ANTIMICROBIAL PEPTIDES AND THE INTERPLAY BETWEEN MICROBES AND HOST

Towards preventing porcine infections with *Streptococcus suis*

Rogier A. Gaiser

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Antimicrobial peptides and the interplay between microbes and host

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Rogier A. Gaiser

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Voor diegenen die ik liefheb

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CHAPTER 1

General Introduction

Antibiotic discovery and the emergence of antibiotic resistance

Antibacterial chemotherapy is an important component of modern medicine and it has drastically improved human health and life expectancy worldwide, bringing benefits to the individual and society as a whole [1]. Use of antibiotics in livestock and poultry, initially mainly for growth promotion but also to control endemic disease among increasingly larger groups of animals, has also revolutionized animal production agriculture [2]. This started with the discovery and development of sulphonamides and penicillin during the first half of the twentieth century, followed by the so called Golden Era of drug discovery, which accelerated the discovery of many novel classes of antibiotics [3] (Figure 1).

However, extensive (over- and mis-) use of antibacterial drugs over the past decades have favoured the selection and spread of resistant bacteria [4]. The high level of antibiotics utilized in the livestock and poultry industry is considered to have contributed to the global spread of antibiotic resistance [5]. As a consequence, there are plans to further reduce antibiotic use in livestock across Europe [6]. Nevertheless, resistance of clinically relevant bacteria to commonly used antibiotics combined with the current void in discovery and market approval of successful novel antibacterial drugs remains a global health emergency [7, 8]. The rising prevalence of antibiotic resistance in bacteria is a leading healthcare priority worldwide, with drug resistant pathogens causing increased morbidity, mortality and overall healthcare costs in both human and veterinary medicine [8, 9]. Annually, 25.000 Europeans and 23.000 Americans are estimated to die as a direct result of infections with multidrug-resistant bacteria [8, 10]. Although it is difficult to estimate, bacterial antimicrobial resistance has been reported to cost the European Union economy approximately €1.5 billion and the US health-care system between $21 billion to $34 billion per year [7, 11]. Yet, it is not so much the current impact of antibiotic resistance but the potential future implications that are worrisome. Antibiotic resistance is expected to lead to increased morbidity and mortality, with an associated exorbitant increase in health-care costs and indirect economic impacts worldwide [7, 9, 12].

Compounds that were found in the Golden Era of antibiotic discovery (1940s to 1960s) or derivatives of these compounds encompass practically all currently used antibiotics and despite the enormous high-tech discovery efforts which started in the 1990s only two new classes of antimicrobials have been approved in the last 20 years [3, 13] (Figure 1). History has shown that the introduction of every new antibiotic was rapidly followed by the emergence of resistance in the target microorganism, and the required steady flow of novel classes of antibiotics and derivatives has been outpaced by the emergence and spread of antibiotic resistance [13, 14]. Although there are hundreds of essential proteins in bacteria, the number of targets of currently used antibiotics is relatively small [13]. The most successful antibiotics hit only three targets or pathways: the ribosome (affecting protein synthesis), DNA gyrase or DNA topoisomerase (affecting DNA synthesis), or cell-wall synthesis. Other antibiotics target RNA synthesis, folic acid metabolism pathways and the bacterial cell membrane (Figure 2).

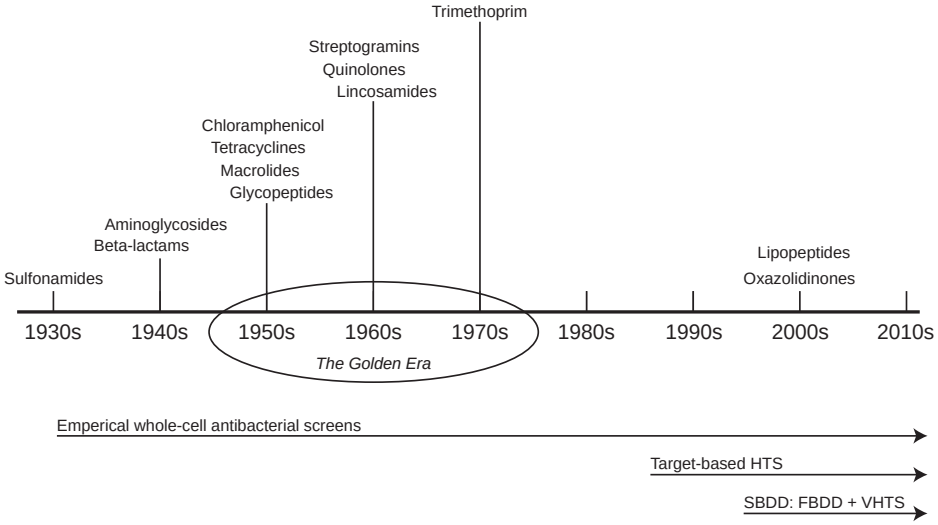

Figure 1. History of antimicrobial drug discovery, showing the "Golden Era" of antimicrobial drug discovery from ~1945 – 1976. Market approval of different classes of antibiotics per decade are indicated above the timeline. The principal strategies employed in antimicrobial drug discovery are displayed below the timeline (HTS: High-Throughput Screening, SBDD: Structure-Based Drug Discovery, FBDD: Fraction-Based Drug Discovery, VHTS: Virtual High-Throughput Screening). Figure is adapted from [15] and [14].

Antibiotic resistance is a normal ecological phenomenon which is widespread in the environment. Over billions of years bacteria have evolved to resist the action of naturally occurring antibacterial compounds [16]. It has been shown that antibiotic resistance in bacteria already existed before the modern selective pressure caused by human use of antibiotics was introduced [17, 18]. It is this dramatic increase of selective pressure caused by the extensive use of antibiotics over the last 70 years that has accelerated the evolution and spread of bacteria that are resistant to antibiotics. Although antibiotic resistance is a complex phenomenon, its mechanisms can be classified in three categories, namely intrinsic, acquired and adaptive resistance. Some common antibiotic targets and mechanisms associated antibiotic resistance are shown in Figure 2.

