binds the virus and deactivates it (Yolken et al. 1992).

2.1.2. Small peptides in milk and colostrum

Milk and colostrum both contain short peptide sequences embedded within various proteins that are inactive when part of the proteins, but become active when released during food processing, fermentation, and gastrointestinal digestion by pepsin, trypsin, and chymotrypsin (Korhonen & Pihlanto 2006; Zimecki & Kruzel 2007; Korhonen 2010).

During fermentation of cheese, yoghurt, and sour-milk, bioactive small peptides derived from milk proteins are released (Korhonen & Pihlanto 2006; Erdmann et al. 2008). These peptides have antihypertensive effects by inhibiting the activity of ACE and therefore reducing blood pressure. ACE-inhibitory peptides are only a few amino acids long and are mainly derived from casein and whey proteins (Fitzgerald et al. 2004).

The most well described ACE-inhibitory peptides are VPP and IPP derived from β- and κ-casein by *Lactobacillus helveticus* in fermented sour milk. Both of these tripeptides have been tested in clinical trials (Korhonen & Pihlanto 2006; Zimecki & Kruzel 2007; Sharma et al. 2012). Antihypertensive peptides are found in cheese (αS_1- casein fragments 1-6, 1-7, and 1-9 and β-casein fragments 58-72 and 60-68) and in sour milk (β-casein fragments 74-76 and 84-86 and κ-casein fragment 108-111). Yoghurt also exhibits weak ACE-inhibitory activity, however, the peptide fragments have not yet been identified (Korhonen & Pihlanto 2006).

Moreover, milk peptides have other health promoting activities. For example, the glycomacropeptide (GMP) is derived from κ-casein after enzymatic digestion. Chymosin cleaves κ-casein between Met ^{105} and Phe ^{106}, thereby releasing para-κ-casein fragment 1-105 and GMP fragment 106-169 (Saito 2009). GMP prevents cavities, clotting of blood, but also provides antiviral and antibacterial activity (Shah 2000; Korhonen & Pihlanto 2006; Zimecki & Kruzel 2007). These GMPs are also active in the intestines of infants and induce growth of bifidobacteria (Idota et al. 1994; Park 2009).

In addition, para-κ-casein is an important source of disulfide/sulfhydryl groups derived from cysteine/cysteine residues that promote bifidobacterial growth (Poch & Bezkorovainy 1991). A flourishing commensal microbiota effectively inhibits the growth of invasive pathogens and prevents development of gastrointestinal diseases of the newborn (Brody 2000). Furthermore, small peptides released from caseins have antioxidative effects via free radical scavenging and thereby prevent lipid peroxidation (Korhonen & Pihlanto 2006).

Small peptides, such as immunopeptide (LLY) and β-Casokinin-10 (YQQPVLGPVR), derived from β-casein have immunostimulatory effects (Park 2009). Moreover, casein fragments can have both opioid agonist or antagonist activity, meaning that these peptides have morphine-like effects that either activate or block opioid receptors (Shah 2000). For example, some opioid agonists are derived from β-casein (YPFPG; YPFVE; YPFP-NH_2) and opioid antagonists from κ-casein (YPSY(O-CH_3); YPSY-GLNY; YPYY(O-CH_3); YIPIQYVLSR) (Shah 2000; Park 2009).

Other milk proteins also contain sequences of small active peptides, such as α-lactalbumin$^{50-53}$ (YGLF) and β-lactoglobulin$^{142-148}$ (ALPMHIR), that have ACE-inhibitory activity (Shah 2000). Small bioactive peptides within functional foods may strengthen resistance towards cardiovascular (Erdmann et al. 2008) and gastrointestinal diseases (Donnet-Hughes et al. 2000; Playford et al. 2000), prevent arthritis (Korhonen 2010), and are used in the therapy of both psoriasis (Pouliot & Gauthier 2006) and Crohn's disease (Fell et al. 2000).

2.1.3. Growth promoters in milk and colostrum

Colostrum may be the most important source of bioactive peptides in the early stage of newborn development and may provide growth promoting and health-strengthening components (Fig. 2).

The main growth factors in bovine colostrum are the epidermal growth factor (EGF) family that includes beta cellulin, fibroblast growth factors (FGF1 & FGF2), insulin-like growth factors (IGF-I & IGF-II), transforming growth factors (TGF-β1 & TGF-β2) and platelet-derived growth factor (Gauthier et al. 2006; Korhonen 2010). These growth factors are between 6-30 kDa, and are found in higher concentrations in colostrum (up to 40 mg/L) compared with milk (up to 2 mg/L). Their concentrations are highest during the first hours after calving and drop dramatically afterwards. These bioactive molecules may contribute to cell growth, induce immune system responses and protect and repair tissues (Korhonen 2010). It has been shown that growth factors will survive heat treatment, pasteurization, and strong denaturants, which indicates that these peptides are stable and may retain their functionality in processed colostrum and milk products (Gauthier et al. 2006).

2.1.4. AMPs in milk and colostrum

AMPs in milk and colostrum provide protection for the mammary glands of lactating mammals (Murakami et al. 2005; Armogida et al. 2011) and are probably important for defense against mastitis (Roosen et al. 2004; Swanson et al. 2004). However, AMPs also provide growth-inducing and immune-enhancing molecules for the newborn (Bosire et al. 2007; Wang et al. 2014). Milk and colostrum contain two major groups of AMPs: defensins and cathelicidins. These are either secreted into the milk by epithelial cells of the mammary gland or by immune cells, e.g. neutrophils, present in both milk and colostrum (Reddy et al. 2004). The research that explores the roles of these AMPs in both milk and colostrum is analyzed in the following sections.

2.2. Peptides with antimicrobial activity

There are two major classes of antimicrobial peptides. The first class is mainly produced by bacteria and fungi and represents non-ribosomally synthesized peptide antibiotics, which contain non-proteinogenic, D-amino acids and often have a cyclic or branched structure (Wiesner & Vilcinskas 2010).

These antimicrobial peptides, such as tyrocidine, bacitracin, bacillibactin, vancomycin, cyclosporine, and surfactin, are produced by non-ribosomal peptide synthetases (Marahiel 2009; Wiesner & Vilcinskas 2010).

The second class is gene encoded and synthesized by ribosomes in a wide variety of organisms. Proteomic studies in mammals show that from all identified proteins and peptides, between 15 to 30 % are involved in various defense mechanisms (Ibeagha-awemu et al. 2010; Addis et al. 2011; Hettinga et al. 2011). This class of peptides can be subdivided into subgroups (Fig. 1). Cryptic peptides are one such group and are produced by enzymatic degradation of proteins (Zimecki & Kruzel 2007). Another subgroup, termed lantibiotics, contains members that are widely used as preservatives in the food industry. The most well-known lantibiotic is nisin from *Lactococcus lactis* (Liu & Hansen 1990; de Vos et al. 1993; Willey & van der Donk 2007), which contains unusual residues of dehydroalanine and dehydrobutyrine and has Gram positive antimicrobial activity already at nanomolar concentrations (Yeaman & Yount 2003; Reddy et al. 2004). The third subgroup are AMPs which include defensins and cathelicidins that are specifically expressed in immune-competent cells and released in response to infection or injury (Hancock & Lehrer 1998). It is this group that is the primary focus of the work presented herein.

2.2.1. Host defense antimicrobial peptides (AMPs)

AMPs are also termed host defense peptides (Brown & Hancock 2006) and are produced by immune-competent cells to provide a rapid and efficient defense against a wide variety of pathogens. AMPs are present in a wide variety of organisms, including, vertebrates (Andreu & Rivas 1998; Brogden et al. 2003; Zairi et al. 2009; Wang et al. 2011), invertebrates (Andreu & Rivas 1998; Dimarcq et al. 1998; Reddy et al. 2004; Li et al. 2012), bacteria (Aymerich et al. 1996; Corr et al. 2007), fungi (Gun Lee et al. 1999; Rodríguez-Martín et al. 2010), and plants (García-Olmedo et al. 1998; Zasloff 2002).

For example, AMPs have been investigated in the mammary glands and milk of several domestic ruminants, including, sheep (Addis et al. 2011; Addis et al. 2013), goat (Zhang et al. 2014), and buffalo (Das et al. 2009). Australian mammals, such as the tammar wallaby, produce a promising antimicrobial cathelicidin WAM1 which works effectively against treatment-resistant *P. aeruginosa* without affecting the viability of human red blood cells (Wang et al. 2011). Moreover, frogs from tropical areas produce dermaseptins and magainins in their skin glands which could be used in the future as spermicidal contraceptives and for sexually transmitted infection control (Zairi et al. 2009). Piscidins, a family of AMPs derived from fish and used as model AMPs during my study, have strong bacterial cell disruption activity yet do not harm eukaryotic cells (Campagna et al. 2007; Noga et al. 2009).

AMPs kill invasive and hazardous parasites, bacteria, fungi, and viruses and are present throughout the body, from the stomach, intestines, and kidney to saliva, sweat, blood, and milk, to small cells such as epithelial and mast cells, neutrophils, and lymphocytes (Bals et al. 1998; Reddy et al. 2004; Zanetti 2005). AMPs are particularly abundant in cells exposed to the external environment, e.g., lungs, the gastrointestinal tract, and skin, because this is the first line of defense and also highest numbers of pathogenic insults occur in these tissues (Bals et al. 1998; Cole et al. 2000; Dorschner et al. 2003).

Most AMP genes are clustered in one region of the genome and are translated as prepropeptides. The N-terminal pre-segment is responsible for forming stable secondary polypeptide structures via disulfide bonds. The mature C-terminal and often cationic antimicrobial segment acquires its activity only after cleavage of the pro-sequence (Reddy et al. 2004). The antimicrobial part could be cleaved off during subsequent intracellular processing or as part of the secretion process of the mature protein into the extracellular space (Bals 2000).

AMPs usually contain between 12 to 50 (less than 100) amino acids (Wiesner & Vilcinskas 2010). AMPs are typically cationic and amphiphilic and contain several positively charged amino acids and a clear segregation of hydrophilic and hydrophobic parts in their structure. However, there are also reports of anionic AMPs that contain no basic amino acids (Lai et al. 2002; Lai et al. 2007). The structure of AMPs varies from a linear cysteine free α-helix and β-sheet with stabilized disulfide bridges to unusual structures with an excess of a particular amino acid (Brogden et al. 2003; Wiesner & Vilcinskas 2010), e.g., tryptophan or proline. Human and bovine defensins and cathelicidins are two main antimicrobial families with several members.

2.2.1.1. Defensins

Defensin peptides are between 18-45 amino acids long, amphiphilic, and positively charged (Fig. 4) (Selsted & Ouellette 2005). Defensins contain six conserved cysteine residues, and the position of these in the sequences divides the group into α- and β-defensins that form triple-stranded β-hairpin structures with three or four disulfide bridges (Boman 2003; Wiesner & Vilcinskas 2010; Li et al. 2012). α-defensins are only found in mammals, whereas β-defensins are expressed in many different vertebrates, invertebrates and plants (Boman 2003). Some primates also express θ-defensins, which are modified α-defensin genes (Selsted & Ouellette 2005). θ-defensins contain double-stranded β-sheets, made from two separate defensins and united with disulfide bonds (Boman 2003).

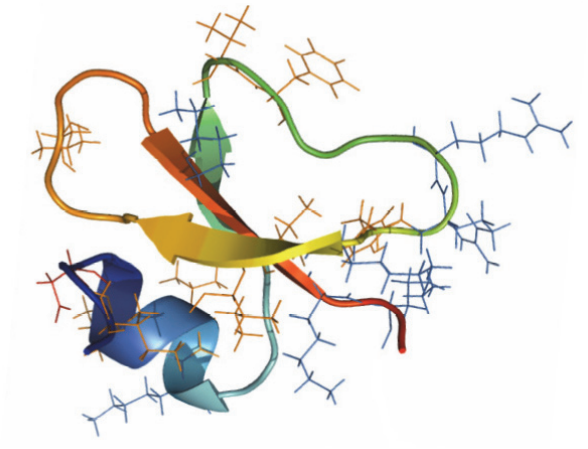

Figure 4. Monomeric structure of human β-defensin 2 (HBD-2). Rainbow coloring indicates transition from the N- to C-terminus. Cationic residues are marked with blue, hydrophobic residues are orange, and anionic residues are red. Adopted from (Suresh & Verma 2006).

Human α-defensins are mainly located in neutrophils and macrophages, but are also found in other leukocytes and small intestinal Paneth cells (Grigat et al. 2007). Human β-defensins are mainly produced by epithelial cells (Yang et al. 2002).

Defensins have activity against several pathogens, including, viruses, protozoa, fungi, and bacteria (Yang et al. 2002; Selsted & Ouellette 2005). The direct killing effect of defensins is due to electrostatic binding to negatively charged bacterial membrane components, followed by membrane permeabilization and cell death (Yeaman & Yount 2003). Additionally, defensins take part in innate and adaptive immune defenses through toll-like receptor pathways, including, attracting lymphocytes, inducing epithelial cell growth, and participating in wound healing (Zasloff 2002; Wiesner & Vilcinskas 2010).

2.2.1.1.1. Defensins in the bovine mammary gland, milk and colostrum

A study of the *Bos taurus* genome indicates that several β-defensins (Table 2) are encoded in a wide range of body parts, like the digestive and respiratory tracts and reproductive system (Cormican et al. 2008). However, there is no evidence in the literature or proteomic databases that cows express α-defensins (Fjell et al. 2008).

Mastitis and rise in somatic cell count (SCC) are the main accelerators that enhance the expression of AMPs (Isobe et al. 2013; Kawai et al. 2013). Quantitative reverse transcription polymerase chain reaction (RT-PCR) analyses show expression of the β-defensin family: lingual antimicrobial peptide (LAP), tracheal antimicrobial peptide (TAP), bovine β-defensin 4 and 10 in different parts of the bovine udder (Tetens et al. 2010; Whelehan et al. 2011). These AMPs provide protection against invasive pathogens (Fig. 5).

Other β-defensin family members are expressed at different pathological stages during infection of the cow's udder, such as neutrophil β-defensins (BNBD3, BNBD9, BNBD12). A novel β-defensin, DEFB401, has only been found in lactating cows that have mastitis caused by *Actinomyces pyogenes* (Roosen et al. 2004). Some novel β-defensins (BBD119, BBD120, BBD122, BBD122A BBD123, BBD124) have also been found in humans, however, none of these are produced by the mammary gland (Semple et al. 2003; Radhakrishnan et al. 2005). In contrast, these defensins are detected in both bovine mammary glands and mammary cells and are secreted into milk (Cormican et al. 2008).

For example, neutrophil β-defensin 5 (BNBD5) is detected in both uninfected (Cormican et al. 2008; Compton et al. 2009) and mastitic glands, however, the concentration is more than tenfold higher in the latter (Goldammer et al. 2004). During bacterial infection, which triggers the expression of β-defensin 5, αS_1-casein expression is shut down completely.

Table 2. Bovine defensins and sequences of the antimicrobial part of propeptides (combined using UniProt BLAST database and CLUSTAL O (1.2.1) multiple sequence alignment). Red letters indicate positively charged amino acids at pH 7.4 (Lysine – K; Histidine – H; Arginine – R).

```
β-defensin 119     --RRHMLRCMGDLGICRPACRQS-EEPYLYC-RNYQPCCLPFYVRIDISGKEGKNDWSRENRWPKVS

β-defensin 1       ----DFASCHTNGGICLPNRCPGHMIQIGICFRPRVKCCRSW-------------------------

β-defensin 11      ----GPLSCRRNGGVCIPIRCPGPMRQIGTCFGRPVKCCRSW-------------------------

β-defensin 12      ----GPLSCGRNGGVCIPIRCPVPMRQIGTCFGRPVKCCRSW-------------------------

β-defensin 13      SGISGPLSCGRNGGVCIPIRCPVPMRQIGTCFGRPVKCCRSW-------------------------

β-defensin 4       QRVRNPQSCRWNMGVCIPFLCRVGMRQIGTCFGPRVPCCRR--------------------------

β-defensin 5       QVVRNPQSCRWNMGVCIPISCPGNMRQIGTCFGPRVPCCRRW-------------------------

β-defensin 10      QGVRSYLSCWGNRGICLLNRCPGRMRQIGTCLAPRVKCCR---------------------------

β-defensin 6       QGVRNHVTCRIYGGFCVPIRCPGRTRQIGTCFGRPVKCCRRW-------------------------

β-defensin 2       --VRNHVTCRINRGFCVPIRCPGRTRQIGTCFGPRIKCCRSW-------------------------

β-defensin 3       QGVRNHVTCRINRGFCVPIRCPGRTRQIGTCFGPRIKCCRSW-------------------------

β-defensin 9       QGVRNFVTCRINRGFCVPIRCPGHRRQIGTCLAPQIKCCR---------------------------

β-defensin 7       QGVRNFVTCRINRGFCVPIRCPGHRRQIGTCLGPRIKCCR---------------------------

β-defensin 8       --VRNFVTCRINRGFCVPIRCPGHRRQIGTCLGPQIKCCR---------------------------

LAP                --VRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK-------------------------

TAP                ----NPVSCVRNKGICVPIRCPGSMKQIGTCVGRAVKCCRKK-------------------------

                              *    *.*             *       **
```

Defensin	AMP position in protein sequence	MW of peptide	
		MW of AMP, Da	MW of total pro-peptide, Da
β-defensin 119	21-83	7583	9805
β-defensin 1	1-38	4278	4278
β-defensin 11	23-60	4163	6507
β-defensin 12	1-38	4106	4106
β-defensin 13	1-42	4450	4450
β-defensin 4	23-63	4783	7161
β-defensin 5	23-64	4808	7228
β-defensin 10	23-62	4527	6928
β-defensin 6	1-42	4839	4839
β-defensin 2	1-40	4649	4649
β-defensin 3	16-57	4834	6325
β-defensin 9	16-55	4559	6049
β-defensin 7	23-62	4573	6964
β-defensin 8	1-38	4359	4359
LAP	25-64	4464	7041
TAP	27-64	4091	6954

This demonstrates that when some defense mechanisms are initiated, nutrient production is delayed. This causes a reduction in the yield of milk (Yang et al. 2006; Petzl et al. 2008).

Mammary epithelial cells express the β-defensin LAP in both healthy and mastitic bovine mammary glands (Roosen et al. 2004; Isobe et al. 2009a) where they are further secreted into milk (Petzl et al. 2008). During mastitis, mammary epithelial cells increase expression of LAP 50 fold compared with healthy tissue (Tomasinsig et al. 2010). However, the concentration and kinetics of the expression of LAP mRNA is independent of the particular bacteria that caused mastitis and LAP exhibit a late immune response that does not depend on the pathogen load (Günther et al. 2010).

Furthermore, LAP may be activated by pro-inflammatory cytokines because defensins have been expressed during cytokine-induced fever during mastitis (Petzl et al. 2008). More precisely, the LAP concentration in milk increases 2 h after *E. coli* infection and persists at a high concentration for 12 h up to a few days (Isobe et al. 2009b; Huang et al. 2012). After this time the immune-stimulating activity of LAP may have initiated an appropriate immune response (Isobe et al. 2009b).

Comparing LAP expression with other widely ubiquitous defense proteins in milk such as lactoferrin, it seems that LAP and lactoferrin are localized in different parts within mammary glands. Furthermore, LAP expression in the epithelial cells of the alveolar epithelium eliminates the presence of lactoferrin and vice versa. Moreover, when analyzing milk samples, lactoferrin expression was detected two days after infection, whereas LAP expression was observed shortly after infection (Huang et al. 2012). These observations indicate that AMP response may be biphasic with a rapid response of LAP aimed at killing as many bacteria as possible and thereby attracting neutrophils to the mammary gland, and subsequently increase in the expression of an iron-sequestering protein for bacteriostatic purposes.

β-defensin TAP is found in healthy cow's udder (Roosen et al. 2004), whereas expression of TAP mRNA has been detected in both bovine mammary gland tissue and mammary epithelial cells (Cormican et al. 2008). Moreover, TAP expression increases in mammary gland infected by *S. aureus* (López-Meza et al. 2009; Yang et al. 2013). However, in an attempt to avoid mastitis in ruminants, a mammary gland tissue-specific expression vector carrying a bovine TAP gene was successfully transferred into the lactating mammary gland of goats. Milk samples from this system display antimicrobial activity against both *E. coli* and *S. aureus.* The milk samples from negative control animals did not suppress the growth of bacteria (Zhang et al. 2007).

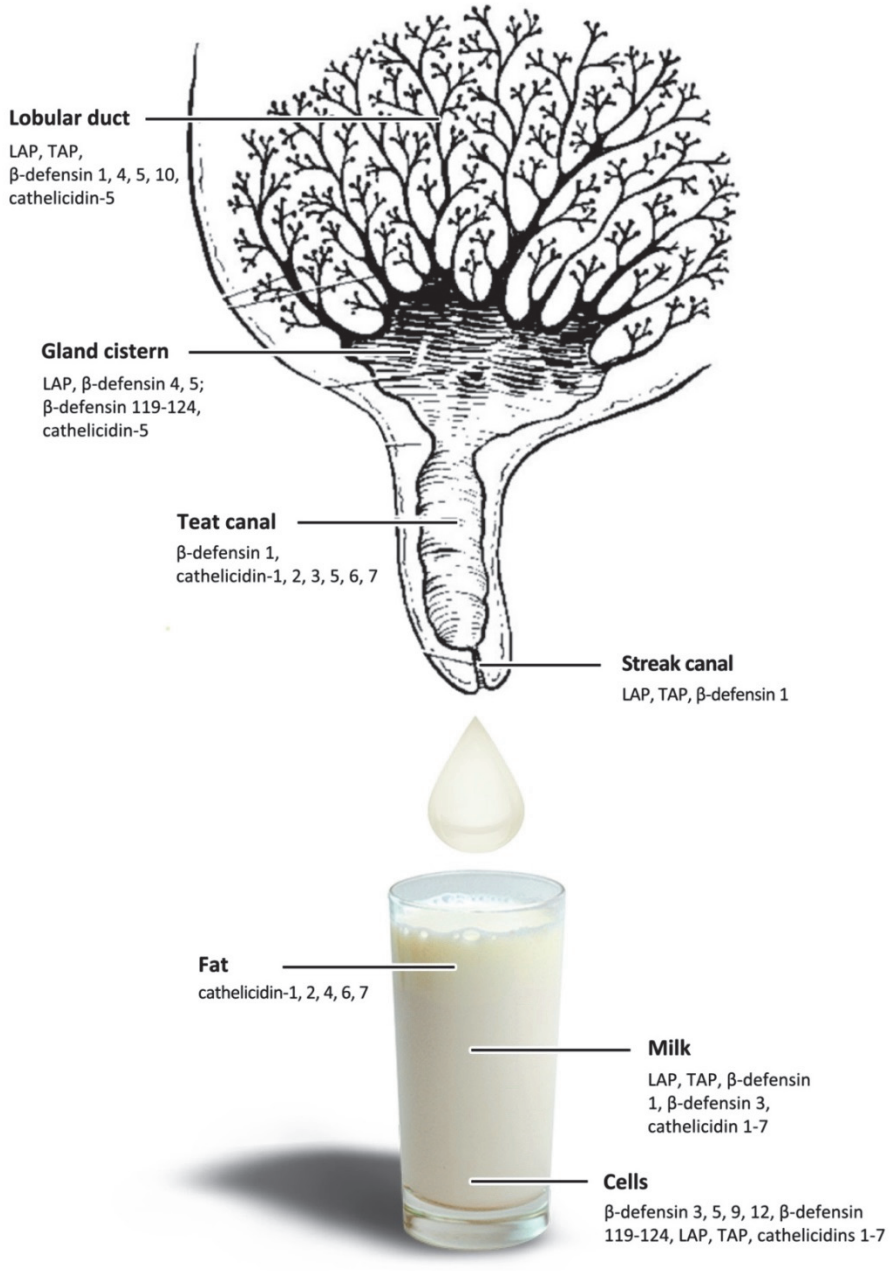

Figure 5. Main defensins and cathelicidins expressed in the udder and secreted into milk. The illustration provides a cross-section of the bovine udder that indicates the location of primary defensins and cathelicidins. These AMPs are secreted into milk and may be found in the fat, skimmed, and cell fractions of milk.

2.2.1.2. Cathelicidins

Cathelicidins are well characterized antimicrobial peptides that are expressed and stored in cells as two-domain proteins (Zanetti 2005). Cathelicidin genes contain four exons, where exons 1 to 3 encode a highly conserved N-terminal cathelin-like domain and exon four encodes the antimicrobial domain that constitutes the mature protein (Whelehan et al. 2014).

The cathelin-like domains are 99-114 amino acids long and their name is derived from cathepsin L, because cathelicidins display cathepsin L inhibitor activity (Bals & Wilson 2003). The antimicrobial C-terminus (Fig. 6), typically between 12 to 100 amino acid residues long, is released via extracellular proteolysis from the holoprotein to become active (Heilborn et al. 2005; Takagi et al. 2012).

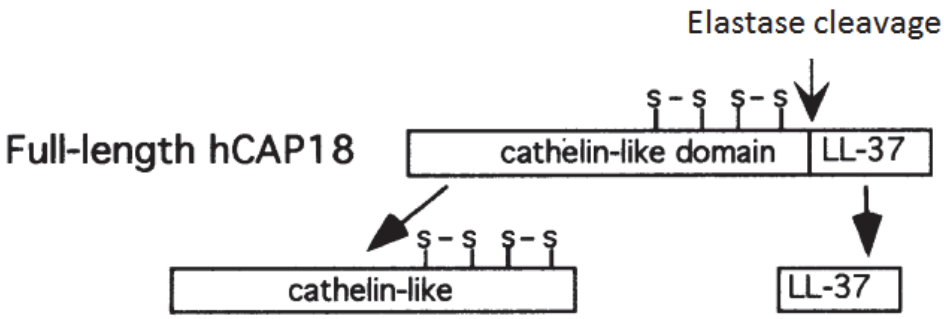

Figure 6. Schematic representation of cathelicidin cleavage by elastase. An inactive full-length precursor is cleaved by enzymes to release two distinct peptides with complementary actions. S-S indicates disulfide bridges (adopted with modifications from (Zaiou et al. 2003)).

The mature cathelicidin peptides are divided into three groups: 1) 23-40 amino acids with a linear α-helix structure (Fig. 7) and no stabilizing disulfide bridges, examples include human LL-37 and bovine BMAP-28 (Zanetti 2005; Wiesner & Vilcinskas 2010); 2) peptides with stabilizing disulfide bridges that form β-hairpin structures (Fig. 7) such as a porcine cathelicidin peptide (Bals & Wilson 2003); 3) peptides enriched with one particular amino acid, such as the proline-rich bovine Bac 5, and 13 residues long indolicidin (Fig. 7) with five tryptophans (Bals & Wilson 2003; Zanetti 2005; Wiesner & Vilcinskas 2010).

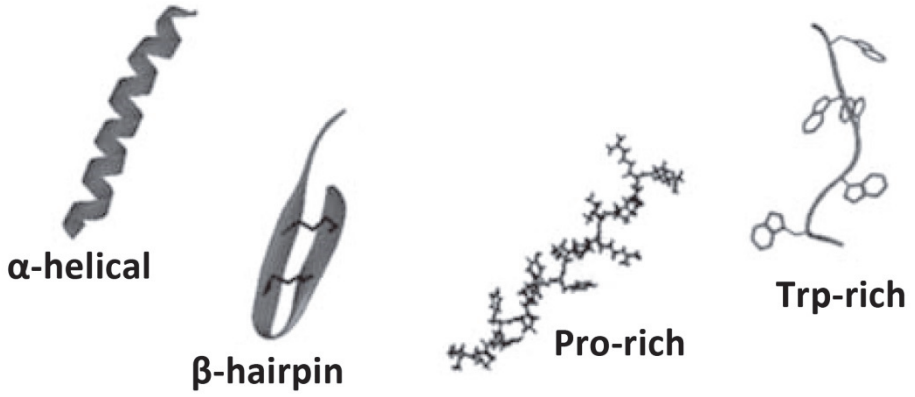

Figure 7. Major structural classes of cathelicidins as exemplified by (left to right) human LL-37; porcine cathelicidin; bovine Bac 5 and bovine indolicidin. Adopted from (Tomasinsig & Zanetti 2005).

Antimicrobial cathelicidins are mainly found in leukocytes, various epithelial cells and granules of macrophages (Boman 2003; Wiesner & Vilcinskas 2010) where they are released from the N-terminus by neutrophil elastase (Hettinga et al. 2011). The main function of cathelicidin is direct killing of invasive pathogens. The electrostatic interactions between the cationic cathelicidins and negatively charged bacterial membrane components leads to cell disruption and death (Tomasinsig & Zanetti 2005).

However, cathelicidins enriched in proline also attack intracellular targets, inhibit DNA and protein synthesis, and thus affect the viability of bacteria (Tomasinsig & Zanetti 2005).

Humans have only one cathelicidin, LL-37, and the content of this AMP is almost five times higher compared with defensins in milk (Bals & Wilson 2003; Murakami et al. 2005; Steinstraesser et al. 2011). Mice also have a single cathelicidin, CRAMP, that has a sequence homologous to human LL-37 (Boman 2003). Contrary to human and mouse, the bovine genome contains several cathelicidins (Mollé et al. 2009; Hettinga et al. 2011; Le et al. 2011). Regardless of species, all cathelicidins display antimicrobial activity against both Gram positive and Gram negative bacteria (Murakami et al. 2005; Wang et al. 2011).

2.2.1.2.1. Cathelicidins in the bovine mammary gland, milk and colostrum

The *Bos taurus* genome has cathelicidin genes clustered in 100 kb long regions located in the long arm of chromosome 22. Seven protein encoding genes, termed CATHL 1-7 (Table 3), are expressed in the mammary gland and in leukocytes isolated from milk (Fig.5) (Whelehan et al. 2014). More precisely, cathelicidins are expressed by epithelial cells in the mammary gland in ruminants (Addis et al. 2013; Whelehan et al. 2014; Zhang et al. 2014) and are up-regulated after infection by coliform pathogens (Ibeagha-awemu et al. 2010). Also, neutrophils in the mammary gland contain cathelicidin peptides, and mRNA expressions for these are increased after *E. coli* treatment (Tomasinsig et al. 2010).

Bovine cathelicidins 5 and 6, also called myeloid antimicrobial peptides (BMAP-27 and 28) and cathelicidin 2 and 4 (Bac 5 and Indolicidin, respectively), show broad antimicrobial activity against 28 mastitic pathogens with varying minimum inhibitory concentration (MIC) values from 0.5 to 32 μM. However, Tomasinsig and colleagues (2010) proposed that lipids and caseins bind AMPs, which hinders the direct killing activity of peptides against pathogens.

Their results show that adding lipid components inactivates Bac5 and indolicidin and suppresses the activity of BMAP-27 and 28. Casein also inactivates Bac5 and indolicidin but did not affect the activity of BMAP peptides (Tomasinsig et al. 2010). The same phenomenon is shown in my publication II, where the antimicrobial activity of piscidin was completely abrogated *in vitro.* This was attributed to electrostatic interactions with caseins (Kütt & Stagsted 2014).

The presence of immune defense peptides, including cathelicidin-1, 3, 5 & 6 in colostrum, indicates that AMPs are present in the early stages of lactation to provide host defense effects to both mother and infant against invading pathogens (Le et al. 2011). In milk and colostrum, cathelicidins (cathelicidin 1; 2; 4; 6; 7) have been detected in the milk fat globule membrane (MFGM) (Smolenski et al. 2007; Affolter et al. 2010; Hettinga et al. 2011). For example, a proteome analysis of MFGM proteins using nano LC-ESI-linear ion trap (LIT)-MS/MS revealed that cathelicidin-1 is only present in pasteurized and ultra-high temperature treated milk, and is not present in either raw milk or nutritional powder for infants (Arena et al. 2011). Therefore, heating of milk may break molecular interactions that prevented detection, and the apparent concentration of cathelicidin peptides increased.

Proteomic analysis of bovine milk samples revealed that in the absence of infection, AMPs occur only to a small extent and the most abundant proteins in milk are caseins, β-lactoglobulin, α-lactalbumin (Boehmer et al. 2008) and bovine serum albumin (BSA) (Boehmer et al. 2010a). With the first signs of mastitis, such as an increase of SCC (Boehmer et al. 2010a; Danielsen et al. 2010), several immune-regulating pathways will be activated and cathelicidin AMPs will be expressed and detected in the milk (Boehmer et al. 2008; Boehmer et al. 2010a; Danielsen et al. 2010; Ibeagha-awemu et al. 2010; Alonso-Fauste et al. 2012).

Transgenic cows that can produce extra AMPs and secrete these into milk may provide additional immune protection and might be beneficial also for human. A study conducted with transgenic cattle that express specific gene(s) from human mammary-glands, shows upregulation of cathelicidin-1 in milk without affecting overall protein profile compared with control (Zhang et al. 2012).

