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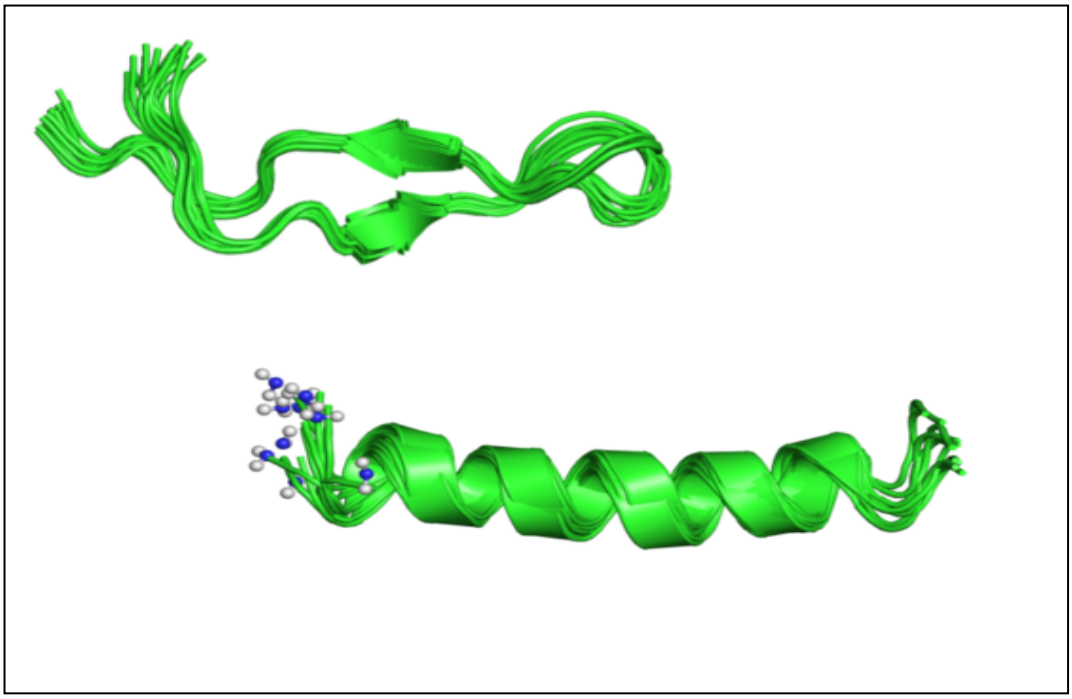

Antimicrobial Peptides and the Potential in Hygiene Products

Master of Science Thesis [in the Master Degree Program, Biotechnology]

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Department of Chemical and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden, 2012

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Cover:
[NMR-images of β-sheet lactoferricin B and α-helical magainin 2. These are two AMPs that are representative for the two major classes]

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The project was carried out at SCA Hygiene products under the supervision of Ulrika Husmark (SCA), Carolyn Bernard (SCA) and Christer Larsson (Chalmers)

Abstract

Antimicrobial peptides (AMPs) are essential components of the defence system spanning virtually every kingdom of life. The peptides are relatively small, amphipathic molecules of variable length, sequence, charge and structure. AMPs have been shown to possess activity against a wide range of microorganisms, including bacteria and fungi, and kill their targets by multiple mechanisms mostly through membrane disruption. The field of AMPs has lately received an increased attention, much due to the serious issue of resistance development of microorganism strains against current antibiotics. However, because of the extensive range of microorganism related problems, the field of AMPs is also of interest in other areas such as hygiene applications, which is the main focus of this thesis. The use of AMPs in hygiene products could prevent common pathogen related problems involved in hygiene. Conditions such as fungal and bacterial infections are related to diapers and panty liners, also it is of importance to keep skin and surfaces hygienic. The suggested AMP applications in hygiene products are, AMP expressing probiotics, AMPs in various materials and substances that induce the body's production of AMPs. The main purpose of this thesis is to provide an understanding of the field of antimicrobial peptides and to evaluate the potential in hygiene applications. Both a practical and theoretical approach was applied for this purpose. The theoretical part included an extensive literature study as well as interviews with active scientists and companies. Besides the possibilities of AMPs, there are several challenges involved such as specificity, resistance, cost and stability. However, it should be noted that there are AMPs in commercial products such as pharmaceuticals, food preservatives and wipes, suggesting a possibility for use in hygiene products. The practical part of the thesis involved the testing of Nisin A, Lactoferricin B, Magainin 2, Buforin II and Histatin-5 to evaluate their antimicrobial activity against three relevant pathogens: *Escherichia coli, Staphylococcus aureus* and *Candida albicans*. The antimicrobial activity was measured by an agar diffusion assay named inhibition zone assay. It was examined how the antimicrobial activity varied between microorganisms, different concentrations of sodium chloride and also between different strains. From the practical results it could be concluded that with the right conditions, most of the peptides showed activity depending on the microorganisms. It was also demonstrated that many peptides are salt sensitive, and finally, when tested against different strains of the same microorganism the peptides did not show much difference in activity. To sum up, there is a potential for AMPs in hygiene products but many challenges needs to be considered.

Keywords: Antimicrobial peptides, Inhibition zone assay, Antimicrobial, Hygiene, Nisin, Magainin 2, SCA

Table of Contents

Abbreviations

SCA	Svenska Cellulosa Aktiebolaget
AMP	Antimicrobial peptides
MIC	Minimum inhibitory concentration
HBD	Human β-defensin
HD	Human defensin (α-defensins)
HNP	Human neutrophil peptide (α-defensins)
LPS	Lipopolysaccharide
PAMPs	Pathogen associated molecular patterns
PRRs	Pattern recognition receptors
TLR	Toll-like receptor
LAB	Lactic acid bacteria
25(OH)D	25-hydroxyvitamin D
1,25(OH)D	1,25-dihydroxyvitamin D
PG	Phosphatidylglycerol
IU	International Unit
EDTA	Ethylenediaminetetraacetic acid
VVC	Vulvo-vaginal Candidiasis
UTI	Urinary tract infection
BV	Bacteria vaginosis
PEG	Polyethylene glycol
FDA	Food and Drug Administration

1 Introduction

Living organisms are constantly exposed to microorganism through contact, ingestion and inhalation [1]. The ability of an organism to protect itself from the competitive biological environment is a crucial factor for survival. The innate defence system against invading microorganisms is a complex process that involves a group of molecules named antimicrobial peptides (AMPs), which given by their name, are small protein molecules also called peptides (<100 amino acids) [1] [2]. Besides the AMPs antibacterial and antifungal activity the peptides can also have antiviral and antiprotozoal activity [3] [4]. The research of AMPs began in 1960s when a group of researchers, Spitznagel and Zeya, discovered that basic peptides in white blood cells (later named AMPs) showed antimicrobial activity [5]. The field of AMPs grew further when Hans Boman, Michael Zasloff and Robert Lehrer independently isolated and purified insect cecropins, amphibian magainins and mammalian defensins, respectively [6]. Since the first discovery more than 2000 AMPs have been isolated from bacteria, insects and other invertebrates, amphibians, birds, fishes, mammals and plants [2]. There is a large diversity between AMPs, both in amino acid sequence and in physical properties [3]. AMPs however are in this thesis divided into four major classes, these are: β-sheet, α-helical, AMPs that are rich in a certain specific amino acid and bacteriocins (AMPs produced by bacteria [7]). AMPs display multiple modes of action including bacteriostatic, microbicidal and cytolytic properties. It is generally accepted that AMPs selectively disrupt the cell membranes, creating pores and enhance the membrane ion permeability, leading to cell death [1]. However, other mechanisms such as intracellular killing and immunomodulatory effects exist [8] [6]. It is the amphipathic structural arrangement, positively charged and hydrophobic surfaces of the peptide that are considered to play an important role in the mechanisms [9].

Lately AMPs have received a lot of attention due to the increased resistance of microorganisms against current antibiotics, which is a serious health threat in today's society [1]. It is therefore important to find new antimicrobial alternatives, which microbes are less prone to develop resistance towards. Unlike conventional antibiotics which microbes readily circumvent; resistance development by a sensitive microbial strain against antimicrobial peptides is less probable [3]. This is mainly due to the deep changes in the membrane structure needed to confer resistance [4]. Besides the pharmaceutical sector, the field of antimicrobial peptides has a great potential in many other application areas. The positive aspects of AMPs have resulted in active AMP related companies and research that believe in the field. Pathogens are a large issue in various sectors, such as food, health and hygiene. Current research and many companies focus on direct use of AMPs in various pharmaceuticals (Appendix A- Interviews). Another direction of AMP related pharmaceuticals is to boost the body's own

production of peptides by inducing the host's AMP genes, e.g. vitamin D and butyrate (Appendix A- Interviews). However, other application areas of AMPs such as AMP-wipes and food preservative exist [10] [11]. In this thesis potential hygiene related applications such as probiotics, inducers of AMPs and AMPs in materials will be presented and discussed.

The use of AMPs in different products has many challenges that need to be considered. Publications have shown that there are bacterial species that have developed resistance against AMPs, even though it is rare [2] [3]. This resistance can be a result of different mechanisms such as increased levels of proteolytic enzymes and membrane modifications [6] [2]. Another challenge with AMPs, especially in the pharmaceutical industry, is the risk of cytotoxicity when administering large doses of AMPs [12]. The main differences between the microbe and mammalian cells are the membrane composition, architecture and energetics (e.g. membrane charge and potential) [9]. However, some AMPs can have difficulties to discriminate between mammalian and microbial cells, which could lead to toxicity towards human cells [4] [2]. Because of the risk of cytotoxicity and stability factors when used systemically, much of the research of AMPs is focused on topical applications [3]. Moreover most of the AMPs are broad-spectrum antimicrobials, which could be an issue in some applications where it is important not to disturb the normal micro flora of the body. Further there is a financial challenge in the production of AMPs. The peptides can be produced in two ways, either through chemical synthesis or by expression using biological systems. Both ways of production are in most cases time consuming and expensive [4] [2]. Understanding the resistance mechanisms, factors in target selectivity, increasing stability and minimizing the production cost are all important aspects in the development of AMPs. The on-going research has potential in solving many current and future challenges.

The main purpose of this thesis is to provide an understanding of the field of antimicrobial peptides and to evaluate their potential use in hygiene applications. Firstly, there will be a thorough description of what AMPs are and how they function, including the different structures, properties and mechanisms of action. Further much focus will lie on potential applications and challenges. Another part of the project will be to practically demonstrate the antimicrobial activity, salt-sensitivity, target selectivity and strain-dependency of five different AMPs. Since the project provider SCA focus on everyday hygiene products, the aim will be to study AMPs that are active against certain pathogens related to conditions of interest.

The initial step in the project is to perform a literature study. Besides gathering literature from databases, information was also obtained through interviews with active research groups at universities and companies. The information obtained from the interviews gave a more updated and improved understanding of the field. The experts that were interviewed are Professor Birgitta Agerberth, Professor Martin Malmsten, PhD/MD

Peter Bergman, PhD-student Andreas Cederlund and CEO Sigridur Olafsdottir. These experts are involved in both research at institutions and also in AMP related companies (Appendix A- Interviews, the cited researcher have all read and approved the text concerning their own statements and ideas). Also, based on the information gained from the literature study and interviews, AMPs used in the practical part of the thesis were ordered. The peptides that were ordered are nisin A, lactoferricin B, histatin-5, buforin-II and magainin 2. The antimicrobial activity of these peptides was then measured through a screening method called inhibition zone assay. The method is conducted on agar plates and reveals generally how well the antimicrobial activity of the peptide is. The minimum inhibitory concentration (MIC) is a common measure of the antimicrobial activity and can be calculated through the inhibition zone assay method. From the practical results it could be concluded that with the right conditions, most of the peptides showed activity depending on the microorganisms. It was also shown that many peptides are salt sensitive. Additionally when the peptides were tested against different strains of the same microorganism there was not much difference in activity.

2 Background

The background is aimed to provide a wide understanding of the field and a foundation for the practical part of the thesis. Firstly a general understanding of AMPs such as classification, role in the human innate immune system and mechanisms of action will be described. Next the challenges of using AMPs in applications will be presented. Finally the method and the peptides selected for the practical experiments will be described.

2.1 Antimicrobial peptides – Description and Classification

Antimicrobial peptides are essential components of the innate immune system (described below) in almost all forms of life, and provide a natural defence against invading pathogens [2]. Currently, around 2000 peptides have been discovered from various organisms ranging from insects to plant and animals [13]. AMPs have also been found in a wide range of prokaryotes [14]. Despite the many different origins, the peptides share many biophysical properties. AMPs are generally short peptides composed of between 12 and 60 amino acids, positive net charge and the secondary structure is mostly of amphipathic nature, consisting of both a hydrophilic and a hydrophobic part [14]. However, antimicrobial peptides are a diverse group of molecules and it is therefore not entirely straightforward to divide them into different classes. In this report the AMPs will be divided into groups based on their structure and amino acid composition as suggested by Pasupuleti *et al.* [2].

2.1.1 Classification

Antimicrobial peptides include a large variety of structural motifs; the most common and well-studied being the α-helical structure. Other common groups are β- structured peptides, AMPs that are rich in a certain specific amino acid and bacteriocins [15].

2.1.1.1 α-Helical AMPs

The group of α-helical AMPs is well studied and the peptides are generally known as cationic, amphipathic peptides that form helices [2]. The α-helical peptides can either be linear or contain disulphide bridges that form looped structures, and normally consist of less than 40 amino acids [1]. An interesting property is that in aqueous solutions many of these peptides are disordered; however in the presence of certain hydrophobic solvents, an environment resembling the microbial membranes, the molecule adopts an α-helix structure [6]. The degree of α-helicity correlates with the antibacterial activity, increased α-helical content gives stronger antimicrobial activities [16]. Besides the cationic amphipathic peptides there are groups of anionic or hydrophobic α-helical

AMPs [6]. One of the most studied α-helical peptides is magainin [15]. Other peptides in this class are cecropins, dermaseptins, buforin-II and LL-37 [14].

2.1.1.2 β-Structured AMPs

Only a small number of peptides form β-structures in membrane environments. The formation of anti-parallel β-sheet, β-hairpin and β-turn structures gives the peptide amphipaticity due to that the hydrophilic and hydrophobic residues are located on different sides of the peptide [17]. The peptide lactoferricin B, which is derived from the protein lactoferrin, has been found to adopt a β-sheet structure. This structure makes lactoferricin more amphipathic than lactoferrin, thus more efficient [18]. Compared to α-helical peptides, β-sheet peptides are more ordered in aqueous solution and membrane environments, due to constraints imposed by disulphide bonds or cyclization of the peptide backbone [9]. For example, the secondary structure of tachyplesin, a cyclic β-sheet peptide, is much unchanged as the peptide moves from an aqueous environment to that of a membrane-mimetic [9]. This demonstrates that secondary structures of cysteine-stabilized β-sheet peptides are relatively stable upon interaction with target cell membranes [9]. The β-sheet subgroup is diverse but some common characteristics are that the peptides are short, have a net positive charge and are amphipathic [14]. Peptides in this subgroup also include the different defensins, which play an important role in the human innate immune system (see innate immune system) [14].

2.1.1.3 AMPs with irregular amino acid composition

Many antimicrobial peptides have unusually high proportions of certain amino acids. This often results in peptide structures that differ from regular α-helices or β-structures [18]. The dominant amino acid residues in this group are often proline, arginine, histidine or tryptophan residues. Examples of AMPs in this subgroup are PR-39 from pigs, with an overrepresentation of proline and arginine residues [14]. The peptide histatin, which is produced in saliva, is rich in histidine residues [15]. Other peptides such as indolicidin and tripticin are rich in tryptophan residues [15].

2.1.1.4 Lantibiotics - bacteriocins

There are several antimicrobial peptides produced by bacteria (bacteriocins) [19]. Lantibiotics, which is a class of bacteriocins, are peptides that are composed of rare modified amino acids and thioether bridges [1]. This group of AMPs target a broad range of other Gram-positive bacteria [7]. Some of the general properties of these peptides are cationic, amphiphilic and small [19]. Lantibiotics produced by lactic acid bacteria (LAB) have long been used in the preservation of meat and milk [7]. The lantibiotics are subdivided into two groups: type A and B. Type A lantibiotics are small proteins that contain positively charged molecules, and kill via membrane polarization [19]. Type B lantibiotics are even smaller and kill by interfering with cellular enzymatic

reactions such as cell wall synthesis [19] [7]. Nisin is type A lantibiotic and one of the well-studied lantibiotics and is being used in large scale [20] [21].

2.2 Human innate immune system and AMPs

Almost all multicellular organisms defend themselves continuously against potentially harmful microbes. The most common sites for invading microbes in mammals are the epithelial surfaces such as the skin, the moist surfaces of the eyes, nose, airways, lungs and mouth and also the digestive tract and urinary system [22]. The human defence or immune system is divided into two parts, the adaptive and the innate. The innate immune system is fast and unspecific in action, while the adoptive immune system is a slow process [14]. The rapid innate immune defence includes physical barriers and epithelial cells, neutrophils and macrophages, proteins and AMPs [14]. The innate immune system protects us from microbial invasion by recognising the microbes and then rapidly eliminates and/or impedes their spread until the slower-acting adaptive immune system is mobilised [8]. There is also growing evidence that antimicrobial peptides can activate the adaptive immunity [22]. The invading microorganisms are recognized by receptors that sense certain molecules found only on the surface of microbes and not on host tissue. These molecules are called pathogen associated molecular patterns (PAMPs) and the receptors are named pattern recognition receptors (PRRs) [14]. In either part of the immune system, the response includes mobilization and production of antimicrobial peptides [14].

The highest concentrations of AMPs are found in tissues exposed to microbes or cell types that are involved in host defence. Epithelial surfaces secrete antimicrobial peptides from both barrier epithelia and glandular structures [22]. Phagocytic cells such as neutrophils and macrophages also contain several types of granules for antimicrobial substances and digestive enzymes [22]. In the process of phagocytosis, granules fuse to lysosomes that contain microbes, thereby exposing them to very high concentrations of microbicidal substances such as AMPs [22].

There are two main groups of peptides in humans, defensins and cathelicidins [8]. Other human peptides are lactoferricin [23] and histatin [24], which will be described in section 2.7 Peptides.

2.2.1 Cathelicidins

The cathelicidins form a large family of antimicrobial peptides found in all mammalian species [25]. The peptides are grouped together as a family based on a conserved region (the cathelin domain) of about 100-120 amino acid residues [26] [25]. The cathelin domain is flanked by a signal peptide domain (approximately 30 residues long) on its

N-terminus, and by an antimicrobial peptide region on its C-terminus (*Figure 1*) [26] [25]. Cathelicidins are synthesized in this form and are named pre-proteins. The signal peptide is cleaved off once it has fulfilled its purpose of targeting the cathelicidin to storage granules or to the exterior of the cell [26]. The cathelin and AMP complex are denoted pro-protein since it does not represent the active, but rather a storage form. In most cases the cathelin domain must be removed to obtain the mature and antimicrobial form [25]. Cathelicidins are found in cells of the immune system including neutrophils and macrophages [25]. They are also found in cells that coat the epithelial surfaces like the skin respiratory tract, and gastrointestinal tract, since these surfaces are often exposed to potential pathogens [27]. Cathelicidin has a broad anti-microbial activity against gram-positive and -negative bacteria, as well as certain enveloped viruses and fungi [25]. The killing mechanism of cathelicidin involves bacterial lysis through membrane permeabilization (section 2.4.1 Mechanisms of Action) [25]. Only one human cathelicidin has been isolated; hCAP-18 (unprocessed form), or LL- 37 (processed and active antibacterial form) (*Figure 1*) [8]. Professor Birgitta Agerberth was one of the persons involved in the discovery of LL-37, which is a 37 amino acids long amphipathic, α-helical molecule (Appendix A.1 Interview with Birgitta Agerberth). Human neutrophils contain large amounts of hCAP-18 and the concentration can be up to 5 μg/ml in the bulk of different body fluids such as blood plasma, airway surface fluid, wound fluid and blister fluids formed during infection or inflammation [8].

Figure 1. Image of the human cathelicidin hCAP-18. Reprinted from [26] *with permission from Elsevier (2006).*

2.2.2 Defensins

The defensins are a family of antimicrobial peptides that are widely spread in nature and found in mammals, plants and insects [8]. Like cathelicidins, the defensins are synthesized as pre-pro-peptides, which are then processed to various extents to release the active peptides [14]. Defensins are small cationic peptides and are categorized in three subfamilies based on size and organisation of disulphide bonds, α, β-, and θ-defensins [28]. In humans only α- and β-forms are found. There are 6 different human α-defensins HNP 1-4, HD 5 and HD 6, which are found in neutrophils and monocytes [14]. The importance of α-defensins was demonstrated in an experiment where transgenic mice with α-defensins deficiency showed higher susceptibility to infections with *Salmonella typhimurium* compare to wild type mice [29]. Human β-defensin HBD

1-4, are found in a wide verity of cells including keratinocytes, epithelial cells, monocytes, dendritic cells and mast cells [8]. Genomic research does predict 28 additional human β-defensins [17], however, to date these have not been verified on the protein level [28].

2.2.3 Inducers of AMPs in the innate immune system

On-going research is focusing on finding inducers of AMPs (Appendix A.1 Interview with Birgitta Agerberth). Inducers that have been studied are for example vitamin D, litocholic acid and butyric acid (Appendix A.1 Interview with Birgitta Agerberth). Inducing the AMP production in the body is a field with interesting applications. According to Olafsdottir, the development of resistance might be less probable compared to using single peptides in applications (Appendix A.4 Interview with Sigridur Olafsdottir).

2.2.3.1 Vitamin D

In the 19th century it was discovered that a number of factors such as dry and warm climates with plenty of sunshine were beneficial for patients suffering from infectious diseases such as tuberculosis [30]. A recent study has now shown that vitamin-D induces antimicrobial peptide gene expression, thus partly explaining the antibiotic effect of vitamin D [27]. The regulation of AMPs is biologically important for the response of the innate immune system to wounds and infection. It follows that vitamin-D deficiency could therefore have a role in the weakened response towards invading bacteria, viruses, protozoa, and fungi [27]. It has been demonstrated that wounding of the skin is a potent inducer of LL-37 expression in normal skin and vitamin D is recognized as a positive regulator of the expression [31].

Humans can obtain vitamin D in two different forms, vitamin D3 and vitamin D2. Both forms can be found in foods or supplements but only vitamin D3 is produced in skin upon exposure to ultraviolet B radiation [27]. The vitamin D-compounds undergo two modifications to become biologically active. First vitamin D compounds are hydroxylated mostly in the liver to form 25-hydroxyvitamin D2 or D3 (25(OH)D2 or 25(OH)D3), these are the major circulating forms. These compounds are then hydroxylated locally by an enzyme to produce the hormonal 1,25(OH)2D2 or 1,25(OH)2D3 [32]. It has been demonstrated in a wide range of studies that 1,25D treatment up-regulates cathelicidin mRNA in several cell lines and primary cultures including keratinocytes, neutrophils, and macrophages [33] [34]. It has been reported that vitamin D response element is present on the promoter of genes coding the anti-microbial peptides, thus regulating the expression [34]. To sum up, the findings suggest that 1,25D up-regulates anti-microbial peptide production, mainly cathelicidin, on a

variety of different cells [35]. Also studies have found an association between vitamin D and incidence of several infections such as bacterial vaginosis [35].

2.2.3.2 Sodium butyrate

Professor Birgitta Agerberth, which is involved in the research about inducers of AMPs, mentions that butyric acid is another inducer of LL-31 in colonic epithelial cells (Appendix A.1 Interview with Birgitta Agerberth). In a study it was demonstrated how treatment with oral sodium butyrate during early phases of an experimental Shigella infection resulted in the induction of cathelicidin expression, which in turn reduced the numbers of Shigella in the stool, and enhanced recovery [36].

2.3 Microorganism overview

Microorganisms are defined as single cell organisms and are divided into different main groups, among which are bacteria and unicellular fungi [37]. Bacteria are in turn generally classified in what color they receive when stained with gram stain. The gram stain discriminates between two categories of bacteria: Gram-positive bacteria, where the color is violet/blue and Gram-negative bacteria, where the color is red/pink. The difference in how the cells react to gram color is believed to have its explanation in the variation between the cell wall structures [38] [37]. The group fungi include a wide range of eukaryotic organisms. The description will be limited to yeast, which is a single cell fungus [39]. Each group of microorganisms will be described from a structural point of view and the structure will be explained from the outside to the inside of the cell. Fungi will be described together with mammalian cells in order to point out the similarities. The scope of this section is to provide a foundation for the understanding of antimicrobial peptides in their interaction with the outer parts of the cell.

2.3.1 The Gram-negative cell-envelope

Three layers surround the cytoplasm of Gram-negative bacteria (*Figure 2*): The cytoplasmic membrane, the peptidoglycan layer and the outer membrane. These layers together with the periplasm are called the Gram-negative cell-envelope. The peptidoglycan layer and periplasm is located in between the outer and the cytoplasmic membrane [40] [41].

The major constituents of the outer membrane are lipoproteins, lipopolysaccharides (LPS) and phospholipids. Lipopolysaccharides are composed of a hydrophobic part called lipid A and a hydrophilic part that consist of various carbohydrates. Phospholipids and LPS molecules are mainly negatively charged at neutral pH, which

results in that the outer face of the outer membrane is highly charged [41] [40]. The inner leaflet of the outer membrane has most of the phospholipids [40]. Apart from phospholipids and LPS, the outer membrane also consists of lipoproteins that have functions such as stabilizing the membrane and to attach it to the peptidoglycan layer. There are also proteins functioning in the facilitation of the permeation of nutrients and small molecules which makes the outer membrane relatively permeable in comparison to the cytoplasmic membrane [37] [40]. The porins may be a possible pathway for the AMPs to cross the outer membrane [42].

Inside the outer membrane is the periplasm, which is 15nm wide and has a content of proteins that the outer membrane is preventing from diffusing away [37]. In the middle of the periplasm is the peptidoglycan layer, which is about 2nm thick. The peptidoglycan layer is consisting of a glycan backbone of alternating repeating sugar units cross-linked with certain amino acids. This forms a mesh-like structure that supports the structure of the cell [38] [37]. The cytoplasmic membrane, which is the inner layer of the cell envelope, is consisting of mostly negatively charged phospholipids [2].

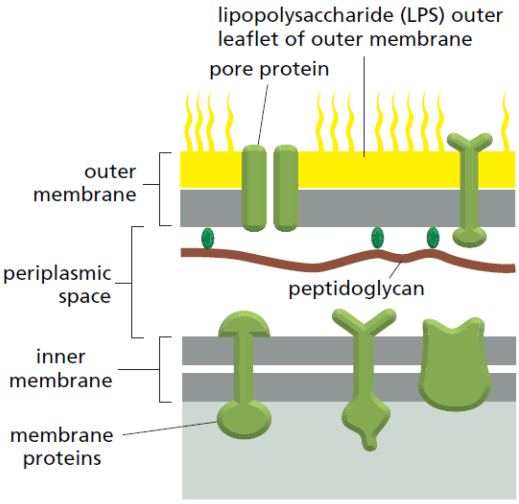

Figure 2. Representation of the gram-negative cell envelope. Copyright 2008 from [43], *reproduced by permission of Garland Science/Taylor & Francis Books, LLC*

2.3.2 The Gram-positive cell-envelope

Unlike Gram-negative bacteria, Gram-positive bacteria lack an outer membrane (*Figure 3*). The cell wall of Gram-positive bacteria is situated at the outer most part of the cell and consists, like Gram-negative bacteria, of peptidoglycan. The amino acids in the peptidoglycan are, however, often linked in a different way [38]. The peptidoglycan layer is 20-40 nm wide, which is wider than the peptidoglycan layer of Gram-negative bacteria [38]. Similar to Gram-negative bacteria the cytoplasmic membrane is

negatively charged [2] [38]. Moreover, Gram-positive bacteria also have teichoic acids embedded in their cell wall. Teiochoic acids are negatively charged which further contribute to the negative charge of the cell surface [37].