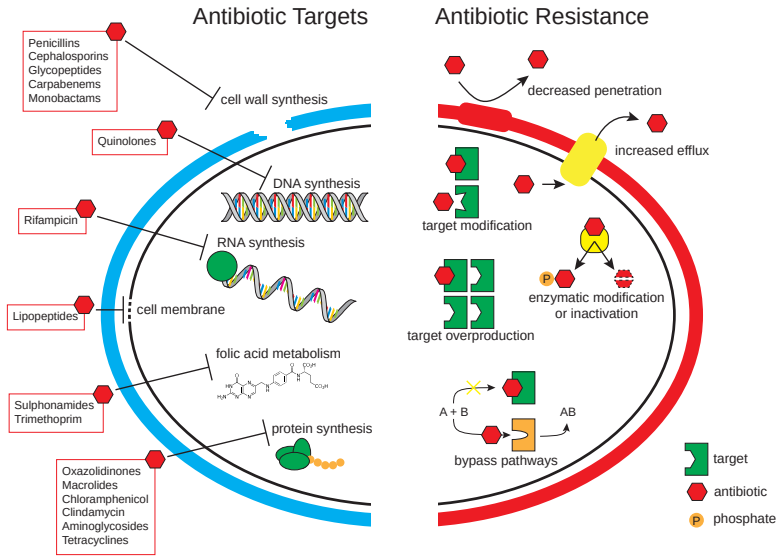

Figure 2. Antibiotic targets and mechanisms of resistance. Figure adapted from Wright (2010)[19] and Lewis (2013) [13].

Alternatives to conventional antibiotics

The alarming problem of antibiotic resistance has prompted renewed interests in alternative strategies to combat pathogenic microbes, such as the use of bacteriophages [20], synthetic antimicrobial peptides and peptoids [21-24], and naturally occurring bacteriocins [25-27]. Renewed optimism about natural product discovery and the overwhelming number of uncharacterized biosynthetic gene clusters (BGCs) has also accelerated efforts to find novel antimicrobial secondary metabolites from microbes [28-30]. Vaccination, often being relatively cheap and highly effective, is another historically successful strategy against bacteria causing infectious disease. Although new tools and vaccination strategies are continuously being developed, still a myriad of challenges remain, such as persistent, highly variable and/or novel pathogens, complex (e.g. polymicrobial) infections, and target populations that are difficult to reach [31]. The work in this thesis will focus on antimicrobial peptides as an alternative strategy to reduce the risk of infections by pathogenic bacteria.

Antimicrobial peptides

As mentioned above, the use of antimicrobial peptides (AMPs) is one of the alternatives to conventional antibiotics that is currently being explored and which has received considerable interest from the scientific community. Peptides with antibacterial and antifungal activity have a very ancient evolutionary origin and are widely distributed throughout all domains of life; currently, sequences of 2692 antimicrobial peptides originating from six kingdoms are deposited in the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/) [32]. Their efficacy in antimicrobial activity against pathogens has likely contributed to their ubiquity

throughout many lifeforms. AMPs have been found in numerous multicellular organisms, and are an essential component of innate immunity and form a first line of defence against pathogenic microbes [33]. In mammals, cathelicidins and defensins are two major classes of AMPs that are often produced by epithelial cells, circulating neutrophils, and tissue macrophages which are important in protecting against pathogenic invasion. For example, studies in knockout mice have shown that the murine cathelicidin-related antimicrobial peptide (mCRAMP) mediates defense against intestinal colonisation with epithelial adherent pathogens such as *Citrobacter rodentium* [34]. The protective role of human defensin 5 was demonstrated by its expression in Paneth cells of transgenic mice, which led to increased resistance to oral challenge with virulent *Salmonella typhimurium* [35]. Expression of human antimicrobial peptides is greatly reduced in skin lesions of patients suffering from atopic dermatitis, which is characterized by increased bacterial, fungal and viral skin infections [36]. Similarly, amphibian skin AMPs have been shown to effectively inhibit skin infections by the pathogenic fungus *Batrachochytrium dendrobatidis* [37].

Being an integral component of innate immunity, AMPs usually act non-specifically (broad-spectrum activity) and rapidly (within minutes) [38]. There is an enormous diversity of known peptide sequences and typically little sequence similarity is seen between peptides isolated from different species [33]. This thesis is mostly centred around the antimicrobial activity of AMPs. However, in addition to direct pathogen killing, many AMPs have been shown to possess other activities such endotoxin neutralization, immune cell recruitment, modulation of cytokine production, suppression of potentially harmful inflammation, induction of cell differentiation, and enhancement of adaptive immune responses, cell survival, wound healing and angiogenesis (Figure 3), and thus are also commonly referred to as host-defense peptides (HDPs) [21, 39-41].

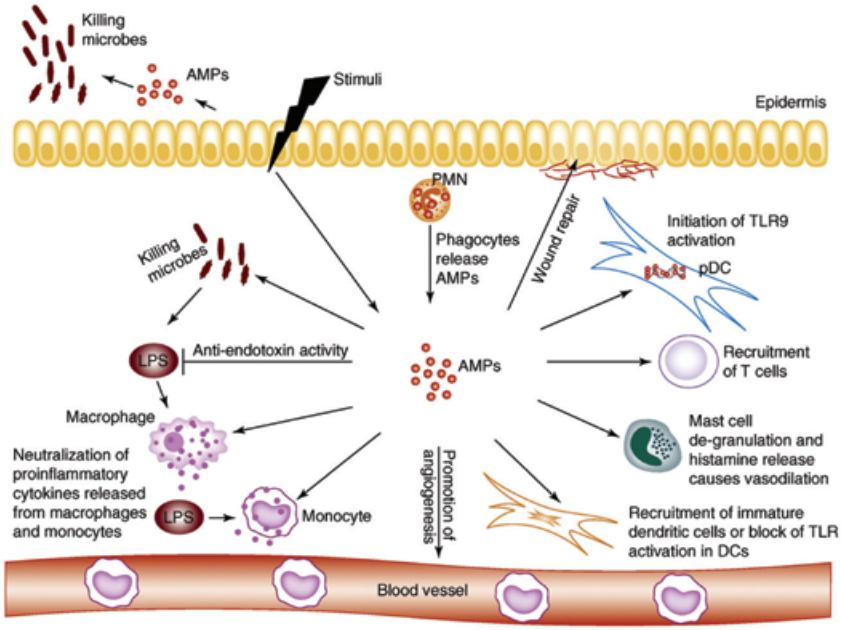

Figure 3. Multiple functions of antimicrobial peptides in host defense. Source: Lai & Gallo (2009)[40]

Structures of AMPs

Over the last two decades multiple peptide databases have been established in an attempt to classify the wide range of AMPs based on characteristics such as origin, secondary structure, mode of action, or post-translational modification [42]. Traditionally, AMPs are categorized regarding the most common three-dimensional secondary structures: α-helices (e.g. LL-37, magainin, cecropin), β-strands having multiple stabilizing disulphide bridges (e.g. human α- and β-defensin, protegrin), loop structures with one internal disulphide bridge (e.g. bactenecin), and extended structures rich in glycine, proline, tryptophan arginine, or histidine (e.g. indolicidin)[39] (Figure 4).