Table 3. Bovine cathelicidins and amino acid sequences of the antimicrobial part of propeptides (combined using UniProt BLAST database and CLUSTAL O (1.2.1) multiple sequence alignment). Red letters indicate positively charged amino acids at pH 7.4 (Lysine – K; Histidine – H; Arginine – R).

```
Cathelicidin-1(Bactenecin-1)  -------RLCRIVVIRVCR-------------------------------------------------------
Cathelicidin-3(Bactenecin-7)  -------RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLP--FPRPGPRP---IPRPLPFPRPGPRPIPRP-
Cathelicidin-2(Bactenecin-5)  DINCNELQSVRF------RPPIRR--------------PPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFP
Cathelicidin-7(BMAP-34)       ---AGLFRRLRDSIR---RGQQKIL----EKARRIGERIKDIFRG----------------------------
Cathelicidin-6(BMAP-27)       ----------------------------GRFKRFRKKFKKLFKKLSPV--------IPLLHLG---------
Cathelicidin-5(BMAP-28)       ----------------------------GGLRSLGRKILRAWKKYGPI-------IVPIIRIG---------
Cathelicidin-4(Indolicidin)   ----------------------I--------------LPWKWPWWPWRRG----------------------
```

Cathelicidin	AMP position in protein sequence	MW of peptide	
		MW of AMP, Da	MW of total pro-peptide, Da
Cathelicidin-1 (Bactenecin-1)	144-155	1486	17600
Cathelicidin-3 (Bactenecin-7)	131-189	6911	21567
Cathelicidin-2 (Bactenecin-5)	131-173	6265	20030
Cathelicidin-7 (BMAP-34)	131-164	4210	18848
Cathelicidin-6 (BMAP-27)	132-158	3283	17852
Cathelicidin-5 (BMAP-28)	132-159	3132	17616
Cathelicidin-4 (Indolicidin)	131-143	1964	16479

2.2.1.3. Piscidin

AMPs such as cathelicidins and defensins have also been found in fish (Uzzell et al. 2003; Chang et al. 2006; Zou et al. 2007). Fish are constantly exposed to bacteria and AMPs are found in their skin, gills, and in the epithelial cells of their intestines (Campagna et al. 2007; Corrales et al. 2010). AMPs are also expressed in leukocytes (Salerno et al. 2007), skin cells (Cole 1997), and immune cells, including mast cells (Noga & Silphaduang 2003).

Piscidins form a family of gene-encoded antimicrobial peptides, so named because they were found in fish (Pisces). Piscidin-1, with a highly conserved amino terminus, contains 22 amino acids, of which seven are positively charged at a neutral pH (two arginines, one lysine and four histidines (Noga & Silphaduang 2003; Campagna et al. 2007). Piscidin-1 has a linear α-helical structure (Fig.8), where polar residues cover one side of helical cylinder and hydrophobic residues are located on the other side (Noga & Silphaduang 2003).

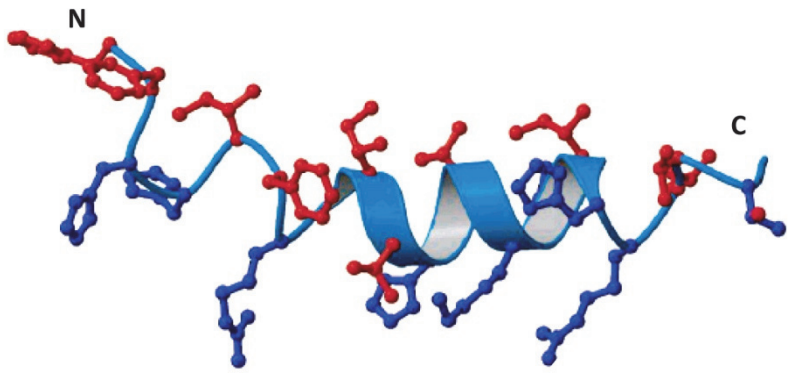

Figure 8. Structure of piscidin-1. Hydrophilic and hydrophobic side chains are colored blue and red, respectively. N- and C-terminal ends of the peptide are indicated (adopted with modifications from (Campagna et al. 2007)).

To date, four piscidins have been identified (Fig. 9). Piscidin-1 and piscidin-2 are 95% homologous, whereas piscidin-3 is more divergent and shows only 70% homology (Noga & Silphaduang 2003). Recently, piscidin-4 was discovered, which has a C-terminal extension compared to piscidins 1-3 (Noga et al. 2009; Corrales et al. 2010).

```
Piscidin-1      FFHHIFRGIVHVGKTI------HRLVTG----------------
Piscidin-2      FFHHIFRGIVHVGKTI------HKLVTG----------------
Piscidin-3      FIHHIFRGIVHAGRSI------GRFLTG----------------
Piscidin-4      FFRHLFRGAKAIFRGARQGXRAHKVVSRYRNRDVPETDNNQEEP
                *::*:***     :         :.::
```

Figure 9. Amino acid sequences of piscidins 1, 2, 3 and 4. CLUSTAL O (1.2.1) multiple sequence alignment. Red letters indicate positively charged amino acids at pH 7.4 (Lysine – K; Histidine – H; Arginine – R).

Piscidins are preserved in mast cells and obtained mainly from fish gills, skin, mucus, and the gastrointestinal tract (Noga & Silphaduang 2003; Campagna et al. 2007). One study found that the cells that express piscidins are all mast cells, but not all mast cells express piscidins (Corrales et al. 2010). The expression of piscidin was affected by age, size, and physiological conditions in various fish species (Corrales et al. 2010). Moreover, piscidins have mainly been studied in marine bass and are some of the most salt tolerant antimicrobial peptides that also possess antifungal, antiviral, and antiparasitic activity (Noga & Silphaduang 2003).

Piscidins are able to permeabilize bacterial cells because they have both positively charged residues and amphiphilic helical structures (Campagna et al. 2007; Noga et al. 2009). Piscidins disrupt the cell membrane by forming toroidal pores that lead to lysis of the pathogen (Campagna et al. 2007). Much like neutrophils in higher vertebrates, phagocytic granulocytes in fish contain piscidin peptides which may play important roles in both extracellular and intracellular killing of bacteria (Mulero et al. 2008).

These antimicrobial peptides work against multi-drug resistant bacteria at relatively low concentrations without affecting the viability of eukaryotic cells and should therefore be considered novel antibiotics able to fight mammalian pathogens (Noga & Silphaduang 2003; Noga et al. 2009).

2.3. Mechanisms of action of AMPs

AMPs are host defense molecules that protect the host against invasive pathogens. The host defense action of AMPs can be divided into two different mechanisms (Fig. 10) – AMPs may either directly kill bacteria or they may function as immune system enhancers (Hancock & Sahl 2006; Steinstraesser et al. 2011).

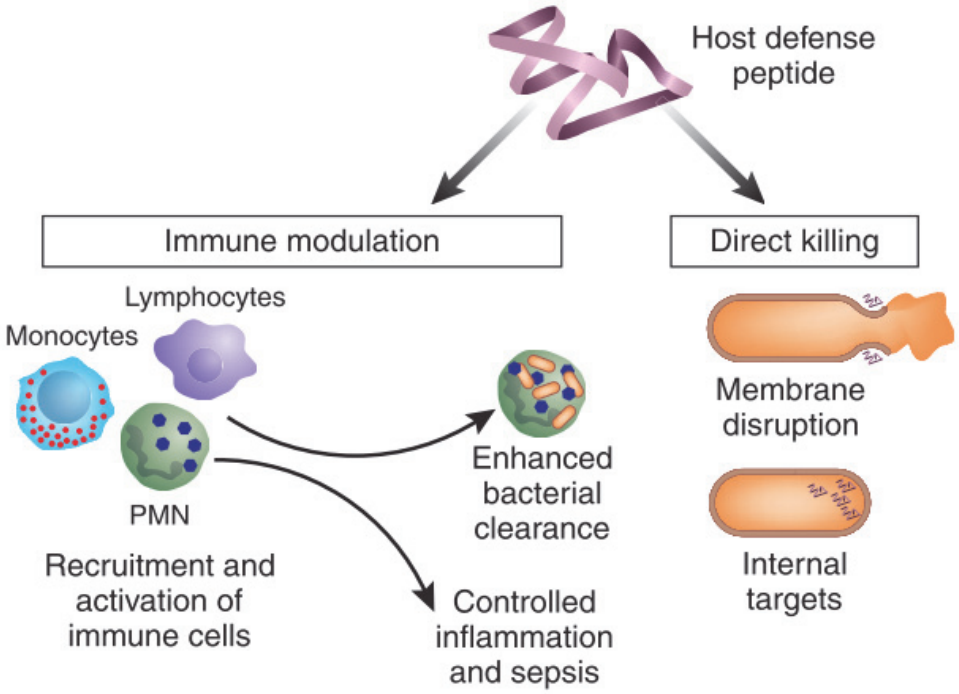

Figure 10. Biological roles of AMPs that either directly kill bacteria or provide innate immune modulating activity (adopted from (Hancock & Sahl 2006).

2.3.1. Direct killing effect

Host defense AMPs are active against both Gram positive and Gram negative bacteria (Brogden et al. 2003; Reddy et al. 2004). Moreover, AMPs are also effective against mycobacteria, which resemble Gram positive bacteria but have a thicker and more hydrophobic cell wall rich in mycolic acids (Miyakawa et al. 1996). While AMPs kill Gram positive and negative bacteria through membrane disruption, *Mycobacteria tuberculosis* is killed through phagocytosis by neutrophils facilitated by AMPs that adhere to the waxy surface of these bacteria (Kisich et al. 2002; Ramón-García et al. 2013).

The positively charged amino acids of AMPs mediate binding to the anionic cell membrane of bacteria (Matsuzaki 1999). Gram negative bacteria contain lipopolysaccharides (LPS) as part of the outer membrane, which are strongly negatively charged due to sulfonic acid moieties in the structure that can be neutralized through interaction with cationic amino acids in the molecules of defensins and cathelicidins (Matsuzaki et al. 1999). Similar to LPS, AMPs interact with teichoic acids, lipoteichoic acids, or lysyl phosphatidylglycerol from Gram positive bacteria (Fig. 11) (Lai & Gallo 2009).

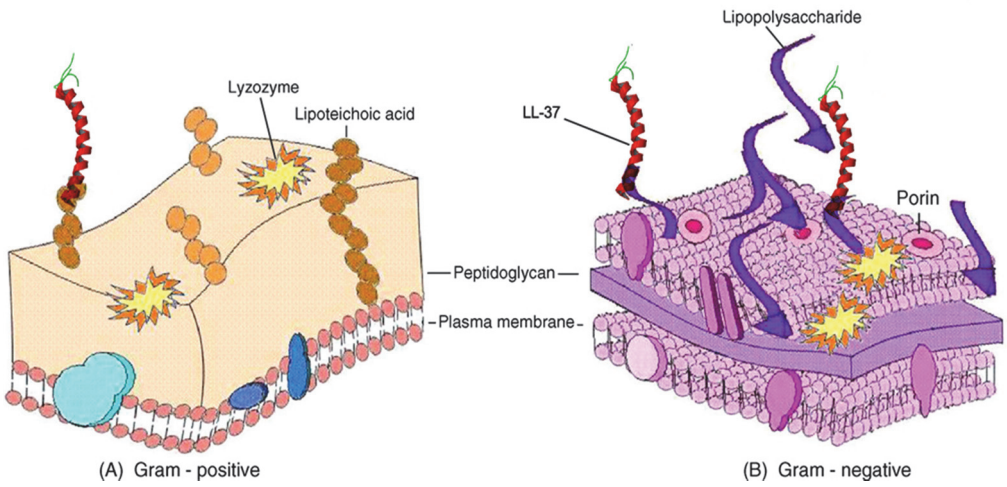

Figure 11. Crosscut of Gram positive (A) and Gram negative bacteria (B) showing both the inner membrane and mechanisms of AMP action. (A) AMP binds to negatively charged lipoteichoic acid, neutralizes the wall charge and allows other antibacterial compounds to act, such as lysozymes, which in turn disrupt the bacterial cell wall. (B) AMP binds to LPS causing it to be liberated thereby damaging the cell membrane (adopted with modifications from (González-Chávez et al. 2009)).

AMPs do not harm eukaryotic body cells. One explanation is that they possess cholesterols while bacterial cell membranes are devoid of these compounds (Boman 2003; Lai & Gallo 2009). Another explanation is that eukaryotic cell membranes contain more neutral phospholipids that cannot interact with AMPs (Lai & Gallo 2009).

There are three main models for how antimicrobial peptides rupture the bacterial membranes. One is termed the barrel-stave model, where the hydrophobic part of the peptide aligns with the acyl chains of the membrane lipids, while the hydrophilic peptide region forms an inner layer that allows pore channels to form in the bacterial membrane (Fig. 12A) (Brogden et al. 2003; Wiesner & Vilcinskas 2010). The toroidal-pore forming mechanism (Fig. 12B) differs from the barrel-stave model in that peptides associate over their full-length with the charged lipid head groups even when they are perpendicularly inserted into the membrane.

In forming a toroidal pore, the polar surfaces of the peptides associate with the head groups of the lipids. Both peptides and lipid head groups line the pore, with the lipid monolayer bending through the pore thereby forming a toroidal hole (Brogden et al. 2003). In the carpet model (Fig. 12C) peptides lay on the bacterial membrane in parallel due to the electrostatic interactions between cationic AMPs and anionic phospholipid head groups. Local accumulation of peptides then leads to disruption of the membrane and formation of peptide-lipid aggregates (Brogden 2005; Wiesner & Vilcinskas 2010).

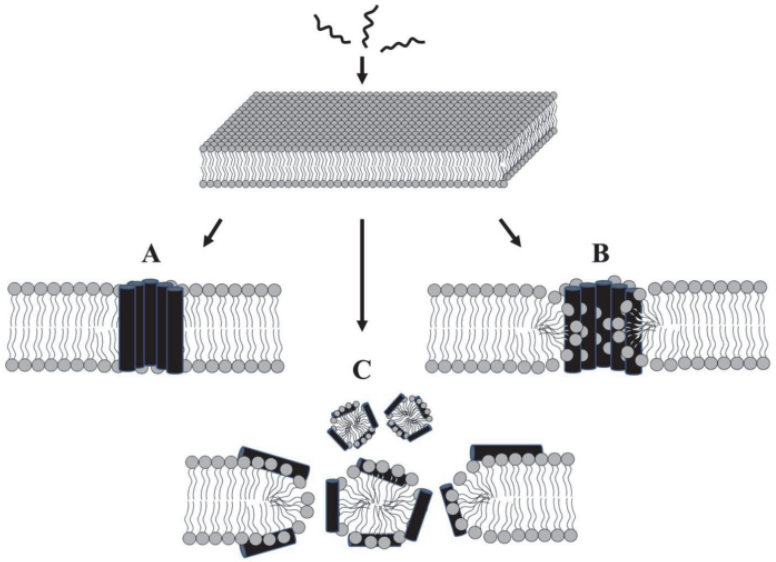

Figure 12. Three modes of action how AMPs insert into lipid bilayers and cause membrane disruption. (A) barrel-stave model; (B) toroidal pore model; (C) carpet model (adopted from (Park et al. 2011)).

It has also been speculated that in addition to extracellular membrane disruption AMPs also have intracellular targets. It has been suggested that some AMPs could inhibit cell wall synthesis, modify bacterial cytoplasmic membranes (Yeaman & Yount 2003), bind with DNA, prevent protein synthesis, or inhibit the activity of various enzymes (Brogden 2005; Wiesner & Vilcinskas 2010).

Furthermore, AMPs are capable of disrupting organelles, for example mitochondria, within fungal pathogens much as they do with bacteria and thereby cause cell death (Yeaman & Yount 2003). In summary, the precise mechanism of the action of AMPs is not clear, however, AMPs may also use multiple mechanisms to cause bacterial cell death.

2.3.2. Immune enhancing effect of AMPs

In addition to the "direct killing" of microorganisms, AMPs also have indirect immune system enhancing properties. AMPs are capable of modifying mammalian cell membranes and can induce receptors that accelerate intracellular processes, including mediation of host gene expression (Lai & Gallo 2009). Furthermore, it has been proposed that host defense molecules are able to function as chemokines themselves or induce production of chemokines, release cytokines that control or regulate T cells and dendritic cells of the adaptive immune response (Lai & Gallo 2009). Moreover, AMPs have the ability to affect and accelerate the process of wound repair (Heilborn et al. 2003).

After attack by an antagonist, the innate immune system of mammals depends on the activation of receptors that recognize various pathogen-associated components such as LPS in Gram negative bacteria (Matsuzaki et al. 1999) or lipoteichoic acids in Gram positive bacteria (Lai & Gallo 2009). Of specific interest are members of the Toll-like receptor (TLR) family, which are generally membrane-anchored cell-surface receptors that recognize specific molecular structures within bacteria, viruses, fungi, and protozoans (Mogensen 2009). For example, LPS from Gram negative bacteria electrostatically attracts the CD14 protein in the relation with Toll-like receptor 4 (TLR-4), which in turn signals expression of nuclear factor κB (NF-κB) which up-regulates proinflammatory genes that cause sepsis (Fig. 13) (Mookherjee et al. 2006; Lai & Gallo 2009). Moreover, LPS also induces overproduction of TNF-α in macrophages, which leads to septic shock or even cell death (Zhang et al. 1999; Scott et al. 2002).

Still, several cationic AMPs, such as human cathelicidin LL-37 (Scott et al. 2002; Mookherjee et al. 2006) or defensins from insects (Saido-Sakanaka et al. 2004), recognize bacterial LPS and form strong electrostatic interactions that do not allow LPS to bind to CD14 and therefore do not trigger TLR-dependent inflammatory responses in cells (Lai & Gallo 2009).

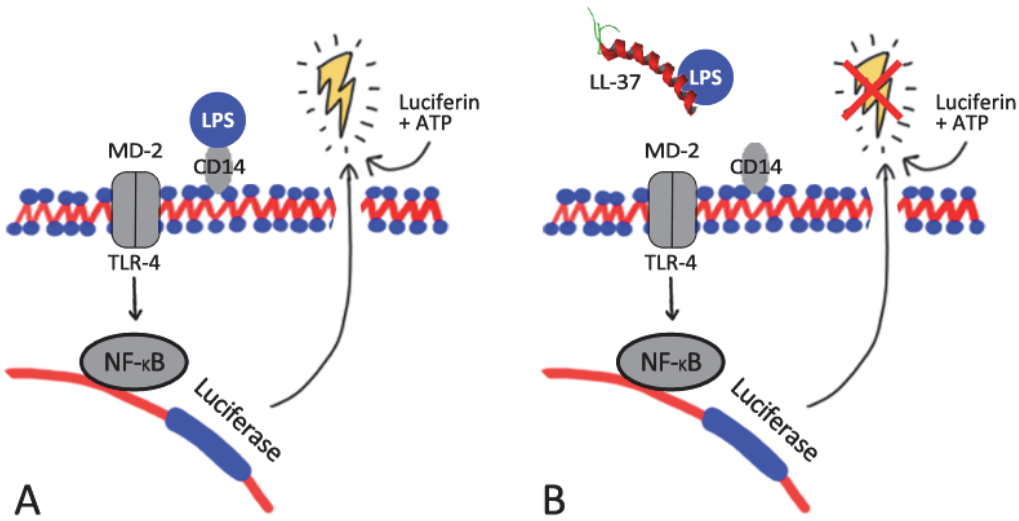

Figure 13. Bacterial LPS induces NF-κB response in mIC_{C12} cells. A) LPS binds to CD14 on the cell surface to accelerate the inflammatory response. B) LPS interacts electrostatically with AMPs and binding to CD14 is eliminated. Luciferase was cloned after the NF-κB promoter and transfected into mIC_{C12} cells. An increased or suppressed expression of luciferase can then be determined as a measure of TLR-4 activation after lysis of cells using cycles of freezing and thawing by addition of ATP and luciferin as substrates and measured as luminescence.

While newborns lack CD14, human colostrum supplies sufficient quantities of this protein, and is one reason why it is extremely important for infants to receive mother's milk to build up a functional innate immune system (Walker 2010).

Therefore, milk and colostrum are not only the first nutritionally balanced food for newborns, but they also aid in immunological protection (Joss et al. 2009; Armogida et al. 2011; Senda et al. 2011). In cattle, NF-κB is essential for inducing the β-defensin 5 gene in mammary epithelial cells triggered by invasive pathogens. One study determined that heat-killed *E. coli* and LPS increased the expression of NF-κB by threefold, which was equivalent to the threefold increase in expression of the β-defensin 5 encoding gene. On the other hand, RT-PCR results revealed a 25 fold difference in expression of endogenous β-defensin 5 between uninfected and infected udders. This means that even though the transcript factor NF-κB is required for stimulating the expression of AMPs, the β-defensin 5 expression could also be dependent on other factors (Yang et al. 2006). These aforementioned host defense properties illustrate only a minority of the applications of AMPs assist innate and adaptive immunity.

2.4. Methods used to identify AMPs

Antimicrobial activity is often measured visually using a radial plate diffusion assay. It is a quick test to semi-quantitatively determine antimicrobial activity through the appearance of clear zones in agarose plates seeded with bacteria (Isobe et al. 2009c; Wang et al. 2011). Antimicrobial activity may also be measured in a liquid growth medium assay to detect the lowest concentration of AMP that reduce the growth of the specific microorganism by roughly 50% (Andrews 2001; Tomasinsig et al. 2010). Surprisingly, there are very few data sets that measure the direct antimicrobial activity of colostrum and milk. AMPs in both of these are typically found by investigating different milk fractions using specific immunoassays and novel peptide sequences are verified by searching for them within the genome of the target organism.

The most widely used method to detect AMPs is enzyme-linked immunosorbent assay (ELISA), which is able to detect AMPs at picogram levels present in both healthy (Wang et al. 2014) and diseased milk samples (Bosire et al. 2007). Western blotting has also been used to quantify the concentration of β-defensin in human milk (Jia et al. 2001) or cathelicidin in LPS-infected goat milk (Zhang et al. 2014). Although, ELISA is often employed, only a small number of peptides have been quantified using this technique due to lack of specific antibodies. Immunostaining has been used to detect AMPs in tissues to reveal the precise localization of defensins and cathelicidins in the mammary gland (Tunzi et al. 2000; Welsch et al. 2007).

Defensins and cathelicidins have also been investigated in epithelial cells of the mammary gland and milk using genomic approaches such as RT-PCR (Tunzi et al. 2000; Tomasinsig & Zanetti 2005; Petzl et al. 2008; Das et al. 2009; Wang et al. 2014), which allows for high throughput screening to detect the mRNAs that code for different AMPs and can be used to determine their location in the mammary tissue. PCR is viewed as a useful tool to detect defensin mRNA expression as a biomarker for non-infected and mastitic mammary gland epithelial cells (Roosen et al. 2004; Swanson et al. 2004; Alva-Murillo et al. 2013; Yang et al. 2013). PCR has also been used to screen the profiles of AMPs during different periods of lactation, both in humans and in ruminants (Murakami et al. 2005; Zhang et al. 2014).

Both the precision and accuracy of detecting AMPs using indirect mRNA based PCR methods have been surpassed by novel proteomics methods. Mass Spectrometry (MS) is a widely used proteomic approach that is able to identify proteins, peptides, and their fragments by matching their precisely determined mass-to charge (m/z) ratios using databases that contain either known or predicted masses. Mass-spectrometric analyses are robust assays that are able to detect several AMPs from samples that are much smaller in comparison with those used in quantitative ELISA methods that require antibodies (Boehmer et al. 2010a).

The two most often employed soft desorption/ionization techniques for protein and peptide analysis in complex biological matrices are electro spray ionization (ESI) and Matrix Assisted Laser Desorption Ionization (MALDI) (Shevchenko et al. 2001; Trauger et al. 2002; Coon et al. 2005; Jørgensen et al. 2010). ESI allows one to analyze biomolecules that are associated through non-covalent interactions, including protein-peptide complexes (Glish & Vachet 2003). MALDI is very sensitive method that works without destroying the sample and has been used to detect a wide variety of biomolecules (Go et al. 2003). ESI is less stable than MALDI, however, it is also less affected by signal suppression and can be directly interfaced with liquid chromatography (Trauger et al. 2002; Ahmed 2008).

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is a popular tool to analyze the protein profiles in various milk fractions (Boehmer et al. 2010b; Hettinga et al. 2011). LC-MS/MS has been used to screen AMPs throughout the lactation period (Liao et al. 2011a; Palmer et al. 2006). The human milk proteome shows that different fractions of colostrum contain various AMPs and proteins that are involved in immune responses and these are almost twice as high in whey (34.8 %) compared with MFGM in the fat fraction (19.9 %) (Liao et al. 2011b; Liao et al. 2011a). Moreover, cathelicidins have been found in considerable amounts in bovine MFGM fractions using the peaks heights within mass spectra (Smolenski et al. 2007; Hettinga et al. 2011). Analysis using a nano LC-ESI-LIT-MS/MS on various bovine milk samples revealed that cathelicidin-1 is only present in MFGM fraction of pasteurized and ultra-high temperature milk and not in fresh milk and nutritional powder for infants (Arena et al. 2011). We could speculate that heating irreversibly dissociates some molecular interactions in milk and thereby increases the apparent concentration of cathelicidin peptides detected in the samples.

An MS approach has been developed to determine the concentrations of AMPs in food matrices and complex mixtures (Domon & Aebersold 2006; Affolter et al. 2010) that makes use of isotopically labeled internal standards to avoid many quantification errors introduced by variable matrix effects. Stable isotopes from C, O, and N are the most commonly used isotopic labels and allow one to determine the ratios of C^{12}/C^{13}; O^{16}/O^{18}, and N^{14}/N^{15} (Werner & Brand 2001). Using nuclear magnetic resonance (NMR) combined with isotopic labelling with C^{13} or/and N^{15} allows one to determine the three-dimensional structures and molecular interactions of bioactive peptides in their natural cellular state (Moon et al. 2007). One milk study used stable isotope dilution to determine the concentrations of seven proteins from the MFGM fraction of bovine milk. Their concentrations are obtained by spiking milk samples with heavy peptides. This allows one to assess the immunity and defense potential of various milk fractions (Affolter et al. 2010). Moreover, isotopic labeling has been used to screen for mastitis and follow LPS challenges in bovine milk samples (Boehmer et al. 2010b).

3. Aims of this dissertation

This dissertation aims to:

I. Develop strategies to isolate and identify components from bovine colostrum with antimicrobial activity.
II. Test the potential immunomodulating properties of colostral AMPs using a murine intestinal cell line.
III. Identify and characterize AMPs in colostrum using proteomic tools.
IV. Measure the consumption patterns of free and peptide-bound amino acids by *L. lactis subsp. lactis IL1403* to gain further insight into the role of peptides as a source of amino acids.

4. Materials and methods

Detailed descriptions of the materials and methods are available in the publications reproduced in the appendix that form the basis of this dissertation. The following sections are provided as an aid to the reader. A scheme depicting the overall workflow is provided in Fig. 14.

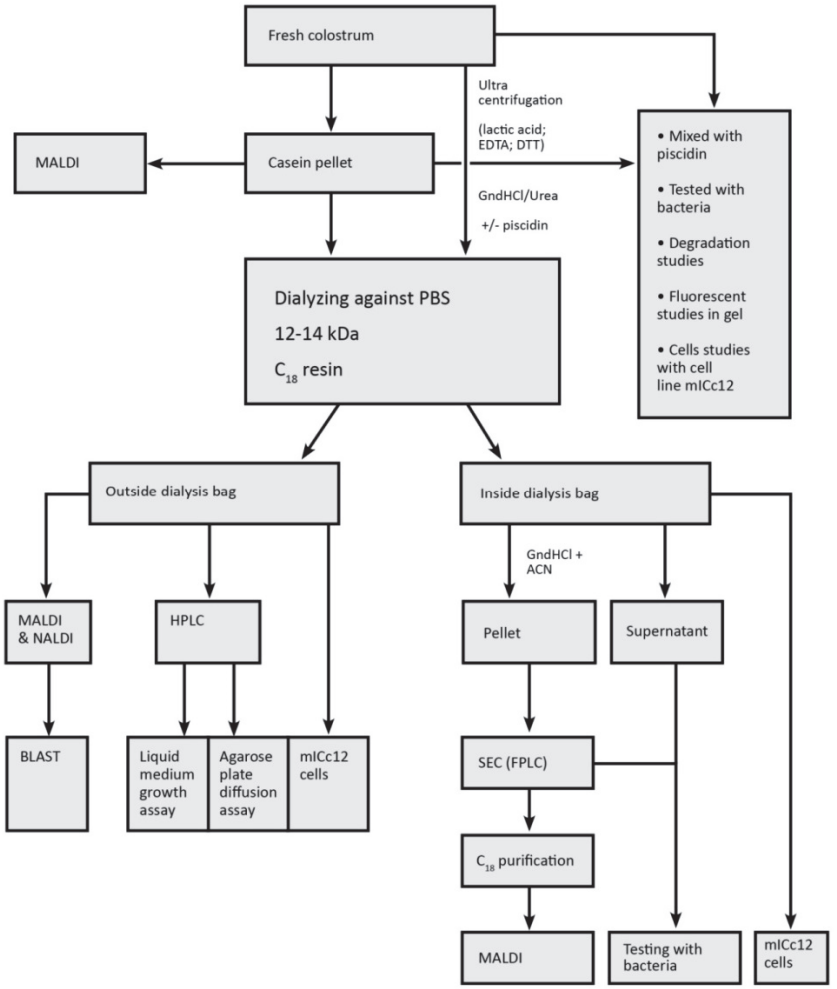

Figure 14. Overall workflow and design of experiments.

4.1. Bacterial and mammalian cell cultivation

Cultures of *Escherichia coli* and *Staphylococcus aureus* strains isolated from milk were obtained from the Estonian University of Life Science, Tartu, Estonia. The *E. coli K-12* strain used was kindly provided by Ole Højberg, Department of Microbiology, AU, DK. Bacteria were grown on agar plates. Single bacterial colonies were picked and pre-grown overnight in Luria Broth (LB) (Fluka Analytical, MO., USA) at 37°C, washed with phosphate buffer saline (PBS) and diluted to an optical density (OD) of 0.05 at 600 nm.

Lactococcus lactis IL1403 was pre-grown from freeze-dried stock culture and transferred into a chemically defined medium (Lahtvee et al. 2011) that was supplemented with 2 g/L of ^{15}N-labelled yeast hydrolysate. Fermentations were carried out under surface aeration with N_2, at 34 °C, with pH controlled at 6.4 and an agitation speed of 300 rpm. A more detailed description is available in Publication III.

We made use of the murine epithelial cell line mIC_{C12} kindly provided by Professor Mathias W. Hornef. The mIC_{C12} cell line is derived from small intestinal crypt cells that express some of the TLR innate immune receptors, particularly TLR-4. They have been transfected with a luciferase reporter gene that is under the control of the NF-κB promoter such that stimulation of NF-κB signaling results in luciferase activity.

mIC_{C12} cells were grown in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, Life Technologies, Grand Island, NY) with GlutaMAX/glucose/Phenol red containing: Insulin ($9x10^{-7}$ M); Dexamethasone ($5x10^{-8}$ M); Sodium selenite ($6x10^{-8}$ M); Transferrin (5μg/ml); Triiodothyronine (10^{-9} M); EGF (10 ng/ml); D-Glucose ($1.3x10^{-2}$ M) (Sigma-Aldrich, MO., USA); L-glutamine ($2x10^{-3}$ M); HEPES ($2x10^{-2}$ M); Pen/strep/neo solutions (1%); Gentamicin solution (1%) (Gibco, Life Technologies, Grand Island, NY); heat inactivated fetal calf serum (2%).

Confluent cells from flasks were harvested, pelleted by centrifugation (100 x g, 10 min), and re-suspended in culture media. Cells were seeded in 96 well plates at ~5000 cells/well (100 µl) and allowed to settle in the plates at room temperature for 30 min before placing in the incubator (37 °C, 5.0 % CO_2). When cells reached ~90% confluence after a few days, they were used in the immunoassays that followed.

4.2. Obtaining the bioactive fractions

4.2.1. Colostrum or casein LMW fraction

Freeze-dried colostrum powder (a mixture collected and pooled from different cows and herds) was kindly provided by Biofiber Damino, Gesten, DK. Fresh colostrum and milk samples from different cows were kindly provided by Mette-Marie Løkke, Department of Food Science, AU, DK. Low molecular weight (LMW) fractions were obtained by dialysis from either fresh colostrum, reconstituted colostrum (prepared from freeze-dried colostrum powder), or washed casein pellets prepared from fresh colostrum by ultracentrifugation (100 000 g, 60 min, 4˚C). Dialysis was performed according to Jørgensen et al. (Jørgensen et al. 2010) with some modifications. Fresh colostrum was used directly; colostrum powder and caseins were re-suspended with either MilliQ water or 6 M of guanidine hydrochloride (GndHCl) or 8 M of urea (Sigma-Aldrich, MO., USA). 12-14 kDa cut-off dialysis tubing (Spectrum Laboratories, Inc., CA) was used with addition of 10 ml of appropriate solvent inside and colostrum or casein solution outside the bag or vice versa. In some experiments we used a C_{18} reverse phase resin (Waters Corporation, Milford, USA) conditioned with methanol and washed with 0.1% trifluoroacetic acid (TFA). The dialysis bags were incubated on a rotator overnight (or longer) at 4˚C (or at room temperature or 37 °C if indicated). In some cases, 1% pen/strep/neo solution or 0.1% of sodium azide was included to prevent bacterial growth.