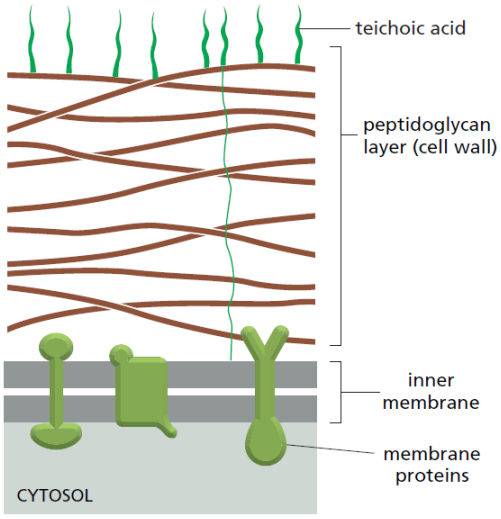

Figure 3. Representation of the gram-positive cell envelope. Copyright 2008 from [43]*, reproduced by permission of Garland Science/Taylor & Francis Books, LLC*

2.3.3 Eukaryotic cell-envelope

Yeast and mammalian cells are both eukaryotes, which mean they have a nucleus. Yeast, like bacteria, also has a cell wall, although consisting of other structural elements such as chitin [39]. The structural elements of the cell wall are polysaccharides that differ depending on the taxonomic group [44]. Mammalian cells and fungi have sterols incorporated in their cytoplasmic membranes. Mammalian cells have the sterol, cholesterol, which is uncharged whereas yeast uses the sterol, ergosterol, which also is uncharged [39] [44]. The yeast membrane as well as the mammalian membrane is mainly composed of zwitterionic phospholipids, which are neutral in charge, thus the membrane of yeast and mammalian cells both have a more neutral charge in comparison with bacteria [45].

2.4 Mechanism of antimicrobial peptides

The mechanisms underlying the antimicrobial activity of AMPs can vary greatly. In this section a detailed explanation will be given for all of the known mechanisms concerning antimicrobial activity. As previously stated the mechanism may involve both extracellular and intracellular variants. Most of the focus will be kept on the more common extracellular mechanisms. Firstly the mechanisms of action will be described and thereafter parameters affecting the antimicrobial activity and selectivity of AMPs.

2.4.1 Mechanisms of Action

In this section the mechanism regarding the antimicrobial activity will be presented. It should be noted that peptides could kill microorganism indirect trough immunomodulatory effects, or by direct killing through membrane disruption or internal targets (*Figure 4*) [46].The main focus will be on the direct killing mechanism, however, immunomodulatory effects will be briefly described at the end of this section.

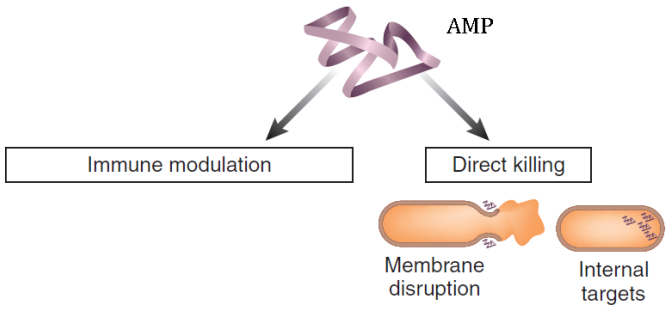

Figure 4. AMPs can kill microbes indirect through immune modulation and direct through membrane disruption and internal targets. Reprinted by permission from Macmillan Publishers Ltd: [Nature Biotechnology] [47] *copyright (2006)*

Although AMPs belong to innate immunity, the mechanism by which they kill the microbes differs from that of cytokines and phagocytes. The AMPs specific antimicrobial mode of action is however not fully understood [2]**.** The main idea is that peptides kill microorganisms by causing several defects in target microbial cell membranes [9]. Peptides are able to form transmembrane pores, which in turn leads to the destruction of the membrane electrochemical potential and pH gradient which leads to altered osmotic regulation, inhibited respiration and eventually cell death [14]. Besides this mechanism much research now supports additional or complementary mechanisms. In these cases, cell killing proceeds with relatively little impact on membrane disruption and instead on intracellular processes such as inhibition of protein or cell-wall synthesis, interaction with DNA/RNA or inhibition of enzymatic activity [9]. Regardless of the mechanism, the peptides need to interact with the microbe membrane either to pass it or rupture it. The mechanisms by which a peptide interacts and causes damage to the microbial membrane can be divided into three steps namely attraction, attachment and permeabilization [6].

2.4.1.1 Attraction, Attachment and permeabilization

The initial mechanism by which AMPs target microbes occurs through electrostatic interactions [2]. As an example, cationic AMPs are attracted to the anionic outer envelope, LPS or phospholipids, of Gram-negative bacteria and to the teichoic acids of Gram-positive bacteria [14]. Much of the research regarding mechanism of action has focused on interactions with bacterial or bacteria-like membranes and studies examining

fungal membrane interactions are very few [17]. However, it is thought that the pore-forming mechanism is similar for fungi and bacteria [17]. In order for the AMPs to reach and interact with the microbe membrane, the peptides must first pass through the extensive cell wall [6]. For Gram-negative bacteria AMPs need to pass through the layers of LPS, and then possibly through the porins [42]. For Gram-positive bacteria the AMPs have to pass through polysaccharides and teichoic acids [14]. The antifungal AMPs need to pass through fungal cell wall, which mainly consists of chitin and glucans [17] (see section 2.3 Microorganism overview).

The mechanism where peptides permeabilize the microbial membranes differs between peptides [9]. There are three suggested models that are common when describing membrane permeabilization by AMPs. These are: the barrel-stave model, the toroid-pore model, and the carpet model [9] [14] [6].

2.4.1.1.1 Barrel-stave model

In the barrel-stave model bundles of peptides form transmembrane pores [14]. The mechanism involves the following: The initial step in pore formation involves peptide monomers binding at the membrane surface in a helical structure (*Figure 5*A) [18]. Upon binding, the peptides force the membrane polar-phospholipid head groups aside and induce membrane thinning. Next, bound peptides reach a threshold concentration, at that stage peptide monomers self-aggregate and at least two monomers insert deeper into the hydrophobic membrane core to initiate the formation of a pore (*Figure 5*B) [9] [18]. Additional peptide monomers are then recruited and attached to the bundle leading to an increase in the size of the pore. Thus, several peptides are needed to form a pore. The fungal peptide alamethicin is the best studied AMP that uses the barrel-stave type of pore [14]. Since these peptides can insert into the hydrophobic core of the membrane, their binding to the target membrane is mainly driven by hydrophobic interactions. As a consequence, they can bind to both zwitterionic and charged phospholipid membranes [18].

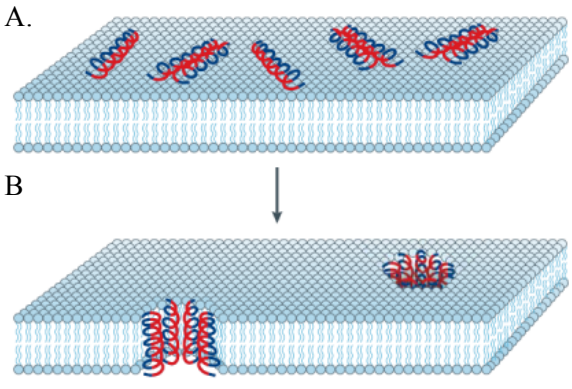

Figure 5. An illustration of the barrel-stave model: A) Attachment to the membrane and B) formation of pores. Reprinted by permission from Nature Reviews Microbiology **[6]** *copyright (2005)*

2.4.1.1.2 Carpet model

In the carpet model, peptides initially accumulate parallel to the membrane surface in a carpet-like manner (*Figure 6A*) [6]. This initial interaction with the negatively charged target membrane is electrostatically driven, and therefore the active peptides are always positively charged. The positive charges of the basic amino acids interact with the negatively charged phospholipid head groups or water molecules [18]. At high peptide concentrations, surface-oriented peptides are thought to disrupt the bilayer in a detergent-like manner, eventually leading to the formation of micelles. At the threshold concentration, the peptides form toroidal transient holes in the membrane, allowing additional peptides to access the membrane (*Figure 6B*). Lastly the membrane breaks down and micelles are formed (*Figure 6C*) [6]. As with any mechanism of action, it is possible that alternate results may be obtained using different membrane models or assay conditions [9]. The carpet model describes the action of for example dermaseptin S, cecropin, and melittin [14].

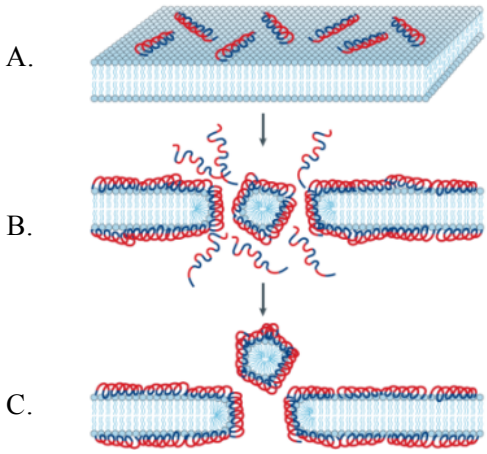

Figure 6. An illustration of the carpet model: A)Attachment to the membrane surface, B) disruption of the membrane and micelle formation and C) disintegrration of the membrane. Reprinted by permission from Nature Reviews Microbiology [6] *copyright (2005)*

2.4.1.1.3 Toroidal-pore model

One of the most well characterized peptide-membrane interactions is that of the toroid pore [9]. In forming a toroidal pore, the polar side of the peptides associate with the polar head groups of the lipids *(Figure 7A*) [6]. When the peptides insert into the membrane they induce the lipids to tilt and connect the two layers of the bilayer membrane, thereby forming a bend continuously through the pore (*Figure 7B*). Both the lipid head groups and the inserted AMPs make a hydrophilic lining all through the pore (*Figure 7C*) [14]. The toroidal model differs from the barrel-stave model as the peptides are always associated with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer. Magainin-induced toroidal pores are larger and have a more variable pore size than alamethicin-induced (barrel-stave mechanism) pores [6].

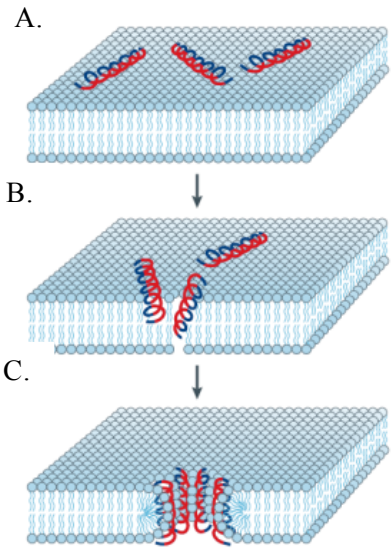

Figure 7 An illustration of the toroidal model: A)Association with polar head groups, B) formation of a bend, which connects the two layers of the bilayer membrane and C)lipid head groups and inserted AMPs form the pore. Reprinted by permission from Nature Reviews Microbiology [6] *copy right (2005)*

2.4.1.2 Models of intracellular killing

Although membrane destruction contributes to AMP mechanisms of action, recent studies suggest that disruption of different key intracellular processes may contribute or be required for cell death [9]. Peptides with these mechanisms can for example bind and inhibit DNA (buforin II and tachyplesin), which is suggested to be due to the negative charge of nucleic acid attracts the cationic peptides. Other peptides inhibit cell-wall synthesis (the lantibiotic mersacidin), or inhibit enzymatic activity (histatins) [18]. AMPs may also target and inhibit intracellular organelles found within fungal pathogens. It has been shown that exposure to the cationic peptide histatin-5 caused damage to the mitochondrial membrane in *C. albicans* [9]. From these perspectives, antimicrobial peptides may have multiple and complementary mechanisms of action necessary to inhibit or kill a wide variety of pathogens in diverse physiologic settings while suppressing the ability of the pathogen to avoid these mechanisms [9].

2.4.1.3 Immunomodulatory effects

There is evidence that clearly demonstrates that AMPs are important in the immune response, for example animals with decreased AMP expression or activation are more prone for specific infections [47]. However it is being questioned what part the direct antimicrobial activity of the peptides play in the protective role. An increasing number of studies are focusing on the immunomodulatory effects of AMPs, or Host-defence peptides, as they are named in this context [48]. The immunomodulatory effects have not been clearly demonstrated, however it has been suggested that host-defence peptides have immunomodulatory properties such as modulating the expression of hundreds of genes in monocytes and epithelial cells, direct attraction of immune cells, induction of chemokine's, wound-healing responses, and resolution of infections [46]. As a result reports have shown that too high levels of peptides such as LL-37 may result in

inflammatory pathogenesis [8]. In a study it was specifically demonstrated that LL-37 was expressed at very high levels in the skin of patients suffering from the inflammatory skin disease rosacea [49]. Other studies are saying that host-defence peptides in fact suppress TLR signalling responses, and thus might not be pro-inflammatory (see section 2.2 Human innate immune system and AMPs) [46].

The immunomodulatory effect of AMPs within the body is strengthened by studies showing that at physiological concentrations most AMPs are not direct antimicrobial [47]. However, it should be noted that at physiological conditions in the skin peptides such as LL-37could be directly antimicrobial [8].

2.4.2 Parameters that affect activity

Parameters that affect the antimicrobial activity and the selectivity of AMPs include: conformation, peptide length, charge, polar angle, hydrophobicity and amphipathicity. These parameters are often co-dependent, thus changing one parameter may affect the other. This needs to be considered when trying to increase the activity or the selectivity of AMPs [9].

2.4.2.1 Peptide conformation

Different peptide conformations have already been mentioned in previous section (section 2.1.1 Classification). In this section the focus will however be on the effect of the peptide conformation. Cyclic or linear conformations of AMPs can influence the activity of the AMP. Linear AMPs have shown to have a higher antimicrobial activity than cyclic AMPs [16]. It is believed that the bulky nature of a cyclic AMP prevents it from penetrate into the phospholipid membrane and thus results in a decreased binding to the phospholipid membrane. Moreover, experiments with two magainin 2 analogues, where one of the analogues was linear and the other was cyclic, showed that the linear analogue had a higher antimicrobial activity [16].

Most of the linear AMPs form an α-helical structure in hydrophobic or amphipathic environments such as the phospholipid bilayer [41]. Most α-helical-peptides have amphipathic characteristics. This makes it possible for the polar residues to interact with the phospholipid head-group (the charged end of the phospholipid) and the nonpolar residues to interact with the uncharged chains of the phospholipids [50]. Additionally, it has been shown that increased helicity increases the antimicrobial activity of the peptides. However, increased helicity also gives rise to increased cytotoxicity. Thus, when trying to synthesise peptide analogues with decreased cytotoxicity one approach is to destabilize the helices of the peptide [16]. Less is known about β-sheet peptides, it has however been speculated that the disulphide bonds can be important for the antimicrobial activity as well as for the specificity [2]. Further, it has been shown that

maintenance of a suitable hydrophobic and hydrophilic balance and cyclization is important for β-sheet peptides [2].

2.4.2.2 Peptide charge

As have been mentioned AMPs are usually cationic. The cationic charge is an important parameter when AMPs initially bind to the negatively charged cell membranes [9]. The charge can vary from +2 to +9 net charge but most of the natural peptides have a charge of +4 to+6 [51]. The charge is a result from an overrepresentation of the positive charged amino acids lysine and arginine. Studies conducted on the AMP magainin have showed a direct correlation between the peptide charge and its potency [2]. In some cases the relationship is not entirely linear and the effect can follow an indirect or reversed relationship [9]. If the net charge exceeds +7, no increase in the antimicrobial activity is observed. The reason for this is that the strong interaction between the peptide and the phospholipid head group prevents translocation into the deeper layers of the membrane [2]. Still, within a certain range the effect is in general linear [9]. A high cationic charge is however not effective when the target microorganism have a neutral or low membrane charge [16].

2.4.2.3 Peptide length

It has been shown that the length of the peptide affects the antimicrobial activity [16]. Decreasing peptide length results in a decreased membrane binding. This is because the AMPs need to span the thickness of the membrane, which usually is around 40 Å, otherwise the pore formed by the peptides will not be stable enough. A decreasing peptide length also results in decreased tendency to form secondary structures like α-helices, which have been shown to increase the antimicrobial activity of the peptides [16]. However, it has been demonstrated that in some cases it is possible to shorten a peptide to a certain length [16]. One example is the peptide LL-37 that after half of the peptide removed still has retained antimicrobial activity. If LL-37 becomes sufficiently short the activity will however be lost [26].

2.4.2.4 Polar angle

Polar angle is related to hydrophobicity and amphipaticity. It is a measure of the relative proportion of polar versus non-polar facets of a peptide conformed to an amphipathic helix [2] [9] [51]. The polar angle can be represented with the aid of a helical wheel in which the alpha helix of a protein or peptide is plotted in a rotating manner. The amino acid that follows in the sequence is plotted 100° from the closest neighbouring amino acids. The plot reveals whether hydrophobic amino acids are concentrated on one side of the helix, with polar or hydrophilic amino acids on the other side [52]. The angle of the polar amino acids represents the polar angle. As example, if peptides that are composed of only hydrophobic residues on one face and hydrophilic residues on the other face, the polar angle will be 180° (*Figure 8*). Most natural helical AMPs have a

polar angle of 140°-180°. The polar angel is important in the overall stability and the half-life of AMPs induced pore angel [2] [9] [51]. The rate of pore formation has been shown to be faster for peptides with small polar angle, but the same is true for the pore collapse. Peptides with smaller polar angles are thus believed to achieve less stable pore formation than peptides with a larger polar angle [9].

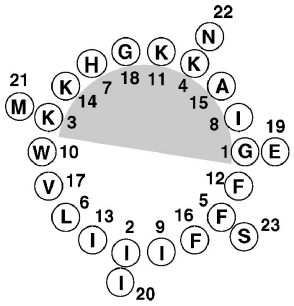

Figure 8. Helical wheel of an analogue to magainin (MG-H1) The shaded area represents the polar surface of the amphipathic helix. Reprinted from [155] *with permission from Elsevier (2006)*

2.4.2.5 Peptide Amphipaticity

Amphipaticity is a parameter that reflects the relative abundance and polarization of hydrophobic and hydrophilic domain within a protein [51]. The most common protein structure that gives rise to amphipaticity is the α-helix. The α-helix has an optimal amphipaticity to interact with the amphipathic bio membranes. The parameter affects both negative charge membranes as well as uncharged membranes but the effect on the latter one may be even greater. A high amphipaticity is therefore often correlated to increased toxicity towards cells with neutral phospholipids [51] [9]**.** As well as the α-helix, the β-helix is also amphipathic [9].

2.4.2.6 Peptide hydrophobicity

When the AMPs adsorb to the membrane both electrostatic interactions and hydrophobic interactions are important. The hydrophobic interactions are responsible for the deeper penetration into the membrane [16]. AMPs are usually consisting of approximately 50 % hydrophobic residues. The hydrophobicity of a peptide affects both the antimicrobial efficiency and the specificity of α-helical peptides. If the hydrophobicity parameter is over optimal levels the antimicrobial activity will decrease and the cytotoxicity increased. The increased cytotoxicity is due to the poor solubility in aqueous solutions, which promote the peptide to bind to eukaryotic cell membranes. It is therefore a strong correlation between hydrophobicity and cytotoxicity [2] [16] [51].

2.4.2.7 Summary of activity parameters

Overall it has been shown that linear AMPs have a higher antimicrobial activity than cyclic AMPs [16]. Further, increased helicity have shown to increase the antimicrobial

activity of the peptides. However, increased helicity also gives rise to increased cytotoxicity against mammalian cells [16]. Less is known about β-sheet peptides, it has however been speculated that disulphide bonds can be important for the antimicrobial activity as well as for the specificity [2]. Most of the natural peptides have a charge of +4 to+6 [51]. It has been demonstrated that AMPs, such as magainin have a direct correlation between the peptide charge potency [2]. However, if the charge exceeds +7, no increase in the antimicrobial activity will be observed. By decreasing the peptide length a decrease in membrane binding and activity is observed [16]. AMPs usually consist of approximately 50 % hydrophobic residues. If the hydrophobicity parameter is higher than optimal levels the antimicrobial activity will decrease and cytotoxicity against mammalian cells will increase [2]. Amphipaticity is important for the antimicrobial activity and both α-helix and β-structured AMPs can be amphipathic. The α-helix has an optimal amphipaticity to interact with the amphipathic bio membranes. A higher amphipaticity is correlated to increased toxicity towards mammalian cells with neutral phospholipids [51]. Moreover, the polar angle is important in the AMP pore formation. A small polar angle is believed to result in less stable AMP pores [9].

2.5 Challenges

There are many challenges involved in development of AMP applications. The major considerations will be described in this section, beginning with production and economic considerations. Other important challenges described are resistance development, specificity, toxicity and stability. The stability includes several aspects such as salt, pH and temperature sensitivity; however, in this thesis the main focus lies on salt-sensitivity. Finally, it should be noted that the immune-modulatory effects might cause inflammatory responses [49].

2.5.1 Production and cost aspects

One of the major problems in the use of AMPs is the high cost. The peptides can be produced either through chemical synthesis or by expression using biological systems. Either way of production can be both time-consuming and expensive [2] [4]. Natural sources are not cost effective with the exception of the AMP nisin, which can be produced naturally by fermentation by Lactococcus lactis [53].

When using chemical synthesis to produce AMPs expensive synthesis procedures is needed and a variety of purification steps need to be added after each step of the synthesis procedure, which together contributes to a high cost [2]. A lot of industrial research has been able to lower the production cost. The cost for synthetic peptides would still be several times higher than that of conventional antibiotics existing on the market [2] [4] [53].

A promising alternative to synthetic production is the production by recombinant expression methods. The fact that the AMPs can be toxic to the expression host makes the process complicated. To solve this problem the peptides can be expressed as fusion proteins in bacterial strains lacking the enzyme protease [2]. This method both increases the solubility, avoids degradation, and toxicity to expressing cells. However it leads to other complications such as waste of protein and energy as well as the extra costs related to the cleavage and purification of the protein. The production of AMPs by bacterial expression systems is in many cases still an expensive production method with a lot of technological difficulties when it comes to large-scale production. Research is being conducted to make bacterial expression methods more cost effective [2] [4]. Apart from bacterial expression systems, a fungal expression system has been developed. The company Novozymes (Bagsvaerd, Denmark) is currently using this system to produce the AMP plectasin that is produced in high yields [4].

2.5.2 Specificity

Specificity is in general an important attribute when it comes to the development of new therapeutics. A substance with a high specificity against its target will have a lower probability of being toxic against human cells. This is also a challenge when using AMP in a variety of applications [2].

One common way for the screening of toxicity is to use human red blood cells. With this assay the haemolytic activity of the peptides can be determined and used as a measure for the cytotoxic activity [2] [54]. The toxicity of a peptide can be determined by the haemolytic activity. It follows that a low haemolytic activity indicates a larger specificity of the peptide. It has been shown that certain physical parameters of AMPs can affect the toxicity. As already been mentioned; high amphipaticity, high hydrophobicity and high amount of α-helices or β-sheet structure are parameters that all have been shown to increase the toxicity of AMPs [55].

Apart from toxicity towards mammalian cells, low specificity of AMPs can also result in counteracting the non-pathogenic microorganisms of the body. This is a concern when using AMPs in topical applications where for example the peptides may attack the normal flora of the skin [2]. The selective toxicity between different bacterial species is explained by that an antimicrobial agent can have a greater affinity for a certain lipid species over another, which can vary between microorganisms [38]. Mechanisms involving specificity of AMPs are described in further detail in the section below.

2.5.2.1 Mechanisms behind specificity

There are different explanations in how an antimicrobial peptide can distinguish between pathogens and host cells. The structure of the host cell and the microbe are the most common theme for the selectivity [9]. Another factor behind selectivity is high regulation of the peptides or limited access of the peptides to sensitive tissue of the host organism [9].

An important property that influences selectivity is membrane charge. As been mentioned, (Section 2.3 Microorganism overview) the prokaryotic membrane is negatively charged unlike the eukaryotic membranes that usually are neutral in charge. The charge difference is mainly due to the differences in composition of the phospholipid bilayers. The phospholipids of eukaryotic cells differ significantly from the phospholipid composition of bacteria. Eukaryotic cells are often composed of zwitterionic phospholipids, which have no net charge. The membranes of prokaryotes, however, often consist of negatively charged phospholipids. As mentioned LPS and teichoic acids also contribute to the negative surface charge of Gram-negative and Gram-positive bacteria respectively [9] [2]. As well as the difference in membrane composition, the distribution of the phospholipids can also affect the charge of the cells [9] [16]. Apart from the membrane net charge, the trans-membrane potential has been hypothesized to be another factor to affect the selectivity between prokaryotic and eukaryotic cells. The trans-membrane potential is a result of charge separation between the extracellular and intracellular parts of the cytoplasmic membrane. The trans-membrane potential in prokaryotes is often 50% greater than in mammalian cells which can help some AMPs to distinguish between cells with differing potential [9] [2] [51].

As has already been described (section 2.4.2.1 Peptide conformation), the conformation of the AMP may also affect the toxicity towards the host cell. Moreover it has been shown that a cyclic or a linear conformation can affect the toxicity of the peptide [9].

As mentioned in the beginning of this section, limited access to sensitive tissue may avoid toxicity towards host cells. It has been shown that many AMPs in vertebrates are secreted on epithelial surfaces that are less sensitive such as the skin of amphibians. Moreover, many AMPs can be expressed inside of phagocytes. One peptide common in mammalian phagocytes is defensins [29]. The defensin group is one of the most potent AMP group known but also the group with the least selective toxicity. The isolation of defensin inside phagocytes results in lower risk of toxic effects towards the host organism. [9]

Apart from discriminating between prokaryotic and eukaryotic cells, some AMPs can act with selective toxicity towards single cell fungi. These AMPs can discriminate between the cells based on the sterols, cholesterol and ergosterol [9] [56]. Cholesterol is present in human cells while ergosterol is present in the membrane structure of fungi.

Cholesterol is representing 45% of the total lipids in the human cell membranes [9]. Cholesterol is thought to decrease the membrane association of the host cell with some AMPs and the cholesterol is thought to protect against membrane permeabilization [56]. When searching for antifungal therapeutics, peptides selective for ergosterol are usually possessing lower toxicity towards human cells [16]. In addition the antifungal peptide histatin-5 has shown to bind to a specific protein located in the fungal cell envelope, called heat shock protein Ssa2p. This protein is absent in mammalian cells, and might explain the peptides low mammalian cell toxicity [57].

Finally, it can also be mentioned that although it has been hypothesized that AMPs have a non-receptor type interaction with most pathogenic membranes, studies show that there are exceptions. One exception is nisin, which is believed to have a specific receptor like interaction with lipid II [58]. Lipid II is a membrane-anchored cell-wall precursor that is important for bacterial cell-wall biosynthesis [59]. Experiments have shown that when lipid II is present the activity of nisin was increased 1000-fold [9].

2.5.3 Resistance

It has previously been hypothesized that it is difficult for microbes to build up resistance against AMPs [**2**]. The general belief has been that the main mechanism of AMPs, to attach to the membrane, is too difficult for the microbes to counteract because of the large membrane modifications that would be needed. Moreover, it has been described that the large variability of different AMPs expressed by host cells makes it difficult for microorganisms to evolve resistance mechanisms against a variety of AMPs present at the same time. Nowadays it has however been shown that microorganisms develop resistance in a greater extent and in a more variable way than previously thought [2]. This resistance can be a result of different functions, which can involve mechanisms such as increased levels of proteolytic enzymes and membrane modifications [60] [2]. The general mechanisms of resistance will be explained in greater detail in the following section.

2.5.3.1 Mechanism behind resistance

The resistance mechanisms of AMPs can be divided into two major groups or major strategies, constitutive resistance and adaptive resistance. Constitutive resistance mechanisms are defined by mechanisms that are always active in the cell regardless of the AMP concentration in the surroundings. The opposite apply for adaptive mechanisms, which are triggered when peptides are present. Both strategies include important mechanisms in the protection against AMPs and vary between Gram-positive and Gram-negative bacteria and also between bacteria and single cell fungi [9] [51]. Examples of both adaptive resistance mechanisms and constitutive resistance mechanisms will be given in the sections below.

2.5.3.1.1 Constitutive

Different phospholipid composition of the membranes is a strategy in how microbes shield themselves from invading AMPs. The composition can result in that the microorganisms inherently lack electrostatic affinity or repel AMPs [51] [9]. An example of a microorganism with this type of resistance mechanism is the microorganism *Staphylococcus aureus.* Staphylococcus species normally have membranes that consist mainly of negatively charged phospholipids [61]. *S.aureus* have however been demonstrated to possess a different type of membrane structure than other species of *staphylococcus* [61]. A higher level of the membrane component, lysyl-PG, which is a derivative of the phospholipid PG, is present in a higher level in *S.aureus* than in other staphylococcus species. This component is much less negative than PG, which results in a decreased membrane charge (*Figure 9*). It has been suggested that constitutive alterations of the cytoplasmic membrane could be the key resistance mechanism of *S.aureus* [61].