A commonly found and widely studied class of HDPs is cationic α-helical AMPs. Such linear AMPs often appear in aqueous solution as unorganized coils, but they can adopt an active secondary α-helix structure in the presence of phospholipid membranes [43]. In this shape, clusters of cationic and hydrophobic amino acid residues are organised three-dimensionally in separate clusters of the molecule, resulting in an amphipathic structure. Often, in amphibian skin secretions several cationic α-helical AMPs can be found, such as the magainins being among the first HDPs to be described [44]. The antimicrobial and cytolytic activity of these peptides is determined by cationicity, hydrophobicity, α-helicity and amphipathicity [45], parameters that can be altered by amino acid substitution to change antimicrobial potency or specificity [46]. In this thesis, naturally occurring amphibian AMPs and analogues thereof have been tested for their activity and selectivity against pathogenic bacteria, and the human cathelicidin LL-37 was used as a model cationic peptide.

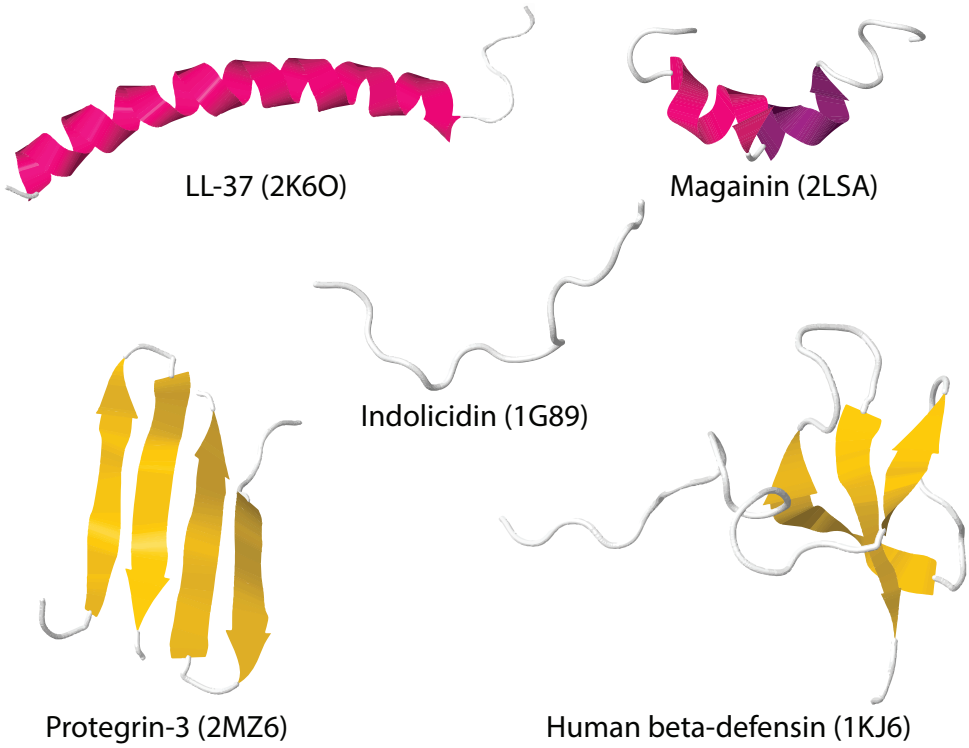

Figure 4. Three-dimensional structures of selected AMPs, showing the structural diversity. Pink colour highlights α-helical motifs, while β-sheets are shown in yellow colour. Protein Data Bank ID of the displayed models is given in parentheses.

Bacteriocins

Ribosomally synthesized AMPs from prokaryote origin are generally known as bacteriocins and they have been proposed as potential alternatives to or synergistic complementary agents with conventional antibiotic therapy [26, 47]. They are usually classified based on their post-translational modification; Class I peptides (e.g. nisin, thuricin CD, bottromycin A2) are modified, whereas class II peptides (e.g. pediocin PA-1, epidermicin NI01, enterocin AS-48) are largely unmodified or cyclic in their active form [26, 48]. Bacteriocins target some pathways or processes in common with conventional antibiotics, such as the inhibition of cell wall biosynthesis and DNA or protein synthesis. Additionally, many bacteriocins have been found to disrupt membrane integrity (see below) [47]. In general, bacteriocins have narrow-spectrum activity against a limited set of species that are phylogenetically closely related to the producer and are therefore thought to modulate the composition of bacterial species colonizing a niche without dramatically disrupting the microbiota [26, 49]. Production of such AMPs is widespread among bacteria and is regarded to contribute to bacterial fitness, colonization capacity, and probiotic functionality by having multiple ecological functions [49]. Commensal production of AMPs specifically targeted against pathogens *in situ* is

considered an attractive strategy to prevent infectious disease [26, 50]. For example, a *Bacillus thurigiensis* isolate from human faeces was found to produce the two-peptide bacteriocin thuricin CD that displays narrow-spectrum antimicrobial activity against *Clostridium difficile*, without significantly disrupting the resident microbiota which might lead to recurrence of *C. difficile* associated diarrhoea [49, 51].

Non–ribosomal peptide biosynthesis

In addition to ribosomally synthesized peptides, many microbes have the ability to produce secondary metabolites of wide structural diversity, including peptide antibiotics, using large multifunctional enzymes encoded by BGCs. One class of BGC is formed by non-ribosomal peptide synthetases (NRPSs), which consist of a linear arrangement of modules, each of which are further subdivided into separate catalytic domains [52, 53]. Each module is responsible for the incorporation of one building block (e.g. amino or hydroxy acid) into the growing peptide chain, with the separate domains catalysing specific substrate activation, covalent binding, and peptide bond formation. In this sense, the NRPS comprises both the assembly machinery as well as the specific template for biosynthesis of the product [52]. The structural diversity of NRPS derived molecules is enormous, because the possible building blocks are not limited to the 20 canonical amino acids [53]. To date more than 530 possible monomers and 1179 different non-ribosomally derived peptides have been identified [54]. Many pharmaceutically valuable antimicrobial compounds that are used in the clinic today are microbial NRPS products or derivatives thereof [55]. Vancomycin, belonging to the glycopeptide class of antibiotics, is a potent drug against Gram-positive bacteria and was already isolated and described in the early 1950's [56]. The cyclic lipopeptide daptomycin was discovered in fermentations of *Streptomyces roseosporus* and is now used as a last-resort antibiotic, for instance against Gram-positive skin infections [57, 58].