LMW components were recovered on small columns from the C_{18} reverse phase resin. After washing with 0.1% TFA, the bound LMW components were eluted with 70% of acetonitrile (ACN) in 0.1% TFA and lyophilized. More detailed descriptions of these methods are available in Publication I and Publication II.

4.2.2. ^{15}N-labelled yeast hydrolysate

Saccharomyces cerevisiae (strain S288c) was grown in fed-batch on a synthetic medium with ^{15}N-labelled (98 %) ammonium chloride (Sigma-Aldrich, MO., USA) as the sole nitrogen source. The yeast biomass was freeze-dried and hydrolyzed with a mixture of proteases (Promod 144GL, Biocatalysts, Cardiff, UK). The extract was purified using an Amicon Ultra 15 centrifugal filter device (Millipore Corp., MA) with a 10,000 cut-off to remove any residual protease activity. The soluble yeast hydrolysate was freeze-dried. A more detailed description of this method is available in Publication III.

4.3. Bioactivity assays

Throughout the study, synthetic amidated AMP piscidin-1 (FFHHIFR-GIVHVGKTIHRLVTG-NH2) and fluorescein-piscidin-1 (N-terminally labeled) (CASLO Laboratory ApS, Lyngby, Denmark) were used as model antimicrobial peptides and are referred to in the text as piscidin and fluorescein-piscidin, respectively.

4.3.1. Radial plate diffusion assay

Autoclaved and cooled LB broth containing 1.5 % agarose (Invitrogen, UK) and protease inhibitors (0.5 mg/ml) (Sigma-Aldrich, MO., USA), was mixed with washed bacteria (OD_{600}=0.05) and poured into Petri dishes. After the medium solidified, 3 mm holes were punched into the gel and filled with ~20 µl of sample. Synulox (Pfizer Italiana, Italy), a mixture of amoxicillin and clavulanic acid, was used as a non-peptidic positive control. Piscidin was used as a model AMP control.

The following day, after overnight incubation at 37˚C, we measured the diameter of each inhibition zone. A more detailed description of this method is available in Publication II.

4.3.2. Liquid medium growth assay

Suppression of bacterial growth was also measured in an assay using liquid LB growth medium. Wells in 96 well micro titer plates were filled with either one or a mixture of two analytes in LB broth together with protease inhibitors (0.5 mg/ml) and washed bacteria (adjusted to 0.05 at OD_{600}). We followed the growth of bacteria for up to 16 h at 37˚C while shaking by measuring the optical density every minute at 600 nm with a Synergy 2 micro plate reader. Piscidin was used as positive control. A more detailed description of this method is available in Publication II.

4.3.3. Induction of luciferase activity in mIC_{C12} cells

In this study we made use of the $mIC_{C12,}$ murine epithelial cell line to study the immune system enhancing activity of AMPs. When AMPs are present, they electrostatically bind with LPS and suppress luciferase activity which is detected by a decrease in the chemiluminescence signal.

Test fractions were made by mixing either colostrum LMW fraction, yeast hydrolysate (Lallemand Inc., Canada), or piscidin, together with DMEM followed by pH adjustment to 7.4. Growth medium was removed from confluent cells and replaced with 100 µl of this test solution both together with and in the absence of LPS (LPS from *E. coli* serotype 0111:B4, Fluka Buchs, Switzerland), followed by incubation for 4 h (+ 37 °C, 5.0 % CO_2). All wells were aspirated and 75 µl of buffer D (ATP Determination Kit, Biaffin GmbH & Co KG) was added followed by lysis using three freeze/thaw cycles (-80 °C/+37°C for 10 min). 100 µl of a mixture of ATP/luciferin/ dithiothreitol (DTT) in buffer D was then added to the cell lysate and 150 µl of this mixture was further transferred to Costar™ 96-Well White Plates (Thermo Fisher Scientific Inc.).

Luciferase chemiluminescence at 560 nm was measured to determine stimulation of NF-κB signaling in a Synergy 2 micro plate reader.

4.4. Electrostatic interaction studies between AMP and colostral components

4.4.1. Binding and release studies with fluorescein-piscidin in milk or colostrum

The binding of fluorescein-piscidin with reconstituted colostrum, fresh colostrum, or milk was studied by fluorescence quenching. The release of fluorescein-piscidin was performed with sequential additions of 6 M of GndHCl (pH 7.0) after every 30 seconds.

Interaction studies with fluorescein-piscidin were also performed in micro titer plates pre-coated with 1% BSA (Sigma-Aldrich, MO., USA) to prevent non-specific adsorption and quenching. Quenching of fluorescein-piscidin was followed after addition of increasing amounts of fresh colostrum or BSA. Quenching of fluorescein-piscidin was competed with increasing concentrations of unlabeled piscidin by first pre-incubating fresh colostrum with unlabeled piscidin before adding fluorescein-piscidin. More detailed descriptions of these methods are available in Publication II.

4.4.2. Cross-titration setup of piscidin and casein

The cross-titration setup was performed in 96 well micro titer plates where piscidin or caseinate (Arla Foods, Holstebro, Denmark) was three-fold serially diluted in PBS and then combined in the square matrix. The highest concentration of both components was at the upper left corner and control (PBS alone) at the lower right corner in the square matrix. The bottom row only contained caseinate dilutions and the right column only contained piscidin dilutions. Various concentrations of piscidin and caseinate were combined in 6x6 squares.

For each combination, *S. aureus* in liquid LB medium with OD_{600}=0.05 was added. The optical density of bacteria was measured every minute at 600 nm with the Synergy 2 micro plate reader at 37˚C with shaking. A more detailed description of this method is available in Publication II.

4.5. Analytical methods (for identification and characterization of bioactive peptides)

4.5.1. FPLC

Fast protein liquid chromatography (FPLC) with size exclusion columns was used to both assess piscidin degradation and fractionate colostrum or caseins. All FPLC experiments were carried out using a ÄKTA FPLC 900 Chromatography System (Amersham Biotech) equipped with a Sephacryl S200 column - 15 mm x 100 mm – internal diameter x length (Pharmacia, Sweden) with either PBS or 3 M of GndHCl (pH 7.0) as the mobile phase, at a flow rate of 1 ml/min, and followed by UV detection at 280 nm. Series of either colostrum or casein fractions were collected in 1 ml Eppendorf tubes and further purified using C_{18} Sep-Pak cartridges (Waters Corporation, Milford, USA). The purified eluates were tested in a liquid medium growth assays or radial plated diffusion assays.

To assess the degradation of fluorescein-piscidin, 300 µl of each fraction eluted from the size exclusion column was collected into Costar™ 96-Well White Plates. The fluorescence of all wells was measured with a Synergy 2 micro plate reader using excitation at 485 nm and emission at 528 nm. Subsequently, pronase E (Merck, Germany) was added to all fractions to release fluorescein followed by an additional fluorescence measurement after 30 minutes. A more detailed description of this method is available in Publication II.

4.5.2. HPLC

Colostrum and casein LMW fractions that had been first dialyzed and then eluted from C_{18} resin were lyophilized to remove ACN and dissolved in 1 ml of 0.1% TFA. Precipitates were removed using centrifugation and 500 µl of the supernatant was purified using a Protein & Peptide C_{18} column, L 150 x ID 2.1 mm, 5µm (Grace Davison Vydac, USA). The chromatographic conditions were as follows: elution with a linear gradient from 0-100% of ACN in 0.1% TFA at a flow rate of 1 ml/min followed by detection at 214 nm. In total, 96 fractions were collected and tested using a liquid medium growth assay. A more detailed description of this method is available in Publication II.

4.5.3. Size Exclusion Chromatography

Size exclusion chromatography (SEC) was used to determine the size distribution of peptides in both a yeast hydrolysate and a colostrum LMW fraction. We used a Waters 1515 HPLC system (Waters Corp., Milford, MA) equipped with a Superdex Peptide 10/300GL column (GE Healthcare, NJ) and a Waters 2414 Refractive Index detector to determine the distribution of peptides in the LMW fractions. The chromatographic conditions were as follows – isocratic elution with 0.1 N ammonium acetate (pH 5), flow rate was 0.5 ml/min. The size distribution was determined based on the following markers: alpha- lactalbumin (14146 Da), aprotinin (6512 Da), nisin (3354 Da) and glutathione (307 Da). A more detailed description of this method is available in Publication III.

4.5.4. Gel-electrophoresis

Agarose gel electrophoresis was used to separate a mixture of piscidin and casein micelles. The gel was prepared with 0.5 % agarose (BioWhittaker Molecular Applications, ME, USA) containing PBS and 3 M of urea at pH 6.0. Casein, obtained from ultracentrifugation, was reconstituted with PBS (final pH 6.35). Three samples were prepared: casein (80 mg/ml); casein spiked with 0.1 mg/ml of fluorescein-piscidin; 0.1 mg/ml of fluorescein-piscidin alone.

All three samples contained PBS and 20% glycerol (Merck KGaA, Germany). 10 µl of each sample were loaded into the wells. The gel was electrophoresed (Bio-Rad, USA) in PBS buffer (pH 6.0) on ice for two hours by increasing the current linearly from 100 mA to 500 mA. Alternatively, 3 M of urea was added to the samples and electrophoresis buffer to improve the separation of each mixture.

4.6. Mass-Spectrometry analysis

4.6.1. Liquid Chromatography Mass-Spectrometry

High performance liquid chromatography (HPLC/ESI-MS) was used to measure the consumption patterns of free and peptide-bound amino acids by *L. lactis subsp. lactis* IL1403. Free amino acids were measured directly from the culture medium and the peptide-bound amino acids were determined after separately hydrolyzing the culture media and biomass in 6 M HCl containing 1 % phenol for 24 h at 105 °C. After hydrolysis, the samples were re-suspended in a methanol-water mixture and filtered through 0.2 µm filters for further analysis. All samples were derivatized with AccQ-Fluor reagent (Waters Corp.) before LC-MS analysis.

The amino acid concentrations and mass spectra of both the biomass and culture medium were determined with a LCT Premier LC–MS system (Waters Corp.) equipped with AccQ-TagTM Ultra column (2.1 x 100 mm) (Waters Corp.). The chromatographic conditions were as follows – column temperature 55 °C; sample run 20 min; flow rate 0.3 ml min-1; connected to a photodiode array detector at 260 nm. Amino acids were separated using a gradient from 0.1 to 59.6 %B (A: AccQTag Ultra eluent A, B: AccQTag Ultra eluent B; Waters Corp.), sprayed directly into the mass-spectrometer operated in positive ionization mode at 300 °C solvation temperature, 120 °C source temperature and 2.5 kV capillary voltage.

MassLynx V 4.1 software (Waters Corp.) was used for data processing. The ^{15}N-enrichment of amino acids in both the biomass and culture medium was estimated using least squares regression to fit the measured mass spectra by theoretical isotope distributions of labelled and unlabelled amino acid species. A more detailed description of this method is available in Publication III.

4.6.2. LC-MS/MS

The colostrum LMW fractions were purified with StageTips and dissolved in 0.5 % formic acid. Purified peptides were analyzed by LC-MS/MS (Agilent Technologies) connected to a LTQ Orbitrap mass-spectrometer (Thermo Electron, San Jose, CA, USA) equipped with self-packed fused silica emitter (150 mm x 0.075 mm, Proxeon) packed with Repropur-Sil C_{18}-AQ 3 um particles (Dr. Maisch, Germany) and a nano-electrospray ion source (Proxeon, Odense, Denmark). The peptides were separated with a 120-minute gradient from 2-40% B (A: 0.5% acetic acid, B: 0.5% acetic acid/80% acetonitrile) using a flow-rate of 200 nl/min and sprayed directly into LTQ Orbitrap mass-spectrometer operated at 180˚C capillary temperature and 2.4 kV capillary voltage.

Full mass spectra were acquired with a mass range from m/z 250 to 1800 Da and ions with a charge state of => 1+ were selected for fragmentation. Raw MS files were analyzed by MaxQuant (version 1.3.0.5). MS/MS spectra were searched against a database containing *Bos taurus* protein sequences downloaded from UniProt (2012.12.28). The search included variable modifications of methionine oxidation and N-terminal acetylation, and as fixed modification of cysteine carbamidomethylation. Peptides with a minimum length of 7 amino-acids were allowed in the analysis and no specific enzyme cleavage was used. A more detailed description of this method is available in the dissertation written by L. Arike (Arike 2012).

4.6.3. MALDI-TOF-MS/MS & NALDI-TOF-MS/MS

To detect AMPs with m/z less than 500-700 Da, we had to overcome problems with interference using the MALDI-TOF MS due to the added matrix (Glish & Vachet 2003; Go et al. 2003). For this we used a novel matrix free application termed Nanostructure-Assisted Laser Desorption/Ionization (NALDI). The NALDI target plate is covered with several layers of nanoscale inorganic materials and then coated with a hydrophobic organic surface that allows peptides to attach to the plate while being simultaneously stimulated by a UV laser. This allows one to ionize the sample without additional matrix. NALDI is suitable for molecules with a mass range between 200-1500 Da and it has been shown that this technique may give up to ten times more intensive analyte peaks than MALDI-TOF-MS (Daniels et al. 2008; Kütt et al. 2011).

The C_{18} Sep–Pak eluates from colostrum LMW fractions were serially diluted up to one million times in HPLC grade water (Baxter, IL, USA) and directly deposited on the MALDI and NALDI plates. The dots of the sample where dried overnight. The MALDI samples also received α–cyano–4– hydroxycinnamic acid (Bruker Daltonics, Germany) matrix.

Both sample plates were analyzed with an Ultraflex IV TOF/TOF, (Bruker Daltonics, Germany) equipped with a 337 nm nitrogen laser in positive mode. Full mass spectra were acquired with a mass range of 10–3510 Da based on the m/z charge ratio. A total of 1000 shots obtained from five different positions of the sample. Spectra were analyzed with FlexAnalysis version 2.4 software (Bruker Daltonics). The resulting fragments of the parent ions were analyzed with *De novo* sequencing software and UniProt BLAST database against the *Bos taurus* genome. More detailed descriptions of these methods are available in Publication I and Publication II.

5. Results and discussion

This section presents the results and discussion relating to the detection of LMW peptides using proteomic approaches. Furthermore, the antimicrobial effects of colostral components and binding studies between colostrum and piscidin, the antimicrobial peptide from fish are presented. Finally, my results for immunoregulating effects of colostral peptides will be discussed together with the growth inducing effects of yeast peptides.

5.1. Mass-spectrometry techniques for detection of peptides in colostrum (Publication I + unpublished results)

The proteomic approaches most often employed to detect proteins and peptides in complex biological matrices are ESI-MS and MALDI-MS (Ahmed 2008; Ahmed 2009). However, there are several problems with matrix effects and interfering compounds that occur when analyzing biological samples and new technologies are applied to eliminate these obstacles. For example, MALDI analyses require an additional matrix to ionize the molecules, however, this additional matrix often suppresses the signal of peptides with m/z less than 700 (Cohen & Chait 1996; Knochenmuss et al. 1996). We thus employed the matrix-free NALDI method to detect and identify small peptides.

Molecules within purified colostral LMW fractions were desorbed and ionized using either MALDI or NALDI steel adapter plates and analyzed using TOF-MS/MS. A dilution series from each sample displayed different peak intensities and even a 10^6 fold dilution provided sufficient analyte signal using NALDI in contrast with MALDI. For further identification, we chose the highest peak obtained with NALDI (1377 m/z Da) whereas intensity of the same peak obtained using MALDI was 30 times lower and was impossible to fragment in MS/MS mode due to matrix effects that suppresses the analyte signal (Fig. 15).

Interestingly, the MS spectra of NALDI and MALDI varied to some extent. The differences of spectra between two ionization methods have been noted by (Vidova et al. 2010), where phospholipids analyzed in the MS spectra of NALDI contained mostly protonated ion adducts, whereas the MS spectra obtained using MALDI also contained alkali-metal ion adducts. It could be that the samples ionized differently with the two methods and therefore both MALDI and NALDI could be applied to obtain more complete proteomic profiles for colostrum and milk.

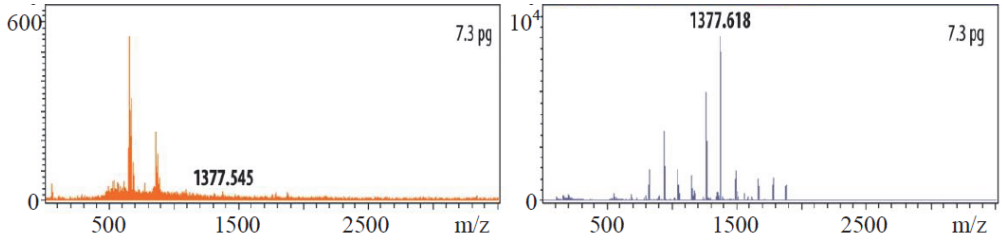

Figure 15. MS spectra using MALDI (left) and NALDI (right) of colostrum LMW fractions obtained by dialysis and compared after 10^6 times dilution.

The peak at m/z 1377 obtained with NALDI was further analyzed and fragmented in MS/MS mode. The peptide search with *De novo* sequencing and Mascot database resulted in a fragment of β-casein (210-222) with an amino acid sequence of EPVLGPVRGPFPI (Fig. 16). Antimicrobial activity (MIC 0.4 mg/ml) against *E. coli* DPC6053 has been observed in two sequences (YQEPVLGPVRGPFPIIV and YQEPVLGPVRGPFPI) cleaved from the C-terminus of bovine β-casein (Birkemo et al. 2009), so it is possible that fragment EPVLGPVRGPFPI from colostrum might also display bioactivity. Thus, it was hypothesized that NADLI may be an appropriate tool to identify and characterize AMPs in colostrum and milk without complex sample pretreatment. Unfortunately, we did not find bovine AMPs in the MS spectra obtained using NALDI. Because there are several reports of AMPs presented in cow colostrum, we suppose that those present may be bound to macromolecules in colostrum and not released during the dialysis method used to prepare the LMW fractions.

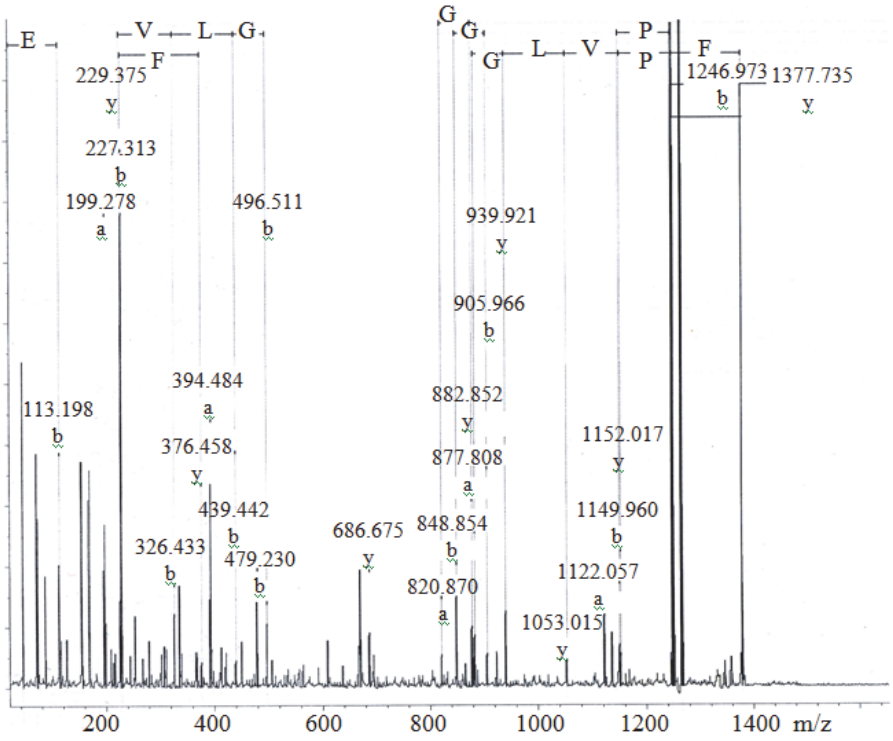

Figure 16. MS/MS spectrum of ion 1377. The figure shows detection of a, b and y ions after fragmentation, together with their fragment sizes. Suggested partial amino acid sequence shown on top.

GndHCl is a powerful denaturant able to dissociate many interactions that occur in complex biological matrices. We used GndHCl in further experiments to liberate AMPs, especially defensins and cathelicidins. Colostrum was dialyzed either against water or 6 M GndHCl, and peptides in the dialysates were recovered with C_{18} columns and analyzed with LC-MS/MS (Fig. 17). This proteomic approach was able to identify a total of 1639 amino acid sequences from 88 different proteins and peptides. However, none of these sequences matched with known *Bos taurus* defensins or cathelicidins. Nevertheless, this does not mean that defensin or cathelicidin peptides (Table 2 and 3) were not present; the concentration of AMPs could have remained below detection limit or salts in the colostrum could have suppressed their ionization (Shadforth et al. 2005).

During our search for bioactive peptides in the dialysate, various sequences from the bioactive protein osteopontin were identified using a BLAST search against the UniProt database. This protein has been found in bovine and human breast milk and also in the intestines of infants where it functions as an important part of the newborn immune system (Schack et al. 2009). Moreover, lactoferrin, the most abundant antimicrobial protein in milk, was detected only in the sample where colostrum was dialyzed against water, but not in the GndHCl treated sample. However, most of the peptide sequences found belong to α-, β- and κ-caseins, α-lactalbumin, and β-lactoglobulin.

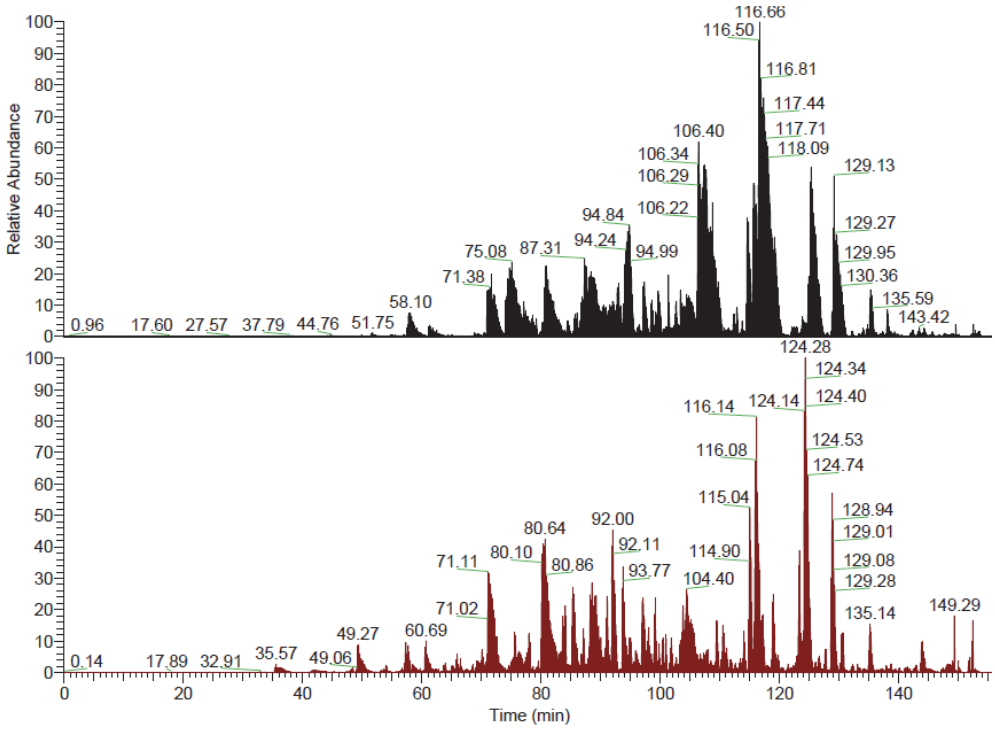

Figure 17. Chromatograms of reconstituted colostrum samples dialyzed against water (black) or 6 M of GndHCl (red) and purified through Sep-Pak C_{18} cartridge. The obtained LC peaks were fragmented and MS/MS spectra were searched against a database containing *Bos taurus* protein sequences.

Colostral AMPs could be bound strongly to caseins. To test this, we analyzed peptides from washed casein micelles. The casein micelles were obtained using ultracentrifugation, treated with GndHCl, dialyzed in 12-14 kDa cut off dialysis bags, and the LMW components were analyzed using MALDI-TOF-MS/MS. The most intense peaks were further fragmented in MS/MS mode and analyzed with the Mascot database. The NF-κB activating and calcium ion binding proteins gave the highest scores. Furthermore, with a lower detection score, we identified both the IL-1 and MUC proteins, which both contribute to the immune system enhancement of mammals (Dowbenko et al. 1993; Newburg & Walker 2007; Garofalo 2010).

Unfortunately, no AMPs were identified with a score of 100%. However, some of the peptides were additionally analyzed using *De novo* sequencing software. For example, a peak with an m/z of 2262 was fragmented and further analyzed using *De novo* tool. The most likely sequence obtained was inserted in a BLAST search against the UniProt *Bos taurus* database and gave a hit for cathelicidin-4 with an identity of 83% and a score of 53. Even though the database provided a hit close to 100%, uncertainty remains because *De novo* searches often conciliates amino acids randomly into the peptide sequence (Shadforth et al. 2005) and thus we question the validity of this result.

5.2. Electrostatic interactions between colostrum and piscidin; a model system to study colostrum antimicrobial activity

5.2.1. Antimicrobial effect of colostrum, colostral LMW components and piscidin (Publication II)

Numerous studies have shown that milk and colostrum from various mammals contain several AMPs (Murakami et al. 2005; Wang et al. 2011).

Even though we did not find any AMPs in bovine colostrum using proteomic tools, we continued the search for antimicrobial activity using bioactivity assays. The antimicrobial effects of colostrum and its LMW components were tested with both Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*) bacteria using both radial plate diffusion and liquid medium growth assays. However, no antimicrobial activity using microbiological assays was detected (Fig. 18).

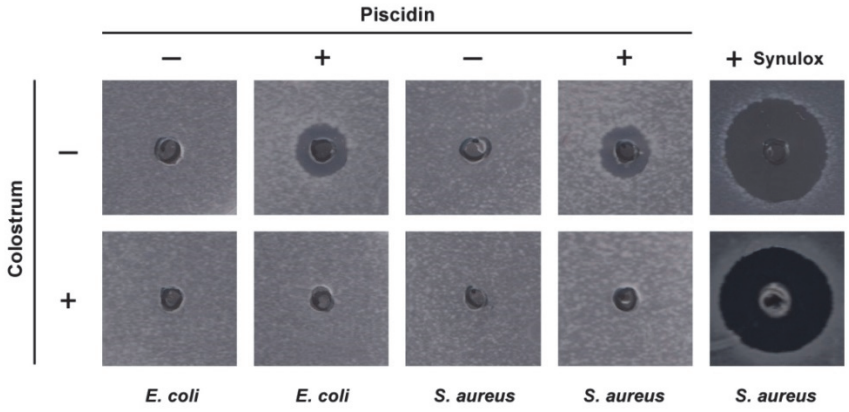

Figure 18. Radial Plate Diffusion Assay for testing antimicrobial activity of colostrum and piscidin. Sections of agarose plates are shown with compounds added in various combinations as indicated. -: sterile PBS, +Synulox were used as a positive control.

The pores of an agarose gel are large enough to allow even very large molecules to diffuse, and therefore should also be suitable for AMPs. Dialysis was performed to obtain colostral LMW fractions lower than 12-14 kDa. Each of these fractions were tested, however, no antimicrobial effect against bacteria was detected (Fig. 19).

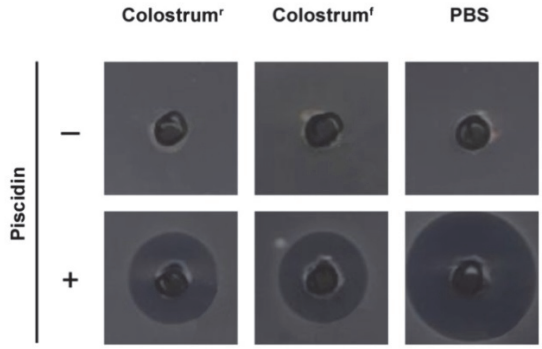

Figure 19. Radial Plate Diffusion Assay for testing antimicrobial activity of piscidin and LMW components obtained from colostrum on *S. aureus*. Sections of agarose plates are shown with compounds added in various combinations as indicated. LMW fractions obtained by dialyzing the reconstituted colostrum (colostrum^r) and fresh colostrum (colostrum^f) with 12-14 cut off dialysis bag. The piscidin concentration in the PBS was 10 fold higher than was added to the LMW colostrum fractions.

To ensure that our experimental design is able to detect the activity of AMPs, we used commercial antimicrobial peptide piscidin (MW=2571 Da) as a positive control. In the radial plate diffusion assay, piscidin had direct killing activity against both Gram positive and negative bacteria (Fig. 18 & 19).

Simply mixing colostrum with piscidin revealed that colostrum completely abrogated the antimicrobial effect of piscidin (Fig. 18). Addition of colostrum to a non-peptide antibiotic (Synulox) had no effect (Fig. 18).

Additional experiments with dialyzed LMW fractions from colostrum spiked with piscidin revealed that LMW fractions do not abrogate the antimicrobial activity of piscidin (Fig. 19). Therefore, one could speculate that either piscidin molecules (or other AMPs) are degraded by enzymes present in the colostrum but not in the dialyzed LMW fractions or that the piscidin was prevented from diffusing through dialysis membrane by high molecular weight (HMW) components in colostrum.

5.2.2. Piscidin degradation in colostrum (Publication II)

To rule out the possibility of enzymatic degradation, fluorescein-piscidin was incubated with fresh colostrum overnight at 37°C with and without bacteria. The results of radial plate diffusion assay indicate (data not shown) that when the colostrum concentration increased, the inhibition zone of bacterial growth decreased thereby demonstrating that fluorescein-piscidin was bound with colostral components and could not diffuse into the agarose to interact with the bacteria.

In addition, the fluorescein-piscidin degradation rate was analyzed with SEC and it was found that ~10% of the fluorescein-piscidin was degraded in fresh colostrum after overnight incubation at 37°C (data not shown). Fluorescein-piscidin degradation was also measured in reconstituted colostrum and less than ~5% of the fluorescein-piscidin was degraded. This reduction in degradation is probably due to the heat treatment that may have denatured and thereby inactivated the colostral enzymes. Furthermore, fluorescein-piscidin degradation was also analyzed in raw and skimmed milk, resulting in degradation of 9.5% and 10% of the fluorescein-piscidin respectively (data not shown). These results clearly show that not enough AMP was degraded during incubation to fully explain the abrogating effect of colostrum.

Further experiments with fluorescein-piscidin and colostrum indicate that colostrum even protects AMP from degradation by the action of various proteases (pronase E).

By first incubating fluorescein-piscidin with colostrum and then adding proteases revealed that fluorescein-piscidin eluted from the size-exclusion column together with caseins.

Afterwards, the degradation level of fluorescein-piscidin was only ~30% and ~10% in reconstituted and fresh colostrum, respectively (Fig. 20A & 20C). Next we changed the order of adding the components such that fluorescein-piscidin was first incubated with proteases followed by the addition of colostrum.

In this case, the degradation of AMP was ~90% in both colostrum samples, as indicated by the later elution time where fluorescein normally elutes from the SEC column (Fig. 20B & 20D).