Membrane energetics is another type of constitutive mechanism. Some AMPs are very sensitive to the transmembrane potential. One example is the AMP defensin II, which increases its antimicrobial activity if the target cells are highly energized [51]. Moreover, it has been shown that *S.aureus* strains with a low trans-membrane potential have a higher resistance to some AMPs [62].

A microorganism can also protect itself by developing a niche specific resistance. The strategy in this type of mechanism is to exploit certain anatomical or physiological niches. Such niches could for example be a surrounding with increased osmotic pressure such as increased salt concentration [51]. The microorganism *P.aeruginosa* has been found to use this type of mechanism. It finds a niche environment in infected tissue, which has an abnormal salt concentration [63]. Because many AMPs often are sensitive to elevated levels of salt, AMPs can be avoided by exploiting this kind of environment [51].

2.5.3.1.2 Adaptive

As explained in the section 2.4.1 Mechanisms of Action, one of the primary mechanisms of the antimicrobial activity of AMPs is the binding of the positively charged AMPs to the often negative membrane of bacteria. Some bacteria, however, have the ability to withstand this interaction by changing their charge characteristics [2]. *S.aureus* has been shown to employ this type of resistance mechanism. Gram-positive bacteria, such as *S.aureus*, lack an outer membrane; instead a dense cell wall consisting partly of teichoic acid is forming the outer part of the bacteria (see section 2.3 Microorganism overview). The charge of the normally polyanionic teichoic acid can be altered by incorporation of positively charged D-alanine amino acids, which in turn

leads to a reduction of the negative cell wall charge. This charge reduction will lead to the repelling of AMPs before they can reach their target of action [64]. Gram-negative bacteria use a similar principle as well. In this case the outer membrane is affecting the ability of AMPs to reach their target. A modification in Lipid A in the LPS molecule reduces the overall negative charge, which in this case also leads to a decreased attachment of AMPs [65]. The above mechanisms can be observed in *Figure 9*.

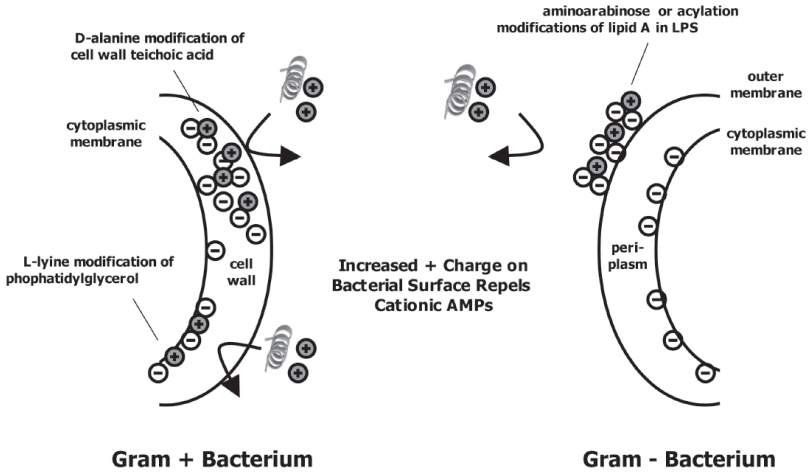

Figure 9. Descriptive picture in how Gram-positive and Gram-negative bacteria can repel AMPs by alteration in surface charge [62].

Another strategy used by some microorganisms is to counteract AMPs before reaching the cell surface. This mechanism can function in different ways. One variant is special proteases that can be produced by certain microorganism. These proteases are able to degrade the antimicrobial peptide before it reaches its target [60]. Both *E.coli* and *S.aureus* employ this type of mechanism, where it has been shown that proteases are responsible for an overall reduction in the activity of lactoferricin B. *E.coli* has for example been shown to have an increased susceptibility when the gene for a periplasmic protease was deleted [66]. Other mechanisms in which proteases do not directly degrade the AMPs have also been found. The microorganism *Pseudomonas aeruginosas* express proteases that degrade the mammalian cells surface proteoglycans [67]. A negatively charged product, called dermatan sulfate, is a result of this degradation. Dermatan sulfate will bind to the common human AMP, α-defensin, before the peptide reaches the surface of the cell. By this mechanism the charge of the AMPs is neutralized which result in the antimicrobial activity being lost [67]. It is also possible for some Group A *streptococcus* strains (GAS) to bind and inactivate AMPs by surface proteins. A surface protein called SIC has been found to inactivate the AMPs LL-37 and α-defensin [68]. The above mechanisms are described in *Figure 10*.

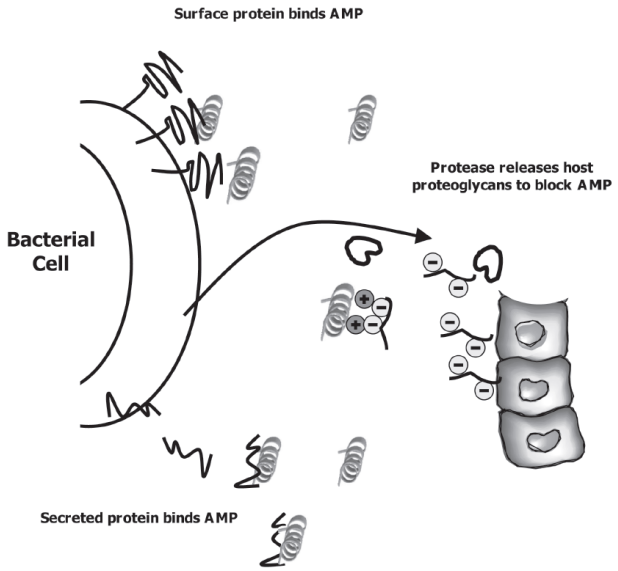

Figure 10. Descriptive picture in how bacteria can inactivate AMPs before reaching the cell surface [62].

In section 2.4.1.2 Models of intracellular killing, it was described how AMPs can sometimes also aim at intracellular targets. It has been shown that some microorganisms can use internal resistance mechanisms to inhibit AMPs with intracellular targets. Another strategy to block AMPs with intracellular targets is the use of energy driven efflux systems to expel the antimicrobial substance. Efflux systems have been associated with both Gram-positive bacterial and fungal pathogens [9] [60]. It has been shown that a type of ABC transporter is active in fungal resistance [69]. Additionally it is possible for some microorganisms to disturb or suppress the AMP response system of the host. This can be conducted by interfering with the signal cascade when a cell reacts to AMP [60]. The skin pathogen *Streptococcus pyogenes* uses this kind of mechanism to down regulate the AMP β-defensin-2, which is a part of the human innate immune system [70]. It is also believed that pathogens can be able to stimulate the mechanism of the host that counter regulates AMP production [60]. Finally, the AMP Histatine-5 can have a reduced activity against some mutants of *C.albicans* because of an adaptive mechanism where *C.albicans* can resist AMPs by lowering its metabolism [71].

2.5.4 Salt sensitivity

Another challenge in the development of AMPs as therapeutics and other applications is salt sensitivity. One group of peptides that have shown high salt sensitivity is defensins, which become inactive in the presence of high salt concentration [72]. In a study it was demonstrated how defensins in the epithelial surfaces of the lung, a high salt concentration environment, became inactive which allowed pathogens to colonize, leading to pulmonary infections [73]. Other AMPs that have shown salt sensitivity are

magainins and buforin [74]. The results from a study by Park *et al* [72] clearly showed that salt sensitivity of antimicrobial peptides is a result of the destruction of α-helical structure and thus loss of membrane binding and permeabilizing activity.

2.6 Method

2.6.1 Inhibition Zone Assay

In order to examine the antimicrobial activity, the method inhibition zone assay was used. It is a method that has been used in several publications for measuring the antimicrobial activity of peptides [75] [76]. The method is described by referring to *Figure 11*. In the method, plates are poured with a liquid mixture of growth medium, agarose and cells of the test microbe (1). The liquid gel is then solidified and small wells are punched in the gel (2). In the next step the wells are loaded with a dilution series of the test- peptide (3). The plates are then incubated overnight. During the incubation the peptides diffuses into the agarose, establishing a gradient; the further the peptide diffuses from the wells, the lower is the concentration of the peptide. At some distance from the well, the effective MIC is reached. Beyond this point the microorganisms grow, but closer to the wells, growth is absent. A zone of inhibition is created and the diameters of inhibition zones are recorded (4) also *Figure 12*. The Minimal inhibitory concentrations (MIC) can then be calculated from the zones. The MIC-value is not constant for a given agent; it varies with the test organism, the composition of the medium, the incubation time, and the conditions of the incubation such as temperature and pH [37]. When different culture conditions are standardized, however, different antimicrobial peptides can be compared to determine which is the most effective against a given organism. The protocols can be found in Appendix B– Materials and Method.

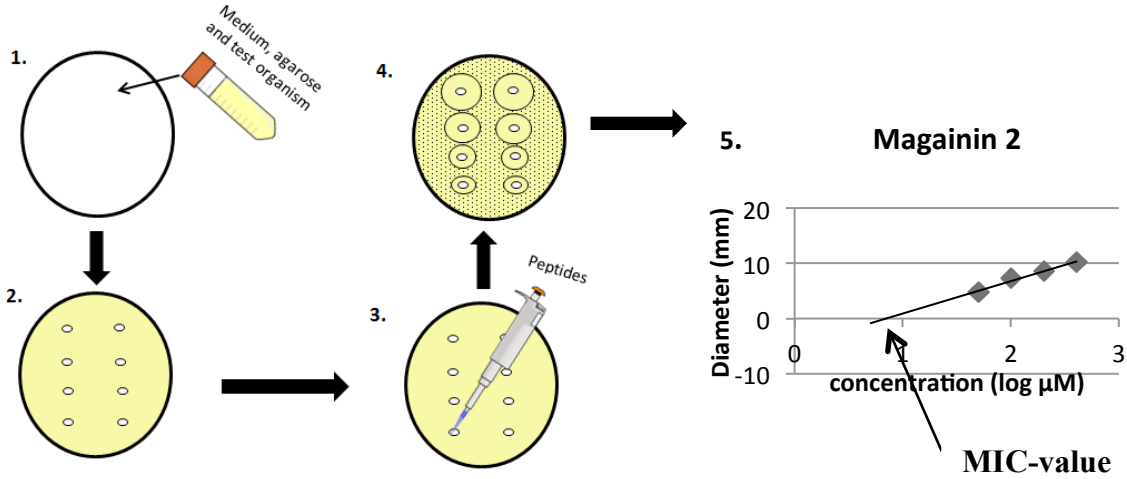

Figure 11. An illustration of the method inhibiton zone assay and a graph showing the MIC-value (see Inhibiton zone assay)

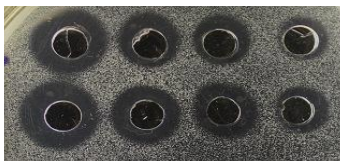

Figure 12. Example of inhibition zones of maginin-2 against C.albicans Appendix C– Laboratory Results

Calculation of MIC value

The MIC values from *Figure 11* (5) are obtained by plotting the diameter of the zone against the log concentration of the peptide. After a linear regression, the MIC is calculated through linear regression. More specifically by determining the intersection of the best-fit line with the x (concentration)-axis, the MIC is obtained.

2.7 Peptides

Below the peptides used in the experimental section are summarized. General mechanisms, classification and target microorganisms are some of the aspects that will be mentioned.

2.7.1 Nisin A

Nisin is a type A bacteriocin [20] and was first discovered in fermenting milk cultures [77]. The peptide is mainly used as a preservative in the food industry and exists in many forms [77]. Two of these forms are nisin A and nisin Z, which are produced by *Lactococcus lactis* [77]. These variants of nisin differ only by a single amino acid, which has no effect on the antimicrobial activity although it has been observed that Nisin Z has higher solubility and diffusion characteristics than nisin A [78] [77]. The commercial form of nisin is called nisaplin which consists of 2,5 % nisin A. The excess material, consisting of salt and milk solids is derived from the fermentation of a modified milk medium by a nisin producing strain of Lactococcus Lactis [21]. The Nisaplin MIC is always stated in IU/ml, IU stands for international unit and according to Sigma Aldrich 1g of Nisaplin corresponds to 10^6 IU. The MIC value of Nisaplin against *S.aureus* has been estimated in a study to 3000-5000 IU/ml [79]. The MIC value against S.aureus of pure nisin Z has been calculated to 75 nM [80].

The peptide length of nisin is 34 amino acids, which includes the two unusual compounds, lanthionine and β-methyllanthione (*Figure 13*) [58]. It therefore belongs to the peptide group of thio ether rings, also called lantibiotics [15]. The net charge of the peptide is 3+ [13]. Nisin have been shown to have two mechanisms to permeabilize membranes. In the first mechanism, nisin binds to anionic lipids on the target membrane

and then inserts between the phospholipid head groups [81]. The aggregation of nisin in the outer lipid monolayer is followed by formation of short-lived pores. The second mechanism is more efficient and is based on the binding of nisin to Lipid-II in the membrane followed by assembly and pore formation [59]. Lipid II is a peptidoglycan precursor and if inhibited it will disturb the cell wall biosynthesis. If nisin can interact with Lipid II in the target membrane, it would follow the Lipid-II pathway for pore formation and not the anionic-lipid pathway. This is because the affinity of nisin for Lipid II is much higher than the affinity of nisin for membranes containing anionic lipids [81] [59].

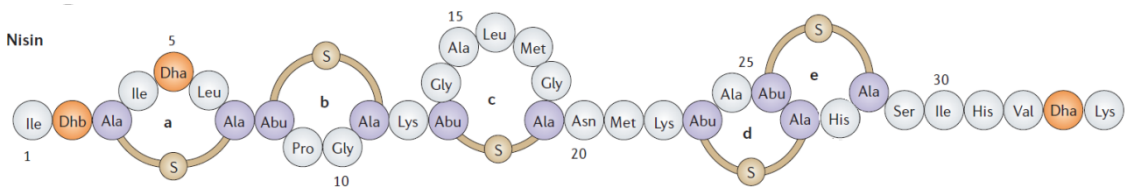

Figure 13. Image of nisin A, where the lanthionine rings are labelled a-e. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Drug Discovery] [59] *copyright (2006).*

Nisin shows activity against a wide range of gram-positive bacteria and has shown little or no activity against gram-negative bacteria [21]. The explanation for this is the LPS composition of the outer membrane of the gram-negative bacteria, which act as a barrier against the action of nisin on the cytoplasmic membrane. It has been shown that addition of chelating agents, such as EDTA, destabilizes this layer and makes it possible for nisin to form pores on the cytoplasmic membrane of gram negative bacteria [78]. It has also been observed that purified nisin Z (not the commercial variant, nisaplin) is active against *E.coli*. [80]

As with many other peptides the antimicrobial activity of nisin is pH dependent. Nisin is most active and stable at pH values less than 5. Nisin considerably loses its activity when it reaches a pH exceeding 7 [82]. It has also been observed that the activity against *E.coli* is salt sensitive while the activity against *S.aureus* is not [80]. The commercial product nisaplin has a relatively high stability. As an example, if stored under dark and dry conditions below 25°C it will show no loss of activity in a period of two years [83]. It has been shown that the stability of nisin used in food systems is affected by three parameters: Incubation temperature, storage time and pH [83]. Nisin has been demonstrated to have a low toxicity towards human cells, if any [83] [84].

Despite the fact that nisin has been used for a long time in the food industry no reports of nisin resistance have yet appeared. It is believed that the dual mechanism of nisin could be an explanation to this fact. It has however in laboratory settings been observed that it is possible to induce nisin resistance in strains [85]. The same study has

demonstrated that there is no difference in the lipid II content of nisin resistant strains of Listeria monocytogenes [85]. The explanation could be that lipid II has a very important function in the cell wall synthesis. Instead nisin resistant strains are thought to reduce the accessibility of lipid II. This can be done by incorporating positive charges in the cell wall which will expel the positively charge nisin from the cell and hence prevent it from reaching lipid II. The main mechanism behind this strategy is believed to be the decrease of the negative charge of the cell wall by the incorporation of D-alanine to teichoic acid. This mechanism has been described in more detail in section 2.5.3.1 Mechanism behind resistance [59].

2.7.2 Magainin 2

AMPs became commercially interesting with the discovery of magainins by Zasloff in 1987 [2]. The name magainin is derived from Hebrew for shield [86], and they are one of the most studied α-helical AMP groups (*Figure 14*) [15]. The magainins involve two 23-amino acid peptides, magainin 1 and 2, which are found on the skin of the African clawed frog, *Xenopus laevis* [87] [88]. Magainins have shown good antimicrobial activity against Gram-negative and-positive bacteria, fungi and protozoa [89] [87]. The MIC value for different microorganisms is typically in the range of 10-100µg/ml [89]. More than 1mg/ml is needed to lyse mammalian cells, thus making magainins selectively toxic against microorganism [87]. Magainins have a net charge up to +4 and binds to negatively charged lipid membranes by electrostatic interactions [90]. Magainin kills microbes through the toroidal pore mechanism [6] (see section 2.4.1 Mechanisms of Action).

Figure 14. The α-helix structure of magainin 2 [91].

Magainins have poorly defined secondary structure in aqueous solutions at neutral pH, but the peptides essentially assume α-helical structures (helicity 60-90%) upon binding to acidic phospholipid bilayers, making them antimicrobial active [87]. Studies have suggested that magainins are sensitive to salt, which destabilizes the helical structure [72] [92]. Lee et al. [92] demonstrated how the antimicrobial activity of magainin-1 against *E.coli* and L. monocytogenes was reduced as the sodium chloride (NaCl) concentration in the antimicrobial assay was increased. In another study the pH-sensitivity was tested, and it was concluded that magainin 2 displayed enhanced in vitro activities against *E.coli* and *P.aeruginosa* at relatively low pH-values [93].

2.7.3 Lactoferricin B

Lactoferricin B is a 25-residue peptide and is released by pepsin cleavage of bovine lactoferrin and has a net charge of 8+ [13]. The human form of lactoferrin is found in different fluids of the body including breast milk [23]. It has been suggested that the human form of lactoferricin, lactoferricin H, can be formed in the stomach of infants where the protein lactoferrin is hydrolysed to lactoferricin H [23]. Lactoferricin B is active against a wide range of gram positive and gram-negative bacteria as well as fungi [94] and belongs to the group of β-sheet structured AMPs (*Figure 15*) [18]. The MIC value for lactoferricin B against fungal species ranges from 3-45 µg/ml; the MIC values for C.albicans ranges from 18-45 µg/ml [17]. It has been shown that its effect against C.albicans can be diminished when Mg2+ and Ca2+ ions are present. Moreover it has been suggested that the highest anticandidal activity is achieved near a pH of 6. [95] One of the proposed mechanisms is the carpet mechanism (see section 2.4.1.1.2 Carpet model) but the main mechanism is believed to be intracellular where the peptide could interfere with cytosolic and nuclear components [17]. Additionally, lactoferricin can also be able to up-regulate the production of reactive oxygen species (ROS) inside the cell [17]. It is also believed that the peptide can inhibit macromolecular synthesis in both gram-negative and gram-positive bacteria [96].

Figure 15. The β-sheet structure of Lactoferricin B [97].

2.7.4 Buforin –II

Buforins are α-helical AMPs isolated from the stomach tissue of the Asian toad *Bufo bufo gargarizan* and they display a broad spectrum of antimicrobial activity against bacteria and fungi [98]. Buforin-II has documented MIC values against microorganisms ranging between 0.1-70 µg/ml [99]. The 21 amino acid Buforin-II is derived from the less potent AMP Buforin I (39 amino acid peptide) [100]. Buforin-II has a net charge of +6 and structure analysis revealed that it has a helix-hinge-helix-structure (*Figure 16*) [13]. The hinge is a proline residue at amino acid position 11 that separates the two helices [101]

Figure 16. The structure of buforin-II. The amino acid residues are colored by the following: positively charged residues, red; other hydrophilic residues, blue; proline, white; other hydrophobic residues, yellow [100].

Buforin II is an interesting example of an α-helical AMP since its mechanism of action does not involve membrane perturbation [101]. Studies have shown that buforin II crosses lipid bilayers in a manner similar to that of magainin 2, via formation of a toroidal pore (see section: mechanism of action) [102]. However, Buforin II only penetrates the membrane, it does not permeabilize it [100]. The key in this property is the proline hinge [102]. Experiments showed that only a single amino acid substitution at the Pro11 position changes buforin II into a membrane-active magainin-like peptide [100]. Also insertion of a proline hinge region into the helix of magainin 2 switches this AMP from a membrane-permeabilizing peptide to a cell-penetrating one [100]. It has been shown that buforin II bind nucleic acids in vitro, which has suggested that buforin II kills bacteria by interacting with their nucleic acids after translocation across the cell membrane [101]. In a patent it was demonstrated that both buforin-II and magainin 2 were clearly sensitive to salt concentrations, as the MIC-value was significantly increased with higher NaCl concentration [74].

2.7.5 Histatin-5

Histatins are a small group of histidine-rich cationic peptides consisting of 7 to 38 amino-acid residues [57]. They can be found in the saliva in man and higher primates [24] [57]. The most common histatins include histatin 1, 3 and 5, which contain 38, 32 and 24-amino acids respectively, with the 22 first amino acids being identical [57]. Histatins 1 and 3 are products of different genes while histatin 5 is a cleavage product of histatin 3 [24]. Histatin-5 has a net charge of +5 and is most well-known for its antifungal activities, but has also showed some antibacterial activity [17] [57] [13]. The oral cavity is susceptible to a range of bacterial and fungal infections and it had been suggested that histatins have evolved to control infection in this region. Histatin-5 is most active against the pathogenic yeast *C.albicans* [57].

In aqueous trifluoroethanol, histatin-5 adopts a weakly amphipathic α-helix, which is thought to associate with receptors on the fungal membrane and then enter the cytoplasm [6] [17]. However, the main antifungal mechanism of action is still not fully understood. It has been shown that Histatin-5 causes cellular leakage of ATP and disruption of membrane, also the peptide interacts with cytosolic components, such as

the mitochondria [57]. The ATP released from the cell can act as a signal for extracellular receptors, which activates a pathway leading to apoptosis [17]. Recent work has shown that histatin-5 binds to a specific protein located in the fungal cell envelope, called heat shock protein Ssa2p. This protein is absent in mammalian cells, and might explain the peptides low mammalian cell toxicity [57].

Synthetic fragments and modification of histatin-5 have been produced, one of these is the peptide p-113, which is a peptide consisting of 12- amino acids. P-113 is more potent against *C.albicans* then Histatin-5 and is considered safe for topical applications [98]. Currently p-113 is an active ingredient in a mouthwash [24]. A challenge to the further development of histatin-5 is the high sensitivity of its antimicrobial action to the presence of small concentrations of salt [17].

3 Applications, Current research and Commercialization

In this section, initially a general view on potential problem areas associated with hygiene products will be presented. There will also be a part introducing how antimicrobial peptides can be used to address these problems. SCA is a leading hygiene company that has a broad product range. Products such as panty liners, diapers, skin-lotions, wet-wipes and tissues are included in their catalogue. Some of these products are associated with potential negative health effects associated with pathogens, such as higher risk for genital infections and dermatitis. AMP-systems such as bacteriocin producing probiotics, AMP-inducers such as vitamin D and AMP-materials are suggested as potential solutions to some of the product-related problems. After the applications section current research and companies associated with AMPs will be presented.

3.1 Conditions and Potential Applications

There are several product related issues. Some are associated with feminine hygiene products, such as panty liners and diapers. These are worn in close contact with the genital areas, and have been suggested to affect these surfaces, through increased dryness, wetness and occlusion. This could lead to changes in the normal physiology of the skin or to changes in the microbial flora, which might increase the risk for infections [103] [104] [105]. Further, other issues may be to keep surfaces and skin clean and hygienic.

3.1.1 Conditions

As been highlighted through the background section, AMPs have the ability to kill and inhibit a wide range of microorganisms. The product related conditions described below, are therefore all related to pathogenic microorganisms.

Vulvo-vaginal candiasis is a relatively common irritation of the genital area. Some authors have suggested that panty liners might trap heat and moisture against the skin, thus possibly increase the risk of infections such as Vulvo-vaginal candidiasis (VVC) [106]; it is believed that modern panty liners with a breathable backsheet do not increase risks. *C.albicans* are the major species associated with VVC and is responsible for approximately 80% of the cases [107]. This statistic is probably the reason why most research is focused on the control of *C albicans* for treating and preventing VVC [107]. It has been suspected that the use of panty liner also can increase the risk of urinary tract infections (UTI) by aiding the transfer of intestinal microbes from the perianal region [106]. The cause of UTI is due to colonization of the vagina and the periurethtral area by pathogens including *E.coli*. The colonization of *E.coli* is characterized by replacement of lactobacilli (see section 3.1.2.1 Probiotics and Urogenital health), which normally maintains the healthy vaginal environment [108]. Another genital infection that could be related to feminine care is bacterial vaginosis, which is associated with disturbance in the vaginal micro flora. The major characteristics are decrease in lactobacilli and an increase of *Gardnerella vaginalis* [107]. In addition BV is associated with a relatively high pH, and a decrease in antimicrobial activity of the vaginal fluid compared to healthy women [19]. With this in mind, maintenance of the dominant bacteria in normal vaginal micro flora, *Lactobacillus*, at a high level is important for prevention of bacterial vaginosis [109]. Dermatitis in the genital area, called diaper dermatitis, is the most common skin disorder of infants. Diaper dermatitis is a condition triggered by irritants affecting the area covered by the diaper [104]. Incontinence among adults may also increase the risk of dermatitis, called incontinence associated dermatitis [105]. The key irritants of the conditions are more or less the same. Moisture such as urine and faeces as well as faecal enzymes are all key irritants. Increased pH of the skin, which can be associated with diaper wearing, particularly non-breathable products, can also increase the activity of the irritant enzymes [104]. Children suffering from diaper dermatitis have a higher risk of infections from pathogens such as *C.albicans* and *S.aureus* [104]. C.*albicans* infections are also common in the case of incontinence dermatitis [105]. One of the precautions for the avoidance diaper dermatitis is cleaning the diaper area with for instance a commercial baby wipe [104].

3.1.2 Potential Applications

AMP-systems such as bacteriocin producing probiotics, AMP-inducers such as vitamin D and AMP-materials are suggested as potential solutions to some of the product-related problems.

3.1.2.1 Probiotics and Urogenital health

SCA and other leading companies are currently researching probiotic bacteria for use in applications such as feminine care applications [110]. One promising probiotic for this purpose is the group of lactic acid bacteria (LAB). In this section a group of AMPs (bacteriocins) produced by LAB will be highlighted for applications in feminine hygiene applications.

3.1.2.1.1 Lactic acid Bacteria and Bacteriocins

The Food and Agriculture Organization of the United Nations and World Health Organization have defined Probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [111]. Examples of such microorganisms are *Lactobacillus, Bifidobacterium* and *Saccharomyces* [107].

Lactobacilli are members of the LAB and are gram-positive bacteria and prefer acidic environments [112]. In general, lactobacilli have not been associated with diseases and have been regarded as non-pathogenic members of the intestinal and urogenital floras [112]. There is a high presence of *Lactobacillus* species in the urogenital micro flora, which plays an important role in reducing the risk of conditions such as bacterial vaginosis (BV), urinary tract infections (UTI) and Vulvo-vaginal candidiasis [19] [112]. *Lactobacillus species* produce organic acids, hydrogen peroxides and bacteriocins, which are major elements found in concentrations that are high enough to be microbicidal [113]. Besides the influence of these compounds, several systems and mechanisms are believed to collaborate against potential pathogenic exogenous microbes [107]. For example, vaginal fluid has a selective antimicrobial activity towards potential pathogenic microbes. Furthermore, the acidic milieu contributes to a stable and specific flora [113] [107]. In addition the epithelial surface of the urogenital tract secretes several other AMPs [114].