AMP modes of action

Although AMPs lack evolutionary conservation of primary sequences and thus have a wide variety of three-dimensional structures, they usually share common features such as cationicity, amphipathicity and short length, which are important for their mode of action [39]. AMPs display a higher specificity to bind and insert into the plasma membrane of bacteria and fungi compared to the membranes of higher eukaryotes. This is primarily due to the higher content of anionic phospholipids exposed on the outer leaflet of bacterial and fungal plasma membranes compared to plasma membranes of plants and animals, where the negatively charged headgroups of the phospholipids are mainly located on the inner leaflet [59]. Other negatively charged components, such as lipopolysaccharide (LPS) or wall- and lipoteichoic acids (WTA, LTA), are also only present on bacterial but not on eukaryotic cell surfaces [60]. Electrostatic interaction between these negatively charged cell envelope components and positively charged amino acids is generally believed to be a main driver for AMP binding and accumulation on membrane surfaces [60-62]. Additionally, a range of

other factors may be involved in the selectivity and activity of AMPs, such as differences in transmembrane potential and the presence of cholesterol in the target membrane altering membrane fluidity and stability [59].

Different models to describe the molecular mode of lethal action of AMPs have been proposed, although the exact mechanisms are not yet fully understood. However, all models agree on the initial key event of stoichiometric accumulation of AMPs on the outer leaflet of the plasma membrane by their binding to phospholipids, and once a threshold is reached, plasma membrane permeabilization is induced [63]. Traditionally accepted models describe permeabilization by micellization ("carpet" model) [64], by formation of pores composed exclusively by peptides organised perpendicularly to the membrane ("barrel-stave" pore model) [65], by accumulation of peptides parallel to the membrane surface causing phospholipid folding and bending leading to mixed peptide-phospholipid pores ("toroidal" pore model) [66] (Figure 5) [63]. Regardless of the mechanism, severe disruption of the internal homeostasis ensues, with a lethal outcome for the targeted microbe [33].

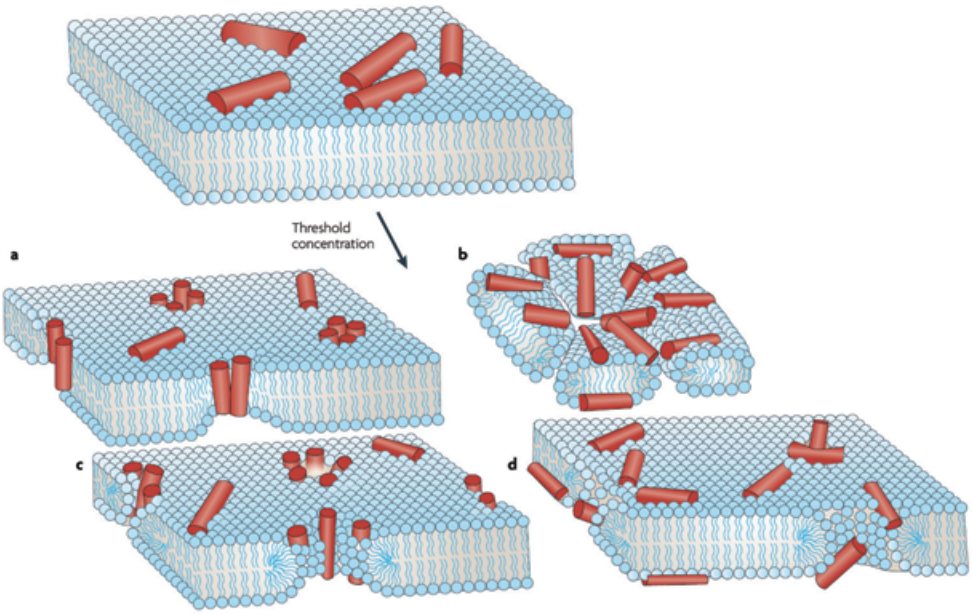

Figure 5. Suggested models for phospholipid membrane disruption by AMPs. Initially, a threshold concentration of AMPs on the membrane interface is required. **a)** Barrel-stave pore, **b)** carpet model, **c)** toroidal pore, **d)** disordered toroidal pore. Source: Melo *et al.* (2009)[63].

It recently became clear that the importance of the non-specific interaction of AMPs with the phospholipid membrane and subsequent membrane disruption might be overestimated, and that the bactericidal activity of many AMPs might be due to interaction with other specific targets [62, 67, 68]. One example is nisin, a lantibiotic produced by strains of

Lactococcus lactis [69] that has been widely used as a food preservative for more than 40 years. Nisin interacts with the membrane-bound cell wall precursor lipid II, thereby inhibiting peptidoglycan synthesis. Additionally, nisin forms membrane pores that consist of lipid II and nisin molecules, causing dissipation of the membrane potential, and rapid efflux of small metabolites [70, 71]. Many defensins from multiple sources have been shown to directly target the bacterial cell wall building block lipid II, thereby specifically inhibiting cell wall biosynthesis [67]. For example, the fungal defensin plectasin showed very potent antimicrobial activity against Gram-positive pathogens, and was able to cure mice from *S. pneumoniae* induced peritonitis and pneumonia without evidence of toxicity to the host [72]. This antibacterial activity is entirely due to lipid II binding and blocking of the peptidoglycan synthesis pathway, without compromising bacterial membrane integrity [73].

Bacterial resistance mechanisms to AMPs

Rapid development of microbial resistance against AMPs due to their mechanism of killing has often been claimed to be unlikely, because bacteria would have to alter the composition and/or organisation of conserved structures like the membrane and cell-wall causing adverse consequences [33]. This notion was until recently supported by the absence of reports of bacterial resistance to AMPs [33, 62] and was one of the reasons for the considerable amount of scientific attention they have received [39]. The enticing idea of developing AMPs as 21st century novel antibiotics that would circumvent the global antibiotic resistance problem now has to be reconciled with recent evidence for several of resistance mechanisms against AMPs [74]. The various mechanisms of resistance to AMP-mediated killing most likely evolved in bacteria due to the selective pressure resulting from exposure to environmental bacteriocins and host AMPs, and include surface charge modification, active efflux, alteration of membrane fluidity, proteolytic digestion, and entrapment by surface proteins and polysaccharides [74]. The current knowledge about strategies employed by bacteria to counteract the lethal action of AMPs is extensively described in recent reviews by Joo *et al.* [75] and Cole & Nizet [74]. Here, some key resistance mechanisms are briefly discussed (Figure 6).