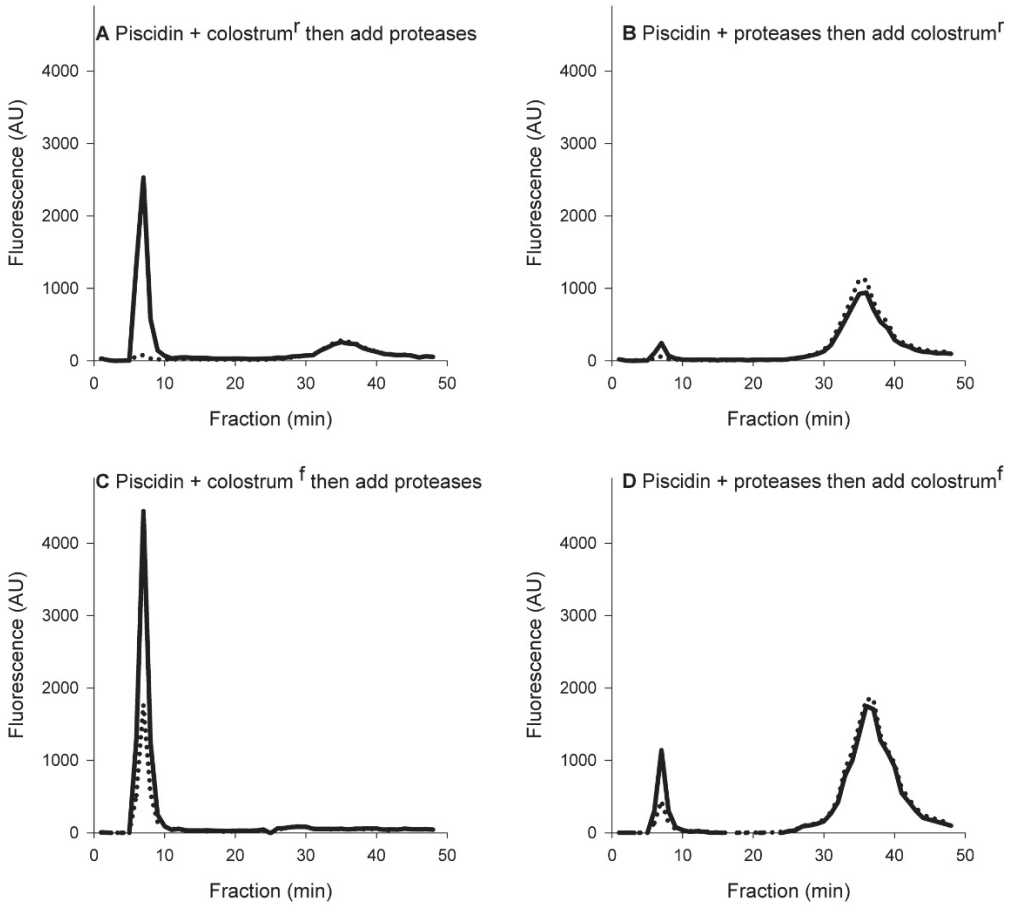

Figure 20. SEC analyses show that colostrum protects fluorescein-piscidin from degradation by proteases. Fluorescein-piscidin was incubated with reconstituted colostrum (A) and (B) or fresh colostrum (C) and (D) and treated with pronase E. The fluorescence of each fraction was measured directly (dotted lines) and after treatment with proteases for 30 min (solid lines) to release fluorescein. Superscript ^{r} and ^{f} stands for reconstituted and fresh colostrum, respectively.

Moreover, to prove that a protective effect of colostrum towards fluorescein-piscidin was specific and not caused due to the saturated amount of proteins that proteases could work with, we performed a parallel test using BSA. By mixing fluorescein-piscidin with BSA first and then adding proteases, the AMPs did not bind with protein and was completely degraded (Fig. 21).

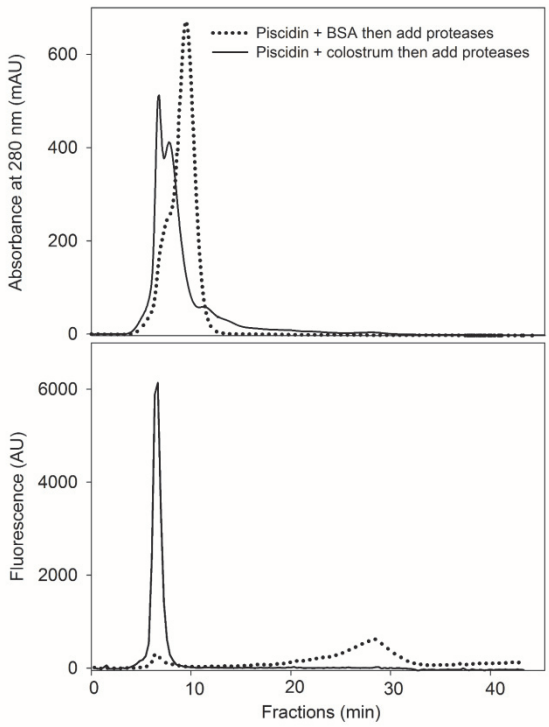

Figure 21. SEC of fluorescein-piscidin incubated first either with colostrum or BSA and followed by the addition of proteases. A) Absorbance at 280 nm of fluorescein-piscidin incubated with fresh colostrum (solid line) or BSA (dotted line). B) Fluorescence was measured 30 minutes after treatment with proteases to release fluorescein. The integrated areas under these four curves are: A) 1642 and 1803 or B) 20037 and 21162 for fresh colostrum and BSA, respectively. These results indicate that in the presence of the same amount of protein, only colostrum is able to protect fluorescein-piscidin against protease degradation.

In contrast, by mixing the fluorescein-piscidin with colostrum we can, presume that the caseins delay or even prevent the degradation of AMPs to some extent. Some studies have also shown that caseins could bind and transport polyphenols (Bourassa et al. 2013) and protect macronutrients (Livney 2010), nutraceuticals (Semo et al. 2007) and insulin (Zhang et al. 2008) from degradation.

5.2.3. Colostrum and casein binding studies with piscidin (Publication II)

The previous section proved that piscidin is not degraded in colostrum. Next, we tested the hypothesis that AMPs bind to HMW components within colostrum. The aim was to find out which specific components in colostrum are responsible for binding AMPs. For this, the fluorescein-piscidin fluorescent signal was measured in a cuvette for 1 min before and after adding reconstituted colostrum.

We observed that upon addition of reconstituted colostrum, the AMP fluorescence signal was quenched within one second (Fig. 22A). Taking into account that fresh colostrum emits a low intensity fluorescent signal, probably due to the presence of riboflavin in colostrum, tests using fresh colostrum resulted in the same quenching effect towards fluorescein-piscidin as observed with reconstituted colostrum. Moreover, raw milk also quenched the fluorescein-piscidin signal, but to a lesser degree. The addition of PBS resulted in only a dilution effect (Fig. 22A). Livney (2010) has reviewed and explained in detail the hydrophobic and electrostatic interactions between proteins and bioactive components in milk (Livney 2010). It is clear that colostrum contains a number of proteins that could interact with piscidin.

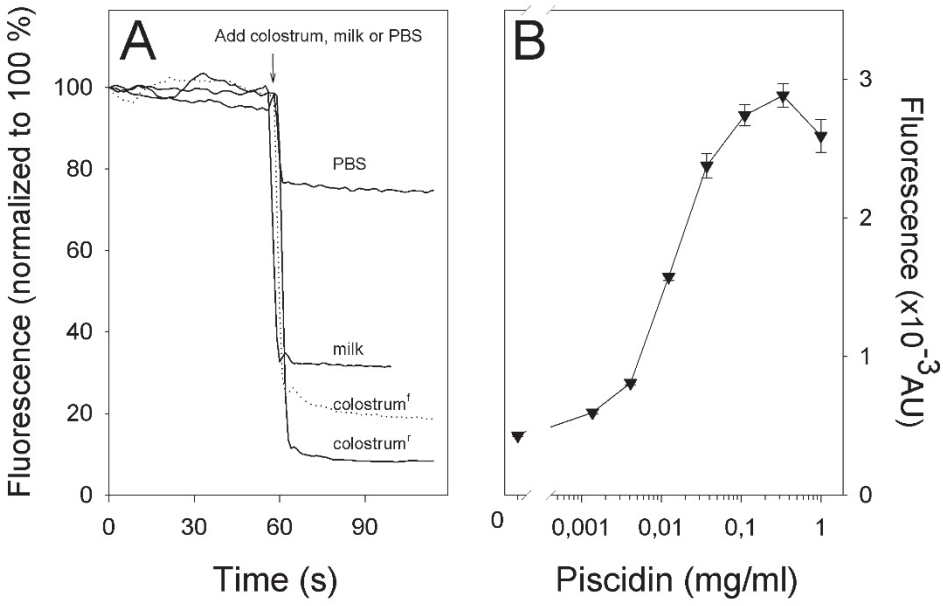

Figure 22. The specific quenching of fluorescein-piscidin fluorescence by colostrum and milk. A) Fluorescence measured before and after mixing fluorescein-piscidin with colostrum^{r} (reconstituted colostrum), colostrum^{f} (fresh colostrum) or milk. The addition of PBS alone shows dilution of fluorescence. The background fluorescence was subtracted using additions in PBS (colostrum^{r}: 5; colostrum^{f}: 108; milk: 93) before normalization of the signal. B) Quenching of fluorescein-piscidin after incubation with colostrum^{r} using different concentrations of unlabeled piscidin as indicated. Fluorescence in B was read after incubation for three minutes.

A control experiment was performed using only fluorescein, however, no quenching of fluorescein signal was observed under the same conditions (data not shown). Specific binding to components within colostrum was verified with additional experiments performed using BSA. BSA is an effective carrier for small molecules and, besides its role in blood, this protein is also found in milk and colostrum (Levieux & Ollier 1999). Nevertheless, BSA did not influence the fluorescent signal of fluorescein-piscidin and no interaction between piscidin and BSA was observed (data not shown).

The binding of fluorescent-piscidin with colostrum could be prevented by saturating the binding sites with unlabeled piscidin molecules. Colostrum incubated with different concentrations of unlabeled piscidin prevented subsequent binding of fluorescein-piscidin in a dose-dependent manner consistent with saturation (Fig. 22B).

To identify which component in colostrum could be responsible for binding piscidin, we performed a series of experiments with caseins. Caseins are the most abuntant proteins in milk and colostrum and contain phosphoserine residues that are capable of binding positively charged components such as calcium or cationic AMPs (Kumosinski et al. 1993b; Farrell et al. 2002; Farrell et al. 2004). One study observed the quenching of fluorescent signal of caseins when positively charged drugs were added; this was probably due to electrostatic interactions with the negatively charged surface of β-casein (Shapira et al. 2010). We began testing caseins because observations from the SEC analyses presented in the previous section also indicate that fluorescein-piscidin elutes from the column in the void volume together with HMW caseins and not at later elution times together with IgG and whey proteins.

As a first step, a cross-titration with caseins and piscidin was performed and various combinations between these two molecules were incubated with *S. aureus* to address both the electrostatic interaction and elminate the antimicrobial effect of piscidin. We found that without adding caseins to piscidin, piscidin maintained an antimicrobial effect towards *S. aureus*. However, piscidin is inhibited by the addition of casein in a dose-dependent manner (Fig. 23A). The highest concentration of caseins (30 μM) completely abrogated the antibacterial activity of the highest concentration of piscidin (54 μM). Interestingly, the interaction between caseins and piscidin always resulted in an all or none response concerning the antibacterial effect (Fig. 23B). Thus, there was a very sharp transition between growth and no growth at a molar ratio around five between piscidin and casein, thus indicating that one casein molecule can neutralize five molecules of piscidin.

As described above, this interaction probably takes place between negatively charged phosphate groups in caseins and positively charged amino acids in piscidin (at neutral pH, piscidin-1 has seven positively charged amino acids (Campagna et al. 2007) (Fig. 23C). Analogous electrostatic interactions have been described between pectin and casein micelles, however pectin is negatively charged and casein is positively charged at pH below 4.6 (Pedersen & Jørgensen 1991).

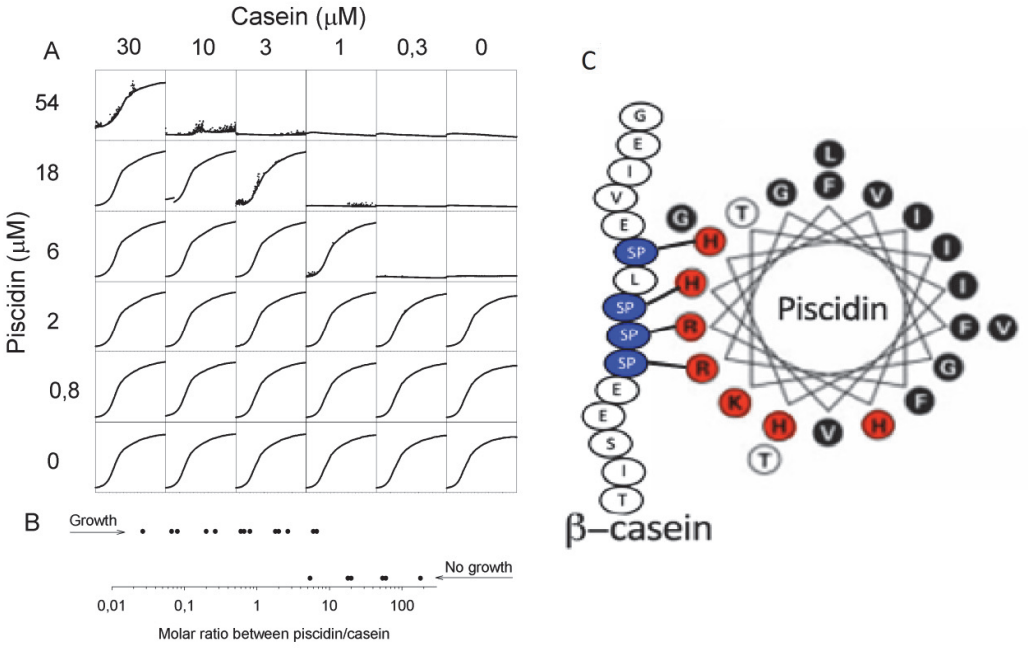

Figure 23. Effect of casein on the antibacterial activity of piscidin. A.) Cross-titration setup where each of the 36 squares representing a 10 h growth curve for S. aureus (time is on horizontal axis and OD600 is on vertical axis). The final concentrations of casein and piscidin mixed with the bacterial culture are indicated. B.) This plot shows the growth or no growth of bacteria as a function of the molar ratio between piscidin and casein based on data in (A). C.) Possible model for interaction through negatively charged phosphoserine residues in β-casein and positively charged amino acids in piscidin.

5.2.4. Disrupting the binding between colostrum and piscidin (Publication II)

The abrogating effect of both colostrum and casein towards piscidin is probably caused by electrostatic interactions. One way to test this is to find ways to dissociate this strong binding so that AMPs originating from colostrum can be released. During this study, several techniques and chemical components were used to dissociate added piscidin from colostrum. The most piscidin was released after adding GndHCl. NaCl and Urea were also tested, however, these were not as effective as GndHCl (data not shown). A follow-up of the previous experiment (Fig. 22A) revealed that after quenching the fluorescein-piscidin signal with colostrum, its fluorescence can be rescued with repeated additions of GndHCl (Figure 24A).

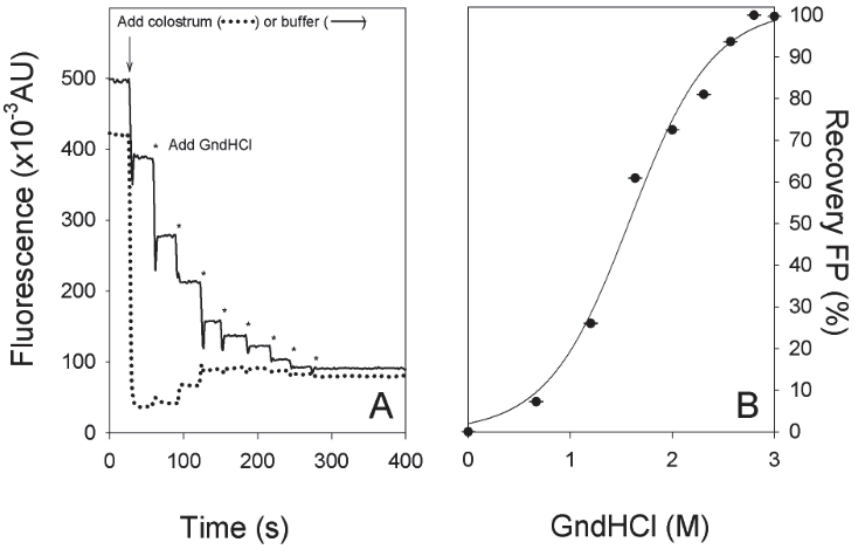

Figure 24. Recovery of fluorescein-piscidin binding to colostrum components using GndHCl. A) Fluorescence was measured as a function of time after mixing fluorescein-piscidin with colostrum^{r} (dotted line) or PBS (solid line) followed by consecutive additions (indicated by asterisks) of 6 M of GndHCl. B) Percent recovery of fluorescein-piscidin from colostrum^{r} data in (A). Superscript ^{r} stands for re-constituted colostrum.

Accounting for the dilution effect of each addition, a concentration of GndHCl up to 3M is able to completely release the added fluorescein-piscidin from the colostrum components (Figure 24B). A control experiment using fluorescein with additions of colostrum or PBS followed by step additions of GndHCl only revealed a dilution effect (data not shown).

The added piscidin was also recovered from colostrum after dialysing the mixture with 6M GndHCl; no recovery was observed in a sample dialysed with water. A purified LMW fraction together with added piscidin was characterized with MALDI-TOF-MS. The results showed that the sample dialysed with GndHCl contained piscidin (2571 m/z) (Fig. 25).

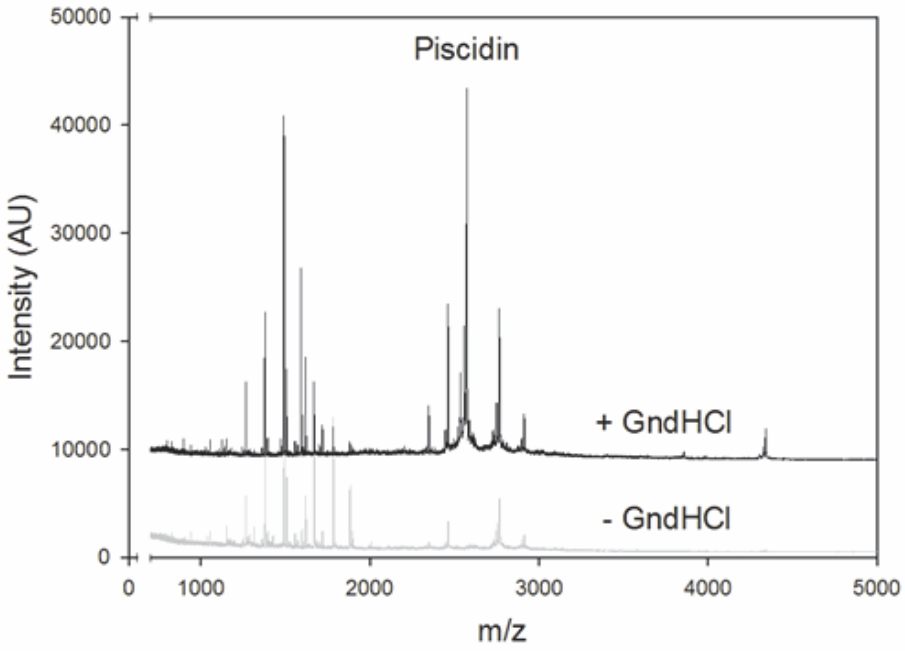

Figure 25. MS spectra of eluates from reconstituted colostrum spiked with piscidin. Grey spectrum of eluates obtained from colostrum in water and black spectrum from eluates obtained in presence of 6 M of GndHCl. Due to matrix suppression, m/z from 0-700 is omitted. The black spectrum is shifted by 10000 AU for clarity.

After using a strong denaturant to dissociate the electrostatic interaction between colostrum and piscidin, we tested the possibility that GndHCl could affect the activity of released piscidin. Allowing piscidin to interact with colostrum or casein first and then adding GndHCl caused a release of piscidin that retained its antimicrobial activity (Fig. 26).

A radial plate diffusion assay showed that without adding GndHCl, colostrum abrogated the effect of piscidin (1 mg/ml), but after adding 3M of GndHCl, ~80% of the piscidin was released and a clear inhibition zone without bacterial growth appeared (Fig. 26A).

There was no inhibition zone observed when only GndHCl was added (not shown) or in presence of colostrum with or without piscidin. In a liquid medium growth assay, the GndHCl alone did not affect the growth of bacteria; instead it dissociated the colostrum and piscidin interaction resulting in a delayed lag phase and suppression of *E. coli* growth (Fig. 26B).

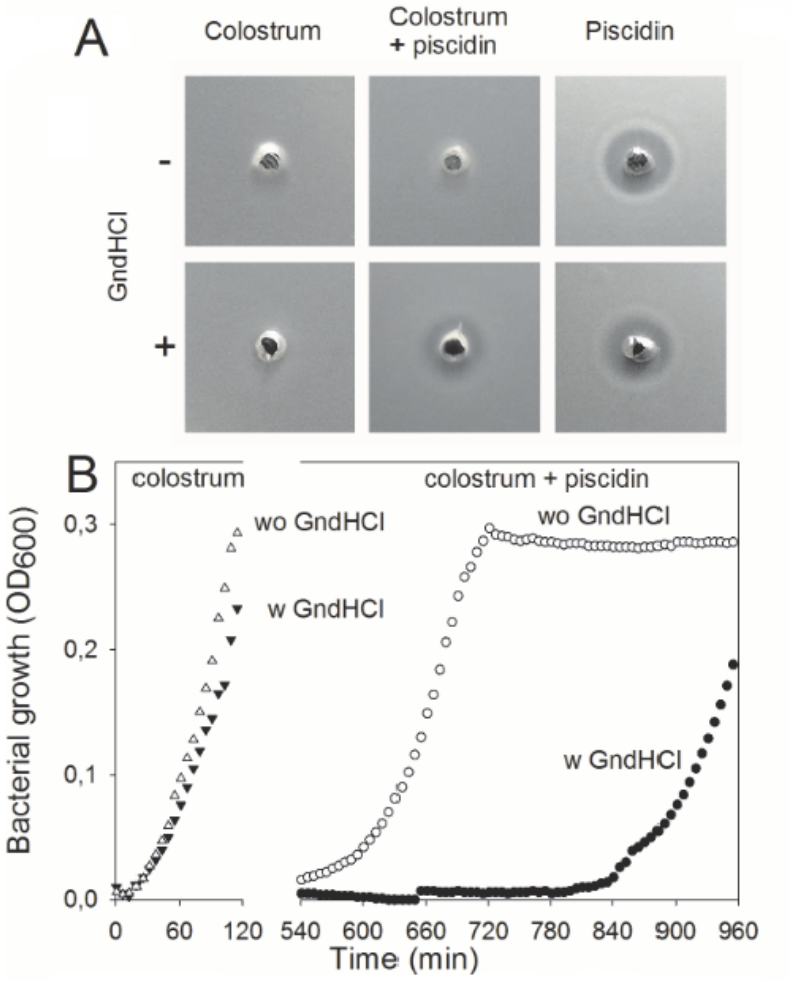

Figure 26. Antimicrobial activity of piscidin against *E. coli* after piscidin was recovered from reconstituted colostrum. A) Inhibition zones observed for piscidin alone or in presence of colostrum obtained either in absence or presence of 3 M of GndHCl. B) Bacterial growth in liquid medium either tested with colostrum alone or colostrum spiked with piscidin in the absence (open symbols) or presence (filled symbols) of 0.5 M of GndHCl. Growth curves with uninterrupted growth (0-120 min) are shown before the break in the time axis and initial suppression of growth by piscidin and piscidin released by GndHCl is shown after the break.

5.2.5. Different approaches to recover AMPs from colostrum and casein micelles (Unpublished results)

To follow up on the model system experiments between piscidin and colostrum presented above, an attempt was made to recover AMPs from colostrum and its HMW components using the same approaches. GndHCl and urea were used to recover colostral AMPs.

5.2.5.1. Obtaining AMPs from colostrum and washed caseins

The most direct way of recovering AMPs from colostrum was to treat fresh colostrum directly with different concentrations of GndHCl and incubate these with bacteria, such that the denaturants do not affect the growth of the bacteria. The best working combination, tested directly in micro titer plate wells, was obtained with 0.5 M GndHCl, which is able to dissociate piscidin from colostrum without affecting bacterial growth. However, despite all efforts, no antimicrobial activity was observed using colostrum treated with GndHCl (Fig. 27).

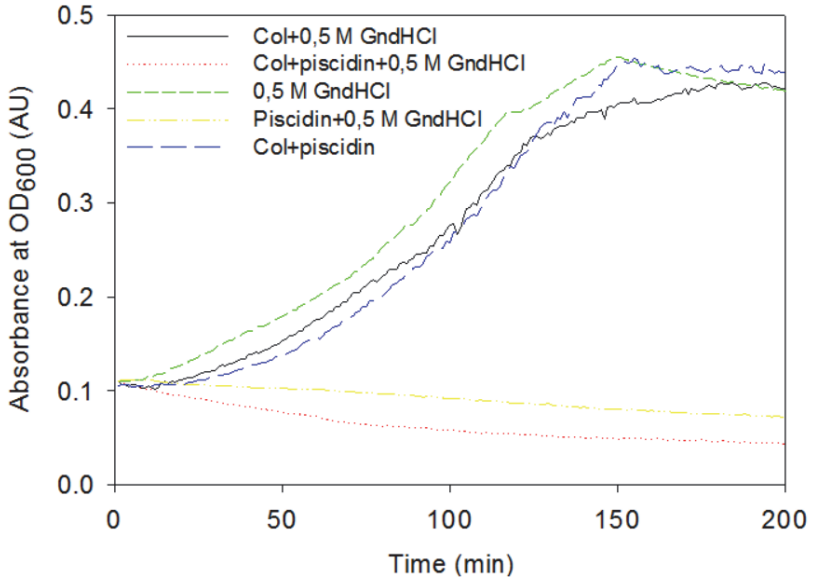

Figure 27. *E. coli* growth curve measured at OD_{600} nm. Fresh colostrum was treated with various concentrations of GndHCl directly on a micro titer plate. 0.5 M GndHCl turned out to be the safest concentration that does not inhibit the growth of a bacterium yet is able to release piscidin (1 mg/ml).

Our previous results show that piscidin interacts with HMW components within colostrum. An attempt was made to recover AMPs from washed caseins obtained by ultra-centrifugation from fresh colostrum. The washed caseins were either spiked with piscidin or not and dialyzed against either 3 M of GndHCl or 4 M of urea. The recovered LMW components, which could possess antimicrobial activity, were further purified in a reverse-phase C_{18} column using HPLC. In total, 96 fractions were collected directly into micro titer plates. All 96 fractions were further tested in a liquid medium growth assay to detect potential antimicrobial activity.

As with the colostrum samples, antimicrobial activity was observed only in the HPLC fractions where piscidin had been added to the washed caseins and either treated with GndHCl (Fig. 28A) or urea (Fig. 28B). The samples without piscidin did not indicate any antimicrobial effect towards growth of *E. coli* (Fig. 28). All of these fractions were also tested with the radial plate diffusion assay and not a single HPLC fraction had any effect on bacterial growth (data not shown). Controls with GndHCl and urea alone also did not affect bacterial growth.

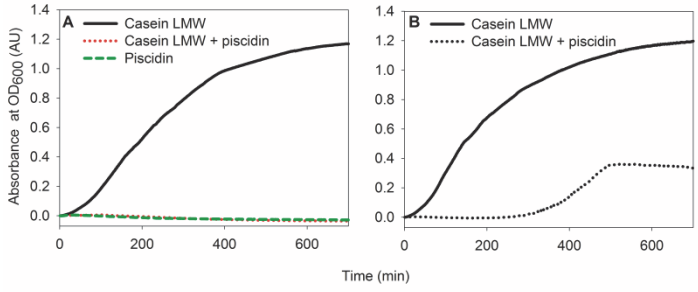

Figure 28. *E. coli* growth curves measured at OD_{600} nm. Fractions collected from an HPLC were tested with bacteria. The growth of *E. coli* was inhibited in the fraction where added piscidin was dissociated from caseins with either 3 M of GndHCl (A) or 4 M of urea (B). Dotted lines indicate the growth inhibiting effect of the recovered piscidin. Solid black lines indicate the effect of these same fractions without piscidin for the growth of bacteria. The positive control of piscidin alone suppressed the growth of bacteria (dashed green line).

Although endogenous AMPs should be in the LMW fractions obtained from washed and denatured casein dialysates, the retentate inside the dialysis bag containing HMW components was also analyzed. The caseins formed a gel when dialyzed against GndHCl or urea and required an extra 3 M of GndHCl to dissolve again. After centrifugation, the supernatant was analyzed with SEC at 280 nm (Fig. 29).

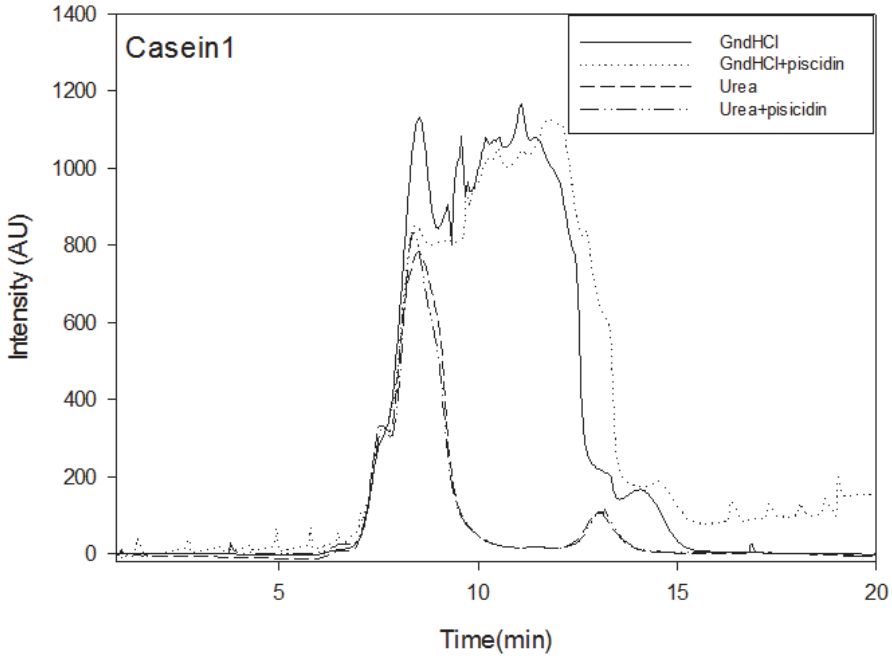

Figure 29. GndHCl and urea treated casein retentates re-solubilized in additional GndHCl and analyzed with SEC FPLC. The mobile phase was 3M of GndHCl. Chromatograms are shown from 0 to 20 minutes. Absorbance was measured at 280 nm.

The chromatograms are almost identical regardless of whether or not the casein sample was previously spiked with piscidin. Fractions eluting at 13-15 minutes into the chromatogram from the urea treated caseins (GndHCl treatment did not result in so clearly separated peaks) had some UV absorbing components, however, no antimicrobial effect was detected (data not shown).

5.2.5.2. Urea gel electrophoresis

Gel electrophoresis has often been used to separate the main milk proteins such as caseins and whey proteins (Kunz & Lönnerdal 1990). Moreover, the presence of urea in the gel allows for better dissociation of α-, β- and κ-caseins (Veloso et al. 2002). Urea gel electrophoresis also has been used to purify AMPs such as piscidin from crude tissue extract (Noga et al. 2009). Combining this knowledge, urea agarose gel electrophoresis was applied to dissociate bioactive LMW components from colostral caseins. Washed caseins were prepared from fresh bovine colostrum by ultra-centrifugation and combined with fluorescein-piscidin before addition to wells of 3 M urea agarose gel in a slightly acidic PBS buffer (pH 6.0). Fluorescein-piscidin alone and casein micelles alone were included as controls. The gel was photographed under UV light (254 nm) before and during electrophoresis. The zero time picture shows a remarkably high fluorescent signal only for the combination of caseins and fluorescein-piscidin (Fig. 30), and a much lower signal from either the casein pellet alone or fluorescein-piscidin alone.

This was a surprising result because we had observed strong quenching of fluorescein-piscidin by the addition of colostrum or caseins when measured in the spectrofluorometer, although we did observe a small background fluorescence signal from colostrum alone. However, it appears that under the conditions of our gel electrophoresis setup, a strong synergistic effect on florescence was observed – this could be due to the different wavelength used for excitation (254 nm) compared with our quenching studies (490 nm). Tryptophans in the caseins fluoresce and can be sufficiently excited at 254 nm, however, the addition of unlabeled piscidin had no effect (this would be expected if piscidin was able to immobilize (some of) the tryptophans – conditions which are known to strongly enhance fluorescence of tryptophans); addition of fluorescein to caseins also had no effect. Immobilizing the fluorescein group of fluorescein-piscidin could also have an effect in shifting excitation/emission towards wavelengths not monitored in the spectrofluorometer.