Bacteriocins are a group of AMPs and amongst the more important antimicrobial substances that protect the urogenital tract from infections [19]. The bacteriocins produced by gram-positive bacteria are similar to the AMPs produced by eukaryotes [7]. Some of the general bacteriocins properties are cationic, amphiphilic and membrane permeabilizing. Bacteriocins produced by LAB have long been used in the preservation of meat and milk. Four main groups of LAB antibiotics have been identified; however the main focus will be on the potential applications of Class I lantibiotics, since it is the most well studied [7].

3.1.2.1.2 Probiotics against genital infections

As been mentioned UTI, BV and VVC are three conditions associated with feminine hygiene products. In the following section it is presented how probiotics and AMPs can be used to prevent or treat these conditions.

3.1.2.1.2.1 Urinary tract infection

Studies have demonstrated that Lactobacilli inhibit the growth and attachment of uropathogenic *E.coli* in vitro [19]. It was shown by Reid et al. [112] that weekly administration of L. rhamnosus GR-1 and L. fermentum B-54 as a vaginal suppository decreased the incidence of urinary tract infection. Probiotic colonization in the vagina might prevent infection by competition for nutrients and mannose and hydrophobic adhesion to receptors and possibly bacteriocin production [19]. It is also accepted that women who are colonized by hydrogen peroxide producing lactobacilli are less prevalent with *E.coli* and thus less prone to UTI [107]. Other studies have demonstrated the presences of other AMPs besides bacteriocins are important in bacterial clearance of the urinary tract [**111**] [114]. In one study it was shown that the cathelicidins LL-37/hCAP-18 are expressed in epithelial cells in the human urinary tract [115]. During infection, epithelial cells rapidly increase the production of cathelicidin peptide to protect the urinary tract from bacterial invasion [115]. Apart from cathelicidins also antimicrobial compounds such as defensins and lactoferrin have shown to be secreted by the genital mucosal epithelia [114].

3.1.2.1.2.2 Bacterial vaginosis

Even though studies have found that hygienic behaviours, such as type of underwear and panty liners used, are less likely to be a direct cause of vaginal symptoms [116], BV is still an interesting condition that could potentially be treated with bacteriocins [19]. Studies of alternative treatments of BV, which include the oral or vaginal introduction of *Lactobacillus* or by buffered vaginal gels with lactate, have been carried out [19]. One of the placebo-controlled studies demonstrated a 37% cure rate by oral ingestion of *L. fermentum* and *L. rhamnosus* [117]. While 60% of BV cases can be successfully treated with antibiotics, about 20% of these cases return with highly developed antibiotic resistance [19]. Also some studies show that antibiotics even inhibit healthy vaginal *Lactobacillus* at concentrations lower than doses topically applied for treatment [19]. The risk of developing antimicrobial drug resistance increases dramatically with increased use of antimicrobial preparations in applications such as, feminine hygiene or perhaps tissue products [118] (Appendix A.2 Interview with Peter Bergman). Therefore, there is an interest in finding alternative treatments against BV, such as antimicrobial peptides that will inhibit BV-associated bacteria without killing healthy *Lactobacillus*. One such alternative that has been suggested is the bacteriocin subtilosin A [19]. In contrast to many bacteriocins, which have an overall positive charge at physiological

pH, subtilosin A is anionic. Because bacterial membranes also have a net anionic charge, it has been hypothesized that subtilosin may not interact solely with the cell membrane, but may first bind a surface receptor prior to insertion into target membranes [119]

3.1.2.1.2.3 Vulvo-vaginal candidiasis

Candida is found in a relatively high frequency (~20%) of vaginal exudates from healthy and asymptomatic women of reproductive age [107]. Thus, *Candida* can be a vaginal commensal as well as a pathogen. *Candida albicans* is the major species associated with VVC [107]. The importance of *Lactobacillus* in the management of vaginal *C. albicans* is not fully understood. It is suggested that specific *Lactobacillus* strains produce metabolites that are toxic to *C. albicans* [107]. However, there is a lack of large clinical studies that investigate the significance of the *Lactobacillus* flora or possible treatment [107].

3.1.2.2 AMPs in materials

In this section a brief orientation will be given about the use of AMPs in solid materials. The AMPs can be attached to a material covalently, thus being immobilized, or dissolved inside of the material. Both solutions will be described beginning with covalently immobilized AMPs.

3.1.2.2.1 Covalent immobilization of AMPs

The immobilization of AMPs could fill a need in the prevention of microbial colonization of biomaterials and thus create a new generation of antimicrobial surfaces. It has been shown that it is possible to covalently attach AMPs to a surface and that the attached peptides, in many cases, retain their activity and can even acquires an increased stability and decreased toxicity [120]. There are different strategies available for the surface attachment of AMPs. Such strategies may involve variations in the surface material, polymer (spacer) or surface concentration of AMPs used [120]. The concept of surface attachment is exemplified in *Figure **17*** The AMP is bound to a polymer but can in some case bind directly to the surface material. The chemistry behind the linkage of AMPs to a surface is out of the scope of this report, and will not be explained [120].

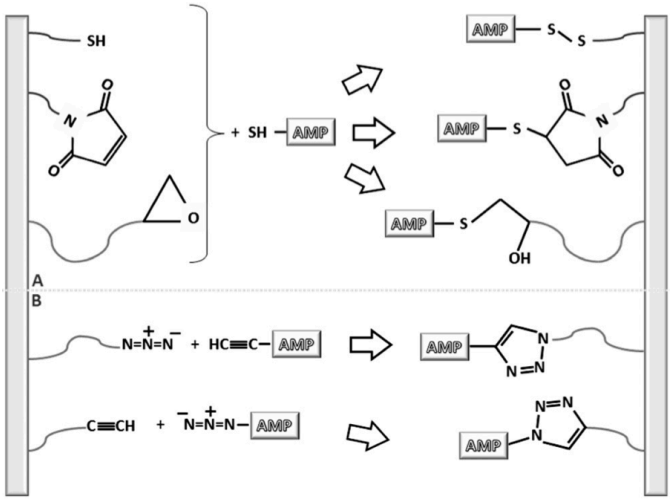

Figure 17 Picture demonstrating chemical strategies of covalent immobilization of AMPs. Reprinted from **[120]** *with permission from Elsevier (2011)*

The variation of parameters can affect whether the AMPs will retain their antimicrobial activity after the attachment to a surface. It has been demonstrated that peptides that are not able to retain their secondary structure lose their activity after the attachment; this applies both to α-helical and β-sheet structures [121]. Other parameters that can affect the activity are length, flexibility, type of polymer (spacer) and the solid material used [120].

AMPs have been assessed at different types of solid supports such as resins, metals and glass [**116**]. The AMP peptides can be either synthesized directly on the surface or be pre-synthesized. The peptides can be attached to a flexible polymer such as PEG (polyethylene glycol) [120]. It has been shown that an increased length in the polymeric chains will increase the antimicrobial activity, which is explained by the increased flexibility that comes with the length [122].

The surface concentration of the peptide has proven not to be as an important factor for the antimicrobial activity [120]. Immobilized AMPs have in many cases been shown to possess a bacteriostatic rather than a bactericidal effect [123]. A lower peptide concentration has, however, shown to give rise to even more of a bacteriostatic effect. This is explained by the fewer peptides able to enter the membrane [120]. Bactericidal effect has been demonstrated on Magainin 2 when attached to a solid surface of polyamide resin [121].

The orientation of the peptide is another factor, which also may affect the antimicrobial activity. A different activity can be achieved depending on how the peptide is attached to the polymer. It has been shown the C-terminal attachment of the peptides gives a higher MIC value [122]. Additionally, it has been suggested that hydrophobic residues

close to the N-terminus as well as cationic residues close to the linker site are important for the activity of the immobilized AMP [124].

It has further been demonstrated that immobilized peptides, in general, give higher MIC values compared to soluble peptides. MIC values have shown to increase 100 fold depending on bacteria [125]. However, as previously mentioned, the cytotoxicity of the peptides decreases with immobilization which are based on results that demonstrates a decrease in hemolytic activity of immobilized peptides compared to soluble peptides [124].

Finally, various studies have examined the stability of immobilized AMPs [120]. A study evaluating how tolerant immobilized AMPs are to washing has shown that AMP attached to resin beads can be washed extensively without loss of antimicrobial activity [121]. In addition some peptides have been tested for their heat stability. The AMP of melimine was shown to maintain its antimicrobial activity during autoclaving [126]. The AMP 6K8L that was immobilized on a PEG-polystyrene resin beads, used on contact lenses, which have been demonstrated to have high pH stability in the pH interval of 3,5-7 [127]. Furthermore it has been shown that AMPs remain active under long time storage up to a period of 6 months, although some of its activity were lost [123]. An additional example of covalently immobilized AMPs, are nisin-coated films, which have the potential to be used in the food industry where it can inhibit bacterial growth and extend the shelf life of foods [128].

3.1.2.2.2 AMPs in Wet wipes

Another application area of AMPs is the use wet wipes. A wet wipe is defined as a small-moistened piece of paper or cloth that is used for cleaning purposes. To demonstrate the use of AMPs in materials such as wet-wipes, a patent with the title "moist bacteriocin disinfectants wipes and methods of using the same [129]" is described.

The patent refers to how dissolved bacteriocins such as nisin can be absorbed into a wipe, how properties such as stability may be improved and possible application areas of the invention. Application areas are stated as disinfection of hands, skin, food lines, hospital surfaces and the prevention of mastitis (a common bovine disease). According to the patent, by adding bacteriocins, the alcohol component of the wipe can be drastically lowered since the alcohol is only needed as a drying component and not as a germicidal component. Other germicidal components may be lowered as well. Different bacteriocins can be used alone, or in combination. It is however stated that nisin is the bacteriocin preferred. One problem when using bacteriocins on a paper or cloth wipe is the process of the release of the bacteriocin from the wipe. A component, such as NaCl, that loosens the binding between the bacteriocin and the surface of the wipe can therefore be added [129]. A chelating agent such as EDTA may also contribute to an

enhanced potency and that a broader range of microorganisms are affected [11]. Moreover a combination of EDTA and citric acid or combinations of methionine and NaCl can enhance the stability of the bacteriocin. The enzyme catalase has also shown to increase the stability [129].

Wipes with bacteriocins incorporated in the wet wipe are currently available in the market. ImmuCell (Portland, USA) is a biotechnology company that develops, manufactures and sells products as to improve animal health and productivity in the dairy and beef industries. Their products focus on bovine diseases such as mastitis. Mastitis is caused by an infection of the cow teat skin and the pathogens *Staphylococcus* and *Streptococcus agalactiae* are responsible for the infection. A product named WipeOut® with the AMP nisin incorporated is sold by the company for the sterilization of the cow teat skin in the milking industry [10]. Another company called Sedna health products and nutritional supplements (Hendersonville NC, USA) also sell antimicrobial wipes containing nisin where another antibacterial ingredient called monolaurin also is added. Mentioned application areas are kitchen and bathroom hygiene as well as hand hygiene. Apart from nisin and monolaurin, other ingredients in the product are Tween 20, EDTA Na2, citric acid, DL-methionine and catalase [130].

3.2 Current research

The following section will cover current research topics by referring to researchers that have been interviewed (Appendix A- Interviews). Also there will be a part covering other interesting research areas.

3.2.1 Experts comments about current research

Antimicrobial peptide research and development has mainly two directions as PhD/MD Peter Bergman points out. One direction is to create prototypes or analogues of naturally occurring peptides, which could then be synthetized and used as pharmaceuticals. However, it is complicated and costly to synthesize these AMPs. Another direction is to induce the peptide production in the body, through certain substances. This is the area, which Bergman has been working with mostly (Appendix A.2 Interview with Peter Bergman). Bergman was active in the same research group as Agerberth, whom is still active in the field and in the approach of inducing AMPs. Agerberth is a professor in medicinal microbial pathogenesis, which studies how microbes, primarily bacteria, cause disease. More precisely the research revolves around the induction of human antimicrobial peptide LL-37 and how it functions and is regulated. The current focus is on the control of *shigella*, which can cause diarrhoea. In fact, Agerberth was one of the people behind the discovery of the human AMP LL-37.

Three inducers that are mentioned are Butyrate, Vitamin D and litocholic acid (Appendix A.1 Interview with Birgitta Agerberth).

According to Bergman the development of using peptides as drugs have reached a high level and it is even possible to dissect which parts of the peptide that is immunomodulatory and antimicrobial. This knowledge makes it possible to design peptides that are either immunomodulatory or antimicrobial or both (Appendix A.2 Interview with Peter Bergman). Professors Martin Malmsten believes that adding AMPs is the simple and straightforward way, compared to inducing the peptides with other substances. Malmsten and the research group at Uppsala University mainly investigate biophysical properties of AMPs. With their research they have been able to see effects of single amino acid modifications to further improve efficiency and selectivity between bacterial and eukaryotic cells. Malmstens research has been published in high profile journals, which has resulted in a number of patent applications, and in the development of some of these peptides towards therapeutic applications through two start-up companies. Past research have led to a good understanding of peptide design, selectivity, and how to get low toxicity and keep the peptide activity. At the moment much research is focusing on the resistance issue. Even though the peptides are not so prone for resistance, the discussion should be more detailed and more research is needed in this area. Malmsten mentions that peptides could be designed with different stabilities, such that it takes a couple of generations for the bacteria to develop resistance (Appendix A.3 Interview with Martin Malmsten).

3.2.2 Examples of research areas

There is on-going research on how to solve some of the challenges involving AMPs such as salt sensitivity and specificity. Some of these areas of research will be presented below.

3.2.2.1 Helix-capping motifs

As been mentioned salt-sensitive peptides is an obstacle for the development of AMPs. A study by Park *et al.* [72] demonstrated how to overcome this problem and develop salt-resistant AMP. In the study it was suggested that loss in helical content was a reason to lowered antimicrobial activity at higher salt concentrations. In order to stabilize the helix, helix-capping motif was incorporated at or near the ends of helices in peptides and provided the necessary intermolecular interactions to stabilize the α-helices. As an example, helix-capping motifs were introduced into magainin 2, a salt-sensitive antimicrobial peptide, to test if it was possible to increase the salt-resistance. The resulting peptide, *N*-Mag-*C*, maintained activity and structural stability at the salt concentrations tested compared to magainin 2. Thus, it is possible salt-resistant

analogues can be derived from a salt-sensitive antimicrobial peptide by the application of helix-capping motifs [72].

3.2.2.2 End Tags

Hydrophobic oligopeptide End Tags is a novel approach of improving the antimicrobial peptides. End tagged peptides have been shown to possess an increased antimicrobial activity which increases with tag length. Tagged peptides have also been shown to hold a lower toxicity against mammalian cells. Additionally, end tagging can increase the potency of the peptide also at high concentration of salt as well as increasing the potency against bacteria of low electrostatic charge. A variety of hydrophobic end tags can be used [45]. Malmsten also mentions in his interview, that end tagging is a way of increasing the selectivity and thus decreasing the toxicity (Appendix A.3 Interview with Martin Malmsten).

3.2.2.3 Genetically engineering of bacteriocins and probiotics

There is on-going research on improving the potency of bacteriocins as antimicrobial agents. Genetically engineering is growing in this area, and it is now possible to modify the bacteriocins and their producing hosts. One improvement that could be achieved is enlargement of the killing spectrum, as the bacteriocins often have a narrow spectrum. Also, bacteriocins can be used as probiotics, with the host designed to produce the specifically required bacteriocins [7].

3.2.2.4 Synergy effects

A variety of AMPs are present in the body and it is believed that this variety is due to the advantage of synergy effects between peptides. Studies have shown that even when AMPs from widely different sources have been combined, synergy effects have been observed [131]. In an experiment, a variety of human AMPs were tested to evaluate the synergy effects by combining peptides with the aim of observing any increase in the antimicrobial activity. The study showed that synergy effects, in many cases, could be detected depending on microorganism and peptide combination studied [132]. Even synergy between AMPs and small molecule antibiotics has been observed. As an example, AMPs can help antibiotics to regain their antimicrobial effect by the blockage of efflux pumps in gram-negative antibiotic resistant strains. Finally, synergy effects can be an approach in the limitation of resistance development [131].

3.2.3 Active AMP related companies and products

In this section active companies in the field of AMPs will be presented. Also some products that have hit the market will be highlighted.

3.2.3.1 DermaGen (Lund, Sweden)

A company that Malmsten founded is DermaGen, which is a company focusing on AMPs in topical substances for use against atopic dermatitis and external otitis. Through DermaGen one peptide, DPK-060, has successfully undergone Phase I/IIa clinical trial. This peptide was for use against atopic dermatitis, and recently a phase 2 study has been initiated also against external otitis. Atopic dermatitis and external otitis are two conditions of high interest since they are both growing problems worldwide (Appendix A.3 Interview with Martin Malmsten). Unlike other conventional antibiotics, DPK-060 has a low potential to induce resistance, and effectively kills MRSA (Methicillin-resistant *Staphylococcus aureus*) [133]. Atopic dermatitis is a common condition during the winter half year, and the current treatments are antibiotics and steroids. Malmsten pointed out that it is not good to use antibiotics and steroids for such a long period. AMPs against atopic dermatitis are of interest since the patients have a lower production of AMPs, and therefore it is appropriate to add AMPs to these areas. Pergamum is the current owner of DermaGen, which is a company focusing on AMPs for topical applications (Appendix A.3 Interview with Martin Malmsten).

3.2.3.2 Akthelia (Reykjavík, Iceland)

Akthelia is a company that develops new products for the treatment of a broad range of infections. However, none of their products have yet reached the market. The CEO of the company Sigridur Olafsdottir points out that their products comprise small, organic molecules that stimulate and restore the expression of AMPs on epithelial surfaces and in phagocytic blood cells. The company have shown that stimulating the expression of AMPs can clear infections in the GI tract and other organs in animal models (Appendix A.4 Interview with Sigridur Olafsdottir). Akthelia have demonstrated that rabbits recovered rapidly from life-threatening gastro-intestinal infection when treated with a selected active substance, AKT10081, while animals in the placebo control group remained morbid or died [134]. Olafsdottir points out that Atkhelia's anti-infective treatments are fundamentally different from conventional treatments in that they induce multiple innate antimicrobial peptides that work via multiple mechanisms rendering it impossible for the microbes to develop resistance. Antibiotics, which are currently used for the same problems, are normally narrow spectrum, and also microbes have developed resistance to all known antibiotics (Appendix A.4 Interview with Sigridur Olafsdottir).

There is a growing interest in Atkhelia's concept, which is reflected in an increase in the reference in the scientific literature to their therapeutic concept (Appendix A.4 Interview with Sigridur Olafsdottir). Akthelia currently have no human efficacy data, therefore investors are hesitant to participate in the project at the moment. However it is investors and grants from public funds to the academic founders that have allowed the company to advance. A current challenge includes seeking further funding for advancement of Atkhelia's clinical programme. Since their concept is entirely research based clinical research is necessary for the advancement of their products (Appendix A.4 Interview with Sigridur Olafsdottir).

3.2.3.3 CytaCoat (Stockholm, Sweden)

Agerberth is active in the company CytaCoat which focus on antimicrobial surfaces. The main idea of the company was to attach AMPs to surfaces and thereby create antimicrobial surfaces. However, Agerberth pointed out that their studies showed that the control polymer (CytaCoat ligand technology) used in the experiments was found to be more active. Therefore their research is now on developing this concept. Potential application areas are surface of a catheter. This solution has been granted a patent. Birgitta has also thought about other application areas for this type of coating. (Appendix A.1 Interview with Birgitta Agerberth).

3.2.3.4 Dipexium Pharmaceuticals, LLC (White Plains NY, USA)

Magainin pharmaceuticals Inc. developed pexiganan, which was the first antimicrobial peptide to undergo commercial development. Pexiganan, a synthetic 22-amino-acid analogue of magainin 2, demonstrated excellent in vitro broad-spectrum activity against many bacterial clinical isolates. Another important aspect is that resistant mutants were not generated following repeated passage with sub-inhibitory concentrations. Pexiganan is formulated as a topical cream and is believed to treat patients with mild diabetic foot infection [135]. However the development of the product was hindered when FDA approval was denied due to the fact that pexiganan was not more effective that other antibiotics used to treat foot ulcers. Later the rights of Pexiganan were sold to the current developer Dipexium Pharmaceuticals, LLC [136] [137]. The FDA requested one additional well-controlled clinical trial to gain approval. Dipexium believes that the potential peak year sales are hundreds of millions of dollars in the U.S. and separately in the EU [136].

3.2.3.5 Demegen (Pittsburgh PA, USA)

Demegen is a company that sells a product called P113, which is an antimicrobial peptide based on histatins. It has been shown to have an excellent in vitro activity against *C.albicans* and common gram positive and gram-negative pathogens [135]. A Demegen licensee is developing P113 in a rinse formulation for the treatment and prevention of oral candidiasis. The product under development is in the form of an

alcohol-free mouth rinse. The benefits of this product compared to other treatments are its demonstrated safety in humans, which is related to its natural origin, and the reduced risk of drug resistance due to P113's unique mechanism of action. P113 is protected with composition of matter and use patents in the USA and abroad [138].

3.2.3.6 ImmuCell (Portland ME, USA)

ImmuCell, which have been mention in previous section, is a biotechnology company that is developing, manufacturing and selling products that improve animal health and productivity in the dairy and beef industries. Their product focuses on prevention, diagnosis and treatment of economically important bovine diseases. The use of the AMP Nisin to clean and sanitize the teat area before and after milking has been proven to reduce the incidence of mastitis in cows. The Nisin-wipe product Wipe Out® has been shown to control Mastitis by killing the two main pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* [10].

4 Results and Discussion

In this section initially a brief motivation of how the peptides were selected will be presented. This is followed by an analysis and discussion of the results and method from the practical part. Finally, the potential of AMPs is discussed based on possible hygiene applications and challenges.

4.1 Peptides

The peptides for the experimental part of this thesis were chosen based on different criteria, which were determined after a primary literature study. The aim was to obtain basic understanding of the field and estimate what criteria that are interesting for the selection of the peptides. The following criteria were found to be relevant: Mechanism of action, classification, availability, cost, microorganism specificity and relevance in hygiene related applications. A secondary literature study was then carried out with the aim of finding peptides that matched the above criteria. Each peptide is motivated below mostly based on specificity and application areas. Mechanisms of action and classification of each peptide are explained in the background section.

In many publications nisin shows a high antimicrobial activity against gram-positive bacteria [59] [139]. This is an interesting property in the targeting of common skin pathogens such as *S.aureus*, which could be an issue in products that increase risk for rashes such as diapers and panty liners. Nisin is produced in an industrial scale and is commonly used as a preservative in the food industry [78]. Therefore it was thought that nisin could be a cheap AMP and thus be suitable for the use in hygiene products. The

commercial form of nisin (Nisaplin) was used in the experiments. Nisaplin was ordered from Sigma Aldrich and the price was significantly lower than the rest of the peptides. The low price made the peptide a good candidate for the initial laboratory experiments. The peptide buforin-II has been shown to be potent against several bacteria and fungi [99]. The peptide was hypothesized to be a good candidate for broad-spectrum applications such as wipes and antimicrobial surfaces. Magainin 2 has low MIC values against gram-negative bacteria and especially *E.coli* [89] [87]. *E.coli* can cause various infections such as urinary tract infections, which is a common condition in feminine hygiene care. Since magainin 2 also is a broad-spectrum AMP, it could also be a good candidate for broad-spectrum types of applications. Lactoferricin B was selected based on its antifungal properties; however it has also been shown to have activity against other pathogens [94]. Lactoferricin B shows relatively good MIC values against *C.albicans* [95], which is a skin pathogen that can cause Vulvo-vaginal candidiasis and other fungal infections for example due to diaper rashes [107] [104]. Another peptide that has a shown good activity against *C.albicans* is Histatin-5 [57]. According to Martin Malmsten, Histatin-5 could possibly be relevant for vaginal applications due to its activity at low pH (Appendix A.3 Interview with Martin Malmsten).

4.2 Results Analysis

In the following section, results from the experiments are presented and discussed. In the first experiment the aim was to investigate the antimicrobial activity of all peptides against selected pathogens. This was followed by experiments where different salt concentrations were compared based on antimicrobial activity. Finally, one different strain of each microorganism was tested in order to demonstrate the peptides strain-dependency.

Besides the AMPs, a negative control (0,01% TFA (Trifluoroacetic acid) and a positive control (AquaStabil (protective media that stops the growth of algae, bacteria and other microorganisms [140]) for bacteria and Nystatin (antibiotic agent against fungi [141]) for *C.albicans*) were tested. The negative control showed no antimicrobial activity in any experiments. The activity of the positive control is presented below. In the abbreviations of the different microorganism strains are defined. The microorganisms that lack an ATCC number have been supplied by the microorganism supplier ESSUM, which provide clinical isolates.

Table 1. Abbreviations of microorganisms used in the experimental part

Notation	Strain
E.coli (1)	*E.coli* (ATCC 10536. Class 2*)
E.coli (2)	*E.coli* (ATCC 8739. Class 2*)
S.aureus (1)	*S.aureus* (ATCC 6538. Class 2*)
S.aureus (2)	*S.aureus III* (ESSUM. Class 2*)
C.albicans (1)	*C.albicans* (ESSUM. Class 2*)
C.albicans (1)	*C.albicans* (ESSUM. Class 2*)

*Class 2 - A pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited [142].

4.2.1 Antimicrobial activity experiment

In the first experiment all of the peptides were tested against *E.coli* (1)*, S.aureus* (1) and *C.albicans* (1)*.* The growth mediums used for bacteria were LB-medium with a salt-concentration of 172 mM. For fungi a SAB-medium was used, which contained no salt. (Appendix B– Materials and Method). Below are the MIC-values and graphs presented, images can be seen in Appendix C– Laboratory Results.

4.2.1.1 *E.coli* (1)

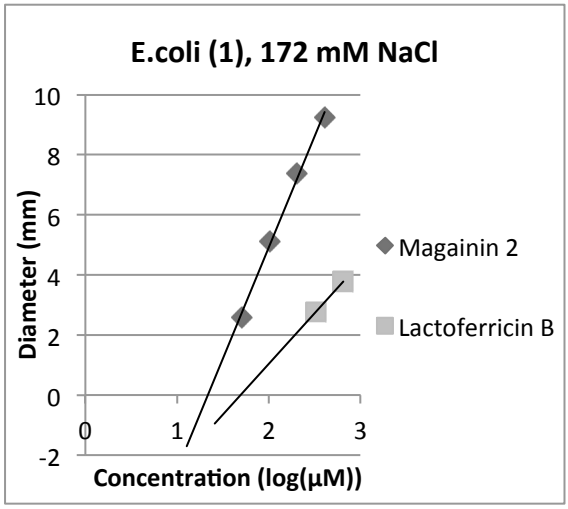

Figure 18. Antimicrobial activity of magainin 2 and lactoferricin B against E.coli (1) at 172mM

Table 2. MIC-values of peptides tested against E.coli (1) at 172mM NaCl

Peptide	MIC (µg/ml)
Nisin (Nisaplin)	>50 (>2000IU/ml)
Lactoferricin B	75,7
Buforin-II	>1000
Histatin-5	>1000
Magainin 2	53,4

As can be seen in *Figure 18* and *Table 2* magainin 2 and lactoferricin B were the two AMPs that showed activity against *E.coli* (1).

Studies have demonstrated that that magainin 2, lactoferricin B, buforin II and histatin-5 are all potent against *E.coli* (1) (see section 2.7 Peptides). It was therefore surprising that only magainin 2 and lactoferricin B gave any results. That nisin showed no activity was however expected, since it is clearly stated in studies that nisin is not active against *E.coli* [59]. As have been mentioned in the section 2.7.1 Nisin A, the LPS of the *E.coli* outer cell membrane hinder the antimicrobial activity of nisin [78]. This is likely to be the reason for the inactivity of nisin against *E.coli*. The MIC-value in *Table 2* suggests that magainin 2 is the most potent peptide against *E.coli* with the given conditions. The strong antimicrobial activity of magainin 2 could be a result of its high helicity upon binding to acidic phospholipid bilayers [87]. Helicity is an important factor for the antimicrobial activity, the higher helicity the stronger the antimicrobial activity [16]. In addition, magainin 2 might be more suitable for the particular antimicrobial assay, which is further discussed in the section 4.3 Method analysis. Finally, it was thought that resistance mechanism of the *E.coli* tested could be an explanation to why certain peptides show no or weak activity. However, later it was shown that when lowering the salt concentration (see salt experiments) all peptides except nisin showed activity against the same *E.coli* strain. This lead to the conclusion that the assay conditions rather than resistance mechanism was the more important factor.