Given that many AMPs are cationic, a well-described resistance mechanism in both Gram-positive and Gram-negative bacteria is modification of anionic cell surface constituents to decrease electrostatic attraction of such peptides [75]. In Gram-positives, the cell-wall components LTA and WTA usually have an overall negative charge, but the gene products of the *dlt* operon confer a positive charge by the incorporation of D-alanyl esters [76]. This reduces the susceptibility of the bacterium to cationic AMPs by electrostatic repulsion [77] and by increasing cell wall density that sterically hinders AMP access to the membrane due to altered conformation of LTA [76]. Another electrostatic repulsion mechanism found in both Gram-positives and Gram-negatives involves the modification of the anionic membrane phospholipid phosphatidylglycerol (PG) by the multipeptide resistance factor (MprF), adding a L-lysine or L-alanine residue, which results in a net-positively charged PG [75, 78, 79].

Secreted or membrane-bound proteases can provide another simple, yet effective resistance strategy against AMPs, by effectively cleaving and inactivating them [75]. For example, proteases from several clinically important human pathogens, such as elastase of *Pseudomonas aeruginosa*, were able to break down the human cathelicidin LL-37 and increase bacterial survival in an *ex vivo* wound fluid model [80].

Aforementioned lipid II is an essential and well conserved component of peptidoglycan biosynthesis pathway required for cell wall formation in eubacteria. Lipid II offers several binding sites for many structurally unrelated antibiotics and AMPs, and their interaction often leads to defunct cell wall biosynthesis with severe adverse effects to the target bacterium [60, 68]. Bacteria may modify lipid II to alter the overall cell surface charge and reduce the binding affinity of AMPs [60]. In contrast to the regulated resistance caused by surface charge modification of proteolytic AMP degradation, structural modifications to the cell wall components is usually constitutive [60].

These and other common mechanisms of resistance to AMPs are highlighted in Figure 6.

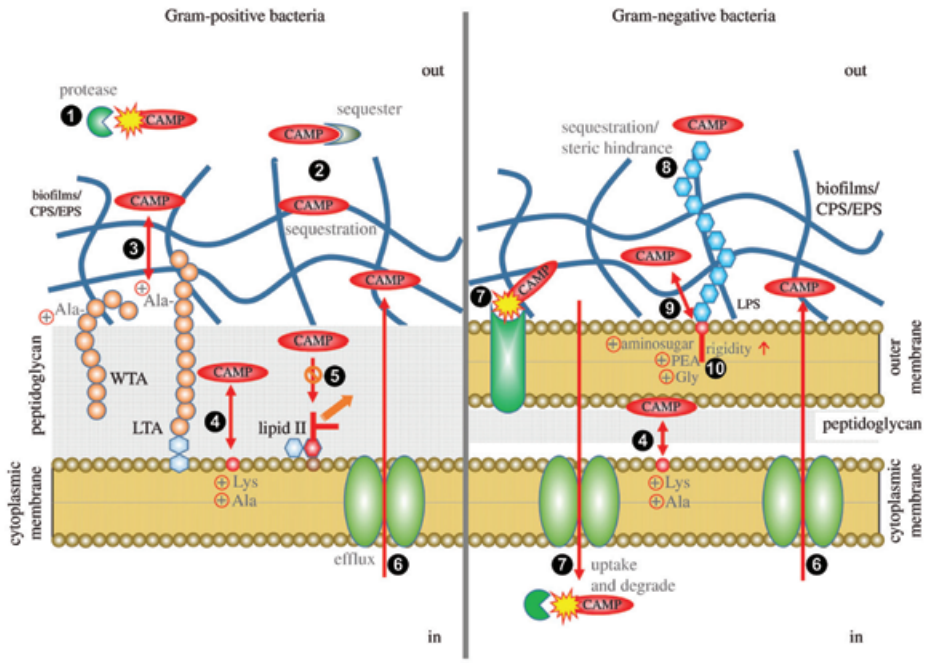

Figure 6. Overview of Gram-positive and Gram-negative resistance mechanisms to cationic AMPs (CAMP). Different counteracting strategies are **1)** proteolytic degradation; **2)** sequestration; **3)** electrostatic repulsion by D-alanylation of teichoic acids; **4)** electrostatic repulsion by amino-acylation of membrane phospholipids; **5)** modification of lipid II to prevent binding; **6)** AMP removal via efflux transporters; **7)** proteolytic degradation by membrane bound or intracellular proteases; **8)** sequestration or steric hindrance by the polysaccharide chain of LPS; **9)** electrostatic repulsion by amine-compound modification of lipid A; **10)** increased membrane by addition of extra acyl chains into lipid A. Source: Joo *et al.* (2016)[75]

Interestingly, AMPs have been proposed to serve as environmental cues to elicit virulence gene expression in the host, leading to bacteria gaining a more "invasive phenotype" [81-85]. Additionally, these AMP induced changes in gene expression could lead to increased adaptive resistance towards conventional antibiotics, such as macrolides, fluoroquinolones, or aminoglycosides [84, 86]. Surprisingly, acquisition of adaptive DNA mutations leading to increased bacterial mucoidy and rifampin resistance were observed upon exposure to sub-inhibitory concentrations of LL-37 [87]. The therapeutic use of AMPs may also compromise natural innate immunity, one example being the exposure of *Staphylococcus aureus* to pexiganan, a synthetic analogue of magainin, leading to cross-resistance to human defensin-1 [88].

Other limitations of AMPs as therapeutic agents

Despite the scientific enthusiasm about AMPs as potent new antibiotic drug candidates and the increasing amount of patents claiming such therapeutic potential, many AMPs do not make it to the market due to failure in clinical trials [62, 89]. This could be due to a number of reasons, such as absorption, low *in situ* peptide stability and proteolytic degradation, haemolytic and cytotoxic activities in patients, or bacterial resistance mechanisms (see below). This emphasizes that there is a gap in fully understanding their plethora of biological functions, the mode of antibacterial activity and the resistance mechanisms developed by bacteria. Thus, there are some major issues that remain to be solved before therapeutic use of AMPs can be achieved.