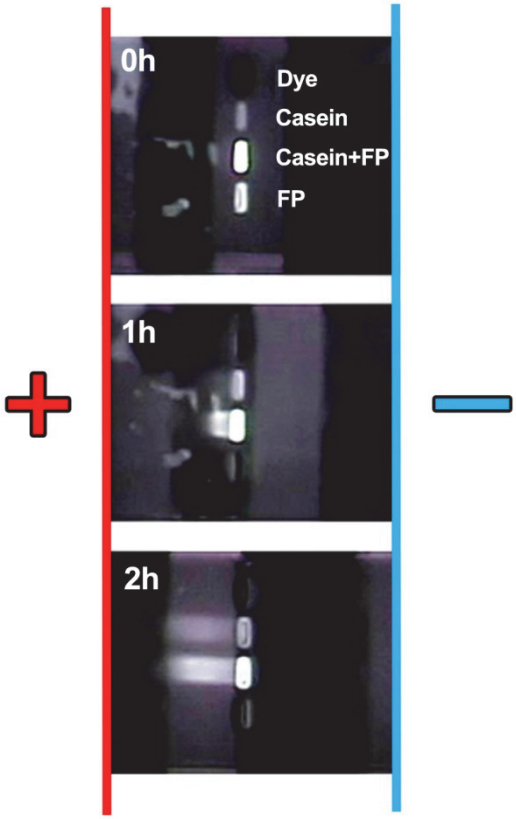

Figure 30. Blue dye; casein; casein spiked with fluorescein-piscidin, and fluorescein-piscidin alone in 3M of urea gel before (0 h), 1 h after, and 2 h after initiation of electrophoresis. The red bar indicates the anode and the blue bar the cathode. Caseins would migrate to the anode, whereas positively charged fluorescein-piscidin would migrate in the opposite direction.

After 1 h of electrophoresis the fluorescein-piscidin was still strongly attached to the caseins, the majority of which had not moved – only a small fraction had moved towards the anode. Presumably, fluorescein-piscidin had not been dissociated from colostral caseins. Interestingly, at the end of the run, the fluorescein-piscidin was not detectable anymore. After several hours, the signal from both components had faded away and isolation of these components from the gel was not possible (Fig. 30).

By examining the gel continuously under UV light, it was observed that fluorescein-piscidin faded without moving significantly. It is clear that even if some casein-associated components would be able to dissociate, probably the majority of AMPs, at least fluorescein-piscidin, would form strong precipitates with casein micelles and become stuck on the bottom of the wells thus preventing their migration even in a strong electrical field.

5.2.5.3. Other dissociation approaches

Apparently, GndHCl and urea are able to dissociate piscidin from colostrum, however, we were not able to dissociate any native AMPs from colostral HMW fractions. Many of the following experiments were performed with bioactivity assays and not with physical binding studies.

Several other attempts were made to try to recover AMPs from colostrum and caseins. First, we reasoned that the positively charged AMPs would bind electrostatically to the many serine phosphate groups in caseins and we thus tested if alkaline phosphatase could cleave the phosphate groups from caseinate and thereby interfere with an interaction between positively and negatively charged components. Unfortunately, alkaline phosphatase also eliminated the antimicrobial activity of piscidin, probably due to the protease side activity of this enzyme preparation.

Next, we tried using chymosin, the protease produced by the calf and used to coagulate the caseins during cheese production, to dissociate AMPs. Unfortunately, chymosin or proteolytic impurities in the preparation (fermentation-produced chymosin by CHR Hansen), eliminated antimicrobial activity of the piscidin added as a positive control. This could be due to side activity of the high concentration of chymosin, because piscidin does not contain the preferred substrate amino acid sequence for chymosin cleavage. Similar results were obtained when applying elastase (data not shown).

One published study has also shown that the human antimicrobial peptide LL-37 is sensitive towards nine commercially available proteases and lost its bioactive function in proteolytically affluent environments (Moncla et al. 2011). Therefore, further attempts to release AMPs with other proteases were deemed to fail, because these enzymes would likely degrade the AMPs and abolish their activity.

We also tried to increase the ionic strength using 2 M NaCl to compete with the electrostatic interactions between piscidin and caseins. However, we were not able to recover spiked piscidin. Surprisingly, NaCl abolished the antimicrobial effect of piscidin in the control sample and yet, despite the high concentration of salt, did not affect the growth of *E. coli K-12*. This result corroborates an observation made in another study, where *E. coli* survived in a medium with high NaCl (20%) for 72 h (Hrenovic & Ivankovic 2009).

EDTA and DTT were also used to dissociate possible interactions between LMW and HMW components from colostrum and caseins. EDTA would chelate calcium and disperse the casein micelles, and DTT would reduce any covalent disulfide bonds between colostral defensins and proteins. A series of experiments tracked by SEC revealed that DTT and EDTA did release some LMW fractions from colostrum, however, upon closer inspection, the apparent antimicrobial activity came from the controls containing EDTA and/or DTT alone (data not shown).

An acidic condition at pH 3 was also tested to try to dissociate AMPs from caseins. However, the spiked piscidin was not recovered and bacterial growth was not suppressed (data not shown). It is possible that AMPs, including spiked piscidin, could be trapped by the large casein aggregates that form when reducing the pH to the isoelectric point (pH 4.6) and beyond. It is also possible that thermal treatment could be used to denature the higher structures of proteins and release AMPs that are potentially bound to them.

To test this, colostrum was spiked with piscidin and heated, however, no antimicrobial activity of piscidin was detected. It is possible that this also was due to non-specific trapping of spiked piscidin and colostral AMPs by the protein aggregates.

Although, we were able to recover spiked in piscidin from colostrum and caseins in a model system, we could not obtain any LMW colostral components that had antimicrobial activity. Neither colostrum itself nor its LMW fractions displayed any inhibitory activity towards bacteria, at least under the conditions employed herein that had worked in the model system with piscidin.

5.3. Other bioactive effects of peptides

5.3.1. Effects of LMW colostrum fractions and yeast extract on murine intestinal cell line mIC$_{C12}$ (Unpublished data)

After attack by an antagonist, the innate immune system of mammals depends on the activation of receptors, such as members of the TLR family that provide a signal via NF-κB activation. A NF-κB signaling assay using a mIC$_{C12}$ cell line was performed to study the immune enhancing system activity of colostrum LMW fractions, which may contain AMPs. The immune system enhancing activity of AMPs could be detected in these cells after AMPs electrostatically bind with LPS and suppress luciferase activity which would be detected by a decrease in the chemiluminescence signal.

First, the assay was evaluated with piscidin that had been incubated with either cells alone or together with LPS to induce the inflammatory response measured through luciferase activity.

We observed that different concentrations of piscidin alone did not cause any inflammatory response in the cells, however, in the presence of LPS, piscidin eliminated LPS-induced expression of luciferase in a dose dependent manner (Fig. 31), probably by binding to LPS and preventing LPS from binding to TLR-4/MD-2/CD14, as described for LL-37 (Mookherjee et al. 2006). However, we cannot rule out the possibility that in higher concentrations the piscidin alone (~ 1 mg/ml) supresses luciferase expression due to a toxic effect that leads to cell death. Still we performed one test with an ATP assay that revealed a high luminescence signal which verified a functional metabolism and thereof viability of the mIC_{C12} cells (data not shown).

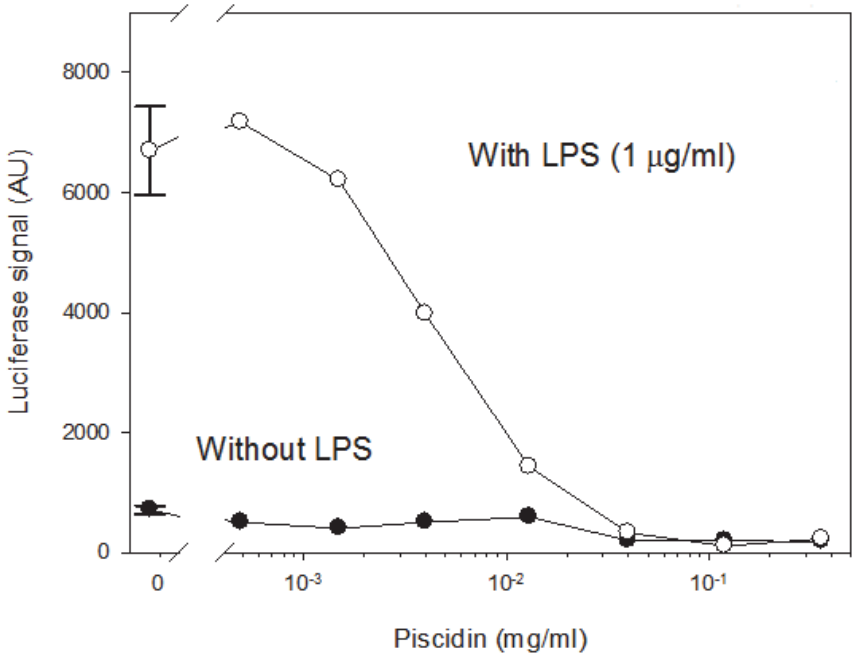

Figure 31. Measuring the NF-κB dependent inflammatory responses in mIC_{C12} cells through expression of luciferase. Three fold serial diluted piscidin was tested with the murine intestinal cell line mIC_{C12} in the presence or absence of LPS. The luminescence was measured from lysed cells in excess of ATP and luciferin.

Next, the LMW dialysates obtained from fresh colostrum were tested on one week old cell monolayers. The pH of the colostrum LMW fractions was always acidic (~6.5, even with the buffer control). However, adjusting the pH to 7.0-7.4, which should be more tolerable for the cells, often caused precipitation, which means that potentially active peptides could co-sediment. On the other hand, keeping a low pH to avoid precipitation affects luciferase activation in these cells (Fig. 32). Therefore, LMW fractions from colostrum were adjusted to pH ~7. Filtered and purified samples were used to avoid insoluble particles, which could give artefacts.

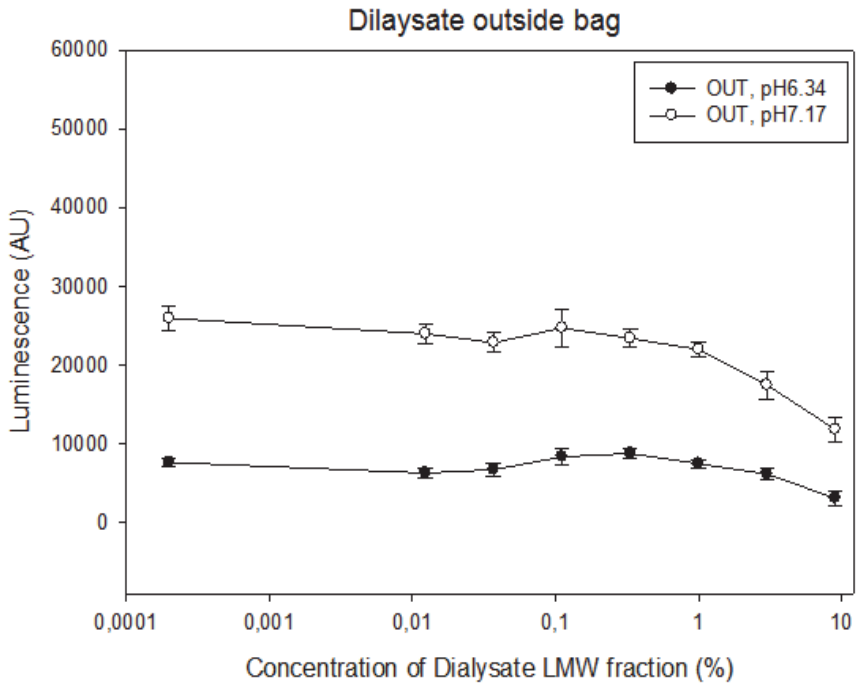

Figure 32. Measuring the effect of colostral LMW fractions at two different pH values. Three fold serial diluted dialysed LMW fractions obtained from fresh colostrum were tested with the murine intestinal cell line mIC_{C12} in the presence of LPS (1 µg/ml).

Further tests with the supernatant of a colostral LMW fraction displayed no promoting effect on luciferase expression (Fig. 32). Adding supernatant to LPS strongly inhibited the luciferase expression, much like piscidin (Fig. 33). Still, at high concentrations, the supernatant of a colostral LMW fraction alone displayed an inhibiting effect which allows us to speculate about the viability of cells incubated with a potentially high concentration of colostral AMPs.

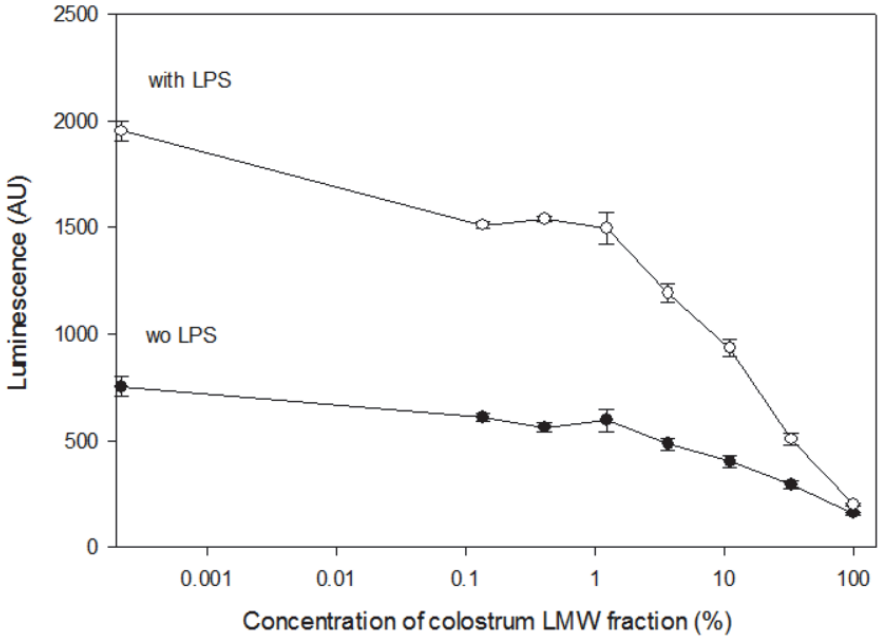

Figure 33. Measuring the effect of colostral LMW fraction on the inflammatory response in mIC_{C12} cells. Serial dilution of a colostrum LMW fraction were tested in the presence or absence of LPS.

Therefore, we performed a control containing 22% of the supernatant of the colostral LMW fraction and observed that with an increase in LPS concentration, the luciferase signal increased and thus the colostral supernatant had no supressing effect.

Moreover, we also used the WST1 Proliferation assay to measure the cell mitochondrial metabolism during incubation with mixture of colostrum LMW fraction (15%) and LPS, and found clear evidence of cell viability in the presence of a high concentration of this colostral fraction (data not shown).

We also performed a series of experiments with colostral HMW fractions. In contrast with LMW fractions, HMW samples induced a luciferase signal. Unfortunately, we observed heavy precipitation of cell wells and therefore probably measured an artefact (data not shown). Still, the results obtained by Jørgensen et al. (2010) showed that intact colostrum decreased the signalling induced by bacterial ligands (Jørgensen et al. 2010). Furthermore, bovine lactoferrin down-regulates the expression of cytokines in the Caco2 cell-line after infection by *E. coli* without causing an inflammatory response (Berlutti et al. 2006).

SEC confirmed that the size distribution of LMW fraction obtained from colostrum was between 400 and 12500 Da, with the most intense peaks were between 700-1000 Da (data not shown). Because the LMW active components from fresh colostrum do not bind to C_{18} columns, one could speculate that they are short hydrophilic peptides, although it has not been verified yet that they are indeed peptides. It would thus appear that colostrum LMW components could have immune-modulating effects.

The NF-κB dependent inflammatory response in mIC_{C12} was also performed using yeast hydrolysates, which are often added as growth promoting components in various food production processes. The rehydration of yeast extract with DMEM did not cause any precipitation and the pH was also acceptable for the cells. The yeast hydrolysate did not inhibit LPS-induced NF-κB activation (Fig. 34).

Moreover, the yeast fractions induced an inflammatory respone and increased the basic level of luciferase expression. This phenomenon can be explained in several ways.

We demonstrated that adding yeast extract to the growth medium increases the specific growth rate and production of the *L. lactis* biomass (Kevvai et al. 2014). This could occur with the mIC_{C12} cells too, so after 4 h of incubation, the biomass of murine intestinal cells may have increased which can explain the observed increase in luciferase expression. Alternatively, the increased expression of luciferase could be explained by β-glucans, which are major component in yeast cell walls that could induce an innate immune response and trigger inflammation (Ortuño et al. 2002; Chan et al. 2009). Although these extracts contain mainly intracellular components of yeast, it is still possible that some cell wall components have not been completely removed. Another explanation could be that the yeast hydrolysates were contaminated with bacterial LPS (Chan et al. 2009), which accelerates the luciferase emission in mIC_{C12} cells.

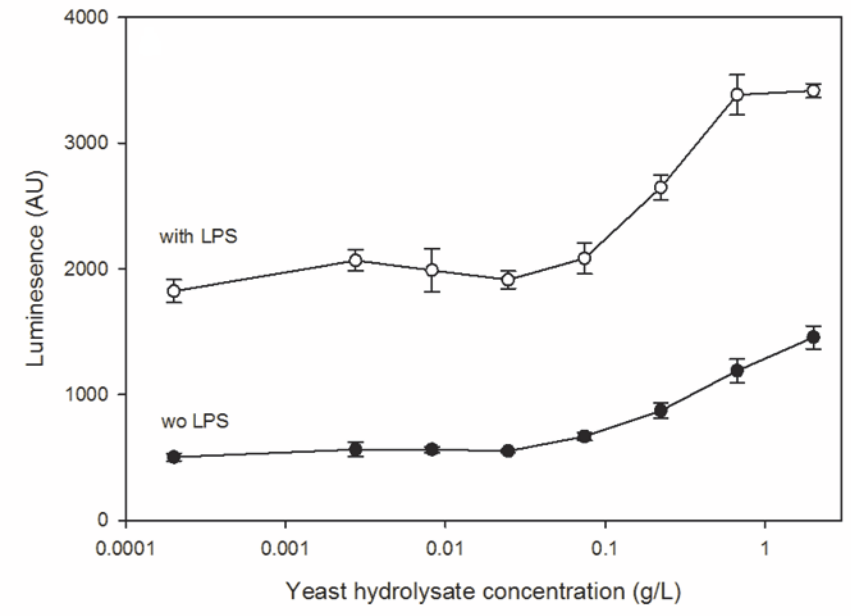

Figure 34. The effect of added yeast extract on luciferase expression in mIC_{C12} cells. Serial dilutions of yeast extract were tested in the presence or absence of LPS.

More studies are needed to characterize and identify the molecules from colostrum that may bind to LPS and prevent the inflammatory response in intestinal cells. Moreover, further experiments should be performed with yeast extracts to determine if the induced immune response could be due to peptides. One possibility is to use proteases to rule out any effects induced by LPS and glucans, which are both polysaccharides and should not be susceptible to enzymatic degradation by proteases. The same experimental design could also be used with colostrum components because the immune enhancing components did not bind to the C_{18} column, wich raises the possibility that these components are not peptides at all. For further identification of active components, the colostrum fractions and yeast extracts should be fractionated and these fractions should be tested one by one with a cellular reporting system and further characterized by proteomic approaches.

5.3.2. Consumption of free and peptide-bound amino acids by lactic acid bacteria (Publication III)

In respect of amino acid consumption, mammalian cells are similar to lactic acid bacteria, and therefore we developed methodology to study the peptide consumption of *Lactococcus lactis* as a model system.

Peptides are important biomolecules with both nutritional and physiological roles in various organisms. More specifically, peptides are a valuable source of amino acids and also play important roles in signaling processes such as gene expression and cell proliferation (Kruzel et al. 2001; Doeven et al. 2005).

One of the most important microorganisms utilized on an industrial scale is *Lactococcus lactis*, which is responsible for the fermentation of various dairy products. *L. lactis*, much like other lactic acid bacterial species, is a fastidious organism and auxotrophic for several amino acids. The strain IL1403 used in the current study is auxotrophic for leucine, isoleucine, valine, methionine, histidine, and arginine (Zhang et al. 2009).

Depending on the growth environment, amino acids could either be obtained directly as free amino acids or from peptides. Therefore, yeast extracts, which are an excellent source of various peptides, could be used as a growth-inducing medium supplements for bacteria.

L. lactis consumes peptides by first taking them into the cells through proton-coupled peptide transporters or ATP binding cassette (ABC) transporters (Foucaud et al. 1995; Lamarque et al. 2011). After translocation into the cell, peptides are hydrolyzed by intracellular peptidases and the resulting amino acids are then used for intracellular metabolic processes, including the synthesis of proteins.

However, studies that have focused on the co-consumption of free and peptide-bound amino acids by *L. lactis* are scarce. We performed a series of batch experiments with the non-pathogenic *L. lactis* strain IL1403 to study the consumption of ^{15}N-labeled yeast hydrolysate peptides as a source of amino acids in the presence of an abundance of free amino acids.

First, we used SEC to determine the size of the peptides consumed during these experiments. Our analysis of the culture media indicated that the yeast hydrolysate used in the experiments contains peptides with molecular weights up to ~5 kDa, whereas peptides with molecular weights up to ~1.7 kDa were consumed in significant amounts by *L. lactis* IL1403 (Fig. 35).

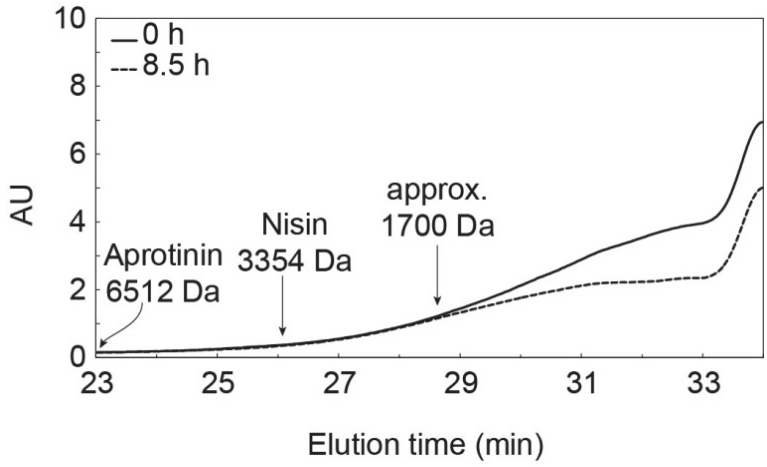

Figure 35. SEC of the culture medium collected during the cultivation of *L. lactis* IL1403 in a synthetic medium supplemented with ^{15}N-labelled yeast hydrolysate. Solid and dashed lines show chromatograms at the beginning and end of the experiment, respectively. The arrows denote the elution times of two marker peptides, and the upper size limit of media components consumed during growth (adopted from (Kevvai et al. 2014)).

The only known transport system for oligopeptides in L. lactis IL1403 is Opt (Lamarque et al. 2004; Doeven et al. 2005; Lamarque et al. 2011), which is a part of the ABC transporter family. Several studies indicate that the Opt system is only capable of transporting oligopeptides with up to six amino acid residues (Juillard et al. 1995; Lamarque et al. 2011). The gradual disappearance of larger peptides from the culture medium could theoretically be explained by extracellular proteolytic activity. However, because *L. lactis* IL1403 does not have extracellular proteases, the only possible explanation is that peptides up to 1700 Da were transported into the cells.

Moreover, the virtue of using heavy isotope labelled nitrogen (^{15}N) in yeast hydrolysate together with unlabeled free amino acids allowed to follow the relative incorporation of free and peptide bound amino acids into the biomass.

The results show that the ^{15}N-labeled-peptides were consumed in parallel with free amino acids and contributed approximately 30 to 60 % to the production of biomass in the beginning stages of fermentation, with the remainder originating from free amino acids (Fig. 36). During later stages of fermentation, the proportion of free amino acids incorporated into the biomass increased, which indicates that the more readily assimilated peptides were likely depleted from the medium.

We observed several exceptions to this rule in the consumption patterns of some amino acids. Both glutamine/glutamate and asparagine/aspartate* were mainly utilized as free amino acids with only 20-25% incorporation of labeled amino acids that originated from the yeast hydrolysate peptides (Fig. 36A).

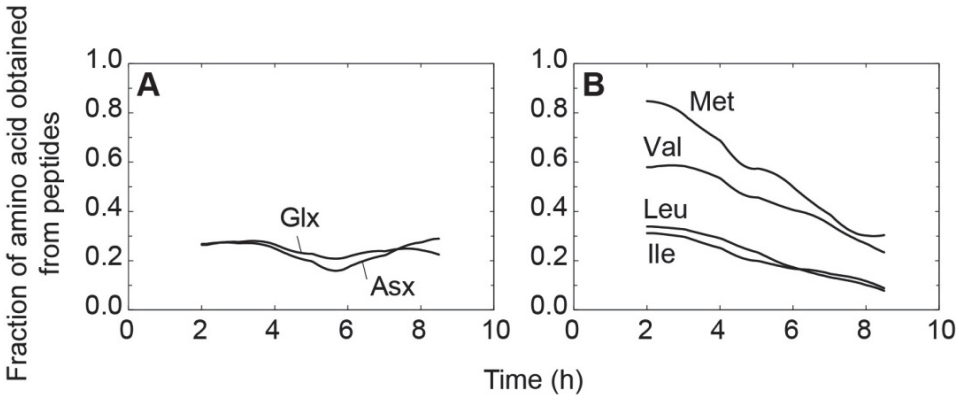

Figure 36. Fraction of amino acids obtained from peptides during the growth of *L. lactis* IL1403 in a synthetic medium supplemented with ^{15}N-labelled yeast hydrolysate. A) The consumption patterns of peptide-bound Glx - Glutamine + Glutamic acid and Asx - Asparagine + Aspartic acid. B) The consumption patterns of peptide-bound methionine and branched-chain amino acids.

* *After acid hydrolysis of biomass samples glutamine and glutamate as well as asparagine and aspartate are pooled.*

In contrast, methionine uptake from peptide bound forms was ~80% during first hours of fermentation (Fig. 36B). One explanation could be that free methionine shares the transport system with branched-chain amino acids (BCAA) (Hengst et al. 2006), which could cause a situation where one amino acid intake into the cell is more preferred compared to another. Even though all four amino acids are essential for IL1403, the specific transporter affinity is higher towards free BCAAs than methionine and, therefore, uptake of free methionine from the culture medium was inhibited thus making it easier for cells to utilize methionine liberated from peptide-bound forms.

The labelling data also revealed that amino acids are transported bidirectionally. Peptides are transported into the cell and digested to obtain amino acids. An excess of amino acids was then released back into the medium. Our results indicate that the amount of amino acids secreted back into the growth medium was of the same order of magnitude as the amino acids theoretically required for biomass production (data not shown). As excess amino acids are ejected into the medium a proton is probably released; this results in the generation of metabolic energy in the form of proton motive force (Trip et al. 2013) and can be used to maintain the intracellular pH.

The study aids in our understanding of the co-consumption of free and peptide-bound amino acids by *L. lactis* IL1403. The exceptions in consumption patterns of several amino acids help to identify potential metabolic bottlenecks. This data could be used to design an efficient growth medium for a specific bacterial strain that would result in increased specific growth rates and production yields during bioprocessing. More specifically, this knowledge could be used to design novel yeast hydrolysates with optimized bioactive peptide content for particular organisms to accelerate and stabilize both laboratory and industrial scale fermentation.

Conclusion

Six main conclusions result from this dissertation.

I. A method to identify and characterize low molecular weight colostral peptides without complex sample pretreatment was developed and tested (Publication I). This preliminary study applied the NALDI method for the first time to detect LMW peptides from colostrum. A comparison between NALDI and MALDI revealed that LMW colostral peptide peak intensities obtained using NALDI were at least ten times higher than those obtained using MALDI. The NALDI method showed high sensitivity in the lower m/z region where MALDI suffers from matrix suppression and does not allow for the detection of small molecules.

II. A model system of bovine colostrum and piscidin was developed to study the interactions between antimicrobial peptides and HMW compounds within colostrum (Publication II). The outcome was that:
    a. Colostrum was capable of completely abrogating the activity of a spiked in antimicrobial peptide (piscidin).
    b. Colostrum did not degrade piscidin and it even protected piscidin against degradation by added proteases.
    c. Colostrum bound piscidin specifically; this effect was not seen with BSA.
    d. Piscidin bound to colostrum casein micelles, but not with IgG or whey proteins. The binding is probably caused by electrostatic interactions between positively charged amino acids in piscidin and negatively charged phosphoserine residues in caseins.

e. The interactions between caseins and piscidin could be dissociated by strong denaturant (GndHCl), without affecting the antimicrobial activity of piscidin against bacteria.

III. Caseins could be carriers of AMPs and could act as a transport vehicle for small molecules that originate from colostrum and milk into the gastrointestinal tract.

IV. Various chemical treatments, such as the addition of denaturants, salts, and enzymes, and the use of methods such as gel-electrophoresis and size exclusion chromatography were used to try to recover AMPs from colostrum and casein fraction of colostrum, however, no antimicrobial activity was detected.

V. Instead, an anti-inflammatory function was observed using a mammalian reporter system. The mIC_{C12} cells were used to detect bacterial LPS-induced proinflammatory responses through members of TLR family that signal via NF-κB activation. The immune enhancing activity could be measured in the cells as a consequence of AMPs that electrostatically interact with LPS and act as anti-inflammatory agents. Moreover, colostrum LMW dialysates also appeared to have this anti-inflammatory effect. Therefore, colostrum could have immune-modulating effects in mammals.

VI. We studied the co-consumption of free and peptide-bound amino acids by *Lactococcus lactis subsp. lactis* IL1403. A novel approach based on the utilization of ^{15}N-labelled yeast hydrolysate was used to gain insight into the role of peptides as a source of amino acids under conditions where free amino acids are abundant (Publication III).

a. During bacterial growth we observed the co-consumption of peptides and free amino acids.
b. A considerable efflux of most free amino acids was observed during growth.
c. The incorporation of a particular amino acid was more dependent on its availability in a readily assimilated form for the cell than the organism's auxotrophy for it. During the fermentation, more readily assimilated peptides were gradually exhausted from the growth medium.
d. Our analysis indicates that the yeast hydrolysate contain peptides with molecular weights up to ~5 kDa. Peptides with molecular weights up to ~1.7 kDa were consumed in significant amounts by *L. lactis* IL1403.

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Curriculum Vitae

Curriculum Vitae

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Kursus:**'' New methods in assessment of food/feed quality and safety''**. Kursuse ettevalmistus. Kursuse läbiviimine, õpilaste juhendamine, analüüside läbiviimine, andmetöötlus.

Appendices

Publication I

Kütt M-L, Malbe M, Stagsted J

Nanostructure-Assisted Laser Desorption/Ionization (NALDI) for analysis of peptides in milk and colostrum

Agronomy Research, 9, 415 - 420 (2011)

Nanostructure-Assisted Laser Desorption/Ionization (NALDI) for analysis of peptides in milk and colostrum

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Abstract. Several bioactive proteins have been identified in colostrum and milk. However there are needs for development of technologies to identify and purify low molecular weight (LMW) peptides with bioactivity. The most used method is Matrix Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS), but matrix suppression often prevents detection of LMW components. Our approach was to work out a suitable method for analysing small peptides in bovine milk and colostrum without extensive sample pre-treatment. Nanostructure-Assisted Laser Desorption/Ionization (NALDI) is a matrix-free method to identify such LMW components. We also made a comparison between MALDI and NALDI for detection of peptides from colostrum samples. Our results show that NALDI provide better intensity compared with MALDI. It allows us to sequence small peptides and to identify a fragment of β-casein from the colostrum sample. Further studies are needed for comprehensive identification and characterization of LMW bioactive peptides from colostrum and milk.

Key words: colostrum, low molecular weight peptides, MALDI, NALDI.

INTRODUCTION

Colostrum and milk are rich sources of nutrients and also assure optimal immune defence in the newborn. It is due to their high content of biologically active compounds protecting against pathogens and diseases. Until now, several bioactive proteins have been identified in colostrum and milk like immonoglobulins, lactoferrin, cytokines, and antimicrobial proteins and peptides, such as defensins and cathelicidins (reviewed by Park, 2009; Stelwagen et al., 2009). Some of the bioactive components may be applicable as food formulations or pharmaceuticals (reviewed by Wheeler et al., 2007). However, a comprehensive description of and knowledge about function of LMW components is limited. More detailed information about individual bioactive components may allow more widespread use and add value to the dairy industry.

To increase knowledge about properties of individual bioactive components there are needs for technologies to identify these and to obtain the components in purified form. One of the possibilities is application of sophisticated proteomics technologies. The most used techniques for analysis of proteins and peptides in different food matrices are electro spray ionization (ESI) and MALDI-TOF MS (Shevchenko et al., 2001; Trauger et al., 2002; Coon et al., 2005; Jørgensen et al., 2010). However, there are also reports available that analysis of low molecular weight compounds with

MALDI can be complicated because of intense chemical noise from the matrix (Cohen & Chait, 1996; Knochenmuss et al., 1996; Glish & Vachet, 2003; Go et al., 2003).

These problems can be reduced by using a matrix-free setup like NALDI which is reported to show good performance for molecules up to 3000 Da (Thomas et al., 2001; Lewis, 2003; Daniels et al., 2008; Dikler & Kowalski, 2009).

Currently there are no scientific reports available about methods for analysis of small peptides from colostrum and milk by NALDI. Thus we started to test suitability of NALDI for identification of bioactive proteins and peptides from colostrum and milk for later application in dairy industry.