4.2.1.2 S.aureus (1)

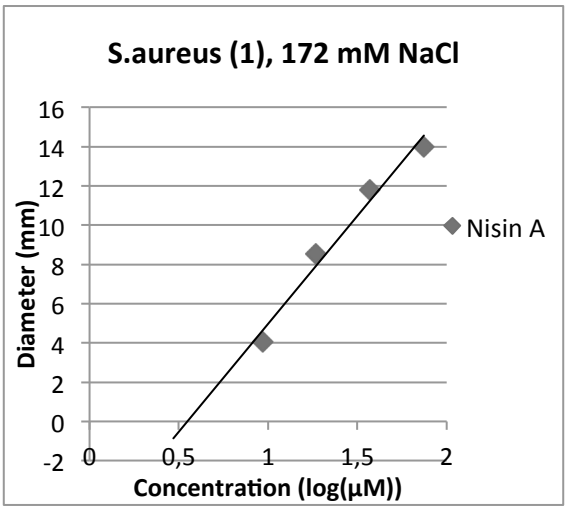

Figure 19. Antimicrobial activity of nisin A against S.aureus (1) at 172mM

Table 3. MIC-values of peptides tested against S.aureus (1) at 172mM NaCl

Peptide	**MIC (µg/ml)**
Nisin A (Nisaplin)	11,8 (473 IU/ml)
Lactoferricin B	>1000
Buforin-II	>1000
Histatin-5	>1000
Magainin 2	>1000

In *Figure 19* and *Table 3*, the antimicrobial activity of nisin, which was the only peptide active against *S.aureus* (1), is demonstrated. Nisin was tested in its commercial form (Nisaplin), which only contains 2.5 %w/w nisin. With this in mind a higher concentration of Nisaplin compared to the other more pure peptides (>95%) was used in this assay (Appendix B.2 Method (protocols)). The MIC-value for both nisin and Nisaplin was calculated. When calculating the MIC-value for nisin, the value had to be correlated with the fact that only 2.5 %w/w of nisin is present in Nisaplin. However, it is not clear if the correct MIC-value of nisin can be calculated in the way that has been conducted (Appendix B.3 Calculation of MIC-values).

Initially the experiment was conducted with lower concentrations, which were in line with the literature protocols [143]. However, since only two zones of inhibition were detected, the concentration was increased in order to receive at least three zones. The results from the increased concentration can be seen in *Figure 19* where four zones were registered. The activity of nisin against *S.aureus* is in accordance with the literature, where it has been clearly stated that nisin is a potent peptide against *S.aureus* [59]. In fact our experiments show a very low MIC-value of Nisaplin compared with other studies where MIC has been estimated to 4000 IU/ml [79]. None of the broad-spectrum peptides showed any activity against *S.aureus*, which again was not in agreement with the literature (2.7 Peptides). An explanation for this could be that peptides use electrostatic interactions to bind to microorganism's cell membrane and since *S.aureus* can develop resistance by altering the charge of the membrane, it might explain the inactivity of the peptides (see section 2.5.3.1Mechanism behind resistance, and 2.4.1 Mechanisms of Action). Nisin however can permeabilize the membrane through binding of Lipid-II and might therefore avoid the above resistance mechanism.

4.2.1.3 C.albicans (1)

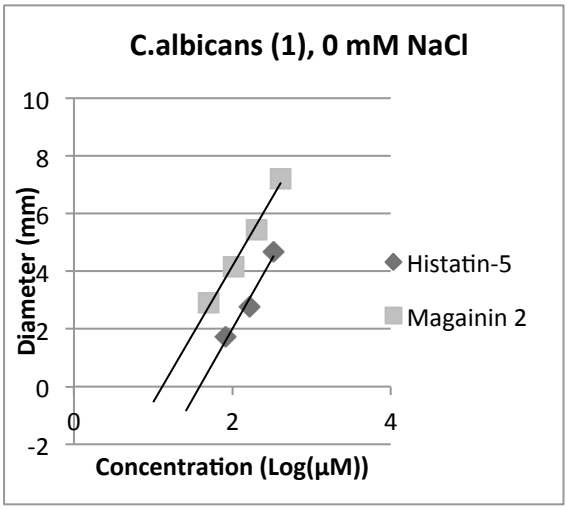

Figure 20. Antimicrobial activity of histatin-5 and magainin 2 against C.albicans (1) at 0mM NaCl

Table 4. MIC-values of peptides tested against C.albicans (1) at 172mM NaCl

Peptide	**MIC (µg/ml)**
Nisin A (Nisaplin)	>50 (>2000IU/ml)
Lactoferricin B	>1000
Buforin-II	500-1000
Histatin-5	117,1
Magainin 2	32,1

When testing the antimicrobial activity against *C.albicans* only histatin-5 and magainin 2 gave zones of inhibition (*Figure 20*). It is clear from the MIC-value that magainin 2 is more potent against *C.albicans* then histatin-5 at the given conditions (*Table 4*). Both histatin-5 and magainin 2 have shown activity against *C.albicans* in other studies, the results are therefore expected [89] [57]. Again, it was anticipated that broad-spectrum peptides such as buforin II and Lactoferrcin B would be active. It was especially surprising that lactoferricin B didn't give any activity, since it has been demonstrated in several studies that the peptide has good activity against *C.albicans* [94]. The lack of activity of lactoferricin B and buforin II could be a result of resistance mechanisms of *C.albicans*. Buforin II, lactoferricin B and histatin 5 have all been suggested to have intracellular antimicrobial mechanisms. Some strains of *C.albicans* have been shown to develop resistance efflux systems against AMPs with intracellular targets [71]. However, since histatin-5 has activity against *C.albicans* (1), the efflux resistance mechanisms would probably not explain why buforin II and lactoferricin B showed no activity. It should also be noted that at high concentrations buforin II showed indications of activity against *C.albicans* could be observed.

4.2.2 Salt-sensitivity experiment

Since some of the peptides did not show any activity in the first experiment, it was speculated that the conditions might not be optimal for the peptides. Conditions that are repeatedly mentioned in the literature, which could affect the activity of peptides, are pH and salt [92]. With this in mind, other LB-mediums such as LB-Luria and LB-Miller, which contains 86 mM and 8.6mM NaCl respectively was tested. The graphs below show the results of the different salt experiments.

4.2.2.1 E.coli (1)

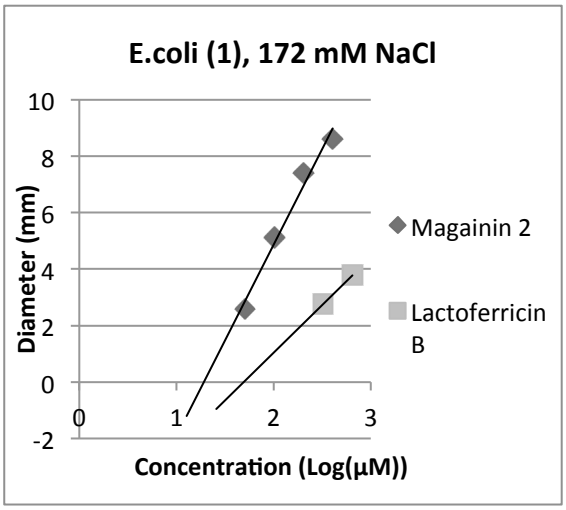

Figure 21. Antimicrobial activity of magainin 2 and lactoferricin B against E.coli (1) at 172mM NaCl

Table 5. MIC-values of peptides tested against E.coli (1) at 172, 86, 8,6mM NaCl

	MIC (µg/ml)		
Peptide	172 mM NaCl	86 mM NaCl	8,6 mM NaCl
Nisin A (Nisaplin)	>50 (>2000 IU/ml)	>50 (>2000 IU/ml)	25-50 (1000-2000 IU/ml)
Lactoferricin B	75,7	74,0	16,2
Buforin-II	>1000	>1000	102,7
Histatin-5	>1000	54,8	57,9
Magainin 2	47,0	17,3	7,1

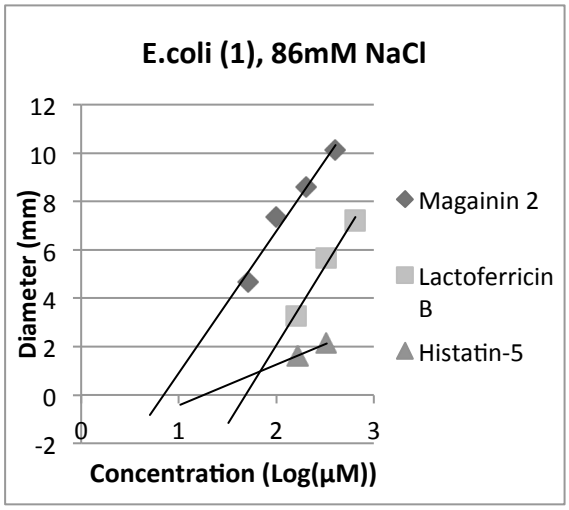

Figure 22. Antimicrobial activity of magainin 2, lactoferricin B and histatin-5 against E.coli (1) at 86mM NaCl

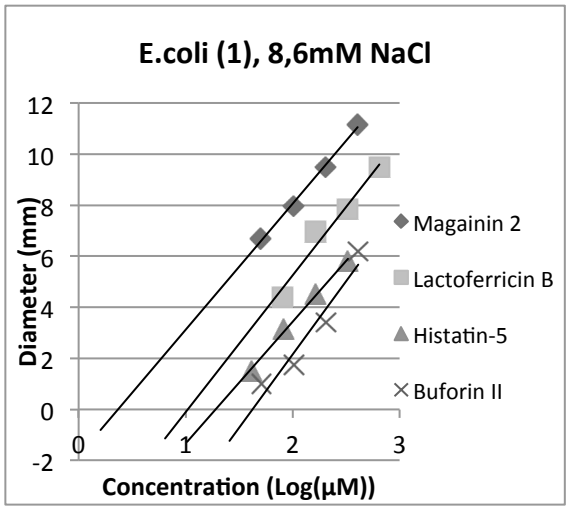

Figure 23 Antimicrobial activity of magainin 2, lactoferricin B, histatin-5 and buforin II against E.coli (1) at 8,6mM NaCl

From the figures (*figure 21-23*) and *Table 5* above it can be seen that lowering the sodium chloride concentration from 172mM to 86mM and 8.6mM activates histatin-5 and buforin II, which showed no activity at 172mM NaCl. The graphs also indicate that lactoferricin B and magainin 2 becomes more potent against *E.coli* at lower NaCl concentrations. The results from these experiments are more in agreement with the literature, since all peptides except nisin are expected to be active against *E.coli*. Even

though buforin II showed activity against *E.coli* at lower NaCl concentration, the MIC it is still very low compared to the literature values [139]. Some of the result from the salt experiments can be confirmed by other studies where magainin 2 and buforin II has shown high sensitivity to salt [74].

Table 6. MIC-values of magainin 2 against E.coli (1) at 172, 86, 8,6mM NaCl

	MIC (µg/ml)		
Peptide	172 mM NaCl	86 mM NaCl	8,6 mM NaCl
Magainin 2	47,0	17,3	7,1

Table 7. MIC-values of lactoferricin B against E.coli (1) at 172, 86, 8,6mM NaCl

	MIC (µg/ml)		
Peptide	172 mM NaCl	86 mM NaCl	8,6 mM NaCl
Lactoferricin B	75,7	74,0	16,2

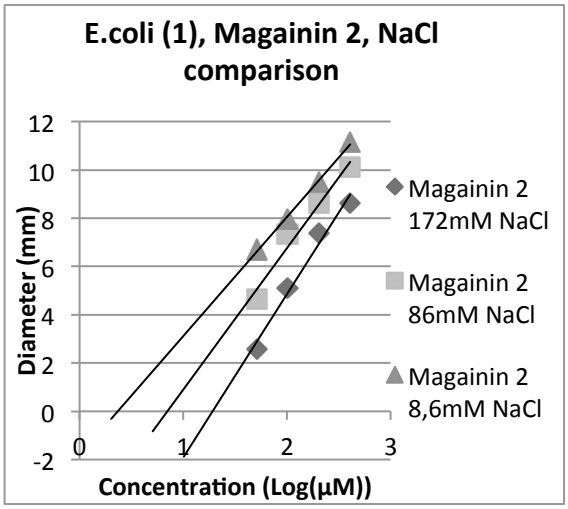

Figure 25. Antimicrobial activity of magainin 2 against E.coli (1) at 172, 86, 8,6 mM NaCl

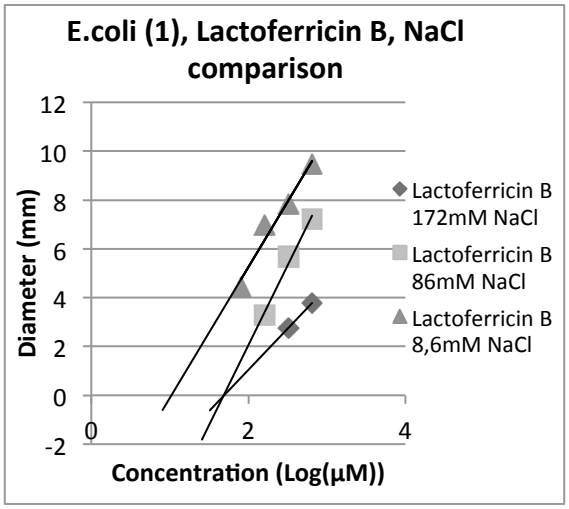

Figure 24. Antimicrobial activity of lactoferricin B against E.coli (1) at 172, 86, 8,6 mm NaCl

In *figure (24-25)* and *table (6-7)*, where all the salt concentrations are compared, it is clearly demonstrated how magainin 2 and lactoferricin B becomes more potent at lower salt concentration. In *Figure 24* a linear relationship exist between salt concentration and the antimicrobial activity of magainin 2. However, this relationship is not as clear in *Figure 25*, which is thought to be due to the fact that lactoferricin B only gave two points at the salt concentration 172mM. As been mentioned two points is not enough for any direct conclusions. However, it is probable that lactoferricin B also shows a linear dependence on salt concentration. The sodium chloride can affect the secondary structure by loss in α-helical structure. Magainin 2, buforin II, histatin-5 and lactoferricin B contains α-helical structures, it is therefore not surprising that these peptides have shown to be salt-sensitive. Nisin (concentration 50 µg/ml) showed some

activity against *E.coli* at a lower NaCl concentration, this action have also been demonstrated in other publications [80].

It was suspected that the bacteria might become weaker at lower salt-concentration. As can be seen in *Table 8* the zone size increased with lower salt-concentration, which suggests that *E.coli* becomes slightly weaker. This factor could have an effect on the MIC values; however the small increase in weakness of the microorganism is presumably low relative to the high increase in peptide activity at lower salt-concentrations.

Table 8. Inhibition zone size of positive control (AquaStabil) against E.coli (1) at 172, 86, 8,6mM NaCl

***E.coli* (1)**	
NaCl concentration (mM NaCl)	**Zone size (positive control) (mm)**
172	22,86
86	27,67
8,6	31,90

4.2.2.2 S.aureus (1)

By lowering the salt concentration it was expected that broad spectrum peptides would show activity against *S.aureus*, since these peptides were "activated" against *E.coli* at lower salt concentrations. Surprisingly the peptides were still inactive and as before only nisin showed activity against *S.aureus*. Below the results for nisin at lower salt concentrations will be discussed.

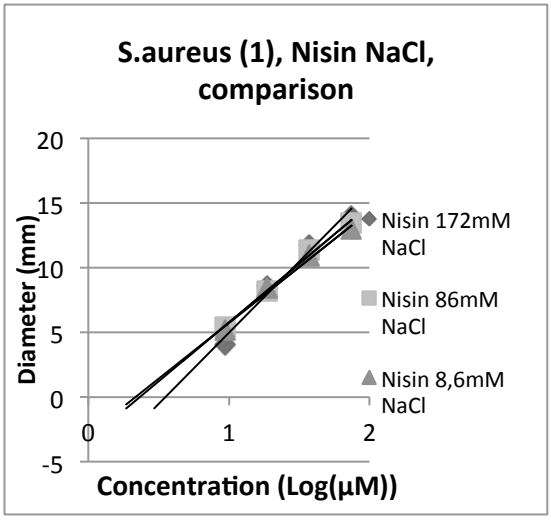

Figure 26 Antimicrobial activity of nisin against S.aureus (1) at 172, 86, 8,6 mM NaCl

Table 9. MIC-values of nisin against S.aureus (1) at 172, 86, 8,6mM NaCl

	MIC (µg/ml)		
Peptide	172 mM NaCl	86 mM NaCl	8,6 mM Nacl
Nisin A (Nisaplin)	11,8 (473 IU/ml)	7,8 (311,3 IU/ml)	7,2 (290 IU/ml)

The results from *Figure 26* and *Table 9* indicate that nisin is not very salt-sensitive. Only small variations can be observed between the tested salt concentrations. Another conclusion that can be made is that salt concentration was not a parameter that affected the other peptides activity against S. aureus. The suggestion that resistance mechanisms may be a cause for the inactivity is therefore strengthened.

According to our results (*Table 10*) *S.aureus* is not salt-senstive. This implicates that the MIC-values from *S.aureus* experiments, should not be affected by weakness of the microorganisms.

Table 10. Inhibition zone size of positive control (AquaStabil) against S.aureus (1) at 172, 86, 8,6mM NaCl

***S.aureus* (1)**	
NaCl concentration (mM NaCl)	**Zone size (positive control) (mm)**
172	30,5
86	30,5
8,6	31,0

4.2.3 Strain-dependency experiment

Different strains of *E.coli*, *S.aureus* and *C.albicans* were tested with magainin 2, nisin A and histatin-5, respectively. This experiment indicates how the antimicrobial activity varies between different strains of the same microorganism.

4.2.3.1 E.coli (1) and (2)

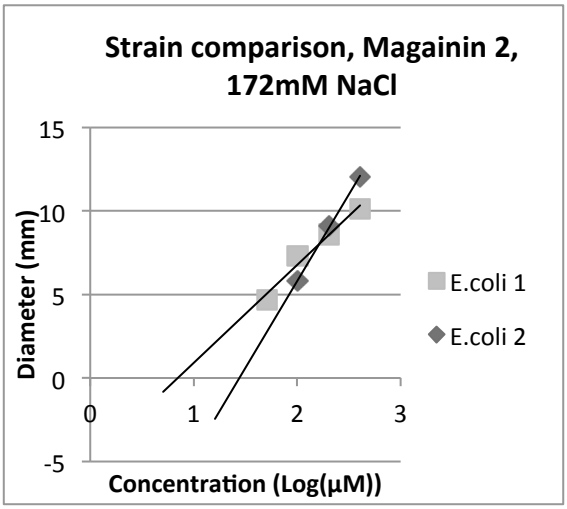

Figure 27. Antimicrobial activity of magainin 2 against E.coli (1) and (2) at 172mM NaCl

Table 11. MIC-values of magainin 2 against E.coli (1) and (2) at 172, 86, 8,6mM NaCl

(Magainin 2)	**MIC (µg/ml)**		
Strain	172 mM NaCl	86 mM NaCl	8,6 mM Nacl
E.coli 1	47,0	17,3	7,1
E.coli 2	68,2	4,1	2,5

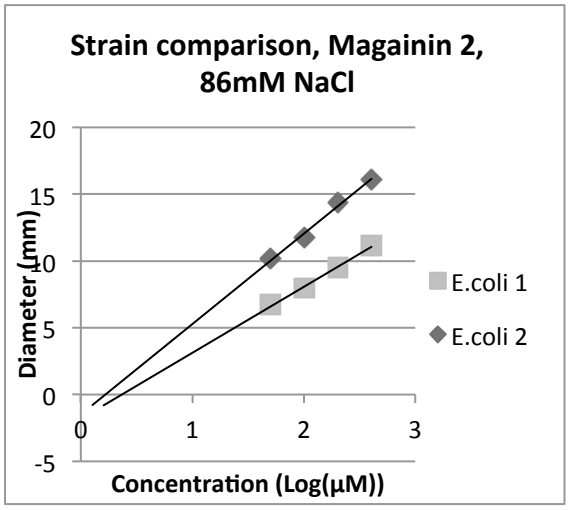

Figure 29. Antimicrobial activity of magainin 2 against E.coli (1) and (2) at 86mM NaCl

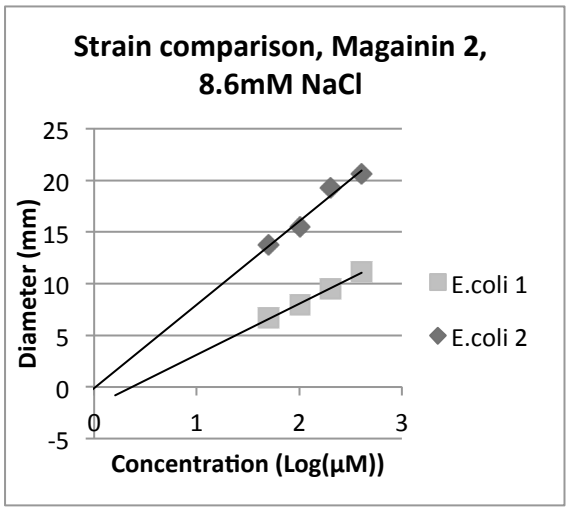

Figure 28. Antimicrobial activity of magainin 2 against E.coli (1) and (2) at 8,6mM NaCl

In the figures above (*27-29*) and *Table 11*, the antimicrobial activity of magainin 2 against *E.coli* (1) and (2) is demonstrated at different salt concentrations. Magainin 2 was selected for this experiment since it showed good activity against the previous

tested strain *E.coli* (1). The aim was to investigate the strain-dependence of the antimicrobial activity. The results from *Figure 28* and *Figure 29* indicate that magainin 2 was more potent against *E.coli* (2). However, this is not the case in *Figure 27* were magainin 2 is more potent against *E.coli* (1). It is therefore difficult to draw any conclusion about which strain that is weaker. However, it can be seen that antimicrobial activity of magainin 2 is strain dependent. Moreover this experiment further strengthens the results that magainin 2 is salt sensitive.

4.2.3.2 S.aureus (1) and (2)

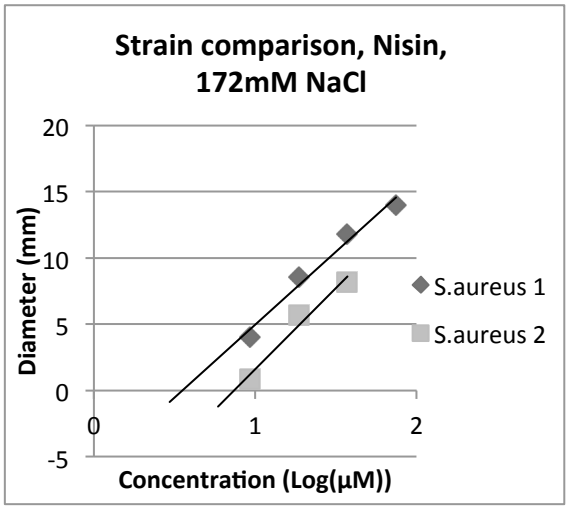

Figure 30. Antimicrobial activity of nisin against S.aureus (1) and (2) at 172mM NaCl

Table 12. MIC-values of nisin against S.aureus (1) and (2) at 172, 86, 8,6mM NaCl

(Nisin A)	**MIC (µg/ml)**		
Strain	172 mM NaCl	86 mM NaCl	8,6 mM Nacl
S.aureus (1)	11,8	7,8	7,2
S.aureus (2)	24,8	21,1	23,5

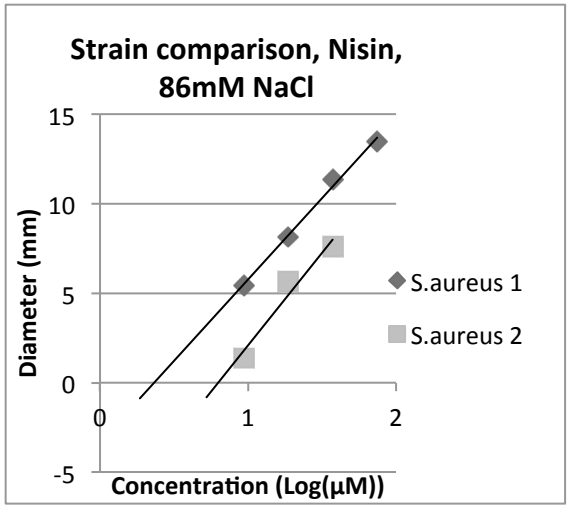

Figure 32. Antimicrobial activity of nisin against S.aureus (1) and (2) at 86mM NaCl

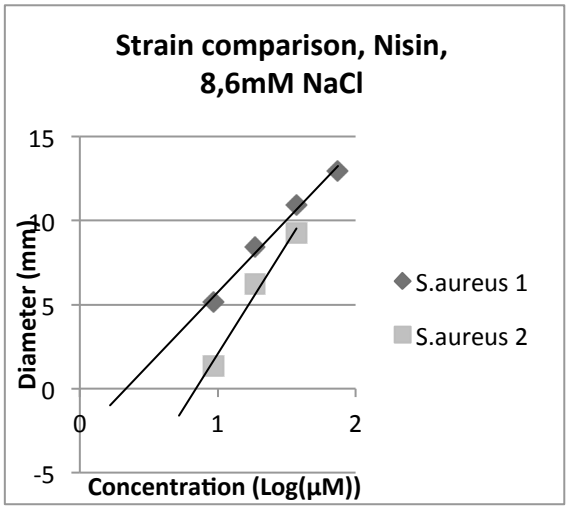

Figure 31. Antimicrobial activity of nisin against S.aureus (1) and (2) at 8,6mM NaCl

Nisin was the only peptide that was active against *S.aureus* (1), and was therefore the only option for testing strain dependence. The nisin concentration was lower in the experiment where *S.aureus* (2) was tested compared to the experiments of *S.aureus* (1). Since the project time was limited, the concentration was not increased further for the *S.aureus* (2) experiment and also three data points were obtained which was considered to be sufficient. This shows that nisin is more active against S. Aureus (1) (figure 30-32 and *Table 12*). The difference in activity against *S.aureus* (1) and (2) could be explained by resistance mechanisms caused by various mutations [144].

4.2.3.3 C.albicans (1) and (2)

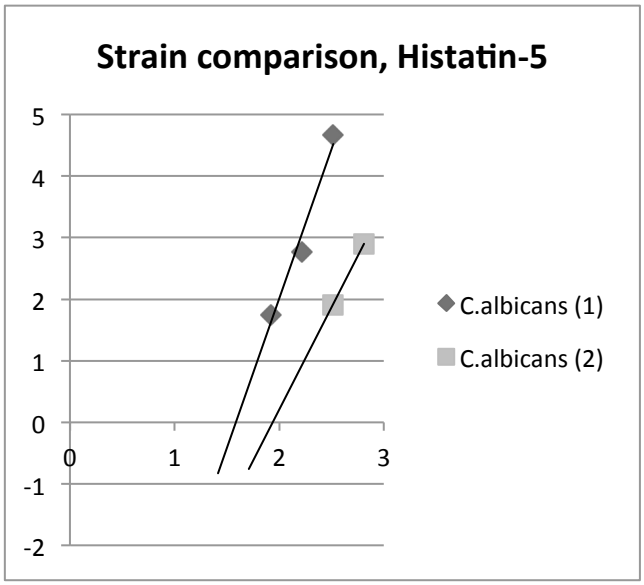

Figure 33 Antimicrobial activity of histatin-5 against C.albicans (1) and (2) at 0mM NaCl

Table. 13. MIC-values of histatin-5 against C.albicans (1) and (2) at 0mM NaCl

Strain	MIC (µg/ml)
C.albicans (1)	117,1
C.albicans (2)	263,3

Result from *Table. 13* and *Figure 33* indicate that histatin-5 is more potent against *C.albicans* (1) than *C.albicans* (2). However there are only two zones of inhibition observed when testing *C.albicans* (2) and therefore the results are less reliable. This suggests that different strains have weaker or stronger tolerance against histatin-5. An explanation could be that certain strains of *C.albicans* are more resistant to the peptide. As have been mentioned strains of *C.albicans* have been shown to develop adaptive resistance mechanisms specifically against histatin-5.

4.3 Method analysis

Inhibition zone assay is in general a good method for screening antimicrobial compounds; however the method also has several drawbacks.