Initially, two methods were available for obtaining antimicrobial peptides: isolation from the host organism or chemical synthesis. Isolation of these defense molecules from host tissues such as frog skins [44] and insect lymph fluid [90] required several fractionation and purification steps, followed by microbiological tests to determine their bactericidal properties. Using this approach many cationic peptides from various species have been discovered. Nevertheless, this method of AMP isolation is tedious, requires large quantities of source material, usually has low yields, and is therefore not suitable for clinical trials [62, 91]. On the other hand, the chemical peptide synthesis using the established solid-phase techniques is faster, easy to automate, and requires simpler purification steps. This technique allows studies on structure-activity relationships without biological restrictions due to toxicity. It also has the possibility of incorporating unnatural amino acids, whereas peptides generated by ribosomes are limited to a subset of α-amino acids and α-hydroxy acids [23, 92]. However, the limitation of chemical AMP synthesis is the high cost and difficulty to incorporate complex posttranslational modifications. For example, the production of peptides containing multiple disulphide bonds and thus complex secondary structures, will substantially increase production costs [91].

Alternatively, the heterologous expression of eukaryotic AMPs by microorganisms, such as lactic acid bacteria, could be a cost-effective tool for functional and clinical research. Production of AMPs by genetically modified microorganisms is interesting due to the low

costs, rapid growth on inexpensive substrates, controllable laboratory conditions, and the generally well-characterized genetic backgrounds and availability of a wide range of vectors and host strains [93]. The use of fusion proteins is common in the production of small, labile AMPs to provide stabilization, shield the AMP from intracellular proteolytic degradation, increase expression and facilitate downstream purification processes, but more importantly to mask the intrinsic toxicity of these peptides to the expression host [91, 94, 95]. However, there are technical problems that currently still persist in optimizing bacteria as AMP producing cell-factories, such as their intrinsic antibacterial activity, lack of post-translational modification machinery in heterologous producers, contamination with bacterial LPS, and the inefficient expenditure of energy and protein synthesis machinery caused by the fusion protein strategy [62]. Despite the feasibility of isolating AMPs, or producing them by chemical synthesis or microbial cell-factories, the cost of manufacturing sufficient quantities for therapeutic use greatly exceeds the costs of currently used antibiotics [62].

From a pharmacological standpoint, AMPs don't follow the Lipinski "Rule of Five" for drug-likeness that predicts good absorption and permeation [96]. Additionally, many natural AMPs are sensitive to enzymatic degradation *in vivo* by host or bacterial (see below) proteases and peptidases. Another limitation of many AMPs is their systemic toxicity and haemolytic activity preventing their use to treat systemic invasive disease. For these reasons AMPs currently hold most promise as topical treatments for skin and wound infections [97, 98].

The importance of the microbiota

Although for several decades there was a strong scientific focus on antibiotic resistance development in specific pathogenic bacteria, there is now concern about collateral effects of the extensive use of broad-spectrum antibiotics on commensal bacteria [99]. Furthermore, the accumulation of antibiotic resistance genes (i.e. the resistome) in commensal microbes and their potential horizontal transfer to opportunistic or pathogenic bacteria is of concern [100]. The diverse and complex communities of commensal microbes that inhabit mammalian external surfaces, such as the gastrointestinal tract, the oral cavity or the skin, are collectively known as the microbiota [101]. The microbiota composition may differ widely between individuals as well as between different niches within an individual [102], and is the result of shared co-evolution, co-adaptation and codependency with the host [103].

It was as early as 1907 that Élie Metchnikoff theorized that beneficial properties of certain commensal lactic acid bacteria could be used to enhance health by manipulating the intestinal microbiota [104]. Methods to study and characterize the microbiota are continuously improving, and especially the advent of cultivation-independent methods, such as the ones targeting the bacterial 16S rRNA gene sequence and random metagenomic DNA approaches, have considerably accelerated scientific research in this field over the last decades [105]. Next generation sequencing (NGS) technologies that allow high-throughput data collection, such as 454 pyrosequencing and Illumina sequencing, have been broadly adopted for microbiota profiling as costs of (outsourced) sequencing continues to decrease.

In addition, studies in gnotobiotic mice have significantly advanced our understanding of the important role of microbiota in the intestinal development and homeostasis and the function of the immune system [106-108].

We now know that the microbiota is involved in various processes that are beneficial to the host [109], such as fermentation and conversion of food or feed components [110, 111], production of essential nutrients and metabolites [109, 112, 113], maturation and modulation of the immune system [107, 114], and colonization resistance against pathogens (see below)[50]. On the other hand, dysbiosis of the gut microbiota and the sudden increase in abundance of specific intestinal pathobionts have been shown to contribute to the pathophysiology of both intestinal and extra-intestinal disorders, such as inflammatory bowel disease, obesity and type II diabetes [115].

It has also become clear that the composition and function of the microbiota can indeed be influenced by environmental factors, and that this may alter the health status of the host. It has been shown that nutrition is an important factor in the modulation of the intestinal microbiota, particularly dietary fibres and probiotics [116, 117]. Several studies have shown that antibiotic chemotherapy can adversely impact on the composition and diversity of the microbiota [118, 119]. Increased susceptibility to pathogenic infections due to loss of colonization resistance after exposure to antibiotics was already demonstrated almost 60 years ago [120, 121] and this phenomenon is now well-accepted [50, 122].

Colonisation resistance

Stable and diverse microbial communities may provide colonisation resistance against pathogenic species by two major mechanisms that are mediated via a variety of molecular microbe-microbe interactions (direct colonisation resistance) and/or microbe-host interactions (indirect colonisation resistance) [122]. It is mutually beneficial to the host and commensal bacteria to reduce or inhibit the establishment of pathogenic bacteria. Such cooperation prevents severe inflammation, tissue damage and associated reduced nutrient uptake for the host and it preserves the balanced niche in which the commensal bacteria thrive [123].

Direct colonisation resistance (i.e. microbial antagonism) may occur through several different mechanisms, including competitive exclusion, nutritional competition and production of metabolites and bacteriocins, thereby conferring a benefit to the host [50, 122, 124] (Figure 7). Competitive exclusion refers to direct bacterial competition for mucosal adherence sites in which several bacterial surface structures, such as adhesins and fimbriae, have been proposed to play an important role [125, 126], although the exact mechanisms behind this remain unclear. Commensal bacteria can also limit the availability of carbon sources and/or micronutrients; for example, *Bacteroides thetaiotaomicron* outcompetes the pathogen *Citrobacter rodentium* for consumption of carbohydrates [127]. Bacterial antimicrobial peptides or compounds might (1) confer a competitive advantage to the producer to facilitate colonization of a specific niche, (2) directly kill or inhibit growth of pathogenic bacteria, and

(3) be involved in signalling within bacterial communities or with the host [25]. Additionally, some commensal microbes are able to neutralize host epithelium damaging toxins secreted by pathogens, such as a protease from *Saccharomyces boulardii* that inactivates toxin A that is produced by *Clostridium difficile* [128].