MATERIALS AND METHODS

Preparation of colostrum samples

Spray-dried colostrum powder (Biofiber Damino, Denmark) was used as a source of colostrum for identification of low molecular weight proteins and peptides. The colostrum sample was prepared as described previously Jørgensen et al., (2010). Briefly, colostrum powder was mixed with ultra pure (MilliQ) water at 200 g per L. Dialysis bags (cut off 12–14 kDa) filled with water were placed in the colostrum mix overnight on a rotor at 4^{o}C. The content of the dialysis bags was subsequently purified through C_{18} Sep–Pak (Waters) columns for further analyses.

Analysis of colostrum samples by MALDI and NALDI

Colostrum samples were analysed as described by Daniels et al., (2008). Angiotensin I and II (Sigma-Aldrich Co) were used as standards for testing the plate and calibrating. The peptides were diluted in ultrapure water to produce a dilution series containing 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg angiotensin I or II.

1 µl of Sep–Pak eluates from the colostrum samples were diluted with 9 µl of HPLC grade water (Baxter, IL, USA) directly deposited on the plates. The samples were serially diluted up to one million times. The dots of the sample where dried overnight. The MALDI samples received 1 µl of a saturated solution of α–cyano–4–hydroxycinnamic acid (Bruker Daltonics, Germany) in 70% of acetonitrile. All samples were analyzed with an Ultraflex IV TOF/TOF, (Bruker Daltonics, Germany) equipped with a 337 nm nitrogen laser in positive mode. The detection was set to 10–3510 Da. The total amounts of shots were 200 at 5 different places on the sample dot. Spectra were analyzed with software's from Bruker Daltonics (Flex-Control and Flex-Analysis version 2.4).

RESULTS AND DISCUSSION

Prior to analysis of colostrum samples, the NALDI plate was tested with the angiotensin I and II. Fig. 1 shows their characteristic ions at m/z 1296.374 and 1046.226.

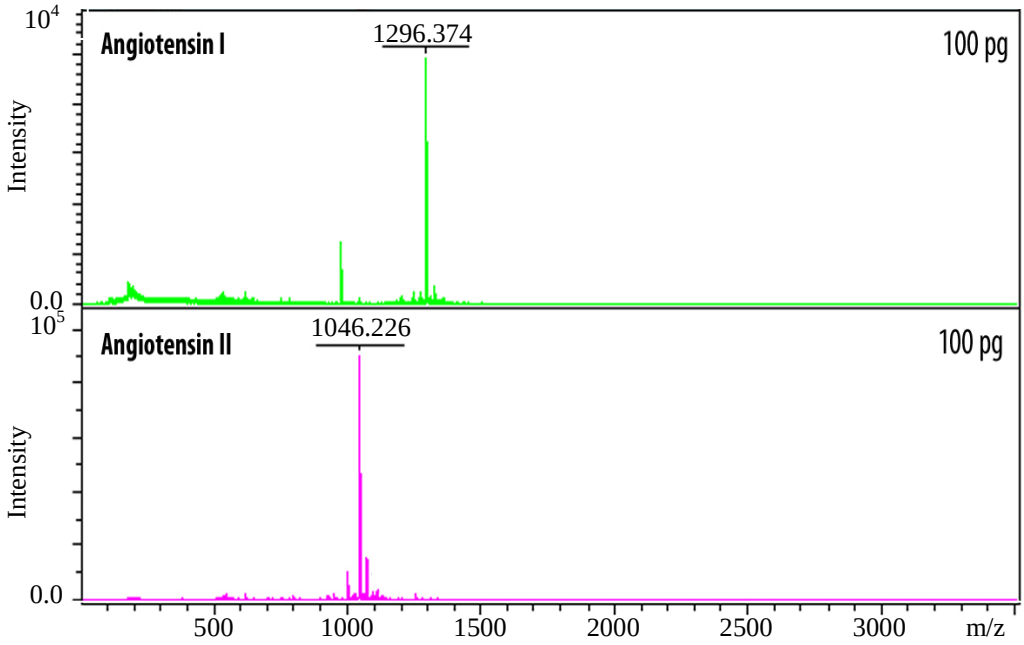

Figure 1. NALDI MS of Angiotensin I and II.

Fig. 2 shows dilution series of colostrum samples analysed by MALDI and NALDI. The results show that even a million fold dilution gives enough signal to detect peptides with NALDI. In contrast, MALDI gives spectra with intensity less than 300, so we are not able to fragment any of these ions. The intensity of the signal from the β-casein fragment is at least 10 times higher with NALDI than with MALDI.

The colostrum sample on NALDI can be diluted even more than million fold. However it can not be done with MALDI because of the increasing intensity of the matrix suppressing the signal from the colostrum sample. Fig. 2 shows that the spectra of NALDI and MALDI are not identical. We assume that this is probably due to differences in ionization between the two methods. Therefore both methods may be used for analysis of peptides in colostrum.

The most intense peak from the NALDI analysis was 1377.570 Da (Fig. 3) and was fragmented with tandem mass spectrometry.

The fragments were analyzed by *de novo* sequencing and Mascot search. The sequence was EPVLGPVRGPFPI (Fig. 4), which is identical to β-casein (210–222).

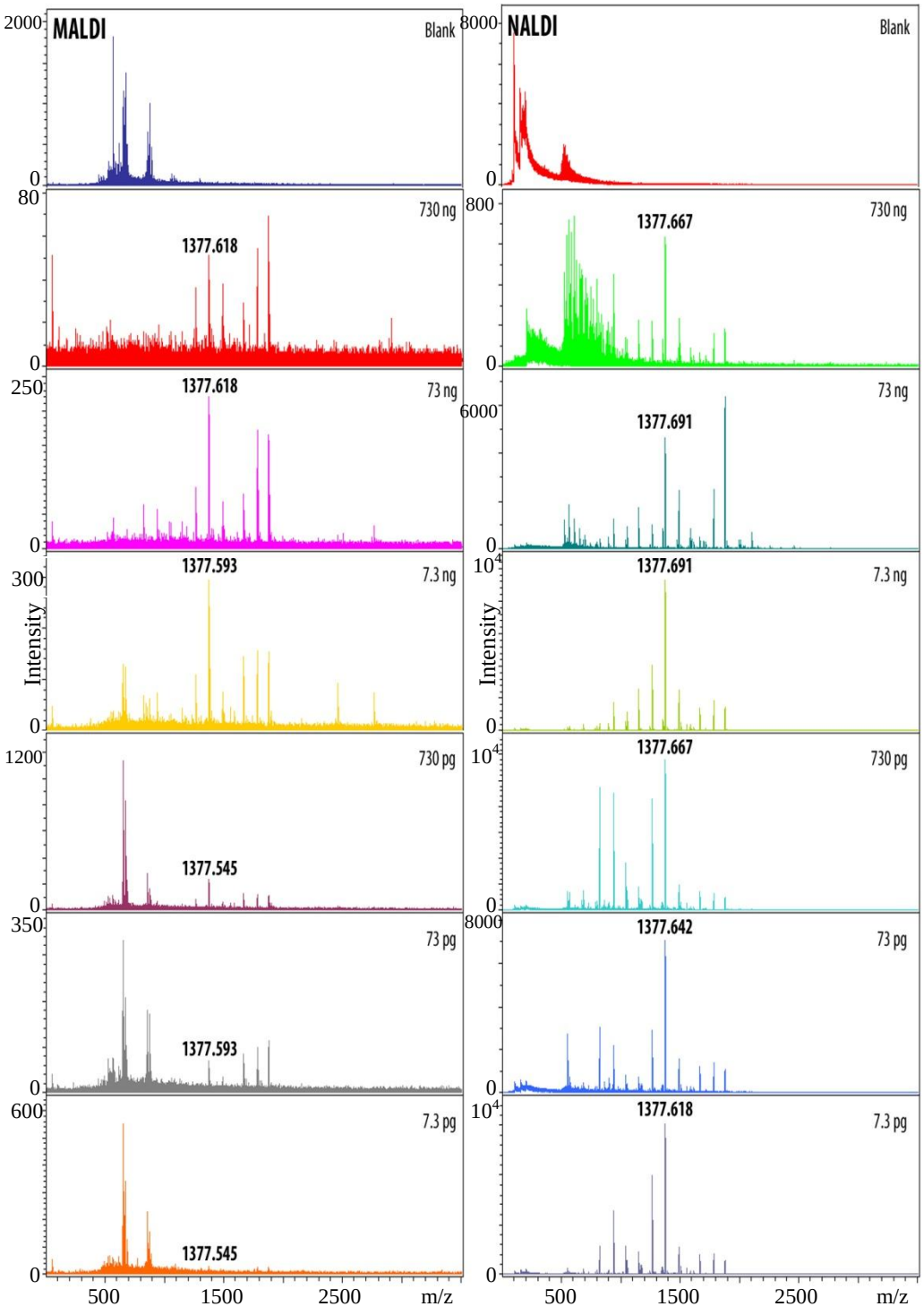

Figure 2. MS spectra of colostrum sample compared by MALDI and NALDI.

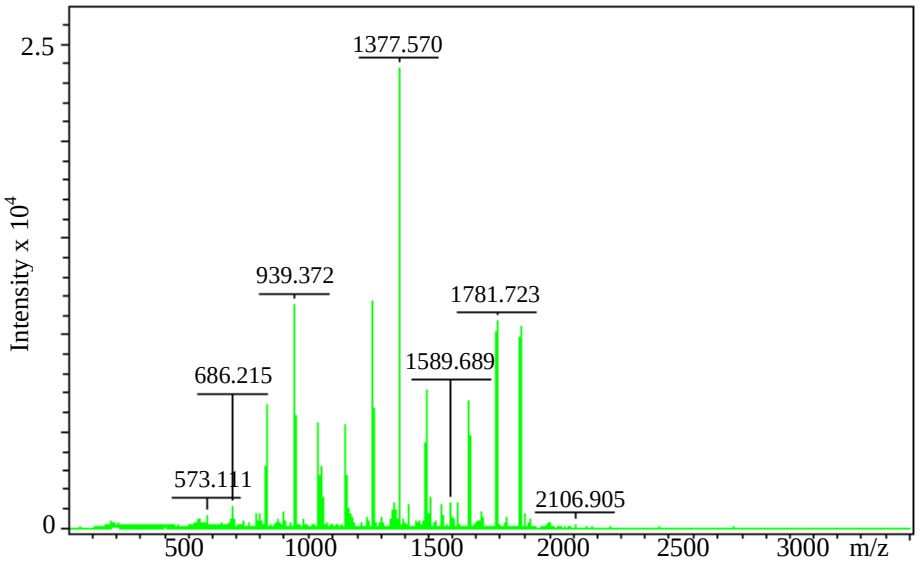

Figure 3. NALDI MS of colostrum sample.

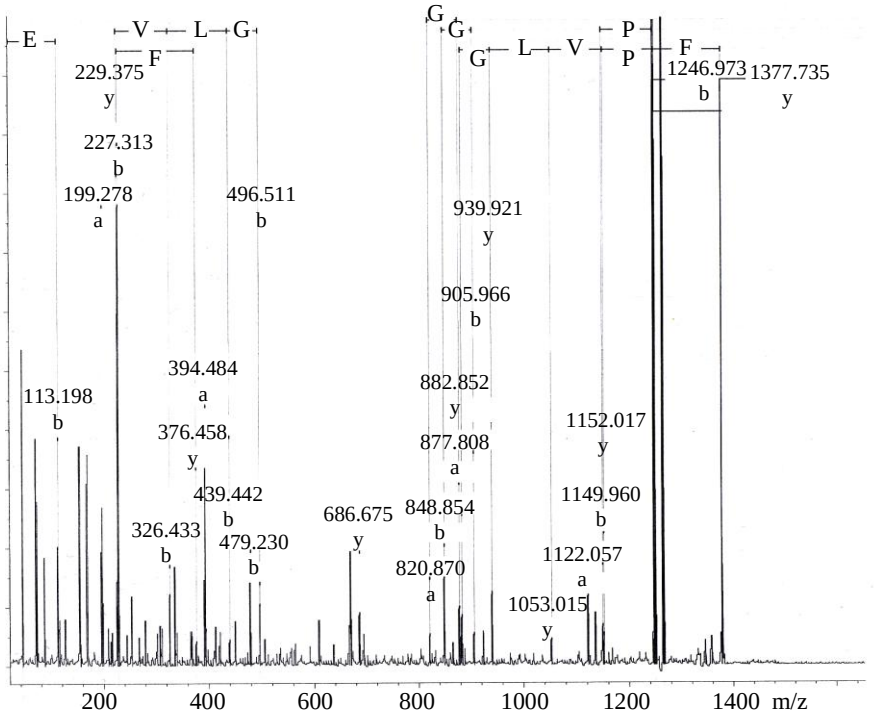

Figure 4. MS/MS spectrum of ion 1377.570. The figure shows detection of a, b and y ions after fragmentation and the fragment sizes.

CONCLUSIONS

This preliminary study was carried out to identify peptides from colostrum with molecular weight under 3000 Da. Our results show that NALDI is useful for analysis

of low molecular weight peptides. NALDI has high sensitivity and is easy to use. Moreover, comparison between NALDI and MALDI shows that peak intensities with NALDI were at least ten times higher than those obtained with MALDI. In this study we were able to detect a β-casein fragment from colostrum. However further research is needed for a comprehensive identification and characterization of low molecular weight bioactive peptides from colostrum and milk.

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Publication II

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Caseins from bovine colostrum and milk strongly bind piscidin-1, an antimicrobial peptide from fish

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Caseins from bovine colostrum and milk strongly bind piscidin-1, an antimicrobial peptide from fish

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ABSTRACT

A model system of bovine colostrum and piscidin, a fish-derived antimicrobial peptide, was developed to study potential interactions of antimicrobial peptides in colostrum. We did not detect any antimicrobial activity of colostrum using the radial plate diffusion assay; in fact colostrum completely abrogated activity of added piscidin. This could not be explained by degradation of piscidin by colostrum, which was less than ten percent. We found that colostrum even protected piscidin against degradation by added proteases. We further observed that colostrum and milk rapidly quenched the fluorescence of fluorescein-piscidin but not that of fluorescein. This effect was not seen with BSA and the specific quenching of fluorescein-piscidin by colostrum was saturably inhibited with unlabeled piscidin. Size exclusion chromatography indicated that fluorescein-piscidin bound to casein micelles with no apparent binding to IgG or whey proteins. Further, addition of pure caseins was able to quench fluorescence of fluorescein-piscidin and to inhibit the antimicrobial activity of piscidin. The interaction between caseins and piscidin could be dissociated by guanidine hydrochloride and recovered piscidin had antimicrobial activity against bacteria. Based on our results we propose that caseins could be carriers for antimicrobial peptides in colostrum and milk.

1. Introduction

During evolution all organisms have evolved various effective strategies to survive and combat invading pathogens, e.g. antimicrobial peptides [1,2]. Colostrum is a complex biofluid, which in addition to an ample supply of nutrients and protective factors, particularly immunoglobulins, contains antimicrobial peptides, like defensins and cathelicidins [3–7]. Recently, genome studies of indigenous Australian mammals, like wallabies [8] and echidnas [9], have revealed expression of cathelicidin genes and other antimicrobial peptides during lactation and point to strong evolutionary selection for these antimicrobial peptides as important defense mechanisms. Antimicrobial peptides display activity against a wide spectrum of pathogens, like bacteria, fungi, parasites and viruses [10].

Although organisms have evolved antimicrobial peptides as part of their innate immune system to provide protection against pathogens, bacteria have also evolved various strategies to resist antimicrobial peptides, like secretion of proteases, modification of cell membrane components or pumping out the antimicrobial peptides [11,12]. For example, in vitro tests with bacterial proteases show that several antimicrobial peptides are susceptible towards enzymatic degradation [13].

Antimicrobial peptides are produced by specialized cells in epithelia [14], such as mammary epithelium that produces colostrum and later milk. A protective transport vehicle in mammalian organisms for antimicrobial peptides could therefore be the casein micelles, which are the major proteins in colostrum and milk. This would ensure that antimicrobial peptides produced in colostrum are at least partly protected and delivered to the newborn.

Caseins are extensively phosphorylated on serine residues in the Golgi complex [15]. Kumosinski and his co-workers have shown through various molecular modeling techniques that all five negatively charged phosphoserine residues located in the N-terminal part of β-casein could potentially interact with positively charged components [16]. α_{S1}-Casein has eight and κ-casein one phosphoserine residue [17,18]. The phosphoserine residues in caseins bind calcium and calcium phosphate and form large molecular micelles in milk and colostrum to encapsulate and to deliver these important macro elements [15]. It is unclear, however, if caseins could function as vehicles for other endogenous

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0141-8130/

components. It has been shown that caseins bind exogenous plant-derived polyphenols through hydrophilic and hydrophobic interactions [19]. Furthermore, β-casein micelles can encapsulate cancer drugs to provide stable combinations for orally consumed medicines for cancer patients [20]. Spray-dried cross-linked casein nanoparticles have been shown to trap alfuzosin hydrochloride for delayed release of the drug [21]. Caseins have also been utilized to protect against enzymatic degradation of small peptides, like insulin in vitro [22].

We have studied the binding and protection of piscidin by bovine caseins as a model to explore the potential of caseins and antimicrobial peptides as a defense mechanism. Piscidins can be obtained from fish gills, skin or mucus and could possibly be used to combat pathogens of humans and other higher vertebrates. Piscidin-1 contains 22 amino acids of which seven are positively charged at neutral pH [23]. The two main characteristics of piscidin, positively charged residues and amphipathic helical structure, cause bacterial cell disruption [24,25].

We surmised that positively charged antimicrobial peptides might bind strongly to negatively charged casein molecules in colostrum and milk and that this interaction might protect the antimicrobial peptides from enzymatic degradation. These peptides could subsequently be released, e.g. through microbial action, in the lower gastrointestinal tract.

2. Materials and methods

2.1. Materials

Reagents and chemicals were purchased from the following vendors: Sigma-Aldrich, MO., USA (Guanidine hydrochloride; Fluorescein; Bovine Serum Albumin; SIGMA*FAST*™ Protease Inhibitor Tablets); Fluka Analytical, MO., USA (LB broth Miller); Invitrogen, UK (Agarose, electrophoresis grade); Pfizer Italiana, Italy (Synulox); AppliChem GmbH, Germany (Trifluoroacetic acid, spectroscopy grade); Merck, Germany (Pronase E, a mixture of proteases from *Streptomyces griseus*); Bruker Daltonics, Germany (α-cyano-4-hydroxycinnamic acid); CASLO Laboratory ApS, Lyngby, Denmark (synthetic amidated piscidin-1 (FFHHIFRGIVHVGKTIHRLVTG-NH2) and fluorescein-piscidin-1 (N-terminally labeled). Throughout the text piscidin-1 and fluorescein-piscidin-1 are abbreviated as piscidin and fluorescein-piscidin, respectively.

Staphylococcus aureus and *Escherichia coli* isolates from milk were obtained from Estonian University of Life Science, Tartu, Estonia. *E. coli K-12* strain was kindly provided by Ole Højberg, Department of Microbiology, AU, DK. Spray-dried colostrum powder (a mixture collected and pooled from different cows and herds) was kindly provided by Biofiber damino, Gesten, DK. Sodium caseinate was from Arla Foods, Holstebro, Denmark. Fresh colostrum and milk samples from different cows were kindly provided by Mette-Marie Løkke, Department of Food Science, AU, DK.

For practical reasons, we primarily used reconstituted colostrum prepared as a 10% (w/v) suspension of spray-dried colostrum powder in water. Some experiments required use of fresh colostrum, e.g. degradation, where the spray-drying process may have inactivated proteolytic activity. Although we have observed only minor quantitative differences in the behavior of reconstituted and fresh colostrum, we have chosen to specifically refer to our use of reconstituted colostrum as colostrum^{r} and fresh colostrum as colostrum^{f} throughout the text.

HPLC grade water (Burdick & Jackson, MI, USA) was used for peptide stock solutions. MilliQ water (Millipore Corporation, MA, USA) was used throughout the study. PBS, ACN, methanol, HCl and NaOH were of analytical grade and commercially available.

2.2. Methods

2.2.1. Bacterial cultures

Bacteria were grown overnight in LB broth at 37 °C. Next day the bacterial cultures were washed three times with PBS (pH 7.0) and diluted in LB to an OD_{600} = 0.05.

2.2.2. Radial plate diffusion assay

Agarose plates were used and growth inhibition was observed as clear zones after application and diffusion of the antimicrobial component from a small hole punched in the agarose. LB broth with 1.5% agarose was autoclaved, cooled to ~40 °C, mixed with washed bacteria to obtain OD_{600} = 0.05 and poured onto Petri dishes. 3 mm holes were punched after solidification and filled with 10–20 μl of sample to be tested. Synulox, a mixture of amoxicillin and clavulanic acid, was used as positive control.

In experiments with guanidinium hydrochloride (GndHCl), we also added protease inhibitors (0.5 mg/ml) in the LB medium. Plates were incubated overnight at 37 °C and next day diameters of inhibition zones were measured.

2.2.3. Degradation of fluorescein-piscidin in colostrum

Colostrum^{f} samples from three different cows and fresh milk from cows in late lactation were centrifuged (~2500*g*, 30 min, 4 °C) to remove fat. All subsequent experiments used skimmed colostrum or skimmed milk. The colostrum^{f} and colostrum^{r} or dilutions thereof were mixed with piscidin (mixture of piscidin and fluorescein-piscidin) or PBS as a control. Parallel samples were then loaded on bacterial plates for measurement of inhibition zones or incubated without bacteria overnight at 37 °C for measurement of degradation of fluorescein-piscidin by size exclusion chromatography using an ÄKTA FPLC 900 System (Amersham Biotech) equipped with column (Sephacryl S200 – 15 mm × 100 mm – internal diameter × length (Pharmacia, Sweden). Chromatographic conditions were as follows – mobile phase: PBS, pH 7.0; flow rate: 1 ml/min; UV detection at 280 nm. Fractions of 1 ml were collected and fluorescence was measured with a Synergy 2 micro plate reader (BioTek Instruments, Inc., USA) using excitation at 485 nm and emission at 528 nm. Subsequently, pronase E were added at 1 mg/ml to all fractions to release fluorescein and fluorescence was measured again after 30 min. Fluorescence was corrected for suppression by a factor of 0.7 as determined for fluorescence intensity of fluorescein before and after addition of pronase E.

2.2.4. Protection of fluorescein-piscidin by colostrum

The degradation of fluorescein-piscidin was analyzed by size exclusion chromatography. fluorescein-piscidin was incubated with colostrum^{r} or colostrum^{f} for 60 s at room temperature. Then pronase E was added and samples were incubated for an additional 60 s under the same conditions. Control samples were prepared under the same conditions, but switching the order of additions. Additional controls with BSA at the same concentration as colostrum^{f} were performed to rule out a general protective effect of protein against degradation of fluorescein-piscidin by pronase E. Samples were then immediately injected and separated by FPLC as described in Section 2.2.3.

2.2.5. Binding of fluorescein-piscidin by colostrum and milk

Binding of piscidin to colostrum or milk was studied using fluorescence quenching. The rapid binding of fluorescein-piscidin was carried out with colostrum^{r}, colostrum^{f} and milk. The signal of fluorescein-piscidin was measured in a quartz cuvette with an LS50B spectrometer (Perkin Elmer, UK) using excitation at 490 nm and emission at 520 nm. After 60 s, colostrum or milk was added and quenching of fluorescein-piscidin was followed for another 60 s.

For some experiments, binding studies were performed in micro titer plates coated with 1% BSA to prevent non-specific adsorption of fluorescein-piscidin. All binding experiments were performed in PBS. Fluorescence of fluorescein-piscidin was measured with the Synergy 2 micro plate reader using excitation at 485 nm and emission at 528 nm. BSA was dissolved in water and used without further treatment. Solutions of fluorescein or fluorescein-piscidin were adjusted to give equal fluorescent intensities and then incubated with increasing amounts of either colostrum^r or BSA as indicated.

Quenching of fluorescein-piscidin by colostrum^r was competed using different concentrations of unlabeled piscidin. Colostrum^r was incubated for 30 min with unlabeled piscidin before addition of fluorescein-piscidin.

2.2.6. Cross-titration setup with piscidin and casein in liquid medium growth assay

Solutions of sodium caseinate were prepared at 50 mg/ml in water and solubilisation was achieved by adjusting to pH ~10 with NaOH and mixing overnight at 4 °C. The solution was then neutralized with HCl.

Piscidin was three-fold serially diluted in rows in a 96 well micro titer plate and sodium caseinate was three-fold serially diluted in columns. This gives the highest concentration of both components at the upper left corner and control (PBS alone) at the lower right corner of the cross-titration squares. The bottom row is therefore sodium caseinate alone and the right column is piscidin.

After combining different concentrations of piscidin and sodium caseinate in 6 × 6 squares, *S. aureus* in liquid LB medium with OD_{600} = 0.05 was added. The bacterial growth was followed for 10 h. The optical density was continuously measured at 600 nm with the Synergy 2 micro plate reader at 37 °C with intermittent shaking.

2.2.7. Release of fluorescein-piscidin from colostrum

The rapid binding of fluorescein-piscidin was carried out with colostrum^r. The signal of fluorescein-piscidin was measured in a quartz cuvette using excitation at 490 nm and emission at 520 nm. After 30 s, colostrum^r or PBS was added and the quenching of fluorescein-piscidin was followed for an additional 30 s. Sequential additions of 6 M GndHCl (pH 7.0) were then performed every 30 s to release fluorescein-piscidin from colostrum^r. Controls with fluorescein or fluorescein-piscidin and PBS were performed under the same conditions.

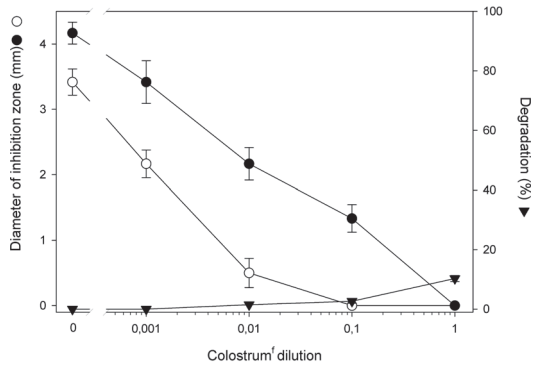

Fig. 2. Different doses of colostrum^r affect the degradation and antimicrobial activity of piscidin (mixture of 300 μg/ml of piscidin and 50 μg/ml of fluorescein-piscidin) against *E. coli* (●) and *S. aureus* (○). Data are from three independent experiments.

2.2.8. Obtaining the low molecular weight fraction from colostrum or caseins

Recovery of low molecular weight (LMW) components, including the added piscidin in spiked samples, from colostrum^r used the method according to Jorgensen et al. [26] with some modifications. Colostrum powder was re-suspended with either MilliQ water or 6 M of GndHCl to obtain a 10% (w/v) homogeneous mixture. Samples were then spiked with piscidin. C_{18} reverse phase resin (Waters Corporation, Milford, USA) conditioned with methanol and washed with 0.1% of TFA was placed in dialysis bags with 12–14 kDa cut-off (Spectrum Laboratories, Inc., CA) followed by addition of 10 ml of water or 6 M of GndHCl. The dialysis bags were then incubated in the colostrum^r suspension and rotated overnight at 37 °C.

In some experiments, we used washed caseins obtained by ultracentrifugation (100,000*g*, 60 min, 4 °C). The casein pellet was washed ten times with ice cold MilliQ water and homogenized with an Ultraturrax homogenizer at 12,000 rpm for 1 min on ice. The washed caseins were then re-suspended in 3 M of GndHCl with PBS (pH 7.0), divided into two equal samples and one sample was

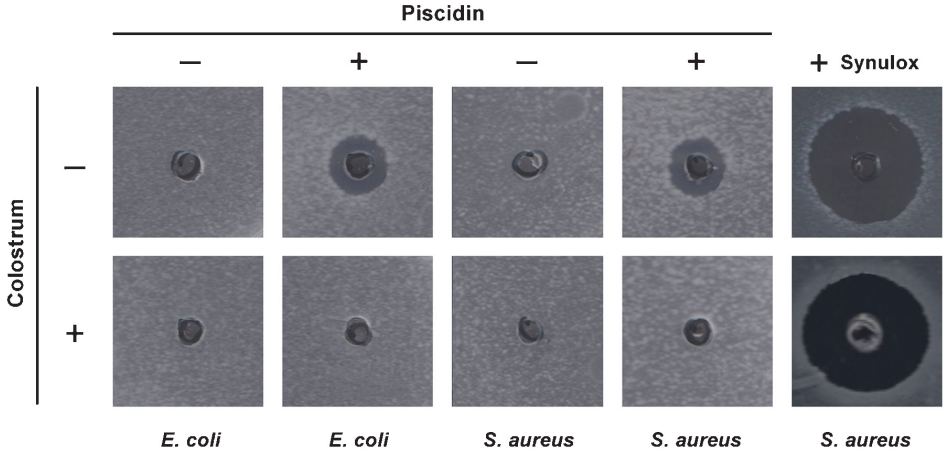

Fig. 1. Radial Plate Diffusion Assay for testing antimicrobial activity of colostrum^r (100 mg/ml) and piscidin (100 μg/ml). Sections of agarose plates with added compounds in various combinations as indicated are shown. **-**: sterile PBS. Synulox, a mixture of amoxicillin and clavulanic acid, was used as a control. Shown are representative sections from plates.

spiked with piscidin. The samples were placed in dialysis bags with 6–8 kDa cut-off and dialyzed against 0.1% of TFA with C_{18} reverse phase resin overnight at 4 °C with continuous magnetic stirring. Note that the resin is outside the dialysis bags to bind small peptides that diffuse out of the dialysis bags.

The reverse phase resin was recovered in small columns, washed with 0.1% of TFA, and bound LMW components eluted with 70% of ACN in 0.1% of TFA. The eluates were frozen in liquid nitrogen, lyophilized and kept at −18 °C for further measurements. In some experiments, ACN was allowed to evaporate at room temperature and the remaining aqueous fraction was tested against bacteria.

Lyophilized eluates were dissolved in 1 ml of 0.1% of TFA, centrifuged (2000*g*, 10 min, 4 °C) and 500 μl of the supernatant was analyzed using a Protein & Peptide C18 column, L 150 × ID 2.1 mm, 5 μm (Grace Davison Vydac, USA) and elution with a linear gradient of ACN in 0.1% TFA. Flow rate was 1 ml/min with detection at 214 nm, and 96 fractions of ~300 μl were collected. 50 μl of the fractions were tested against bacterial growth using 200 μl of diluted bacterial culture with OD_{600} = 0.05.

2.2.9. Identification of piscidin in LMW fractions by MALDI-TOF MS

The LMW eluates were deposited on stainless steel plates and dried at room temperature. Samples received 1 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 70% of ACN and were analyzed in positive mode with an Ultraflex IV (Bruker Daltonics, Germany) equipped with a 337 nm nitrogen laser. The total amounts of shots were 200 obtained from five different positions of the sample. The resulting spectra were analyzed with Flex-Analysis version 2.4 software (Bruker Daltonics).

3. Results

3.1. Antimicrobial activity of colostrum and piscidin

The antimicrobial effect of piscidin alone, reconstituted colostrum (colostrum^r) alone, or the combination of both were tested in the radial plate diffusion assay using *S. aureus* and *E. coli* as examples of Gram positive and Gram negative bacteria, respectively. While there was no antimicrobial activity of colostrum^r alone, and there were clear inhibition zones for piscidin alone, the addition of colostrum^r completely abrogated the effect of piscidin (Fig. 1). This effect of colostrum^r was not seen in the presence of non-peptide antibiotics, a mixture of amoxicillin and clavulanic acid (Synulox). Inhibition of the antimicrobial activity of piscidin was also seen with fresh colostrum from three different cows (Fig. 2) and therefore not specific for reconstituted colostrum.