The main advantage of the method is that it is fast and simple to use. Another advantage is that very low amounts of peptides are needed compared with other antimicrobial assays. These were all important aspects when selecting the method. The main interest in this thesis work was to investigate the antimicrobial activities of the selected peptides. As Malmsten pointed out the diffusion assay method is a good starting point when investigating the peptides antimicrobial activity. Even though the method does not necessarily give an accurate estimate of the MIC values, it is adequate for comparing the activities of the selected peptides (Appendix A.3 Interview with Martin Malmsten).

The drawbacks of this method are several. As Bergman and Malmsten mention, there are challenges such as that different peptides might diffuse differently due to hydrophobicity, size or charge, which will affect the zone size (Appendix A.2 Interview with Peter Bergman, Appendix A.3 Interview with Martin Malmsten). In order to minimize the peptide charge effect, low EEO (Electroendosmosis) agarose was used in the experiments [145]. The low EEO agarose is preferred over agar and other agaroses since it prevents cationic peptides from electrostatically interacting with the matrix [146]. The chosen peptides did differ in hydrophobicity, which might influence the results. According to Malmsten hydrophobicity can give matrix effect and thus affect the diffusion of the peptides (Appendix A.3 Interview with Martin Malmsten). The selected peptides are all in similar size range (21-34), which lowers the possible length effects. Since there are several drawbacks, both Malmsten and Bergman recommend using other methods for a more specific determination of MIC (Appendix A.2 Interview with Peter Bergman, Appendix A.3 Interview with Martin Malmsten). Furthermore many errors can occur during the execution of the method; the most significant ones are discussed below.

One issue is to receive an even distribution of the gel. According to a protocol edited by Anita Boman, this is an important aspect in order to get reliable results [147]. During the experiments it was challenging to get an even distribution since the gel layer had to be very thin. In addition it was difficult to pour the whole amount (6ml) of the gel onto the petri dishes since a small amount of gel was solidified in the bottom of the falcon-tubes *Figure 11*. As a result a convex distribution of the gel was observed, which resulted in that the middle-wells of the petri dish had a smaller volume compared to the wells closer to the edge. Because it took longer time for the peptides in the middle wells to diffuse it was believed that different volumes of the wells could possibly affect the diffusion time. This in turn might affect the zone size. It should be important to let the

peptides diffuse properly into the gel before incubation; otherwise the peptide solvent (TFA) might evaporate and thus also affect the diffusion of the peptides.

As Cederlund mentioned it is important to have the right amount of cell density for the experiments (Appendix A.5 Interview with Andreas Cederlund). This is due to the fact that the peptide to cell ratio will change with higher cell density, and thus affecting the zone size. In order to make the results comparable, similar cell density was used for each microorganism throughout the experiments. Furthermore, during the experiments there was always a risk of microorganism contamination. With that in mind, it was carefully controlled that the petri dishes had a uniform carpet of microorganisms with similar appearance. The different microorganisms had a characteristic appearance, so a contamination was easy to detect. During the experiments contamination was generally not observed, however, in a few experiments some surface contamination was detected, which can be seen in Appendix C.2.2.1 *Figure (A) 45*. However, these contaminations were not believed to affect the results since a uniform carpet inside the gel was observed.

Some other sources of error also exist such as the freezing and thawing of the peptides between the experiments that could possibly weaken peptides thus affecting the activity. Also very small amounts of the peptides were handled, which increases the risk for errors in the pipetting procedure. The measuring of the zones can also be a source of error; to minimize this error only one person was responsible for the measuring.

4.3.1 Possible Improvements of the method

There are studies that suggest several improvements of the method, especially for nisin [143]. Factors that have shown to improve the method for nisin are for example incorporation of the surfactant tween 20, as well as different medium pH. This could also be improvement for the other peptides. When the peptides are bought it could be a possibility that the peptides have been incorrectly synthesized. Therefore a confirmation by an analytic method is recommended and would verify the peptide. However, this was not performed in this thesis since the time was limited.

4.4 Applications discussion

In this part of the discussion, potential applications, challenges and active research and companies will be discussed in order to evaluate the field as well as the potential of using AMPs in hygiene products.

4.4.1 Potential applications in hygiene products

The possible applications of AMPs in hygiene products will be assessed in this section. First the applications of probiotics and bacteriocins are discussed followed by inducers and materials.

4.4.1.1 Probiotics and bacteriocins

Probiotics is an interesting field within hygiene products, mostly feminine care, which is an important product range for SCA and other companies. The antimicrobial property of probiotics is important and bacteriocins are suggested to be a part of the action. With the current research about probiotics and bacteriocins, it is possible to design bacteriocins to match desired applications. This opens a window for probiotics with specific selectivity between microbes or probiotics with a broad-spectrum antimicrobial activity. This in turn could potentially be useful in different kind of applications such as panty liners to prevent genital infections such as UTI, VVC and BV. As an example, the bacteriocin subtolisin A has been suggested to be a good candidate, since it inhibits BV-associated bacteria without killing healthy Lactobacillus [19]. In order to receive probiotics that are efficient in preventing pathogens, understanding and modifying the bacteriocin or lactic acid bacteria might be a solution for new probiotic strains with improved antimicrobial properties.

4.4.1.2 Inducers

As have been mentioned by Olafsdottir and Agerberth, inducing the AMP production, might possibly be a better solution than adding single peptides for reasons such as resistance issues (Appendix A.1 Interview with Birgitta Agerberth, Appendix A.4 Interview with Sigridur Olafsdottir). An interesting possibility is that infections could be prevented or treated by pharmacologically inducing AMPs. As have been mentioned several AMPs such as cathelicidins and defensins have been shown to be important in protecting the urinary tract from microbial invasion [115]. With this in mind, there might be possibilities to prevent or treat genital infections such as UTI by inducing the expression of AMPs. An interesting and well-studied inducer for this purpose might be vitamin D. It can also be speculated if a topical addition of vitamin D would be efficient at other areas, such as wounds. Additionally as Olafsdottir points out, an advantage of

inducing peptides is minimizing the risk of developing resistance compared to adding single peptides (Appendix A.4 Interview with Sigridur Olafsdottir).

4.4.1.3 Materials

The use of AMPs in materials could fill interesting needs in various hygiene products. Materials with covalent attached AMPs have advantages such as decreased toxicity and increased stability that enable long time storage. Moreover, according to Agerberth another possible advantage of covalently attached AMPs is the reduced risk of resistance compared to materials that release antimicrobial agents (Appendix A.1 Interview with Birgitta Agerberth). A material with covalently attached AMPs such as nisin could be used to create antibacterial surfaces that could be relevant for the use in areas exposed to pathogens such as toilet appliances or in materials such as cloths. A recent research has shown that nisin in fact could be attached to materials and act antimicrobial [128]. The low toxicity of nisin and its presumably low cost compared to other AMPs could make it a relevant candidate for the use in hygiene related environments.

Another alternative to the covalent attachment is to use materials with absorbed AMPs. As have been mention in 3.1.2 Potential Applications, there is a patent of moist bacteriocin disinfectants wipes, in which nisin could be used as the bacteriocin. This is an interesting approach of using AMPs in hygiene products such as wet wipes. The product WipeOut® with absorbed nisin, exemplifies this application. The fact that the wipe is used to sterilize cow teat skin indicates that the product could possibly be relevant also in the sterilization of human skin. This type of use is applied by a company, which is selling a wipe containing nisin that is supposed to be used to sterilize human skin and surfaces. The same approach could be used to develop, for example, baby wipes used associated with diaper changes or wipes for hand disinfection.

4.4.2 Challenges in using AMPs in hygiene products

There are many challenges involved in AMP development such as resistance, specificity, stability and cost. Even though there are many application areas the challenges need to be considered in order to evaluate the potential of AMPs.

4.4.2.1 Resistance

As Malmsten mentions, AMPs are in general considered to be a better alternative than antibiotics since the peptides has been hypothesized to be less prone to induce microbe resistance. He also adds that the discussion regarding resistance should be more detailed and more research is needed in this area. Current research demonstrates that microbes as a result of AMPs can develop resistance mechanisms. Resistance is thus a factor that

needs to be considered when using AMPs in products (Appendix A.3 Interview with Martin Malmsten). Bergman is not positive to the idea of overusing AMPs especially in everyday products since bacteria could easily develop resistance. As has been mentioned two directions exist in the development of AMPs either to use the AMP directly or to induce the expression in the body (Appendix A.2 Interview with Peter Bergman). The company Akthelia focus on inducing the expression of AMPs. The CEO Sigridur Olafsdottir highlights that their anti-infective treatments induce multiple innate antimicrobial peptides that work via multiple mechanisms, rendering it difficult for the microbes to develop resistance (Appendix A.4 Interview with Sigridur Olafsdottir). This suggests, at least from the perspective of resistance, that the use of inducers where it is applicable would be a more safe approach then using single AMPs in hygiene products.

4.4.2.2 Specificity

Another challenge is the selectivity of the peptides both the selectivity between microbial and mammalian cells and also between microbial cells. It is very important to consider toxicity in the development of new products such as pharmaceuticals. There is a significant difference between mammalian and bacterial cells such as charge and membrane composition [9]. These are some of the factors that allow AMPs to distinguish between the cells, thus decreasing the risk of toxicity. A strategy to further improve the selectivity could be to introduce end tags. Additionally, since many of the AMPs are broad spectrum the use in hygiene applications could potentially disturb the normal micro-flora of the body.

4.4.2.3 Cost aspect

The cost aspect is one of the major challenges in the commercialization of AMPs. In general AMPs are expensive to produce and therefore an important aspect in the development of AMPs is to reduce the production cost by finding new cheap production methods. Both chemical synthesis and microbial expression systems are methods that are currently being improved which could lead to a reduced production cost in the future. An example of a cheap AMP is the food preservative nisin (section 2.5.1 Production and cost aspects). An interesting point, which also Malmsten points out, is that AMPs partly compete against cheap products that are used regularly; in these applications it is difficult to motivate the use of peptides. However, when the conditions are more severe and the cost is not the main issue, AMPs have a better potential. As most of the interviewed researchers have pointed out and agreed on, the cost would be the main challenge for the use in hygiene products. However there might be a potential in more expensive and niched hygiene products. The use of inducers, such as vitamin D, in hygiene applications could also be an approach to avoid the cost issue (Appendix A.3 Interview with Martin Malmsten).

4.4.2.4 Stability

Stability factors such, as temperature sensitivity, pH sensitivity, storage time and salt sensitivity could be important when developing hygiene products. Several peptides have been shown to be sensitive against some of these factors. From the experimental results it could be observed that peptides such as buforin II and magainin 2 are salt sensitive, which is also confirmed by a patent [74]. This is partly due to the weakened helix stability at higher salt concentrations; this however might be solved by helix-capping motifs. Nisin however is a good example of a peptide that possesses good stability since it is both temperature and salt tolerant and can be stored for a longer period of time.

4.4.3 AMP related companies and products

With the growing number of companies and products, the field of AMPs is getting closer to commercialization. Not many products have yet hit the market and most of the companies are in a research and development stage. This might be due to the challenges that have been discussed. However there is a growing interest in the field, mainly in the pharmaceutical sector. The interest in the pharmaceutical industry is mostly because of the increasing concerns of microbial resistance against current antibiotics. There is a wide research of different application areas such as topical and oral applications. Most of the companies that have been encountered in this thesis focus on single use peptides with topical applications. The companies DermaGen, ImmuCell and Dipexium Pharmaceuticals all use this approach. DermaGen and Dipexium Pharmaceuticals use cream solutions containing AMPs against various skin conditions while ImmuCell uses absorbed AMP in a wipe. Other than topical applications companies such as Demegen uses a mouth rinse of AMP against oral infections. As have been mentioned companies such as Akthelia induce AMPs in the body against infections. Out of these products ImmuCell's nisin wipe is a good example of a topical AMP product that is used in a larger scale outside the pharmaceutical sector.

5 Conclusions

In this section the major conclusions from the practical and theoretical part will be drawn. Firstly the practical conclusions will be presented and thereafter conclusions about the field and the potential of AMPs.

From the practical results it could be concluded that with the right conditions, most of the peptides showed activity depending on the microorganisms. Resistance mechanisms, stability and method problems could be an explanation to why some peptides that were expected to show activity did not. It was also demonstrated that many peptides are salt sensitive, and when tested against different strains of the same microorganism the

peptides did not show much difference in activity. However more strains need to be tested to draw any conclusions of strain dependency.

The method was simple and appropriate for an initial investigation of the antimicrobial peptide activity. However there are some drawbacks that need to be considered. From the experimental and theoretical part it can be concluded that the choice of peptides in most cases was satisfying. The exceptions were lactoferricin B and buforin II, the antimicrobial activity of these peptides varied the most from the literature compared to the experimental results. Also from the information sources taken part of, these were the only peptides not present in any applications. The rest of the peptides or analogues of these were found to be present in various applications.

Currently most companies and their products are in a research and development phase. However a few products have already hit the market, mostly in the pharmaceutical sector. Nisin is an exception since it is used both as a preservative in the food industry and also as an active component in disinfectant wipes. Also many other lantibiotics in general are a part of the probiotics that are being used in many applications. It can be concluded from a variety of information sources that there are two major directions in AMP research and applications, these are either inducing the peptide defence or by applying the peptides directly.

Even though there are many potential applications of AMPs there are also several challenges. The current research of AMPs mainly includes designing peptides with improved properties such as increased selectivity, stability, lower risk of resistance development and also lowering the cost of production. These are among the more critical research areas that need to be further investigated in order to potentially use AMPs in hygiene products and other large-scale applications. Out of the investigated peptides, nisin is the peptide that might be most suitable for this purpose, since it fulfils many of the above criteria. Nisin has a limited activity spectrum that might be either an advantage or a drawback depending on the hygiene application. An advantage could be that it avoids disturbing the normal micro-flora; however, in some applications it is more suitable to have broad-spectrum AMPs. With the current research and development of AMPs there could be a possibility for other peptides in hygiene applications such as magainin 2 and histatin-5, these are currently used in pharmaceuticals as anti-infective agents.

Overall the objective of the thesis has been fulfilled. A thorough investigation of the field has been conducted through literature studies and interviews with both companies and experts, and also the relevance of AMPs in hygiene applications has been examined. Furthermore a method for measuring the antimicrobial activity and selectivity at different conditions has successfully been applied.

5.1 Future

As have been mentioned the method used in the practical part is an initial approach to investigate the antimicrobial activity. From an SCA perspective the next stage of this project could be to evaluate cheaper and improved peptides or peptide analogues with the screening method used. Further, more experiments would be needed to investigate other important factors besides the activity. The toxicity profile could be investigated with test on mammalian blood cells and thereafter on living tissue. Also suitable methods have to be used in order to evaluate different stability factors, mainly temperature-sensitivity, pH-sensitivity and salt-sensitivity. Besides these factors a thorough analysis of the potential resistance development from AMPs needs to be conducted. An evaluation and tests of synergic effects could also be an approach of increasing the activity of the peptide. For example, different peptides could be combined together or with other antimicrobial substances. Finally, the immune-modulatory effect is also a factor that needs to be further evaluated.

Other than the technical aspects of the project, the market potential for a possible AMP product has to be evaluated. This could be performed by various market investigations in order to receive the consumer's opinion about the use of AMPs in hygiene products.

The future use of AMPs in hygiene products can be summarized into three main approaches; these are AMP-producing probiotics, AMP-materials and inducers of AMPs in the body. Probiotics is mainly aimed for use in feminine care and an increased knowledge of bacteriocins could potentially improve the use of probioitcs in different applications. The use of AMPs in materials is also a future potential application. The bacteriocin nisin is being currently used in materials such as wipes. This suggests that out of the AMPs evaluated in this thesis, nisin could be the most relevant AMP for use in large-scale application such as hygiene products. Another future application could be the use of AMPs to create antimicrobial surfaces, which could be used in environments where hygiene is an issue. Interestingly, the company CytaCoat had the approach to use AMPs in materials; however, they noticed that it was more efficient and cheaper to use another ligand instead of using AMPs. With this in mind, CytaCoats solution indicates that there might be cheaper and more efficient approaches in creating antimicrobial materials. Finally it has been speculated that inducers such as vitamin D could be a good approach to avoid some of the challenges associated with peptides such as cost, stability, resistance and toxicity. Inducers could possibly have a potential in applications such as c and wipes in order to boost the human defence system.

5.2 Final Remarks

To sum up the field, antimicrobial peptides have a great potential in many application areas. The positive aspects of peptides are many, which are shown by the increasing number of active companies, and researcher that believe in the field. The on-going research has great potential in solving the current and future challenges. With this in mind, the field is suggested to have a future in many applications. However, the question still remains if the time for AMPs in hygiene products is now.

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Appendix A - Interviews

In this appendix all interviews are summarized. The interviewed researcher have all read and approved the text concerning their own statements and ideas.

A.1 Interview with Birgitta Agerberth

Professor of Medical Microbial Pathogenesis at Karolinska Institute -Department of Medical Biochemistry and Biophysics

Background
Birgitta Agerberth is a Professor of Medical Microbial Pathogenesis at the Department of Medical Biochemistry and Biophysics (MBB) since 2008. Researchers in microbial pathogenesis study how microbes, primarily bacteria, cause disease. The research covers interaction between bacteria and the infected person´s immune defence, how the disease can be diagnosed and how it should be treated. A central issue for Birgitta Agerberth´s research is how the inherited immune defence manages to keep us healthy despite all the bacteria around us. More precisely the research revolves around antimicrobial peptides (AMPs) and the human AMP LL-37 and how it works and is regulated, with the current focus on *Shigella* infection, which can cause diarrhoea. Agerberth was one person behind the discovery of LL-37, belonging to the cathelicidin family.

AMPs in general
Agerberth starts to refer to the general characteristics of AMPs. The peptides are not believed to have as rigid structure as proteins, which leads to, that they can bind to low affinity receptors. The antimicrobial activity is often membrane specific. Other functions among AMPs such as immunomodulation and angiogenesis are more specific and thus receptor mediated. She emphasize that not all of these functions have been shown in *vivo* and have thus not been tested in a physiological environment. AMPs can be both bacteriostatic and bactericidal. One example of antimicrobial proteins is binding to iron that can limit the growth of bacteria and thus act bacteriostatic. Furthermore the concentration of AMPs in the circulatory system is low. However, neutrophils contain both AMPs and different types of antimicrobial proteins that are released at the sites of infection in our body.

Agerberth continues to mention that some AMPs are sensitive against high salt concentrations and she takes defensin as an example. These AMPs are inactivated by an increased salt concentration. The first disease that AMPs was related to was Cystic

fibrosis. This disease is a result of a defect ion channel, which results in a high salt concentration in the lung mucosa. The patients with this disease very often have an infection in the lung. It was found that the defect in the ion channel caused an increased salt concentration that leads to the deactivation of defensins and resulted in infection. It has however later been shown that other factors also affect this disease.

LL-37

Agerberth sees LL-37 as a marker of the AMP system in humans. If LL-37 is present, other AMPs and antimicrobial proteins are often in place. She mentions that the pig has about 10 genes encoding cathelicidins, while humans only have one gene, the CAMP gene encoding LL-37. One of the places LL-37 is present is in the surface epithelia of the intestine where it forms a protective layer. It has also been shown that LL-37 can act pro-inflammatory.

Inducers

Agerberth mentions that the concept of their research is to work with molecules that can induce the production of AMPs. The inducers that are mentioned are butyrate, phenyl butyrate, vitamin D and lithocholic acid. Butyrate is an inducer of LL-37 and is produced by fermentation of dietary fibres in the intestine, where bacteria are present. Butyrate is also utilized by colonocytes and other bacteria in the intestine as a carbon source. Lithocholic acid is produced by bacteria that convert it from bile acid.
It has been shown that it is a connection between the bacterial flora and AMPs. Professor Hans Boman was in the early 90´s claiming that AMPs are important for the regulation of the intestinal flora. It has for example been showed that if one defensin gene from human is cloned into a mouse, its entire microflora is changing. This indicates the importance of AMPs in the composition of the micro- flora. Agerberth also mentions how vitamin D can induce LL-37.

Agerberth gives further details about her research concerning a disease caused by *Shigella* spp, which are responsible for diarrhoea. The disease is common in Bangladesh. Biopsies from patients, suffering from this disease have shown to have a reduced production of AMPs, which most likely facilitate the invasion of *Shigella* and hence more easily can have access to the body. Agerberth suggests that a signalling pathway is affected, resulting in a low expression of AMPs in the intestine. A rabbit model has shown that addition of butyrate will result in recovery of the infection. Agerberth further mentions that it is not suitable to orally administer AMPs because the peptides will be degraded in the gastrointestinal tract. She believes that inducers are the most promising alternative, when it comes to utilize AMPs as therapeutic strategy.

When butyrate was tested on patients in Bangladesh, the results were not as clear as expected. The research was then focused on finding a variant of butyrate, which now has become a registered pharmaceutical. Butyrate has also shown great synergistic

effect with vitamin D. Agerberth adds that one has to be careful when inducing AMP expression because of the possibility of the AMPs to cause inflammation.

CytaCoat

Agerberth is active in the company CytaCoat, which is focusing on antimicrobial surfaces. CytaCoat is a small company with only one employee. In the beginning, reactive AMPs were first attached to a polymer. However, a control polymer was found to be more active. This serendipity discovery has now been granted a patent.

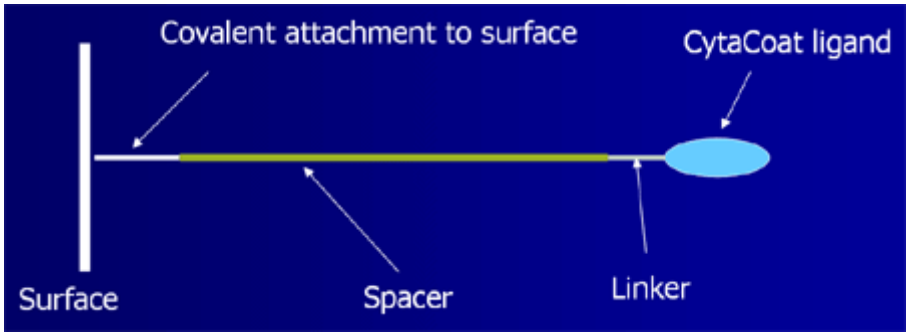


Figure (A) 1. A descriptive picture of the antimicrobial ligand complex, developed by CytaCoat

Agerberth continues to mention that it is the construct of the linker and the CytaCoat ligand that shows antibacterial activity, the ligand alone shows no activity. The coating has been proven to have activity against a wide range of bacteria. The discovery has been tested on different surfaces such as polyethylene, silicon and PVC plastics. . The antimicrobial mechanism is yet not known, but Agerberth mentions that it is believed that the membrane of the bacteria is somehow shattered. Before the invention was patented, much effort was put on elucidating the mechanism. However the mechanism does not have to be known for a patent. Indications show that the CytaCoat ligand work mostly by a bacteriostatic mechanism. Agerberth further mentions that because the coating is covalent attached their solution of an antimicrobial surface is better than the use of silver coated materials, from which the antimicrobial agents are released. Agerberth mentions that the release of antimicrobial substances contributes to resistance in a higher degree than antimicrobial agents that are covalently attached. CytaCoat focuses on medical technology products. Agerberth mentions that although the coating could be used for several applications, CytaCoat is a small company and therefore needs to focus on a certain application. For now CytaCoat works with endotracheal tubes for children. Endotracheal tubes are used outside of the epithelia; therefore fewer rules are involved than when a product is used in the bloodstream. Agerberth adds that for the use in endotracheal tubes, CytaCoat could be able to manufacture the coating by them and there after perform a small pilot clinical study. Private investors have helped CytaCoat financially and CytaCoat was also granted 500 000 SEK from Vinnova. The

private investors also had ideas to use the coating on credit cards. However, Agerberth mentions again that they already have created their niche. She also mentions that it is important for them that the coating is working in a “real” environment.

Agerberth continues to mention that they have been in contact with the neonatal unit at Karolinska University Hospital to see if the coating could be used in incubators for children born preterm. The surfaces used in incubators however, consist of different plastic materials and CytaCoat do not have the resources to develop the technology for so many substrates. In general Agerberth sees the use of the CytaCoat technology instead of an AMP as a better and cheaper solution for an antimicrobial surface. She adds that the CytaCoat ligand is much cheaper than using peptides that are expensive to synthesize. Agerberth was asked if the coating could be used in hygiene products. She mentions that it could work well but that further development work is needed in collaboration with a larger company.

AMPs in materials

Agerberth starts to mention that when peptides are placed with a high density on a surface, it is difficult for the peptides to acquire the right amphipathic structure that are needed for antimicrobial activity. She adds that she doesn’t know if this applies to all kinds of peptides. Agerberth continues with mentioning that immobilized peptides in general are more bacteriostatic because there is no release of the peptide from the surface. . This can be put in contrast to silver coated materials from which the active substances are released. This contributes to an increase in the problem of resistance.
Agerberth mentions that to date they have no research regarding AMPs used in materials since the CytoCoat technology seems to be a better solution.

Inducers in Hygiene products

Agerberth was asked if inducers could be appropriate to be used in hygiene products. Agerberth answers that she thinks it is difficult to use inducers in hygiene products. She sees a problem in how the inducers are supposed to enter the body, which is needed for any induction. When asked if it could be appropriate to use inducers in lotions she mentions that they have not worked so much with the skin. Agerberth continues by mentioning that the skin is producing some peptides in quite high amount and that peptide production is induced by wounds. Agerberth therefore speculates if inducers could be used in some kind of wound care.

A.2 Interview with Peter Bergman

MD, PhD at Karolinska Institute - Department of Laboratory Medicine

Research
Bergman points out that there are different directions in AMP research and development. A lot of the research focuses around peptides which are naturally occurring. The aim is to create prototypes or analogues of these peptides, which could then be synthetized and used as pharmaceuticals. However, it is complicated and costly to synthesise these AMPs. Another direction is to induce the peptide production in the body, through certain substances. Bergman has been working mostly with this area and with different inducers such as vitamin D. The development of using peptides as drugs has reached a higher level, it has been demonstrated that the peptides are not just antimicrobial but also immunomodulatory. It is even possible to dissect which parts of the peptides that are responsible for the specific effects. This knowledge makes it possible to design peptides that are either immunomodulatory or antimicrobial or both. Some even claim that the peptides mostly influence the immune system and the antimicrobial activity is only a secondary effect.

Use in Hygiene products and challenges
Bergman has a background within medical care and says that there is a great need for antimicrobial products in various healthcare applications. He mentions that nosocomial infection is a major and costly problem. There are many companies that focus on antimicrobial silver surfaces, but Bergman was not sure how far the research of AMPs coupled to surfaces has reached. There are many applications for antimicrobial surfaces, but it would be difficult and costly to use peptides for this purpose. Bergman continues by explaining challenges such as the environment that peptides normally function in and also the issue of bacterial resistance. Bergman is not positive in overusing antimicrobial substances, and he underscores the importance of a restricted use of antimicrobial substances in general, since bacteria easily may develop resistance. If the amount of antimicrobial substances in nature rises, so will the selection pressure. For example, if AMPs were to be used in toilet papers, rapid resistance would probably Emerge. You must therefore always be careful and specific when dealing with antimicrobial substances.

In the body, AMPs are found in white blood cells, and as many as 30 different antimicrobial peptides and proteins can be found in one blood cell. When these are released at the same time, microbes are exposed to peptides with many different mechanisms of action. This multi-therapy is making it difficult for the microbes to develop resistance. The multi therapy would not happen if a single peptide with a single mechanism is used as a drug, thus the risk for development of resistance would be dramatically increased.

Practical input

The inhibition zone assay is a familiar method to Bergman. He mentions some challenges with this assay, such as the fact that different peptides might diffuse differently due to different hydrophobicity, size or charge. This will affect the zone size. A more specific determination of MIC is done with broth dilution assay. Inhibition zone assay is mostly use as a screening method, since it is very fast. Another important limitation is that there is no standard protocol for inhibition zone assays; MIC values that are obtained in one lab may differ significantly from those obtained in other laboratories. Bergman recommended using a standard antibiotic as a positive control in our assay.

A.3 Interview with Martin Malmsten

Professor at Uppsala University - department of Pharmaceutical Physical Chemistry

Research

Professor Martin Malmsten and the research group at Uppsala University mainly investigate biophysical properties of AMPs. With their research they have been able to see effects of single amino acid modifications to further improve efficiency and selectivity between bacterial and eukaryotic cells. Their work has also been focusing on studying different mechanism of action. Martin Malmstens research has been published in high profile journals, which has resulted in a number of patent applications, and in the development of some of these peptides towards therapeutic applications through two start-up companies.