As briefly mentioned earlier, the host itself is also an crucial environmental factor shaping local microbial communities, as some bacteria are better adapted than others to withstand the generally non-specific host defenses. Indirect (or immune-mediated) colonisation resistance refers to the microbiota enhancing the mucosal barrier and/or modulating innate and adaptive immunity of the host, which results in reduced susceptibility to (invasive) pathogens [50, 122]. In the intestine, commensal microbes can trigger the secretion of cytokines, chemokines and antimicrobial HDPs [50, 129]. A prime example that illustrates the complexity of such host-microbiota interplay is the secretion of REGIIIγ, a bactericidal C-type lectin peptide. REGIIIγ expression by epithelial cells is upregulated upon exposure to bacterial stimuli, such as LPS, through the Toll-like receptor-MYD88 signalling pathway. Moreover, the cytokine IL-22, which is secreted by innate lymphoid cells (ILCs) and elicits several immune responses essential for mucosal homeostasis and protection against infections, is also required for REGIIIγ expression [130, 131]. Expression of IL-22, in turn, has been shown to be enhanced by activation of the aryl hydrocarbon receptor (Ahr) in ILCs by bacterial metabolites, such as indole-3-aldehyde produced by certain Lactobacillus species, like *L. reuteri* [132]. Increased expression of REGIIIγ in the large intestine is dependent on colonization with commensal bacteria, e.g. *B. thetaiotaomicron*, and through the above mentioned regulatory mechanism, REGIIIγ protects the intestinal mucosal barrier against infections by the pathogens, like *Listeria monocytogenes* [133-135].

The increasing knowledge about the molecular mechanisms underlying the beneficial effects of commensal or probiotic bacteria and their inhibition of proliferation of pathogenic microbes, opens up the way to the development of next-generation probiotics to exploit natural colonization resistance and control potential pathogens [136].

Probiotics

Probiotics are defined as "live microorganisms, which when consumed in adequate amounts, confer a health benefit on the host" [137]. Frequently used probiotic agents include strains of lactic acid bacteria, bifidobacteria, bacilli, non-pathogenic *Escherichia coli*, and yeasts [25]. The beneficial properties of these probiotics may be due to one or more colonisation resistance mechanisms that are described in the previous section, and in this context it has been proposed that bacteriocin production is an important probiotic trait [25]. Currently, the potential to use of (mixtures of) probiotic strains to reduce the risk of infections in humans and animals as an alternative to conventional antibiotic therapy is receiving great scientific and commercial interest [136].

For example, *Lactobacillus reuteri* can produce reuterin (3-hydroxypropionaldehyde), a secondary metabolite with broad-spectrum antimicrobial activity that can modulate the

microbiota composition as well as directly inhibit enteric pathogens [138, 139]. In addition, *L. reuteri* may have immunomodulatory effects by enhancing the intestinal production of IL-22 [132], and by suppressing the production of proinflammatory cytokines such as TNF-α and IL-12 in macrophages, monocytes, and dendritic cells, which can contribute to tolerance and reduction of intestinal inflammation in colitis [139]. Several strains of *L. reuteri* are currently commercially available as food supplements that claim to reduce the incidence and the severity of diarrhea, prevent enterocolitis or reduce infantile colic. Also in livestock, lactobacilli, such as *L. reuteri*, are widely used as probiotics [140, 141]. In pigs, especially neonatal or post-weaning piglets, *L. reuteri* is applied to increase performance (weight gain), prevent of diarrhea, and reducing oxidative stress [141]. While in specific situations probiotic supplementation could reduce or replace antibiotic chemotherapy, in most studies with pigs, the exact underlying mechanism and the specific location where *L. reuteri* exerts its activity are not yet clear. Nevertheless, considering the planned reduction of veterinary antibiotic use, applications of probiotic or commensal bacteria to prevent or minimize infections of piglets by bacterial pathogens would be very valuable for the swine industry. The work in this thesis focusses on alternative strategies against the porcine pathogen *Streptococcus suis*.

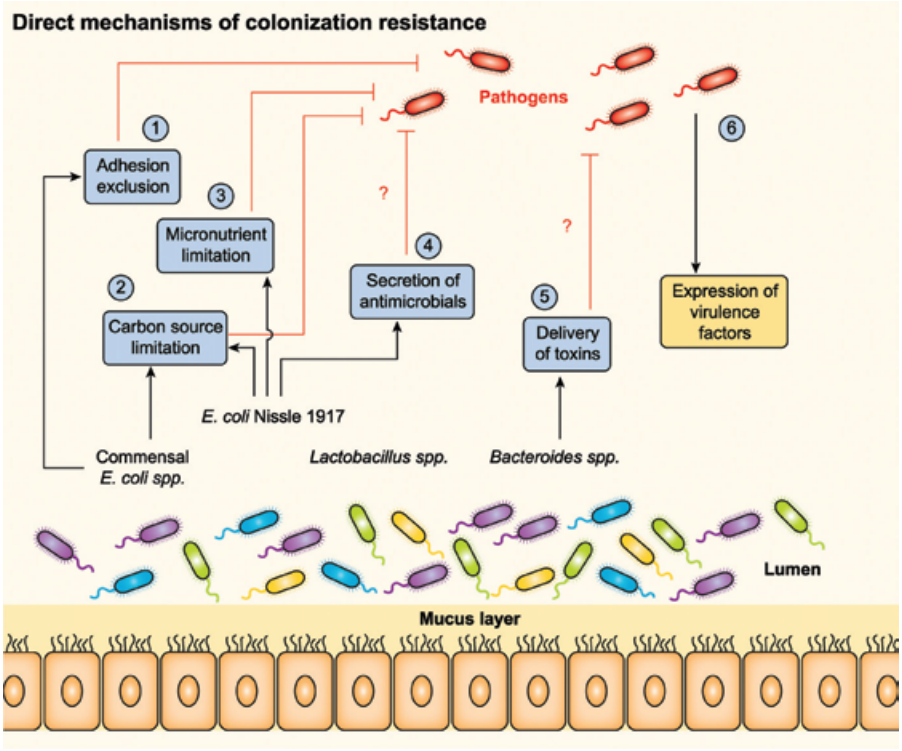

Figure 7. Direct colonization resistance mechanisms. Source: Sassone-Corsi & Raffatellu (2015) [50]

The porcine pathogen *Streptococcus suis*

Streptococcus suis is an encapsulated Gram-positive facultative anaerobic microbe, frequently present as a member of the porcine microbiota of the oro- and nasopharynx, and the intestinal and genital tracts [142-144]. *S. suis* is considered to be an important swine pathogen, causing morbidity and mortality in all countries where pigs are reared on a large scale. As such, infections by *S. suis* cause large economic losses to the global swine industry [145]. Carriage of *S. suis* in adult pigs varies widely among herds, with possible rates of up to 80% of the herd, and is usually asymptomatic, whereas young pigs during weaning (weeks 4 to 12) are most at risk from invasive disease [146, 147]. Disease symptoms such as acute sepsis, meningitis, endocarditis, pneumonia and arthritis are often reported, although in acute cases sudden death may also occur without appearance of obvious symptoms [148, 149]. *S. suis* also contributes to polymicrobial upper respiratory tract infections which may increase susceptibility to subsequent invasive *S. suis* infections [150, 151]. Morbidity due to invasive disease is estimated to be between 1% and 50% although it rarely exceeds 5% when treated promptly, while mortality can increase dramatically up to 20% without rapid and appropriate antibiotic treatment [145].