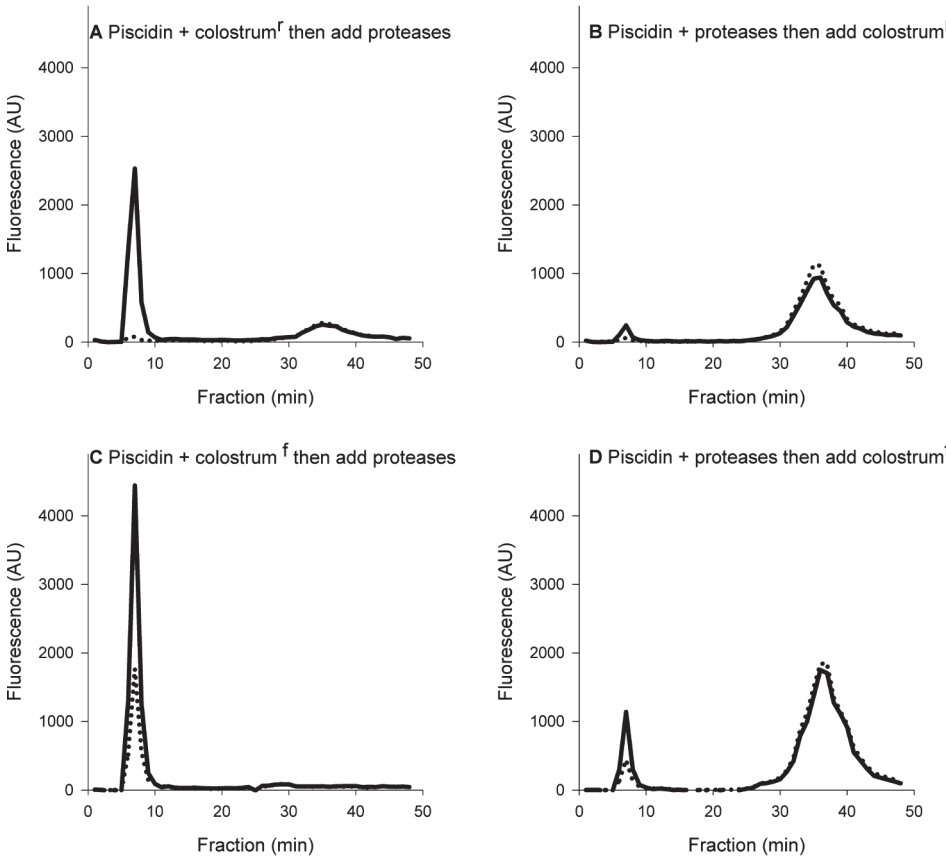

Fig. 3. Size exclusion chromatography shows that colostrum protects fluorescein-piscidin from degradation by added proteases. Fluorescein-piscidin (15 μg/ml) was incubated with 1 mg/ml of colostrum^r (A) and (B) or colostrum^f (C) and (D) and treated with 10 μg/ml of pronase E. Fluorescence of fractions was measured directly (dotted lines) and after treatment with proteases (1 mg/ml) for 30 min (solid lines) to release fluorescein. Representative experiments are shown.

3.2. Degradation of fluorescein-piscidin in colostrum

To investigate degradation of piscidin by colostrum as a possible explanation for the inhibitory effect, we incubated *fluorescein-piscidin in* fresh colostrum (colostrum^{f}) in parallel and under the same conditions (overnight at 37 °C) with and without bacteria and analyzed the integrity of fluorescein-piscidin with size exclusion chromatography.

Fig. 2 shows that the diameters of inhibition zones of piscidin were reduced by increasing concentrations of colostrum^{f}. However, degradation of fluorescein-piscidin as evaluated by appearance of low molecular weight fluorescein components by size exclusion chromatography was only ~10% in undiluted colostrum^{f} and <2% in 10-fold diluted colostrum^{f}, despite complete abrogation of antimicrobial effect in undiluted colostrum^{f} and 10-fold diluted colostrum^{f}, at least for *S. aureus*. *E. coli* was apparently more sensitive and the effect of colostrum^{f} was therefore lower. Degradation of fluorescein-piscidin in colostrum^{r} was ~5% (data not shown). The results clearly show that even the highest concentration of colostrum degraded only a small amount of piscidin and that degradation therefore could not explain the inhibiting effect of colostrum on antimicrobial activity of piscidin.

The effect of colostrum to protect piscidin from enzymatic degradation was further studied after addition of proteases and using size exclusion chromatography. A protease mixture was not able to degrade piscidin, if piscidin and colostrum were mixed for 1 min before addition of proteases (Fig. 3A and C), but almost completely degraded fluorescein-piscidin if added for 1 min prior to incubation with colostrum (Fig. 3B and D). Degraded piscidin eluted from the size exclusion column as low molecular weight fractions. Fig. 3 further shows that fluorescein-piscidin was mainly recovered in the void volume of our size exclusion column (S-200)

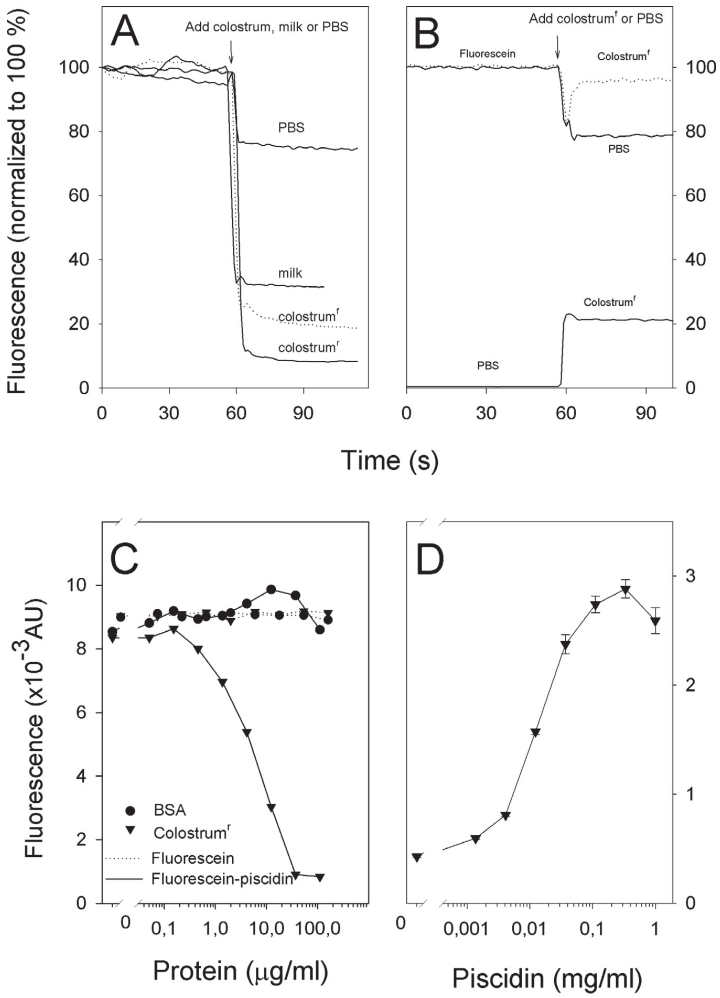

Fig. 4. Fluorescein-piscidin specific quenching by colostrum^{r}. (A) Fluorescence measured before and after mixing fluorescein-piscidin (1 μg/ml) with colostrum^{r}, colostrum^{f} or milk. Addition of PBS alone shows dilution of fluorescence. Background fluorescence was subtracted using additions in PBS (colostrum^{r}: 5; colostrum^{f}: 108; milk: 93) before normalization of the signal. (B) Traces of fluorescein after addition of colostrum^{f} or PBS, and a background trace in PBS of colostrum^{f}. (C) Fluorescence quenching of fluorescein or fluorescein-piscidin by increasing amounts of either colostrum^{r} or BSA as indicated. (D) Quenching of fluorescein-piscidin after incubating colostrum^{r} (100 μg/ml) with different concentrations of unlabeled piscidin as indicated. Fluorescence in C and D was read after three minutes. Representative experiments are shown.

and therefore co-eluted with high molecular weight components in colostrum. Mixing colostrum^r or colostrum^f with fluorescein-piscidin followed by proteases resulted in degradation of ~30% and ~10%, respectively, whereas incubation of fluorescein-piscidin with proteases before addition of either colostrum^r or colostrum^f resulted in degradation of ~90%. To rule out a general protective effect of protein on degradation of fluorescein-piscidin by proteases, controls with BSA showed no effect against proteases compared with colostrum (supplementary Fig. S1).

3.3. Piscidin interaction with colostrum and milk

The interaction between piscidin and colostrum was further studied with fluorescein-piscidin, because addition of colostrum to fluorescein-piscidin quenched the fluorescence signal. Quenching of fluorescein-piscidin occured rapidly after addition of colostrum or milk, in fact the level of quenching was observed immediately after mixing (Fig. 4A). Although some differences between reconstituted and fresh colostrum were observed, both sources of colostrum were better than milk in quenching the signal of fluorescein-piscidin. Quenching of fluorescein was not detected after addition of colostrum^f, in fact colostrum^f displayed a small amount of background fluorescence (Fig. 4B). Bovine serum albumin did not affect fluorescence of fluorescein-piscidin (Fig. 4C) and quenching by colostrum^r was thus apparently specific. Again, quenching of fluorescein did not occur under the conditions of the experiments (Fig. 4C). Quenching of fluorescein-piscidin could be prevented by incubation of colostrum^r with unlabeled piscidin (Fig. 4D). This rapid interaction between piscidin and colostrum^r was therefore saturable and specific.

Size exclusion chromatography experiments (Fig. 3) thus pointed to high molecular weight components eluting in the void fractions as responsible for binding of fluorescein-piscidin. We therefore addressed if purified caseins interact with piscidin and if so, does this interaction also inhibit antimicrobial activity of piscidin towards bacteria.

Cross-titration of piscidin versus sodium caseinate followed by incubation with *S. aureus* showed that casein inhibited antimicrobial activity of piscidin in a dose-dependent fashion (Fig. 5A). The highest concentration of sodium caseinate (30 μM) completely abrogated antibacterial activity of the highest concentration of piscidin (54 μM). Interestingly, the interaction between caseins and piscidin always resulted in an all or none response concerning the antibacterial effect (Fig. 5B). Thus, there was a very sharp transition between growth and no growth at a molar ratio around five between piscidin and casein, indicating that one casein molecule could neutralize five molecules of piscidin.

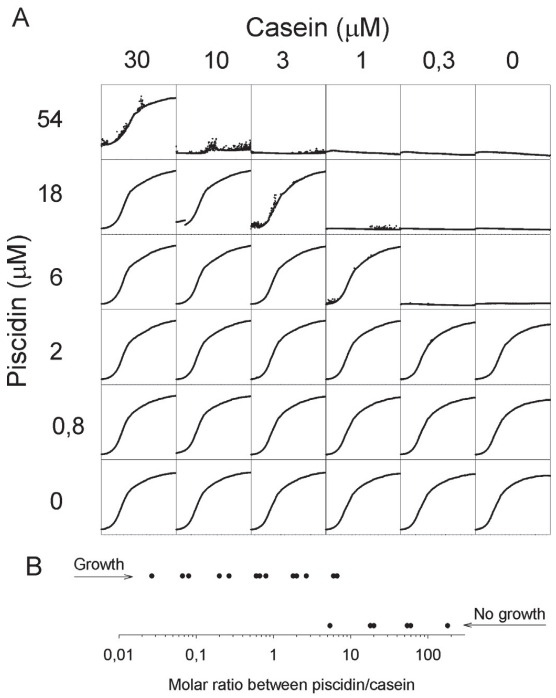

Fig. 5. Effect of casein on the antibacterial activity of piscidin. (A) The graph shows a cross-titration with each of the 36 squares representing a 10 h growth curve for *S. aureus* (time is on horizontal axis and OD_{600} is on vertical axis). Final concentrations of casein and piscidin as mixed with the bacterial culture are indicated. (B) The plot shows the growth or no growth of bacteria as a function of the molar ratio between piscidin and casein based on data in (A). One of three experiments with similar results are shown.

3.4. Dissociating the interaction between colostrum and piscidin

Fig. 6A shows that the fluorescence signal of fluorescein-piscidin quenched by colostrum^r could be recovered by stepwise addition

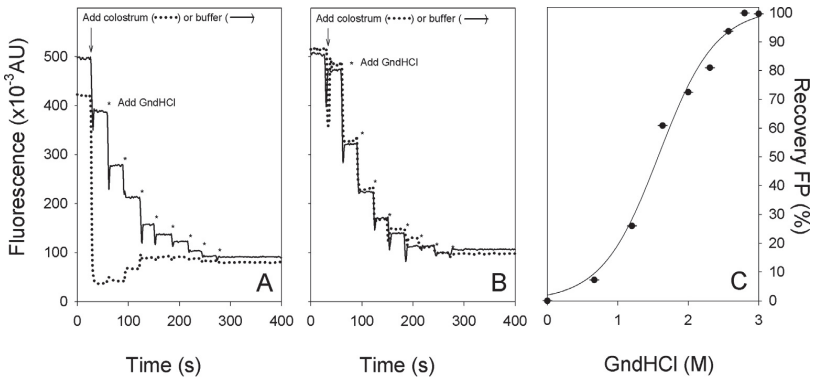

Fig. 6. Fluorescein-piscidin recovery from colostrum quenching by GndHCl. Fluorescence was measured as a function of time after mixing 1 μg/ml of fluorescein-piscidin (A) or 20 ng/ml of fluorescein (B) with 100 μg/ml of colostrum^r (dotted line) or PBS (solid line) followed by consecutive additions (indicated by asterisks) of 6 M GndHCl. (C) Percent recovery of fluorescein-piscidin from colostrum^r data in (A). One of two experiments with similar results is shown.

of GndHCl. In absence of colostrum^r, the apparent decrease in fluorescence intensity was primarily due to dilution as shown by the stippled curve in Fig. 6A. Fig. 6B demonstrates that quenching of fluorescein did not occur under the conditions of the experiments. Fig. 6C shows the concentration-dependent effect of GndHCl to dissociate the complex between fluorescein-piscidin and colostrum^r components.

Subsequent experiments with colostrum^r spiked with piscidin and dialysed against either water or 6 M of GndHCl were analyzed with MALDI-TOF to detect if LMW components, including bound piscidin, could be recovered (Fig. 7). A number of LMW components in the range of 700–5000 Da were observed, some of which only occurred in the presence of GndHCl, including spiked piscidin (m/z 2572.865). This shows that strongly denaturing conditions are required to dissociate binding of LMW components from high molecular weight colostral components, presumably caseins.

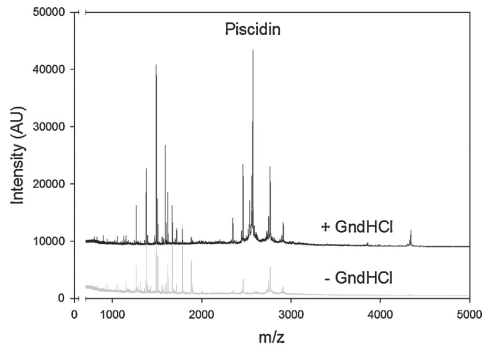

Fig. 7. MS spectra of eluates from colostrum^r spiked with 10 μg/ml of piscidin. Grey spectrum of eluates obtained from colostrum^r in water and black spectrum from eluates obtained in presence of 6 M GndHCl. Due to matrix supression, m/z from 0 to 700 is omited. The black spectrum is shifted 10000 AU for clarity. Representative traces from one of several experiments are shown.

3.5. Antimicrobial activity of recovered piscidin from colostrum and casein

The antimicrobial activity of piscidin after interrupting the interaction with colostrum^r and caseins was tested in agarose plates and liquid growth medium with *E. coli* K-12. Fig. 8 shows

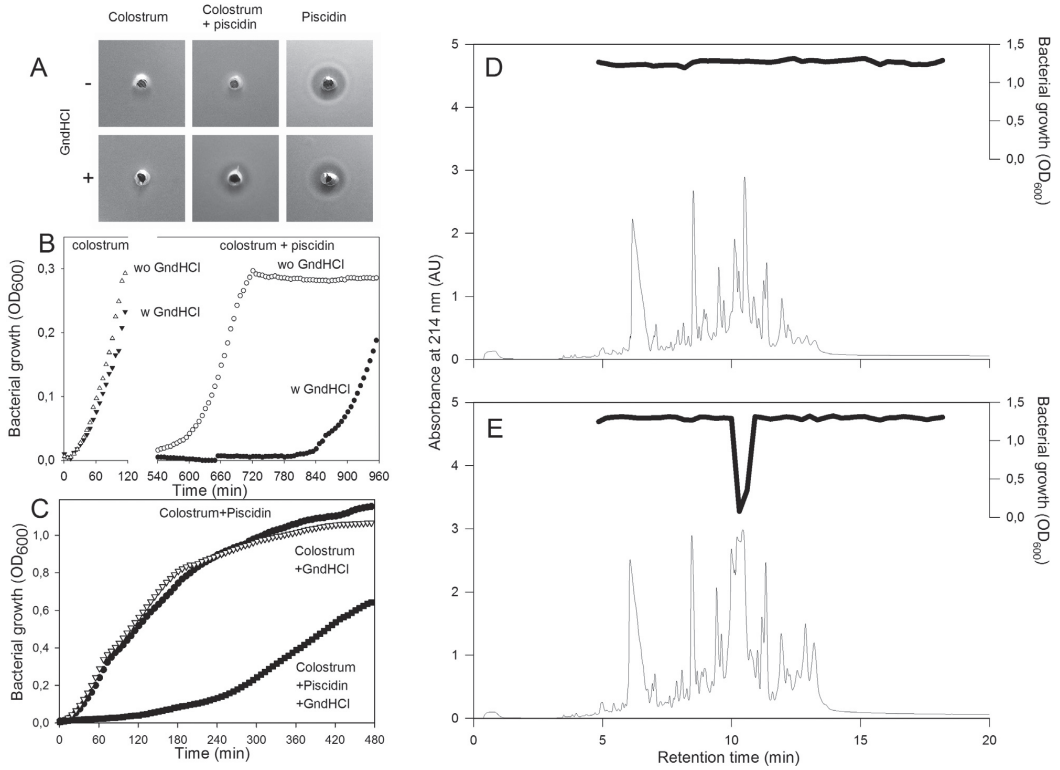

Fig. 8. Antimicrobial activity against *E. coli K-12* of piscidin recovered from colostrum^r or washed caseins. (A) Inhibition zones observed for 1 mg/ml of piscidin alone or in presence of colostrum^r (45 mg/ml) obtained either in absence or presence of 3 M GndHCl as indicated. (B) Bacterial growth in liquid medium either tested with colostrum^r alone or colostrum^r spiked with 250 μg/ml of piscidin in the absence (open symbols) or presence (filled symbols) of 0.5 M GndHCl. Growth curves with uninterrupted growth (0–120 min) are shown before the break in the time axis and initial suppression of growth by piscidin and piscidin released by GndHCl is shown after the break. (C) Growth inhibition by piscidin recovered from dialysates of colostrum^r that had been spiked with piscidin (10 μg/ml) and then either treated with 6 M GndHCl (filled squares) or water (filled circles). Bacterial growth in presence of dialysate from colostrum^r plus GndHCl alone, i.e. without addition of piscidin, is shown as open triangles. (D)–(E) Superimposed HPLC profiles and growth of *E. coli K-12* (thick line; shown as OD_{600} obtained after 12 h) in presence of eluates obtained with GndHCl from washed caseins that had been spiked (E) or not (D) with 50 μg/ml of piscidin. Representative experiments are shown.

that GndHCl was necessary and sufficient to release piscidin from either colostrum or the casein micelles, because we observed again the growth inhibiting effect of piscidin on bacteria. Fig. 8A shows that the antimicrobial effect of piscidin is abrogated by colostrum^r, but that addition of 3 M of GndHCl could release functionally active piscidin resulting in clear inhibition zones in the agarose plate corresponding to recovery of ~80% of added piscidin when compared to standard curves using piscidin alone. There was no effect of GndHCl alone (not shown) or in presence of colostrum^r or piscidin. Similar results were obtained in the liquid medium growth assay (Fig. 8B) with a recovery of added piscidin ~90%. This result showed that GndHCl was able to release piscidin resulting in at least one log order of killing *E. coli*. Furthermore, antimicrobial activity of piscidin that had been added to colostrum^r and recovered by our dialysis setup is shown in Fig. 8C with an estimated recovery of ~60%. Antimicrobial effect was specifically and only detected in the sample spiked with piscidin and treated with GndHCl (Fig. 8D and E) as obtained after separation of the recovered LMW fraction by HPLC. Recovery of piscidin was ~80% in these experiments, and retention time for elution of the component(s) with antibacterial activity was identical to that of piscidin.

4. Discussion

Although our primary interest was to detect antimicrobial activity of colostrum, the radial plate diffusion assay did not show any growth-inhibiting effect of colostrum against bacteria. Furthermore, colostrum completely abrogated the effect of added piscidin, an antimicrobial peptide from fish that we used as positive control. We speculated that this effect could be either due to degradation of piscidin by the colostrum or that non-diffusible components in colostrum interacted with piscidin and prevented diffusion of piscidin into the agarose plates.

Therefore we tested the degradation of piscidin after incubating overnight in reconstituted colostrum. The results revealed that less than five percent of piscidin was degraded under these conditions. The spray-drying process for producing the colostrum powder could, however, have denatured and inactivated proteases present in colostrum. We therefore determined degradation in fresh colostrum and fresh milk, which was less than ten percent and could not explain the abrogating effect.

We reasoned that cationic piscidin would bind to colostral components, which would make piscidin unavailable for antibacterial effects. Colostrum and milk contain many proteins that conceivably could interact with piscidin. Livney [27] has thoroughly reviewed both electrostatic and hydrophobic interactions with cationic and anionic biopolymers in milk.

Our studies with fluorescein-piscidin showed rapid quenching by colostrum and milk. The largest effect occurred when we added reconstituted colostrum to fluorescein-piscidin. Fresh colostrum and milk quenched fluorescence of piscidin to a smaller extent. In fact colostrum^f and milk displayed a small amount of background fluorescence, which could be due to endogenous riboflavin.

Shapira et al. [20] found similar results with positively charged drugs showing that fluorescence quenching is probably due to electrostatic interactions with the negatively charged surface of β-casein. Our size exclusion analyses indicated binding of piscidin to casein micelles. Using different size exclusion columns, we always observed that fluorescein-piscidin eluted in the void volume together with the casein micelles and there was no binding to either IgG or whey proteins, which eluted later in the chromatograms. We further showed that sodium caseinate inhibited the antimicrobial activity of piscidin in a dose-dependent fashion, implying that free piscidin alone inhibits growth of *S. aureus*, whereas piscidin bound to casein is inactive under the conditions of our experiments. Binding of fluorescein-piscidin to casein could be entirely electrostatic due to the negatively charged phosphate groups of caseins and positively charged amino acids in piscidin (piscidin-1 has seven positively charged amino acids in the sequence at neutral pH [24]). Similar electrostatic interactions have been described between pectin and casein micelles, however pectin is negatively charged and casein is positively charged at pH below 4.6 [28].

BSA is a good carrier for small molecules, but it was unable to quench fluorescein-piscidin and we conclude therefore that interaction between piscidin and BSA does not occur. Eventough BSA is mainly found in blood, it is also present in milk and colostrum [29].

It follows that if we want to recover antimicrobial peptides from colostrum we need to dissociate the binding between peptides and colostral components. We attempted to disrupt the interaction between caseinate and piscidin (colostrum and piscidin) using up to 2 M NaCl or lowering the pH to 3, but our results were, however, inconclusive because the conditions either promoted bacterial growth or precipitated the caseins. Also experiments with alkaline phosphatase to remove the phosphate groups from sodium caseinate or chymosin, which specifically cleaves caseins, apparently did not disrupt the interaction between piscidin and colostrum (data not shown). The present study shows that rather harsh conditions, like unfolding caseins with GndHCl, was required to dissociate the strong interaction between colostrum and piscidin.

Proteases will degrade piscidin and therefore antimicrobial effects will be lost. Still, our results show that binding of piscidin to colostrum can delay or even prevent degradation to some extent. Other studies have also shown that caseins can bind and protect polyphenols [19], macronutrients [27], insulin [22] and nutraceuticals [30].

Recovery of piscidin added to colostrum^r samples was only possible in the presence of GndHCl. A number of other LMW components were detected by MS, some of which appeared to show much greater intensity when eluted with GndHCl. Characterization of these components is in progress.

Moreover, piscidin released with GndHCl from either colostrum^r or washed caseins was still able to kill *E. coli* K-12 both in agarose plates and in liquid medium growth assays, as crude eluates in presence of other LMW components released by GndHCl, or following separation and purification by HPLC.

We therefore speculate that caseins, in addition to being important carriers of calcium and phosphate, could have important immune functions as vehicles for antimicrobial peptides in mammalian organisms.

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The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2014.06.063.

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Publication III

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Utilization of ^{15}N-labelled yeast hydrolysate in *Lactococcus lactis* IL1403 culture indicates co-consumption of peptide-bound and free amino acids with simultaneous efflux of free amino acids.

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ORIGINAL PAPER

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Abstract *Lactococcus lactis* subsp. *lactis* IL1403 was grown in medium containing unlabelled free amino acids and ^{15}N-labelled yeast hydrolysate to gain insight into the role of peptides as a source of amino acids under conditions where free amino acids are abundant. A mathematical model was composed to estimate the fluxes of free and peptide-derived amino acids into and out of the intracellular amino acid pool. We observed co-consumption of peptides and free amino acids and a considerable efflux of most free amino acids during growth. We did not observe significant differences between the peptide consumption patterns of essential and non-essential amino acids, which suggests that the incorporation of a particular amino acid is more dependent on its availability in a readily assimilated form than the organism's auxotrophy for it. For most amino acids the contribution of peptide-bound forms to the formation of biomass was initially between 30 and 60 % with the remainder originating from free amino acids. During the later stages of fermentation we observed a decrease in the utilization of peptide-bound amino acids, thus indicating that the more readily assimilated peptides are gradually exhausted from the medium during growth.

Keywords ^{15}N-labelling · *Lactococcus lactis* · Yeast hydrolysate · Peptides · Amino acids

List of symbols

C^i_{pool}; C^i_{prot}	Concentration of amino acid *i* in the intracellular pool of free amino acids and in the protein fraction, respectively (mol g^{-1}_{DW})
C^i_{AA}	Concentration of free amino acid *i* in the culture medium (mol L^{-1})
φ^i_{pool}; φ^i_{prot}	Fractional labelling of amino acid *i* in the intracellular pool of free amino acids and in the protein fraction, respectively
φ^i_{pep}; φ^i_{AA}	Fractional labelling of peptide-bound and free amino acid *i* in the culture medium, respectively

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$r^i_{pep}; r^i_{AA}; r^i_{-AA}; r^i_{prot}$	Flux of peptide-bound amino acid *i* from the culture medium into the intracellular pool; of free amino acid *i* from the culture medium into the intracellular pool; of *i* from the intracellular pool into the culture medium; of *i* from the intracellular pool into the protein fraction (mol g^{-1}_{DW} h^{-1})
X	Concentration of biomass in the culture medium (g_{DW} L^{-1})
$\mu = \frac{dX}{Xdt}$	Specific growth rate (h^{-1})
P^i	Fraction of amino acid *i* obtained from peptides during growth
$\Delta_t C^i_X$	Concentration of amino acid *i* required for biomass synthesis (mol L^{-1})
$\Delta_t C^i_{-AA}$	Concentration change of free amino acid *i* in the culture medium as a result of its efflux from the cell (mol L^{-1})
Y_{XGlc}	Biomass yield relative to glucose consumption (g_{DW} g^{-1})
t	Experiment time (h)

Introduction

Lactic acid bacteria (LAB) are an important group of microorganisms widely used in both the food industry and in the production of various metabolic products and recombinant proteins. They are fastidious organisms that require an external source of essential amino acids and other nutrients. The supply of amino acids is facilitated by both a well-developed proteolytic system comprised of enzymes that degrade proteins and peptides, and transport systems for free amino acids and peptides.

In *Lactococcus lactis*, the cell wall-bound PrtP is considered to be the only proteinase responsible for the extracellular breakdown of proteins into peptides. These range from 4 to 30 residues with a minimal amount of smaller peptides and free amino acids (Juillard et al. 1995a; Konings 2002). The *prtP* genes can either be plasmid- or genome-encoded (Nissen-Meyer et al. 1992; Savijoki et al. 2006). However, some strains, including the plasmid free strain IL1403 used in this study, do not encode PrtP (Xie et al. 2004; Lamarque et al. 2011).

All strains of *L. lactis* are auxotrophic for a number of amino acids; for IL1403 these include leucine, isoleucine, valine, methionine, histidine, and arginine (Zhang et al. 2009). Both these as well as nonessential amino acids are transported into the cells in free and peptide-bound forms. In *lactococci*, the uptake of free amino acids is accomplished by means of symport systems (Leu, Ile, Val, Met, Ala, Gly, Ser, Thr, Lys, His, Pro, Tyr, Phe, and Cys), antiport systems (Arg/Orn, Lys), and ABC transporters (Glu and Gln, Asn, Asp, and Pro) (Konings et al. 1989; Konings 2002). In recent years, several of those (GlnPQ, BcaP, LysP, HisP, AcaP, and FywP) have been cloned and characterized (Schuurman-Wolters and Poolman 2005; den Hengst et al. 2006; Trip et al. 2012). In spite of the large body of knowledge about the free amino acid transport systems, the uptake mechanisms for a number of amino acids remain poorly understood. Also, several amino acids may compete with each other for the same transport system, e.g., Ile, Leu, Val, and Met in the BcaP system (Driessen et al. 1987; den Hengst et al. 2006).

Three peptide transport systems have been described in *L. lactis*: DtpT, Opp, and Opt. DtpT is a proton motive force (PMF)-driven transporter that catalyses the uptake of di- and tripeptides (Doeven et al. 2005). The Opp system belongs to the superfamily of ABC transporters and consists of five proteins (OppA, OppB, OppC, OppD, and OppF). It mediates the transport of peptides from 4 to at least 18 or, according to some data, up to 35 amino acid residues. The substrate specificity of the Opp system is determined by the receptor protein OppA (Tynkkynen et al. 1993; Detmers et al. 2000). Despite the presence of *opp* genes it was discovered that *L. lactis* IL1403 does not express OppA under any conditions and thus the Opt system is the only known transport system used for the utilization of oligopeptides in this strain (Doeven et al. 2005; Lamarque et al. 2004, 2011). Opt also belongs to the ABC transporter family but differs from Opp by the presence of two peptide-binding proteins OptS and OptA and peptide uptake specificity. Initially, the role of Opt was thought to be limited to the transport of hydrophobic di- and tripeptides (Foucaud et al. 1995). However, later studies implicated the role of Opt in the transport of oligopeptides with three to six amino acid

residues (Lamarque et al. 2011). After uptake into the cell, peptides are broken down into free amino acids by various intracellular peptidases that include endo-, amino-, tri- and dipeptidases (Christensen et al. 1999; Christensen and Steele 2003).

Peptides are usually considered nutritionally superior to free amino acids since the uptake of oligopeptides is believed to be energetically more favourable than the uptake of individual amino acids via their dedicated transport systems (Christensen and Steele 2003). In addition, a number of amino acids, released in excess during the hydrolysis of oligopeptides, are excreted from cells via specific amino acid transporters (Konings 2002). Because this efflux may occur by proton symport transporters it could also contribute to the generation of PMF. The capacity of cells to transport peptides is limited and, similar to some free amino acids, different peptides compete for the same transport systems (Smid and Konings 1990; Helinck et al. 2003). Thus, balancing the supply of amino acids and peptides during bioprocessing may improve the overall efficiency.

Yeast hydrolysates are an excellent source of amino acids, peptides, vitamins, and other nutrients and are often used as complex additives in the industrial cultivation of LAB. However, the performance of each type of hydrolysate is highly dependent on the nutritional requirements of a particular species or strain (Ummadi and Curic-Bawden 2010). Additionally, lot-to-lot variations of the same hydrolysate may also significantly influence the performance of a bioprocess, despite their apparently stable composition (Lobo-Alfonso et al. 2010). The reasons for such variations are poorly understood. Moreover, we currently have limited knowledge regarding the interplay of the various components within the proteolytic systems of LAB during growth in industrially relevant media (Benthin and Villadsen 1996), which often makes it challenging to optimize the media composition for a given bioprocess with a specific LAB species.

The aim of the present work was to measure the consumption patterns of free and peptide-bound amino acids by *L. lactis* subsp. *lactis* IL1403 grown on a medium containing a mixture of free amino acids supplemented with ^{15}N-labelled yeast hydrolysate and then use these consumption measurements in a flux model (Fig. 1) to gain further insight into the role of peptides as a source of amino acids under conditions where free amino acids are abundant.