Applications

One of the companies that Malmsten started was DermaGen, which is a company focusing on AMPs in topical substances for use against atopic dermatitis and external otitis. The company was founded in 2004; however Malmsten is no longer an owner, DermaGen was sold Pergamum about a year ago. Through DermaGen and Malmsten, one peptide has successfully undergone Phase I/IIa clinical trial. This peptide was for use against atopic dermatitis, and recently a phase 2 study has been initiated on a peptide against external otitis. Atopic dermatitis and external otitis are two condition of high interest since they are both growing problems worldwide. Atopic dermatitis is a common condition during the winter half year, and the current treatments are antibiotics and steroids. Malmsten pointed out that it is not good to use antibiotics and steroids for such a long period. AMPs against atopic dermatitis are of interest since the patients have a lower production of AMPs, and therefore it is appropriate to add AMPs to these areas. Malmsten believes that adding AMPs is the simple and straight forward way, compared to inducing the peptides with other substances. When Malmsten was active in the company he pointed out that investors showed great interest in their products.

Challenges

Past research have led to a good understanding of peptide design, selectivity, and how to get low toxicity and keep the peptide activity. At the moment much research is focusing on the resistance issue. Even though the peptides are not so prone for resistance, the discussion should be more detailed and more research is needed in this area. Malmsten mentions that peptides could be designed with different stabilities, such that it takes a couple of generations for the bacteria to develop resistance.

Another challenge with AMPs is the cost issue. AMPs partly compete against cheap products that are used regularly; there it is difficult to motivate use for peptides. However, when the conditions are more severe and the cost is not the main issue, AMPs have a better potential. Last challenge that was discussed was the uptake of peptides at certain infections. It is thought that peptides might break down and thus lose its activity.

Use in Hygiene Products

When the question was asked regarding use in hygiene products, the main challenge was the cost. Malmsten speculated if there are niched hygiene products which could be more expensive. If the concept is right, he thought that there is absolutely a potential. There were also some discussions regarding the possibilities to bind peptides chemically to different surfaces and the potential use of antimicrobial surfaces. Another concern was the AMPs selectivity between microbes, which is not well understood. This might be an issue in hygiene products, where you want the normal flora to be intact.

Practical input

Questions regarding what type of experiments, peptides and other practical details were asked before starting the experiments. Malmsten agreed that diffusion assay method was a good starting point when investigating the peptides antimicrobial activity. But he also pointed out that some peptides might be more hydrophobic and longer, which in turn could affect the results. The peptides diffuse differently in the gel. If you suspect these effects there are other methods like viable count.

The peptides we order were as good as any he said, except Histatin, which might be more complicated. The data of Histatin varies more and are more pH sensitive. Histatin is more potent at low pH, and might be of interest in urogenital products. Histatin can also bind to zinc, which boosts the effect.

A.4 Interview with Sigridur Olafsdottir

CEO of Akthelia (Reykjavik, Iceland)

Akthelia and its products

Sigridur Olafsdottir is the CEO of the company Akthelia which develops new products for the treatment of broad range infections. Olafsdottir starts with mentioning that the products of Akthelia comprise small, organic molecules that stimulate and restore the expression of AMPs on epithelial surfaces and in phagocytic blood cells. However, the company has no products in the market yet. Olafsdottir continues to mention that they have shown in animal models that stimulating the expression of AMPs can clear infections in the GI tract and in other organs. She mentions that products that solve the same problem comprise multiple antibiotics available on the market. She mentions that antibiotics however have two main problems. They are normally narrow spectrum, and microbes have developed resistance to all known antibiotics. Furthermore Olafsdottir mentions that their products hold promise of being different from the conventional treatments in both of these aspects which makes their products unique.

The interest for Akthelia

Olafsdottir mention that she feels that it is a growing interest to their concept and that it is reflected in the increase in the reference to their therapeutic concept in the scientific literature. She underscores that their proposed treatments are fundamentally different from all other treatments used in the infectious disease area. She continues to mention that the company currently have no human efficacy data and that investors are hesitant to participate in the project at this stage. The investors tells them to come back to talk to them when they have clinical data.

The future and challenges for Akthelia

Olofsdottir comment that Akthelia plans to license specific indications to other pharmaceutical companies that possess the capacity to support Phase III clinical trials and have marketing departments as well as sales force. Akthelia will continue developing new treatments for infectious disease through Phase II based on the core concept of the company. She further mentions that current challenges include seeking funding for advancement of Akthelia's clinical programme. Because the concept of Akthelia is entirely research based clinical research is necessary for the advancement of their products. The financial support are coming from investors and grants from public funds to the academic founders which have allowed the company to advance.

The use of AMPs in products

When asked about the use of AMPs in hygiene products she mentions that in general it does not seem to be a good idea to use single molecule antibiotics for pharmaceutical or disinfectant purposes. This also applies to the use of single antimicrobial peptides. Due to the selection pressure and the fact that single mutations can generate antibiotic resistance, microbes will always find a way to develop resistance. She also mentions that using human antimicrobial peptides in medical treatments of human infections could potentially weaken the innate defences of patients and generate microbial strains that would be immune to the normal defence mechanisms with potentially very serious consequences. The same applies to the use of AMPs from other animals, which may weaken the defences of animals and other multi-cellular organisms, including plants. Olafsdottir underscores that Atkhelia's anti-infective treatment works in a way that induces multiple innate antimicrobial peptides which work via multiple mechanisms and thus rendering it impossible for the microbes to develop resistance.

A.5 Interview with Andreas Cederlund

PhD at Karolinska Institute - Department of Medical Biochemistry and Biophysics

Andreas Cederlund is a PhD Student in the research group of Birgitta Agerberth (for research information see Agerberths interview). Besides the interview, Cederlund has been providing information about the practical experiments and other important inputs throughout the thesis work.

Field of AMPs

First of all, the different aspects of using AMPs in commercial products were discussed. Cederlund mentions that if synthesized AMPs were to be used in a product, short AMPs could be desirable, because of production cost of a synthetic is proportional to its length. He further mentions that if a molecule with antimicrobial properties attached to a surface is sought, a peptide may be a good choice. Cederlund further discusses how surfaces with covalently linked AMPs would not be suitable for reusable products. Since reusable products covered with an antimicrobial surface may be soiled and covered with layers of for example proteins, dirt and fat that microbes can colonize, which is counterproductive to its intended use.

Practical concerns

Cederlund continues by mentioning some practical concerns regarding the method inhibition zone assay. He starts to comment that it is important to have a standard growth medium in order to compare the results. It has not been any standard developed for the method, which makes it difficult to compare MIC values between different studies. Cederlund recommends using a positive control for the microbe used. By using a defined concentration of a standard antibiotic in all experiments it will then be possible to compare the results between experiments run on different occasions. Cederlund also point out that it is important to not use a thick carpet of microorganisms in the plates, as it may lead to a too small inhibition zone diameter, which is explained by an increased ratio between the cells and peptides. This would result in lowered number of cell death. He therefore suggested to some calibration experiments with chosen microorganism in order to find the right cell density. When Cedelund was asked about the pH-effect, he points out that the pH in the gel doesn't necessarily have to be the same at the bacterial membrane surface. This means that even though the gel has a neutral pH, which some peptides are not active in, another lower pH might be present at the microbe membrane surface. Also the pH of the peptide solvent, which is low, will be buffered out once the peptide starts diffusing into the gel.

Appendix B – Materials and Method

The method that was used for testing the antimicrobial activity was inhibition zone assay. In the following section it will be described how the experiments were conducted.

B.1 Materials

Table (A) 1. List over materials used in the practical part

Chemical	**Product no.**	**Company**
Tryptone	T7293	Sigma-Aldrich (Sweden, Stockholm)
Yeast extract	No data	No data
NaCl – sodium chloride	No data	No data
Saboraud (SAB) medium	No data	Department of clinical microbiology, Sahlgrenska university hospital (Sweden, Gothenburg)
SAB-agar	No data	Department of clinical microbiology, Sahlgrenska university hospital (Sweden, Gothenburg)
Tryptic-soy agar (TSA)	No data	Department of clinical microbiology, Sahlgrenska university hospital (Sweden, Gothenburg)
Trifluoroacetic acid (TFA) (0.1%)	No data	No data
Agarose, Type 1, low EEO	A6013	Sigma-Aldrich (Sweden, Stockholm)
Nisin from *Lactococcus lactis* (2.5%)*	N5764	Sigma-Aldrich (Sweden, Stockholm)
Lactoferricin B (>95%)	1SP-LAFB	Innovagen (Sweden, Lund)
Magainin 2 (>95%)	SP-MG2	Innovagen (Sweden, Lund)
Histatin-5 (>95%)	SP-HST5	Innovagen (Sweden, Lund)
Buforin-II (>95%)	SP-5231	Innovagen (Sweden, Lund)
AquaStabil	8940006	Julabo (Germany, Seelbach)
Nystatin	No data	Department of Medical Biochemistry and Biophysics, Karolinska institute (Sweden, Stockholm)

*Assume Nisaplin® – which contains 2,5% nisin (sodium chloride and denatured milk solids)

Peptide sequences

Table (A) 2. Peptide amino-acid sequences

Peptide	Sequence	Length
Nisin A	ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK	34
Lactoferricin B	RRWQWRMKKLG	11
Histatin-5	DSHAKRHHGYKRKFHEKHHSHRGY	24
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	23
Bufoirn-II	TRSSRAGLQFPVGRVHRLLRK	21

Microorganisms

Table (A) 3. List over microorganism strains used in the practical part

Notation	Strain
E.coli (1)	*E.coli* (ATCC 10536. Class 2*)
E.coli (2)	*E.coli* (ATCC 8739. Class 2*)
S.aureus (1)	*S.aureus* (ATCC 6538. Class 2*)
S.aureus (2)	*S.aureus III* (ESSUM. Class 2*)
C.albicans (1)	*C.albicans* (ESSUM. Class 2*)
C.albicans (1)	*C.albicans* (ESSUM. Class 2*)

*All strains were provided by SCA

Materials

- Petridishes 90X15 mm
- Eppendorf tubes 1,5 ml
- Falcon tubes 15, 50 ml
- Gelpuncher (6mm)
- Water bath
- Incubator 35°C
- Sterile polystyrene loops
- Mixer, vortex

B.2 Method (protocols)

Preparation of Medium

The LB-medium (1 litre) was prepared by mixing tryptone (10 g), yeast extract (5 g) and NaCl (10, 5 or 0,5 g) (depending on what LB) in a flask with deionized water (800 ml). The mixture was then autoclaved at 121°C. The SAB-medium was provided by the department of clinical microbiology at Sahlgrenska university hospital and contains peptone, glucose and water.The growth mediums were then stored in the fridge.

Preparation of gel

Low EEO agarose gel (100 ml) was prepared by mixing agarose (1 g) with a growth medium (SAB or LB) (100 ml) and then autoclave. 6 ml of the liquid gel was then evenly distributed in falcon tubes and stored in the fridge.

Calibration experiments

Since there is no standard protocol for inhibition zone assay, initially it is important to find the right concentration of microorganism and peptides that will be incorporated to the agarose gel.

In order to receive a proper growth, and thus detect the zones, different concentration of *E.coli (1), S.aureus (1)* and *C.albicans (1)* were grown and compared. The following concentrations were tested, 10^9, 10^8,10^7 CFU, for each microorganism. These concentrations were acquired by letting the microorganism grow to 10^9 CFU (overnight in growth medium) and then make a ten-fold serial dilution (10^9, 10^8,10^7, 10^6, 10^5,10^4, 10^3). To verify that the cell-density was correct, 0.1ml 10^3 CFU microorganisms was spread on TSA plate and after incubation the cells were counted. The plates were then casted as described below (see casting the plates). After solidification the plates were incubated in 35°C overnight. The plates were then examined based on the density of colonies.

Casting the plates

The plates were created in the following way: The solid gels (agarose + medium) (6 ml) were heated in water bath to 100°C and then cooled down to 46°C. The microorganisms (50 µl) were then added to the gels (in falcon tubes). The gels with microorganisms were swiftly vortex and then poured into petri-dishes. The gel was then allowed to solidify in room temperature for 1h.

Antimicrobial activity experiment

All the peptides were freeze dried, in powder form, (1 mg) and were diluted with 0.01% TFA (1 ml) to create a stock solution (1mg/ml). All peptides, except Nisin, were diluted to a stock concentration of 1mg/ml. Nisin, in 2.5% form (Nisaplin), was diluted to 0,25mg/ml. The peptides were two-fold serial diluted to acquire stock solutions of:

1000, 500, 250, and 125 µg/ml (Nisin: 250, 125, 62,5, 31,25 µg/ml).The gels were prepared as described above (see "casting the plates"). After solidification 6mm wells were punched in the gel and peptides (12 µl) were then added to each well. The peptides were then allowed to completely diffuse (no liquid in the wells) in the gel in RT. The plates are then incubated in 35°C overnight. The zones of inhibition are then measured.
In the first experiment we tested all peptides against *E.coli* (1) and *S.aureus* (2). The microorganism concentration was 10^8 CFU/ml and we used LB-Lennox medium, which has a NaCl concentration of 1%. *C.albicans* (1) was also tested but 10^7 CFU/ml was used and SAB-medium (contains no NaCl).

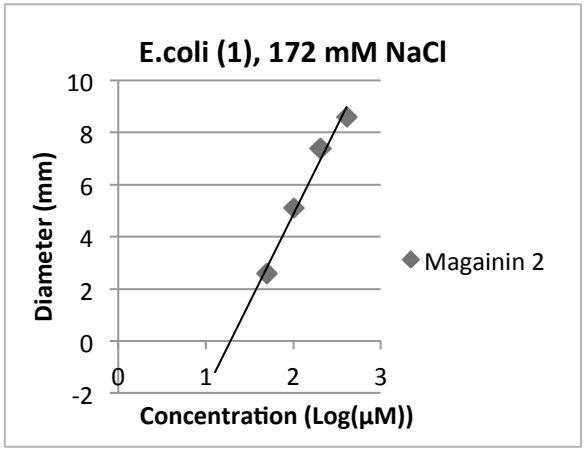
Salt- sensitivity experiments
E.coli (1) and *S.aureus* (1) was the microorganisms tested. In this experiment different types of LB-media were used in the gel. These were LB-Luria with a NaCl concentration of 0.5% and LB-Miller with a NaCl concentration of 0.05%. Otherwise the procedures are the same as above (see "antimicrobial activity experiment).

Strain-dependency experiment
In this experiment magainin 2, nisin and histatin were used against E.coli (2), S.aureus (2) and C.albicans (2), respectively. The procedures were the same as described in "Antimicrobial activity experiment".

Notes
In all experimental parts both a positive control and a negative control was used. The positive control was AquaStabil (12 µl/well) for bacteria and Nystatin (20 µg/ml, 12µl/well) for fungi. TFA (12 µl/well) was the negative control for both fungi and bacteria.

B.3 Calculation of MIC-values

Example, magainin 2 172mM *E.coli* (1)



1. Linear regression results in following equation:

$$y = 6{,}77x - 8{,}66$$

2. The MIC-value is obtained when y(diameter) = 0, which results in:

$$0 = 6{,}77x - 8{,}66 => x = \frac{8{,}66}{6{,}77} = 1{,}28\log(\mu M) = 19{,}05\mu M$$

3. To obtain the MIC-value in the more standard format (µg/ml), following calculation was conducted:

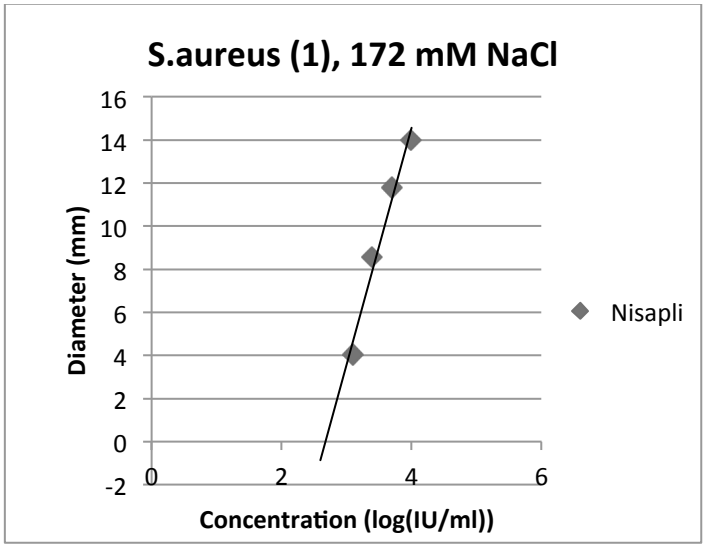
Molar mass of magainin 2 = 2466,93 g/mol

$$\frac{19{,}05 * 10^{-6} mol\ l^{-1} * 2466{,}93 g\ mol^{-1}}{1000} = 47{,}0\ \mu g\ ml^{-1}$$

The identical steps were taken when calculating the MIC for lactoferricin B, histatin-5 and buforin-II.

The calculation of MIC for nisin and Nisaplin was slightly different (see below).

Example, Nisaplin 172mM Nacl, S.aureus (1)



1. Linear regression results in following equation:

$$y = 11{,}01x - 29{,}42$$

2. The MIC-value is obtained when y(diameter) = 0, which results in:

$$0 = 11{,}01x - 29{,}42 => x = \frac{29{,}42}{11{,}01} = 2{,}67 \log(IU\ ml^{-1}) = 467{,}73\ IU\ ml^{-1}$$

3. This MIC-value unit (IU/ml) is the standard unit to express Nisaplins antimicrobial activity

Example, nisin 172mM Nacl, S.aureus (1)

The MIC-value of nisin is derived from the Nisaplin experiments according to the following steps:

Data:

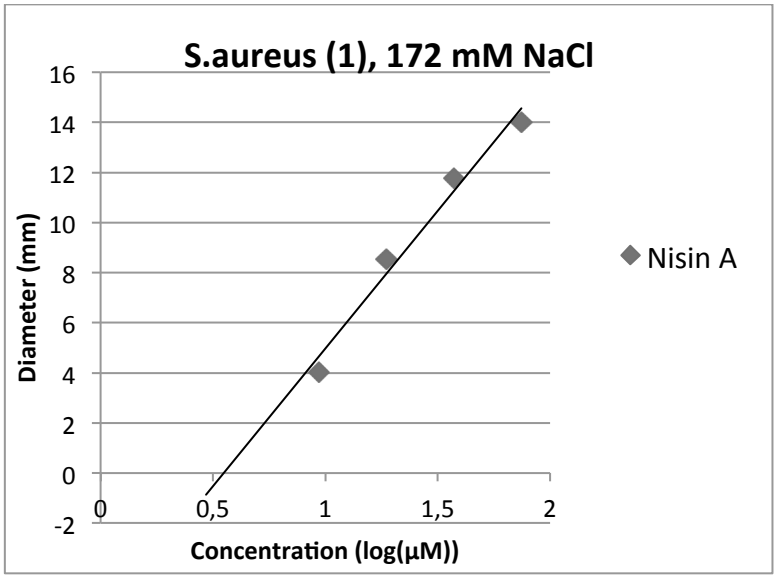
1g Nisaplin = 1000000 IU
Nisaplin contains 2,5 %w/w nisin
Nisin molar mass = 3354,07

The following Nisaplin dilution series was used in the example, 10000, 5000, 2500, 1250 IU/ml Nisaplin. This was converted to 250, 125, 62,5, 31,125 µg/ml nisin according to the following calculation:

Example, conversion of 10000 IU/ml Nisaplin to 250µg/ml nisin:

$$0{,}025 * 10000\ IU\ ml^{-1}\ Nisaplin = 250\ \mu g\ ml^{-1}\ nisin$$

The same calculation was made for the whole dilution series; it was then plotted and gave following graph:



The same steps as for the example for magainin 2 was taken to obtain the MIC-value of 11,84 µg/ml.

Appendix C – Laboratory Results

C.1 Antimicrobial activity experiment

C.1.1 Graphs

C.1.1.1 *E.coli* (1) 172 mM NaCl

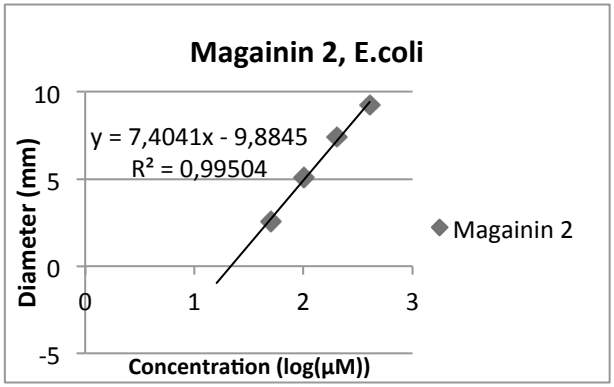


Figure (A) 3. Magainin 2, E.coli(1), 172 mM NaCl

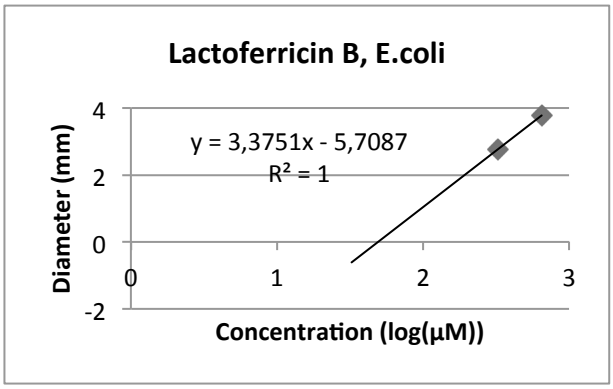


Figure (A) 4. Lactoferricin B, E.coli (1), 172 mM NaCl

C.1.1.2 S.aureus (1) 172 mM NaCl,

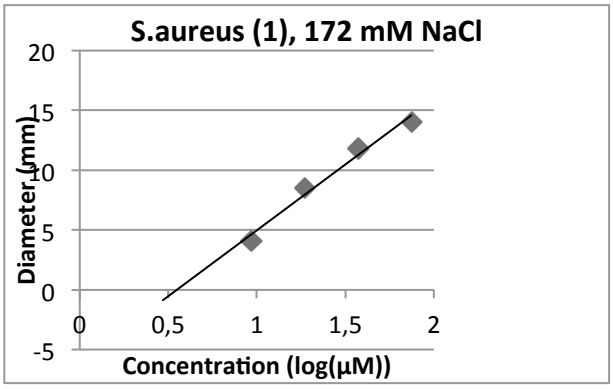


Figure (A) 5. Nisin, S.aureus (1), 172 mM NaCl

C.1.1.3 C.albicans (1) 0 mM NaCl

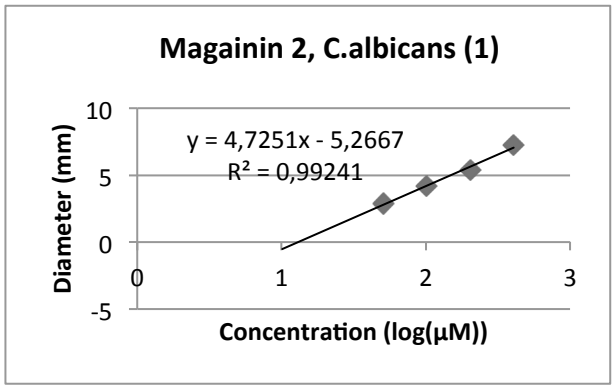


Figure (A) 6. Magainin 2, C.albicans (1) 0 mM NaCl

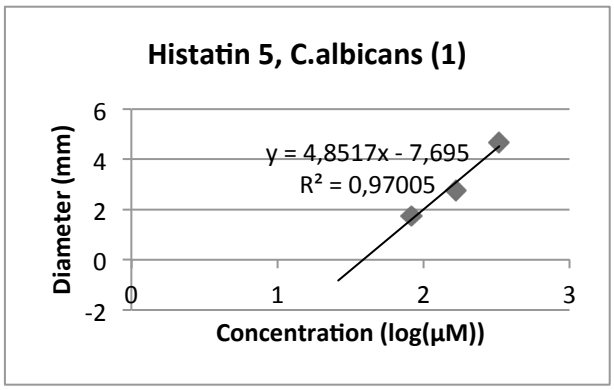


Figure (A) 7. Histatin-5, C.albicans (1) 0 mM NaCl

C.1.2 Images of inhibition zones

C.1.2.1 *E.coli* (1) 172 mM NaCl

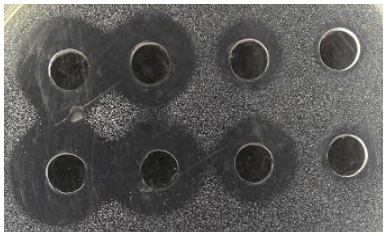


Figure (A). 8 Magainin 2, E.coli (1)

Figure (A) 9. Nisin, E.coli (1)

Figure (A).10 Histatin-5, E.coli (1)

Figure (A) 11. Lactoferricin B, E.coli (1)

Figure (A) 13. Buforin II, E.coli (1)

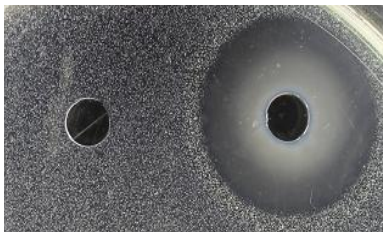


Figure (A) 14. Control E.coli (1)– TFA and AquaStabil

C.1.2.2 *S.aureus* (1) 172 mM NaCl

Figure (A) 15. Magainin 2, S.aureus (1)

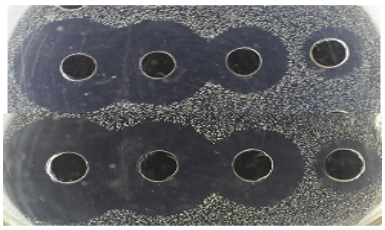


Figure (A) 16. S.aureus (1) 172mM NaCl Nisin

Figure (A) 18. Histatin-5, S.aureus (1)

Figure (A) 19. Lactoferricin B, S.aureus (1)

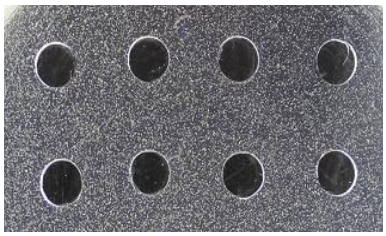


Figure (A) 21. Buforin II, S.aureus (1)

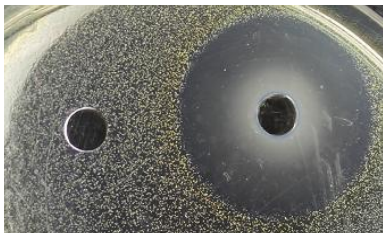


Figure (A) 23.Control S.aureus (1), TFA and AquaStabil

C.1.2.3 *C.albicans* (1) 0 mM NaCl

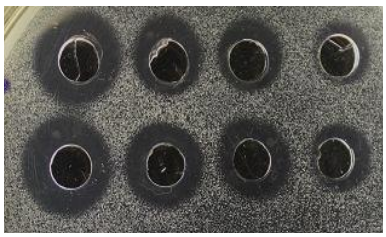


Figure (A) 25. Magainin 2 C.albicans (1)

Figure (A) 27. Nisin C.albicans

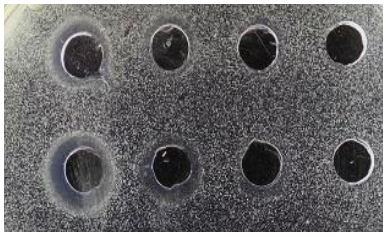


Figure (A) 28. Histatin-5, C.albicans (1)

Figure (A) 29. Figure (A) 30. Lactoferricin B C.albicans (1)

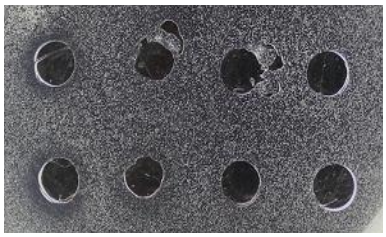


Figure (A) 33. Buforin II, C.albicans (1)

Figure (A) 35.Positive control, Nystatin, C.albicans (1)

C.2 Salt-sensitivity experiment

C.2.1 Graphs

C.2.1.1 E.coli (1)