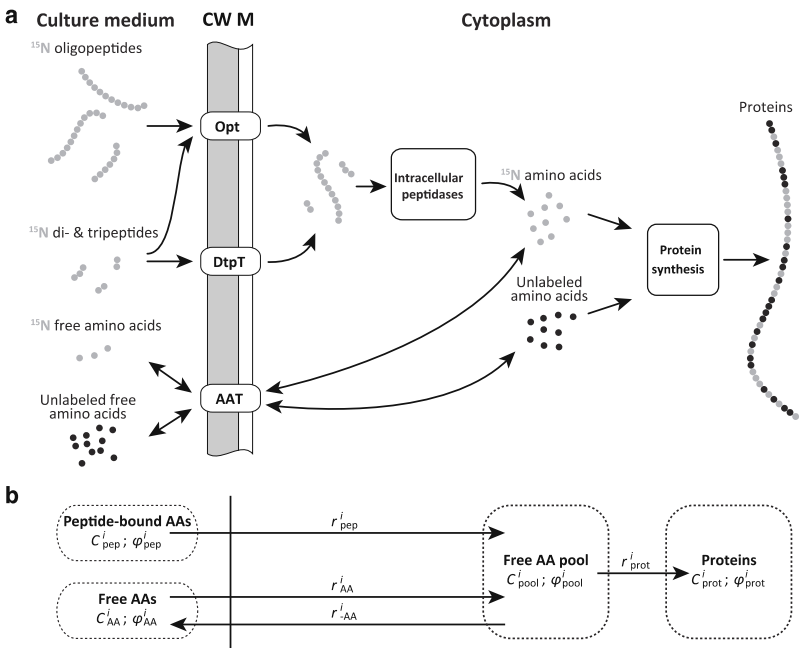

Fig. 1 (**a**) Simplified scheme of the amino acid and peptide utilization systems of *Lactococcus lactis* IL1403. *CW* cell wall, *M* cellular membrane, *Opt* ABC transporter for di-, tri-, and oligopeptides; *DtpT* ion-linked transporter for di- and tripeptides; *AAT* refers to the various free amino acid transporters. Note that efflux of amino acids may occur after their uptake in peptide-bound and free forms. The strain IL1403 lacks cell wall-bound proteinases. (**b**) Schematic representation of the flux model. See the "List of symbols" section for the relevant nomenclature

Materials and methods

Strain and culture media

The *Lactococcus lactis* subsp. *lactis* strain IL1403 used in this study was kindly provided by Dr. Ogier from INRA (Jouy-en-Josas, France). Inoculum was prepared using a freeze-dried stock culture stored at −80 °C which was pre-grown twice on the base medium without yeast hydrolysate.

A chemically defined medium based on the GIBCO™ F-12 Nutrient Mixture (Invitrogen Corporation, Carlsbad, CA) with some modifications (Lahtvee et al. 2011) was used as a base medium which was supplemented with 2 g L^{-1} of ^{15}N-labelled yeast hydrolysate. The concentration was chosen in order to achieve close-to-equal molar concentrations of peptide bound and free amino acids in the culture medium. Refer to Online Resource 1 for detailed medium composition.

Production of ^{15}N-labelled yeast hydrolysate

The ^{15}N-labelled yeast hydrolysate used in the experiments was produced by means of controlled proteolysis from *Saccharomyces cerevisiae* (strain S288c) biomass grown in fed-batch on a synthetic medium (Nisamedtinov et al. 2010) with ^{15}N-labelled (98 %) ammonium chloride (Sigma, USA) as the sole nitrogen source. The yeast biomass was freeze-dried and hydrolysed using a Promod 144GL protease preparation (Biocatalysts, Cardiff, UK). The extract was separated from the insoluble fraction by centrifugation. To remove any residual protease activity, the soluble fraction was further purified using Amicon Ultra 15 centrifugal filter devices (Millipore Corp., MA) with a 10,000 NMWL cut-off (by centrifugation for 45 min at 4,000×*g*). The resulting hydrolysate was freeze-dried. The lack of proteinase activity was confirmed using a Calbiochem Protease Assay Kit (Millipore Corp., USA).

Cultivation system and sampling routines

The cultivation system is comprised of a 1 L Biobundle bioreactor (Applikon, Schiedam, the Netherlands) controlled by an ADI 1030 biocontroller (Applikon) together with BioXpert NT software (Applikon). Cultivations were carried out under anaerobic conditions (surface aeration with N_2) at 34 °C and pH 6.4 (maintained by titration with 1 M NaOH) at an agitation speed of 300 rpm.

The concentration of biomass in the reactor was determined on the basis of the optical density of the culture medium (measured at 600 nm; biomass conversion factor $K = 0.372 \pm 0.005$ g L^{-1} AU_{600}^{-1}). Culture samples were withdrawn at 1 h intervals and collected on ice. The samples were centrifuged (20,000×*g*, 4 min, 4 °C); supernatants were collected and stored at −20 °C until analysis. The biomass pellet was washed twice using ice cold 0.9 % NaCl solution and stored at −20 °C until analysis.

Determination of the size distribution of peptides

The size distribution of peptides in the culture medium was characterized using size-exclusion chromatography (SEC). A Waters 1515 HPLC system (Waters Corp., Milford, MA) equipped with a Superdex Peptide 10/300GL column (GE Healthcare, NJ) and a Waters 2414 Refractive Index detector was used for analysis. Isocratic elution was applied using 0.1 N ammonium acetate (pH 5) as a mobile phase. Data acquisition was carried out using Breeze 3.30 software (Waters Corp.). The size distribution of culture medium components was determined based on the elution time of following molecular markers: alpha-lactalbumin (14,146 Da), aprotinin (6,512 Da), nisin (3,354 Da) and glutathione (307 Da).

Amino acid analyses with UPLC/ESI–MS

Amino acid concentrations in the biomass and culture medium and the respective mass spectra were determined using LCT Premier LC–MS system (Waters Corp.). Samples were derivatised with AccQ-Fluor reagent and loaded on AccQ-Tag™ Ultra columns (2.1 × 100 mm) at 55 °C (flow rate 0.3 mL min^{-1}) connected to a PDA detector ($\lambda = 260$ nm). Amino acids were separated using a 20 min gradient from 0.1 to 59.6 % B (A: AccQTag Ultra eluent A, B: AccQTag Ultra eluent B; Waters Corp.), sprayed directly into the mass-spectrometer operated in positive ionisation mode at 300 °C solvation temperature, 120 °C source temperature and 2.5 kV capillary voltage. Full mass spectra were acquired over the range of 100–1,000 *m*/*z*. MassLynx V 4.1 software (Waters Corp.) was used for data processing.

For measuring protein- and peptide-bound amino acid concentrations in the biomass and culture medium, the samples were hydrolysed with 6 M HCl containing 1 % phenol for 24 h at 105 °C. After hydrolysis the dried samples were dissolved in a methanol-MilliQ water mixture, filtered through Millex 0.2 µm filters (Millipore Corp.) and the free amino acids in the hydrolysate were analysed as described above. The concentrations of peptide-bound amino acids in the culture medium were calculated by subtracting the concentrations of free amino acids from the concentrations of total amino acids.

Calculation of fractional labelling of amino acids

Theoretical isotopic distributions (i.e., the relative abundances of ion species in the mass spectra) of labelled and unlabelled AccQ-Fluor derivatised amino acids were calculated using the algorithm described by Rockwood and Van Orden (1996).

The fractional labelling of each amino acid in the biomass and in the culture medium (i.e., the content of the ^{15}N-labelled amino acid relative to the total content of the respective amino acid) at each sampling point was estimated using least squares regression to fit a measured spectrum by theoretical isotope distribution patterns.

Analysis of peptide consumption patterns

Using the measured enrichment data, a mathematical model was developed to estimate the fluxes of free and peptide-bound amino acids (Fig. 1b; Eq. 1a–1f). The model assumes that no extracellular hydrolysis of peptides occurs during growth and the magnitude of de novo synthesis and turnover of amino acids is negligible.

The system of model equations is presented below:

$$\frac{d(C^i_{\text{pool}} \times \varphi^i_{\text{pool}} \times X)}{dt} = \left(r^i_{\text{pep}} \times \varphi^i_{\text{pep}} + r^i_{\text{AA}} \times \varphi^i_{\text{AA}} - r^i_{-\text{AA}} \times \varphi^i_{\text{pool}} - r^i_{\text{prot}} \times \varphi^i_{\text{pool}}\right) \times X; \quad (1a)$$

$$\frac{d(C^i_{\text{pool}} \times X)}{dt} = \left(r^i_{\text{pep}} + r^i_{\text{AA}} - r^i_{-\text{AA}} - r^i_{\text{prot}}\right) \times X; \quad (1b)$$

$$\frac{d(C^i_{\text{prot}} \times \varphi^i_{\text{prot}} \times X)}{dt} = r^i_{\text{prot}} \times \varphi^i_{\text{pool}} \times X; \quad (1c)$$

$$\frac{d(C^i_{\text{prot}} \times X)}{dt} = r^i_{\text{prot}} \times X; \quad (1d)$$

$$\frac{d(C^i_{\text{AA}} \times \varphi^i_{\text{AA}} \times X)}{dt} = \left(r^i_{-\text{AA}} \times \varphi^i_{\text{pool}} - r^i_{\text{AA}} \times \varphi^i_{\text{AA}}\right) \times X; \quad (1e)$$

$$\frac{d(C^i_{\text{AA}} \times X)}{dt} = \left(r^i_{-\text{AA}} - r^i_{\text{AA}}\right) \times X; \quad (1f)$$

Every model parameter is time dependent and all six sub-equations represent distinct material balances for amino acid i. Two balances were composed for each of three distinct pools of amino acids. These were, the intracellular pool of free amino acids (Eq. 1a, 1b), the amino acids in the protein fraction (Eq. 1c, 1d), and the extracellular pool of free amino acids (Eq. 1e, 1f). In each pool, separate balances were composed for labelled amino acids (Eq. 1a, 1c, 1e) and unlabelled amino acids (Eq. 1b, 1d, 1f).

The discrete measured data points for biomass concentration (X), the concentration (C) of each amino acid, and fractional labelling (φ) of each amino acid in each of the three pools were smoothed and fitted with cubic splines; the resulting continuous functions were used to solve for all model fluxes (r) as a function of time. The resulting solution forms the basis for further calculations.

The fraction of amino acids obtained from the peptides during growth (P^i) can be expressed as a ratio of the flux of peptide-bound amino acid i and the total flux of the respective amino acid into the intracellular amino acid pool:

$$P^i = \frac{r^i_{\text{pep}}}{r^i_{\text{AA}} + r^i_{\text{pep}}}. \quad (2)$$

The concentration of amino acid i required for biomass synthesis during the experiment can be expressed as follows:

$$\Delta_t C^i_X = \int_0^t C^i_{\text{prot}} \times \mu dt. \quad (3)$$

Concurrently, the concentration change of free amino acid i in the culture medium as a result of its efflux from the cell can be expressed as follows:

$$\Delta_t C^i_{-\mathrm{AA}} = \int_0^t r^i_{-\mathrm{AA}} \times X dt. \quad (4)$$

Results

The effect of yeast hydrolysate on the growth characteristics of *L. lactis* IL1403

To see the effect of the addition of yeast hydrolysate on the growth of *L. lactis*, batch experiments at $T = 34$ °C, pH 6.4 on a synthetic medium with and without the added hydrolysate were carried out. The addition of yeast hydrolysate resulted in a significantly higher maximum specific growth rate of the bacteria ($\mu_{\max} = 0.86 \pm 0.02$ h^{-1} compared with $\mu_{\max} = 0.62 \pm 0.01$ h^{-1}; average values of two independent experiments ± standard deviation). Chromatography analyses of the culture media suggested that in both experiments, growth ceased upon the exhaustion of glucose (see Fig. A1 in Online Resource 1 for growth curves). Growth in the medium supplemented with yeast hydrolysate also led to a higher final yield of biomass relative to glucose consumption than in the pure base medium, $Y_{X\mathrm{Glc}} = 0.22 \pm 0.01$ g g^{-1} and $Y_{X\mathrm{Glc}} = 0.14 \pm 0.01$ g g^{-1}, respectively.

Amino acid consumption profiles

In the cultivation with added ^{15}N-labelled yeast hydrolysate, the concentration of free and peptide-bound amino acids in the culture medium were determined at hourly intervals and presented in Fig. 2a. Asparagine, glutamine and arginine were the most intensively consumed free amino acids. Free arginine in particular was completely exhausted at the end of the experiment. The concentration of free serine, threonine, methionine, leucine, and isoleucine also decreased, whereas the concentration of ornithine, glycine, aspartate, and glutamate increased. The increasing concentration of ornithine along with the decreasing concentration of arginine indicates the activity of the arginine–ornithine antiport. Changes in the concentration of other free amino acids were within the range of measurement error. The concentration of all measured peptide-bound amino acids decreased during the experiment (Fig. 2b).

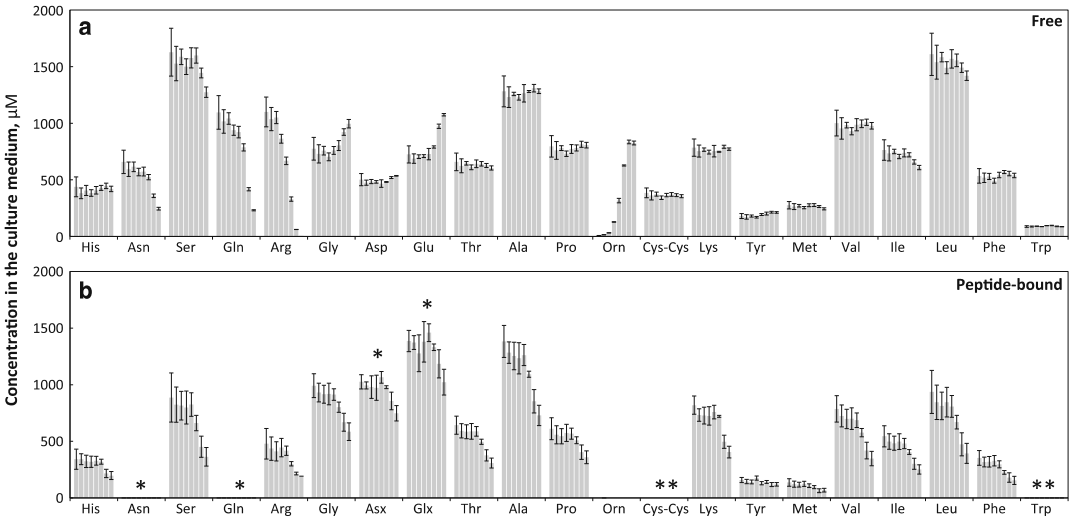

Fig. 2 Concentration of free (**a**) and peptide-bound (**b**) amino acids in the culture medium during the batch cultivation of *L. lactis* IL1403 on a synthetic medium supplemented with ^{15}N-labelled yeast hydrolysate. Data from one of the two independent experiments is shown; each *column* indicates the concentration at hourly sampling points; measurements were carried out in triplicate and the *error bars* represent standard deviation. *asterisk* Glutamate and glutamine, asparagine and aspartate were pooled after hydrolysis; *double asterisk* cysteine and tryptophan were degraded during hydrolysis. The concentration of peptide-bound amino acids is expressed as the differences between the concentration of free and total (free plus bound) amino acids

^{15}N-labelling patterns of amino acids

In order to better understand the role of free and peptide-bound amino acids during growth, analyses of their labelling patterns in both the biomass and the culture medium were carried out. It was found that, with the exception of glutamate, the fractional labelling of all free amino acids in the culture medium increased during the experiment (Fig. 3a, dashed lines). This could result from either (a) extracellular hydrolysis of ^{15}N-labelled peptides (unlikely, because *L. lactis* IL1403 lacks cell wall-bound proteases); (b) efflux of labelled amino acids from the cell (due to excessive accumulation). Considering the latter scenario, the estimated amount of the excreted amino acid (Eq. 4) was in the same order of magnitude with what was theoretically required for biomass production (Eq. 3; see Fig. 3b for tyrosine and histidine as examples; refer to Fig. A2 in Online Resource 1 for other amino acids). From the second sampling point onwards, the fractional labelling of amino acids in the biomass were higher than of those in the culture medium (Fig. 3a), which suggests that the amino acids used for growth were at least partially obtained from (labelled) peptides.

Based on the labelling data presented above, the fractions of amino acids used for biomass production obtained from peptides were then calculated (Eq. 2; Fig. 4). For most amino acids the contribution of peptide-bound forms to the formation of biomass was initially between 30 and 60 % with the remainder originating from free amino acids. During the later stages of fermentation we observed a decrease in the utilization of amino acids originating from peptide-bound forms thus indicating that the more readily assimilated peptides are gradually exhausted from the medium during the cultivation.

Methionine was largely derived from peptide-bound forms during the first hours of both experiments. This suggests that the uptake of this amino acid in free form was inhibited. Both glutamine/glutamate and asparagine/aspartate (Glx and Asx, respectively) exhibited a consistently low incorporation of peptide-bound forms. This agrees with our observation that large amounts of free glutamine and asparagine were consumed (Fig. 2a). During growth, proline, histidine, and lysine were found to increasingly originate from peptide-bound forms.

Estimation of the size of consumed peptides

To estimate the upper size limit of the peptides consumed during the experiment, size-exclusion chromatography (SEC) analysis was carried out on samples of the culture media. The changes in the molecular weight distribution of the culture medium are illustrated in Fig. 5. The results indicated that the yeast hydrolysate used in the experiments contained compounds with molecular weights up to approximately 5,000 Da. However, significant consumption of media components with molecular weight of up to approximately 1,700 Da (corresponding to 15 amino acid residues in case of peptides) occurred.

Discussion

In this study we investigated the role of free and peptide-bound amino acids during the growth of *L. lactis* in a synthetic medium containing a mixture of free amino acids supplemented with ^{15}N-labelled yeast hydrolysate.

First, we analysed changes in the fractional labelling of the amino acids in the culture medium and biomass during growth. Two independent experiments indicated that, with the exception of glutamate, the ^{15}N-enrichment of all free amino acids in the culture medium increased during cultivation (Fig. 3a). This could be explained by extra- and/or intracellular hydrolysis of the labelled peptides. Extracellular hydrolysis is unlikely because strain IL1403 does not encode the cell wall-bound proteinase PrtP (Xie et al. 2004; Lamarque et al. 2011) and potential residual proteolytic activity in the yeast hydrolysate was removed by ultrafiltration (10 kDa cut-off). It could also be speculated that peptides were partially hydrolysed by intracellular peptidases leaking into the culture medium from lysed cells or, e.g., by the surface housekeeping proteinase HtrA (Poquet et al. 2000). However, a significant degree of autolysis is very unlikely under our experimental conditions and the role of HtrA is also probably negligible (Kok and Buist 2003). Thus, it can be assumed that the increasing fractional labelling was mainly caused by the over-accumulation of peptide-derived amino acids in the cells and subsequent excretion of the excess of those amino acids into the culture medium—a

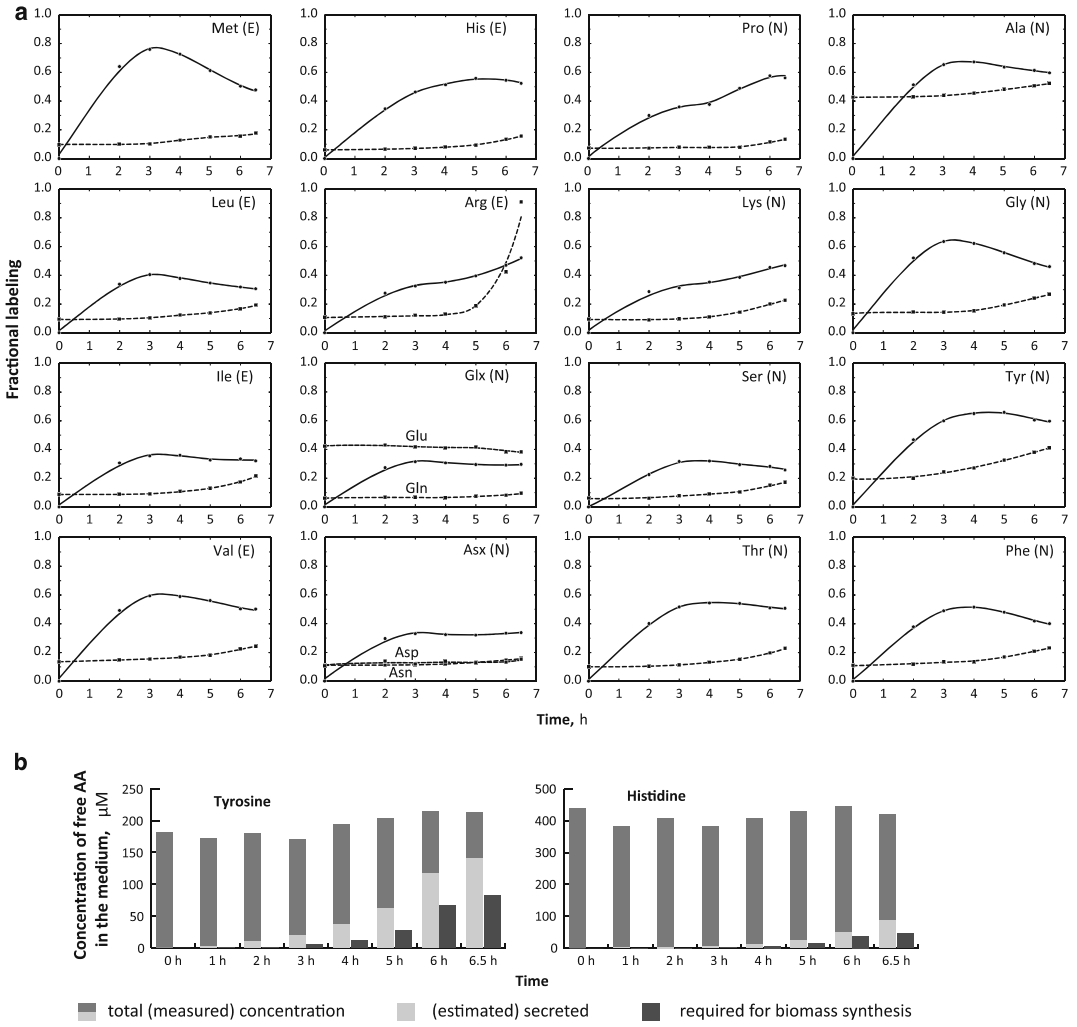

Fig. 3 **a** Fractional labelling of free amino acids in the culture medium (*dashed lines*) and biomass (*solid lines*) determined during the batch cultivation of *L. lactis* IL1403 on a synthetic medium supplemented with ^{15}N-labelled yeast hydrolysate; *E* and *N* denote whether the amino acid is considered essential or non-essential for *L. lactis* IL1403, respectively. **b** Concentration of free tyrosine and histidine in the culture medium. The *total column height* corresponds to the (measured) concentration of the respective amino acid (Fig. 2a) whereas the *light grey area* indicates the (estimated) concentration of secreted amino acid (Eq. 4), and the *black columns* indicate the theoretical concentration of amino acids required for the synthesis of biomass during the experiment (Eq. 3). Data from one of the two independent experiments is shown

phenomenon that has also been observed by authors who studied the growth of *L. lactis* in milk (Juillard et al. 1995b; Cretenet et al. 2011). Assuming that extracellular proteolytic activities were negligible, our calculations indicate that many amino acids are excreted in quantities that exceed the amount required for the formation of biomass (Fig. 3b). The efflux of excess amino acids has been proposed to be coupled to the excretion of a proton, which would result in the generation of metabolic energy in the form of proton motive force (Trip et al. 2012), and provide an additional means to maintain intracellular pH. The

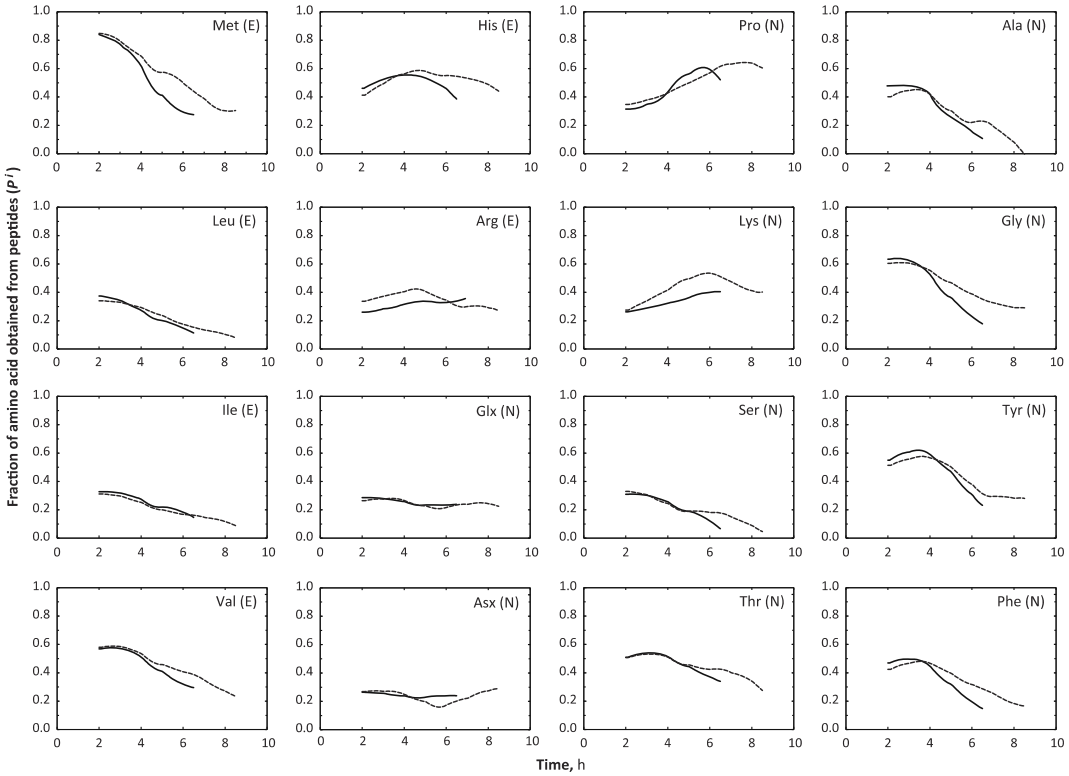

Fig. 4 Fraction of amino acids obtained from peptides during the growth of *L. lactis* IL1403 in a synthetic medium supplemented with ^{15}N-labelled yeast hydrolysate. *Glx* Glutamine + Glutamic acid; *Asx* Asparagine + Aspartic acid. *E* and *N* denote whether the amino acid is considered essential or non-essential for *L. lactis* IL1403, respectively. Data from two independent experiments (indicated by the *solid* and *dashed lines*) with different inoculum sizes is shown

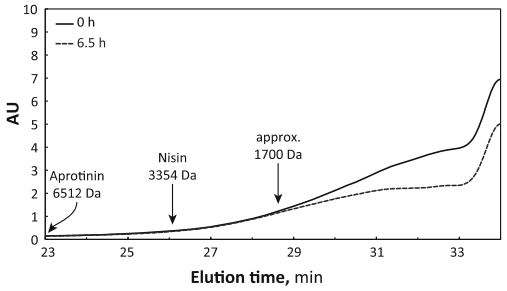

Fig. 5 Size-exclusion chromatograms of the culture medium collected during the cultivation of *L. lactis* IL1403 in a synthetic medium supplemented with ^{15}N-labelled yeast hydrolysate. *Solid* and *dashed lines* show chromatograms at the beginning and end of the experiment, respectively. The *arrows* denote the elution times of two marker peptides, and the upper size limit of media components consumed during growth. Data from one of the two independent experiments is shown

PMF generated in this process could be used to bring in other amino acids and/or peptides. Thus, bidirectional fluxes of amino acids may help balance the intracellular amino acid pools under conditions where the de novo synthesis pathways and their feed-back control mechanisms are not operative.

Excluding extracellular hydrolysis, we estimated the size of peptides utilized during growth. Because the Opt system is the only known transporter in *L. lactis* IL1403 responsible for the uptake of oligopeptides, our results suggest that this system is capable of transporting peptides with a molecular weight up to approximately 1,700 Da, i.e., containing up to 15 amino acid residues (Fig. 5). This extends the findings of Lamarque et al. (2011). They concluded, based on studies with synthetic peptides, that the Opt system in IL1403 can transport peptides containing between

three and six amino acid residues. Because of the combinatorial nature of using synthetic peptides, it is possible that the apparent size limit observed by Lamarque and colleagues may be due to a limited number of tests. Our results suggest that much longer peptides can enter the cell.

Next, we studied the relative incorporation of free and peptide-bound amino acids into biomass. Our calculations assume that all amino acids used for growth are obtained from the culture medium. Jensen et al. (2002) confirmed that under conditions of amino acid surplus in the cultivation media, only aspartate is synthesized de novo by *L. lactis* in considerable amounts (likely from glucose via oxaloacetate). We observed that the consumption patterns of both essential and non-essential amino acids were similar, and co-consumption of free and peptide-bound forms occurs for all amino acids (Fig. 4). This suggests that the incorporation of a particular amino acid is more dependent on its availability in a readily assimilated form than the organism's auxotrophy for it. Nevertheless, the consumption profiles of amino acids between peptide-bound and free forms are noticeably different.

Glutamine and glutamate (Glx; Fig. 4), for example, exhibit a consistently low incorporation of peptide-bound forms. These amino acids are both translocated by the ATP-driven transporter GlnPQ, with glutamate taken up in its protonated form (i.e., as glutamic acid) (Poolman et al. 1987a; Schuurman-Wolters and Poolman 2005). Accordingly, under our culture conditions (pH 6.4), the apparent affinity of GlnPQ for glutamate is an order of magnitude lower than for glutamine, which results in the preferential translocation of the latter into the cells. Interestingly, glutamate was the only amino acid that exhibited no increase in the ^{15}N-enrichment in the cultivation medium during growth (Fig. 3a). This, together with its increasing concentration (Fig. 2a) and the observed low rate of incorporation from peptide-derived forms, suggest that a large part of the intracellular glutamate pool is synthesized from free glutamine (which displayed low albeit slightly increasing fractional labelling in the culture medium; Fig. 3a) and oxoglutarate via the glutamate synthase (gltB) reaction. Excess glutamate, in turn, was then excreted into the culture medium, resulting in an overall increase in the concentration of this amino acid during growth. The efflux of glutamate via the recently characterized AcaP transporter (Trip et al. 2012) may also result in the generation of PMF. The rate of incorporation of peptide-bound forms of asparagine and aspartate was also relatively low (Asx; Fig. 4). Like glutamic acid, aspartic acid is believed to be taken up only in its protonated form and the respective transport kinetics is thus dependent on pH (Poolman et al. 1987b). Thus, one could also speculate that proteinogenic aspartate was (partially) synthesized from asparagine via an asparaginase (ansB) catalysed reaction. These findings suggest that, despite the abundance of glutamate and aspartate (in both free and peptide-bound forms), efficient growth might not be achieved with this strain in case of insufficient supply of free glutamine and asparagine.

In contrast, the rate of incorporation of methionine in peptide-bound forms was over 0.8 during the first hours of the cultivation (Fig. 4). The uptake of free methionine is believed to occur via the secondary amino acid transporter BcaP that is specific for branched-chain amino acids (BCAAs) and (to a lesser extent) methionine (den Hengst et al. 2006). Because the concentrations of free BCAAs in the culture medium were several times higher than that of methionine (and the affinity of BcaP for methionine is lower) its uptake in free form was most likely inhibited by BCAAs. The observed decrease of the incorporation of peptide-bound methionine in the later stages of the cultivation could be explained by the exhaustion of the most readily assimilated methionine-containing peptides. Interestingly, the incorporation of peptide-bound BCAAs into the protein fraction (Fig. 4) was roughly proportional to the affinity constants of the BcaP system (K_t values 6.5, 8.0 and 12 μM for leucine, isoleucine and valine, respectively; Driessen et al. 1987), which is below 0.4 for Leu and Ile, and around 0.6 for Val during the first hours of the experiment. This further supports the notion that in cases where a transport system is shared, the less actively transported amino acids are more readily obtained from peptides. Glycine and alanine are also believed to enter the cell through a shared transport system (K_t values 52 and 330 μM for alanine and glycine, respectively; Konings et al. 1989), and their consumption profiles can be explained using the same principle, although the effect is much less pronounced.

The consumption of peptide-bound forms of a number of amino acids, in particular histidine, lysine, and proline, increased during cultivation (Fig. 4). The exact cause of this behaviour is unclear. The growth environment in batch culture is constantly changing

and transitions in the availability of various peptides could result in a situation where competitive inhibition results in the favoured translocation of peptides with certain characteristics. Helinck et al. (2003) found, for example, that charged casein-derived oligopeptides competitively inhibit the transport of a reporter oligopeptide in *L. lactis*. The increased consumption of peptide-bound amino acids can also be explained by down-regulation of the respective free amino acid transporters. Determining the mechanisms that lead to these peculiar peptide and free amino acid consumption patterns are beyond the scope of this work.

The labelling data reveal that when all amino acids are readily available in both free and peptide-bound forms, co-consumption of these forms takes place, possibly in order to optimally utilize the available cellular transport systems. Our data also indicate that an extensive efflux of amino acids occurs after their uptake from the culture medium. The exact physiological rationale of this phenomenon remains to be elucidated and could be revealed by conducting an accurate mass and energy balance in future studies. The labelling data also suggest that optimization of cultivation conditions and media composition may be possible. One could, for instance, vary both the concentration and composition of free amino acids and/or the labelled yeast hydrolysate in fed-batch or continuous culture experiments while acquiring accurate consumption profiles of free and peptide-bound amino acids. The relative consumption as a function of growth efficiency could be used to adjust the process used to produce yeast hydrolysates to create targeted and optimal yeast extracts for the industrial cultivation of a given microorganism.

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Conflict of interest The authors declare that they have no conflict of interest.

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