C.2.1.1.1 86 mMNaCl

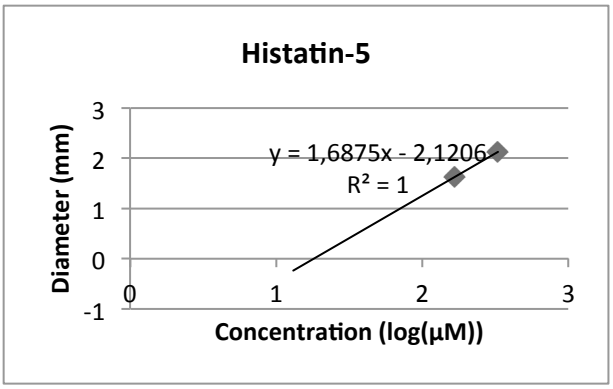


Figure (A) 36. Histatin-5, E.coli (1) 86 mM NaCl

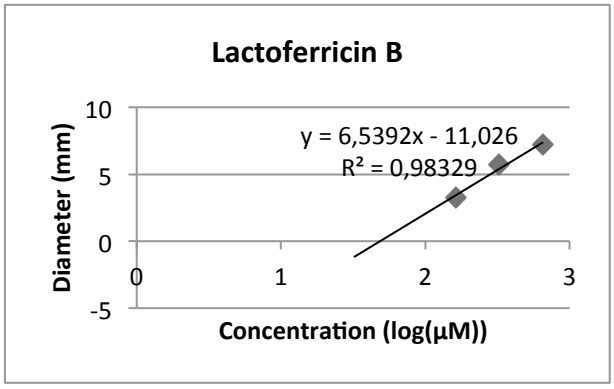


Figure (A) 37. Lactoferricin B, E.coli (1) 86 mM NaCl

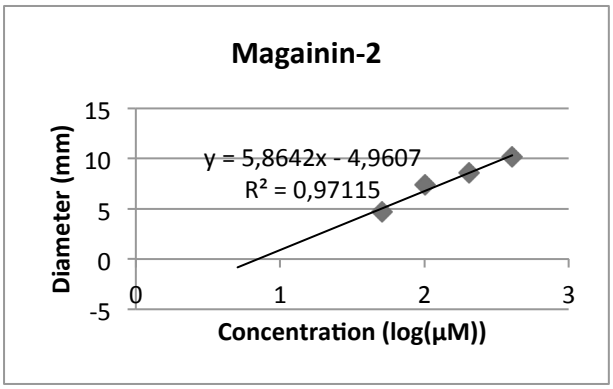


Figure (A) 38. Magainin-2, E.coli (1) 86 mM NaCl

C.2.1.1.2 8,6 mMNaCl

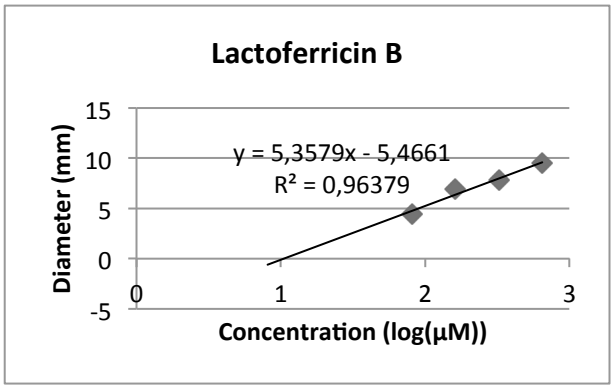


Figure (A) 39. Lactoferricin B, 8,6 mM NaCl, E.coli (1)

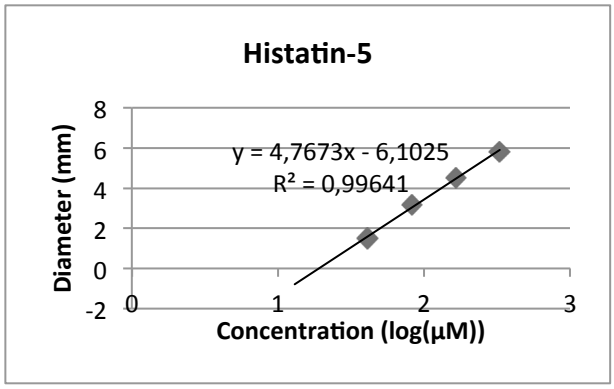


Figure (A) 40. Histatin-5, 8,6 mM NaCl, E.coli (1)

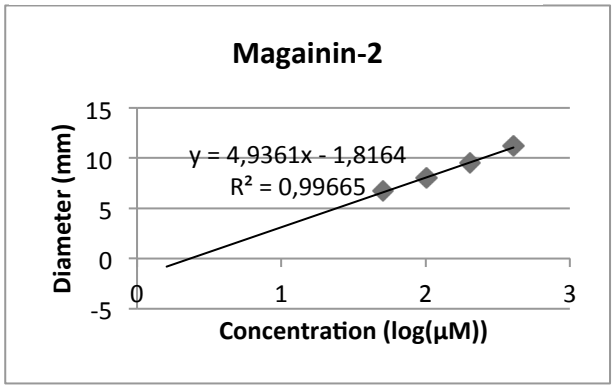


Figure (A) 41. Magainin-2, 8,6 mM NaCl, E.coli (1)

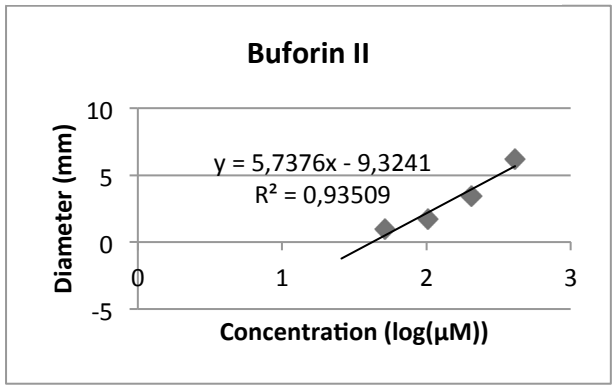


Figure (A) 42. Buforin II, 8,6 mM NaCl, E.coli (1)

C.2.1.2 S.aureus (1)

C.2.1.2.1 86 mM NaCl

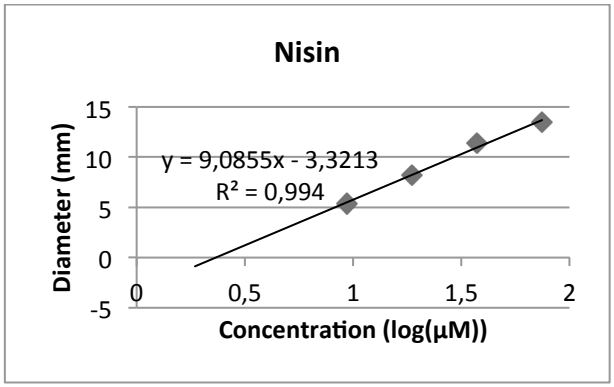


Figure (A) 43. Nisin, S.aureus (1) 86 mM NaCl

C.2.1.2.2 8,6 mM NaCl

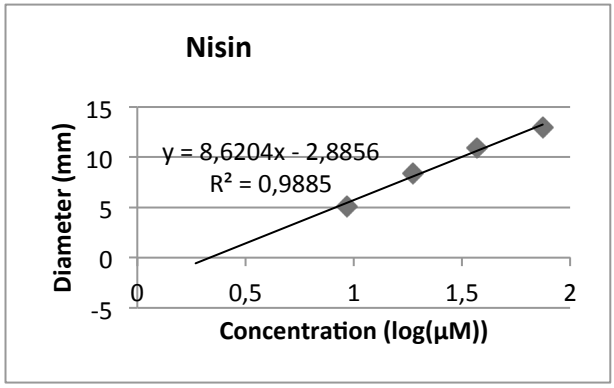


Figure (A) 44. Nisin, S.aureus (1), 8,6 mM NaCl

C.2.2 Images of inhibition zones

C.2.2.1 *E.coli* (1) 86 mM NaCl

Figure (A) 45. Magainin 2, E.coli (1)

Figure (A) 46. Nisin, E.coli (1)

Figure (A) 47. Histatin, E.coli (1)

Figure (A) 48. Lactoferricin, E.coli (1)

Figure (A) 49. Buforin, E.coli (1)

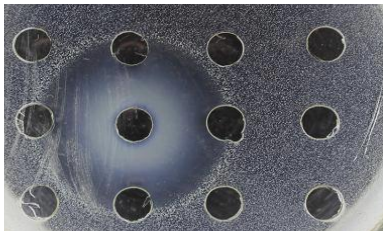


Figure (A) 50. Positive control E.coli (1)

C.2.2.2 *E.coli* (1) 8,6 mM NaCl

Figure (A) 51. Magainin 2, E.coli (1)

Figure (A) 53. Nisin, E.coli (1)

Figure (A) 54. Histatin-5, E.coli (1)

Figure (A) 55. Lactoferricin B, E.coli (1)

Figure (A) 56. Buforin II, E.coli (1)

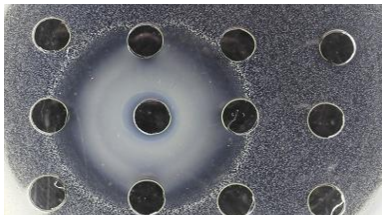


Figure (A) 57. Positive control, AquaStabil, E.coli (1)

C.2.2.3 *S.aureus* (1) 86 mM NaCl

Figure (A) 59. Magainin S.aureus (1)

Figure (A) 58. Nisin S.aureus (1)

Figure (A) 60. Histatin-5, S.aureus (1)

Figure (A) 61. Lactoferricin B S.aureus (1)

Figure (A) 63. Buforin II S.aureus (1)

Figure (A) 62. Positive control, AquaStabil, S.aureus (1)

C.2.2.4 *S.aureus* (1) 8,6 mM NaCl

Figure (A) 65. Magainin 2, S.aureus (1)

Figure (A) 64. Nisin, S.aureus (1)

Figure (A) 66. Histatin-5, S.aureus (1)

Figure (A) 67. Lactoferricin B, S.aureus (1)

Figure (A) 69. Buforin II, S.aureus (1)

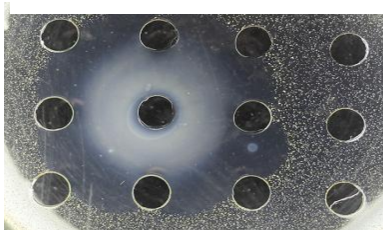


Figure (A) 68.Positive control, AquaStabil, S.aureus (1)

C.3 Strain-comparison experiments

C.3.1 Graphs

C.3.1.1 Nisin, S.aureus (2) (172, 86, 8,6 mM NaCl)

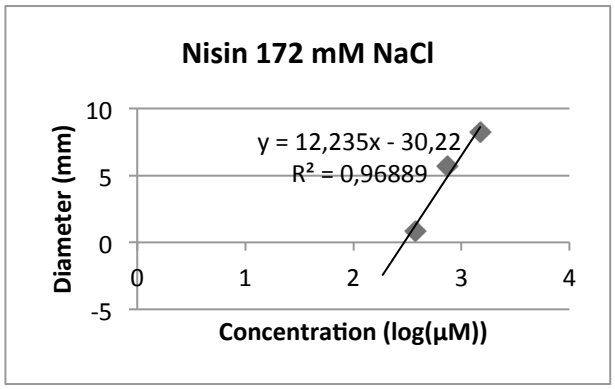


Figure (A) 70. Nisin, S.aureus (2), 172 mM

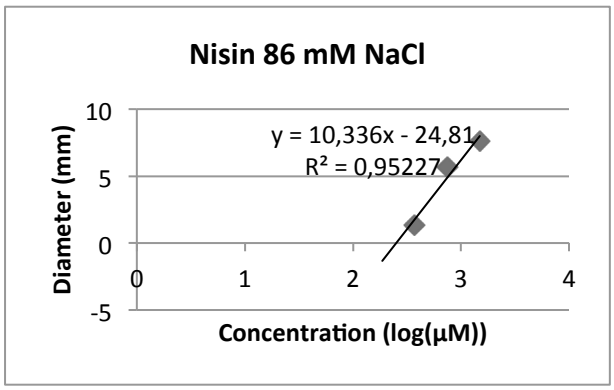


Figure (A) 71. Nisin, S.aureus (2), 86 mM

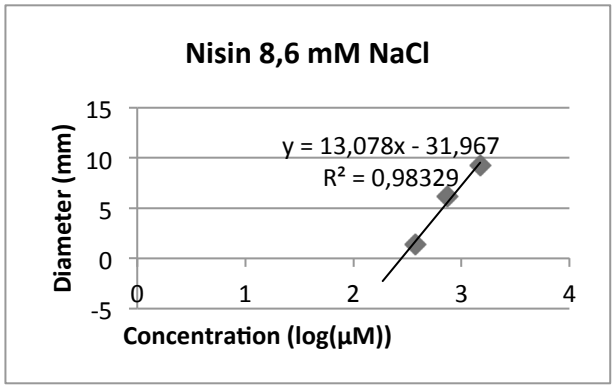


Figure (A) 72. Nisin, S.aureus (2), 8,6 mM

C.3.1.2 Magainin 2, E.coli (2) (172, 86, 8,6 mM NaCl)

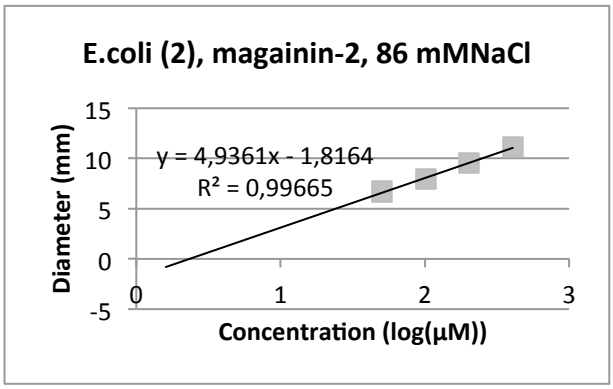


Figure (A) 73. Magainin-2, E.coli (2), 86 mM NaCl

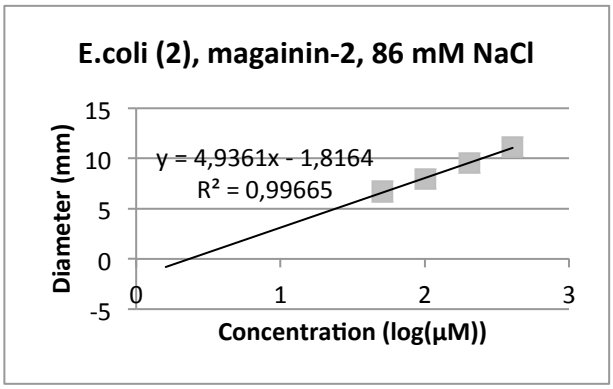


Figure (A) 74. Magainin-2, E.coli (2), 86 mM NaCl

C.3.1.3 Histatin-5, C.albicans (2) 0mM Nacl

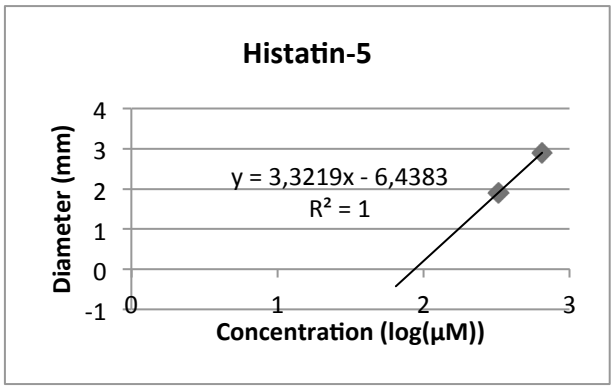


Figure (A) 75. Histatin-5, C.albicans (2), 0 mM NaCl

C.3.2 Images of inhibition zones

C.3.2.1 Magainin 2, *E.coli* (2) 172; 86; 8,6 mM NaCl

Figure (A) 76. Magainin 2, E.coli (2) 172mM NaCl

Figure (A) 77. Magainin 2, E.coli (2), 86 mM NaCl

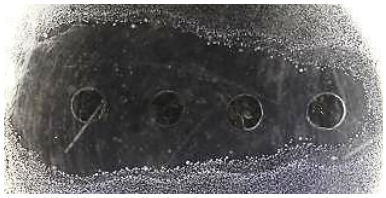


Figure (A) 78. Magainin 2, E.coli (2), 8,6 mM NaCl

C.3.2.2 *Nisin, S.aureus* (2) 172; 86; 8,6 mM NaCl

Figure (A) 79. Nisin, S.aureus (2) 172mM NaCl

Figure (A) 80. Nisin, S.aureus (2)172mM NaCl

Figure (A) 81. Nisin, S.aureus (2) 172mM NaCl

C.3.2.3 *C.albicans* (2) 0 mM NaCl

Figure (A) 83. Nystatin C.albicans (2), 0mM NaCl

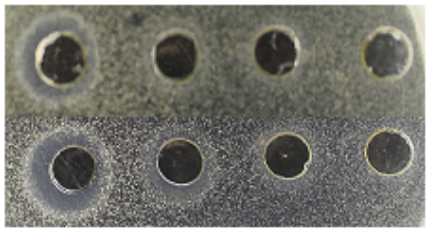


Figure (A) 84. Histatin-5, C.albicans (2), 0mM NaCl

Appendix D – Table of Data

D.1 Peptide concentrations and diameter of inhibition zones

Below are all the measurements of the inhibition zone assay. S.I stands for small indication of a zone.

D.1.1 E.coli (1)

Table (A) 4. Inhibition zone diameters of nisin against E.coli (1)

Nisin	Diameter of Inhibition Zones (mm)		
Peptide concentration (µM)	172mM NaCl	86mM NaCl	8,6mM NaCl
14,9	0	0	1,10
7,5	0	0	0
3,7	0	0	0
1,9	0	0	0

Table (A) 5. Inhibition zone diameters of Lactoferricin B against E.coli (1)

Lactoferricin B	Diameter of Inhibition Zones (mm)		
Peptide concentration (µM)	172mM NaCl	86mM NaCl	8,6mM NaCl
647,3	3,779	7,208	9,494
323,7	2,763	5,684	7,843
161,8	0	3,271	6,954
80,9	0	0	4,414

Table (A) 6. Inhibition zone diameters of buforin-II against E.coli (1)

Buforin-II	Diameter of Inhibition Zones (mm)		
Peptide concentration (µM)	172mM NaCl	86mM NaCl	8,6mM NaCl
410,7	0	0	6,192
205,35	0	0	3,398
102,67	0	0	1,747
51,337	0	0	0,985

Table (A) 7 Inhibition zone diameters of histatin-5 against E.coli (1)

Histatin-5	Diameter of Inhibition Zones (mm)		
Peptide concentration (µM)	172mM NaCl	86mM NaCl	8,6mM NaCl
329,34	0	2,128	5,811
164,67	0	1,62	4,541
82,336	0	0	3,144
41,168	0	0	1,493

Table (A) 8. Inhibition zone diameters of magainin 2 against E.coli (1)

Magainin 2	Diameter of Inhibition Zones (mm)		
Peptide concentration (µM)	172mM NaCl	86mM NaCl	8,6mM NaCl
405,36	9,24	10,129	11,145
202,68	7,3985	8,605	9,494
101,34	5,1125	7,335	7,97
50,67	2,5725	4,668	6,7

D.1.2 S.aureus (1)

Table (A) 9. Inhibition zone diameters of nisin against S.aureus (1)

Nisin	Diameter of Inhibition Zones (mm)		
Peptide concentration (µM)	**172mM NaCl**	**86mM NaCl**	**8,6mM NaCl**
74,53	14	13,45	12,95
37,27	11,8	11,35	10,95
18,63	8,55	8,15	8,4
9,32	4,05	5,4	5,15

Table (A) 10. Inhibition zone diameters of lactoferricin B against S.aureus (1)

Lactoferricin-B	Diameter of Inhibition Zones (mm)		
Peptide concentration (µM)	**172mM NaCl**	**86mM NaCl**	**8,6mM NaCl**
647,3	S.I	0	3,144
323,7	0	0	S.I
161,8	0	0	0
80,9	0	0	0

Table (A) 11. . Inhibition zone diameters of buforin-II against S.aureus (1)

Buforin-II	Diameter of Inhibition Zones (mm)		
Peptide concentration (µM)	**172mM NaCl**	**86mM NaCl**	**8,6mM NaCl**
410,7	0	0	0
205,35	0	0	0
102,67	0	0	0
51,337	0	0	0

Table (A) 12. . Inhibition zone diameters of histatin-5 against S.aureus (1)

Histatin-5	Diameter of Inhibition Zones (mm)		
Peptide concentration (µM)	**172mM NaCl**	**86mM NaCl**	**8,6mM NaCl**
329,34	0	0	0
164,67	0	0	0
82,336	0	0	0
41,168	0	0	0

Table (A) 13. . Inhibition zone diameters of magainin 2 against S.aureus (1)

Magainin 2	Diameter of Inhibition Zones (mm)		
Peptide concentration (µM)	**172mM NaCl**	**86mM NaCl**	**8,6mM NaCl**
405,36	0	0	0
202,68	0	0	0
101,34	0	0	0
50,67	0	0	0

D.1.3 C.albicans (1)

Table (A) 14. Inhibition zone diameters of nisin against C.albicans (1)

Nisin	**Diameter of Inhibition Zones (mm)**
Peptide concentration (µM)	**0 mM NaCl**
14,9	0
7,5	0
3,7	0
1,9	0

Table (A) 15. Inhibition zone diameters of lactoferricin B against C.albicans (1)

Lactoferricin B	**Diameter of Inhibition Zones (mm)**
Peptide concentration (µM)	**0 mM NaCl**
647,3	1,87
323,7	0
161,8	0
80,9	0

Table (A) 16. Inhibition zone diameters of buforin-II against C.albicans (1)

Buforin-II	**Diameter of Inhibition Zones (mm)**
Peptide concentration (µM)	**0 mM NaCl**
410,7	3,07
205,35	S.I
102,67	0
51,337	0

Table (A) 17. Inhibition zone diameters of histatin-5 against C.albicans (1)

Histatin-5	**Diameter of Inhibition Zones (mm)**
Peptide concentration (µM)	**0 mM NaCl**
329,34	4,668
164,67	2,763
82,336	1,747
41,168	0

Table (A) 18. Inhibition zone diameters of magainin 2 against C.albicans (1)

Magainin 2	**Diameter of Inhibition Zones (mm)**
Peptide concentration (µM)	**0 mM NaCl**
405,36	7,208
202,68	5,43
101,34	4,16
50,67	2,89

D.1.4 E.coli (2)

Table (A) 19. Inhibition zone diameters of magainin 2 against E.coli (2)

Magainin 2	Diameter of Inhibition Zones (mm)		
Peptide concentration (µM)	**172mM NaCl**	**86mM NaCl**	**8,6mM NaCl**
405,36	12,05	16,05	20,65
202,68	9,1	14,4	19,25
101,34	5,8	11,75	15,5
50,67	0	10,15	13,8

D.1.5 S.aureus (2)

Table (A) 20. Inhibition zone diameters of nisin against S.aureus (2)

Nisin	Diameter of Inhibition Zones (mm)		
Peptide concentration (µM)	**172mM NaCl**	**86mM NaCl**	**8,6mM NaCl**
37,26815	8,224	7,589	9,24
18,63408	5,684	5,684	6,192
9,317039	0,858	1,366	1,366
5,402392	0	0	0

D.1.6 C.albicans (2)

Table (A) 21. Inhibition zone diameters of histatin-5 against C.albicans (1)

Histatin-5	Diameter of Inhibition Zones (mm)
Peptide concentration (µM)	**0 mM NaCl**
329,34	2,9
164,67	1,9
82,336	0
41,168	0

D.2 Cell-density of microorganisms

Table (A) 22. Cell-density of all tested microorganisms

Strain	Cell-density (CFU/ml)
E.coli (1)	$8{,}8 * 10^8$
E.coli (2)	No data
S.aureus (1)	$4{,}9 * 10^8$
S.aureus (2)	$8{,}9 * 10^8$
C.albicans (1)	$4{,}0 * 10^7$
C.albicans (2)	$2{,}6 * 10^7$

D.3 Positive controls

Table (A) 23. Zone measurements of positive control (AquaStabil) against E.coli (1)

E.coli (1)	**(AquaStabil)**
NaCl concentration (mM NaCl)	**Zone size (positive control) (mm)**
172	22,86
86	27,67
8,6	31,90

Table (A) 24. Zone measurements of positive control (AquaStabil) against E.coli (2)

E.coli (2)	
NaCl concentration (mM NaCl)	**Zone size (positive control) (mm)**
172	No data
86	No data
8,6	No data

Table (A) 25. Zone measurements of positive control (AquaStabil) against S.aureus (1)

S.aureus (1)	**(AquaStabil)**
NaCl concentration (mM NaCl)	**Zone size (positive control) (mm)**
172	30,5
86	30,5
8,6	31,0

Table (A) 26. Zone measurements of positive control (AquaStabil) against S.aureus (2)

S.aureus (2)	
NaCl concentration (mM NaCl)	**Zone size (positive control) (mm)**
172	No data
86	No data
8,6	No data

Table (A) 27. Zone measurements of positive control (Nystatin) against C.albicans (1)

C.albicans (1)	**(Nystatin (20µg/ml))**
NaCl concentration (mM NaCl)	**Zone size (positive control) (mm)**
0	10,8

Table (A) 28. Zone measurements of positive control (Nystatin) against C.albicans (2)

C.albicans (2)	**(Nystatin 20µg/ml)**
NaCl concentration (mM NaCl)	**Zone size (positive control) (mm)**
0	11,25

D.4 Summary of MIC values

In the table below all MIC-values are shown. N.M stands for no MIC-calculation possible. This is due to that only one or no zone was detected for the peptide.

Table (A) 29. Summary of all MIC-values

Type (mM NaCl)	**µM/ml**	**µg/ml**
E.coli (1) 172 mM NaCl		
Nisin (Nisaplin)	N.M	N.M
Lactoferricin B	48,0	75,7
Buforin-II	N.M	N.M
Histatin-5		
Magainin 2	21,6	53,4
E.coli(1) 86 mM NaCl		
Nisin (Nisaplin)	N.M	N.M
Lactoferricin B	49,0	75,0
Buforin-II	N.M	N.M
Histatin-5	18,0	54,8
Magainin 2	7,0	17,0
E.coli (1) 8,6 mM NaCl		
Nisin (Nisaplin)	N.M	N.M
Lactoferricin B	10,5	16
Buforin-II	42,2	103,0
Histatin-5	19,0	57,9
Magainin 2	2,3	7,1
S.aureus (1), 172 mM NaCl		
Nisin (Nisaplin)	3,5 (473,4 IU/ml)	11,8
Lactoferricin B	N.M	N.M
Buforin-II	N.M	N.M
Histatin-5	N.M	N.M
Magainin 2	N.M	N.M
S.aureus (1); 86 mM NaCl		
Nisin (Nisaplin)	2,3 (311,3 IU/ml)	7,8
Lactoferricin B	N.M	N.M
Buforin-II	N.M	N.M
Histatin-5	N.M	N.M
Magainin 2	N.M	N.M

S.aureus (1); 8,6 mM NaCl		
Nisin (Nisaplin)	2,16 (290 IU/ml)	7,2
Lactoferricin B	N.M	N.M
Buforin-II	N.M	N.M
Histatin-5	N.M	N.M
Magainin 2	N.M	N.M
C.albicans (1) 0 mM		
Nisin (Nisaplin)	N.M	N.M
Lactoferricin B	N.M	N.M
Buforin-II	N.M	N.M
Histatin-5	38,6	117,0
Magainin 2	13,0	32,1
E.coli (2) 1%NaCl		
Magainin 2	27,6	68,2
E.coli (2) 0,5% NaCl		
Magainin 2	1,7	4,1
E.coli (2) 0,05% NaCl		
Magainin 2	1,0	2,5
S.aureus (2) 1% NaCl		
Nisin (Nisaplin)	7,4 (989,9 IU/ml)	24,8
S.aureus (2) 0,5% NaCl		
Nisin (Nisaplin)	6,3 (843,1 IU/ml)	21,1
S.aureus (2) 0,05% NaCl		
Nisin (Nisaplin)	7 (939 IU/ml)	23,5
C.albicans (2) 0 mM		
Histatin-5	86,7	263,3