Aside from causing invasive disease in swine, *S. suis* is also considered an emerging zoonotic pathogen [152]. The first human infection was recorded in Denmark in 1968 [153], and since then around 1600 clinical cases have been documented [154, 155]. There have probably been considerably more cases of human infection in countries where *S. suis* is endemic, such as Asian countries with extensive small-scale pig rearing, due to a lack of disease awareness or diagnostic capabilities. Occupational exposure to infected pigs or pork products (a hazard for pig farmers, abattoir workers and butchers) has been shown to be associated with *S. suis* infections in humans [156-158]. Another important risk factor for human infection is the consumption of raw or undercooked pork products, such as meat, blood and organs, which is common in some parts of Asia [157].

S. suis is normally encapsulated and based on the antigenic diversity of the surface-associated capsular polysaccharides (CPS) 35 different serotypes have so far been described. However, it remains unclear whether all serotypes actually belong to *S. suis*; recent analysis of 16S rRNA and *cpn60* genes sequences suggested that serotypes 32 and 34 were phylogenetically more closely related with *S. orisratti* [155, 159]. *S. suis* prevalence is common but the known serotypes seem to have a different geographical distribution worldwide [155]. Mainly serotype 2, but also serotypes 1, 7, 9, and 14, are most frequently isolated from clinical samples of diseased pigs in Europe. Serotype 9 in particular is being increasingly isolated and associated with diseased pigs [160], while serotype 2 strains often are more virulent in pigs and generally considered to be the most common serotype infecting humans [155, 161].

The clinical manifestations of invasive disease by *S. suis* are similar in pigs and humans, with meningitis (68%) being the most common clinical symptom followed by sepsis (25.0%), arthritis (12.9%), endocarditis (12.4%), and endophthalmitis (4.6%), although these reported values may vary per country and depend on study design and used methods [154]. Presence

of *S. suis* serotype 2 is most often confirmed in cases of human infection (74.7%), followed by unknown or unconfirmed serotypes (23%) and serotype 14 (2%) [155].

Challenges in prevention and treatment of *S. suis*

Antibacterial chemotherapy is currently an important tool to treat and control *S. suis* infections in the swine industry. Prompt treatment of animals diagnosed with or suspected of *S. suis* infection with penicillin G, amoxicillin, trimethoprim/sulfamethoxazole, gentamicin, and ceftiofur is recommended [162]. In some countries *S. suis* is the primary cause of mortality and morbidity in young pigs and the most frequent reason to prescribe antibiotics of the amino-penicillin group as a preventative measure. Thus *S. suis* infections on farms indirectly contribute to the spread of antibiotic resistance. Of major concern is the emergence of strains causing more rapid and severe disease [154, 163] and the risk that the burden of both zoonotic and porcine disease will increase as plans for continued reduction of antibiotic use in livestock are implemented across Europe [6]. Prudent use of antibiotics for *S. suis* is essential to preserve the therapeutic efficacy of broad-spectrum compounds and to minimize selection of resistant *S. suis* strains [162]. Studies have suggested a direct link between the use of the antibiotics tylosin and tetracycline and the prevalence of bacterial resistance to these compounds in pigs [164]. Currently, resistance to macrolides, lincosamides, tetracyclines, and sulphonamides has been reported for up to 85% of *S. suis* isolates in many countries [162]. There are also strong indications that *S. suis* could act as a reservoir contributing to the spread of antibiotic resistance to other major streptococcal pathogens, presumably *via* mobile resistance genetic elements [152].

Despite the considerable efforts to develop vaccines against *S. suis* over the last decade, there is currently no efficacious and cross-protective vaccine on the market. Vaccines with heat- or formalin killed bacteria are used in the field with some degree of success and recent studies with CPS specific glycoconjugate vaccines show promising results [165-169]. However, the use of these preventative measures is very limited because protection is restricted to a specific serotype.

For the above mentioned reasons, alternative strategies to control *S. suis* are needed. Bacteriophages, phage lysins [170] [171] and plant extracts [172] are among the alternatives that have been investigated to date. Recently, the production of bacteriocins by selected avirulent strains of *S. suis* that can inhibit virulent *S. suis* of different sequence types was described as a similar strategy [173-175].

Rationale and approach undertaken in this thesis

This thesis studies the potential of naturally occurring host AMPs as therapeutic alternatives against pathogenic bacteria, with focus on *Streptococcus suis* in particular. In order to cause disease, *S. suis* first has to successfully colonize the host's mucosal surfaces, niches that are rich in both host- and microbe-derived AMPs. Hence, it was assumed that *S. suis* is continuously exposed to attack by AMPs, and as a result has likely evolved mechanisms to

defend itself or might sense host-derived AMPs as an environmental cue leading to enhanced colonisation or infection capacity. Another promising approach to reduce *S. suis* infections in pigs could be via administration of bacteria producing AMPs *in situ* to prevent colonization or suppress sudden post-weaning blooming by *S. suis* [123]. Such a strategy may be particularly suitable in young pigs, which are most susceptible to *S. suis* infections after weaning and in which the microbiota is still developing. The feasibility of such an approach was shown for a mixture of five candidate probiotic strains from the porcine intestine that inhibited *Salmonella enterica in vitro* and ameliorated diarrhoea when administered to *S. enterica* infected pigs. The protective effect was proposed to be due to the production of a bacteriocin by one of the isolates [176-178]. As such, the hypothesis was that commensal bacteria displaying (direct) microbial antagonism against *S. suis* may be found in the microbiome of the niches that *S. suis* inhabits. Such AMP producing antagonists could potentially be effective colonisers of these niches and protect against *S. suis* infection when applied early in the life of young pigs.

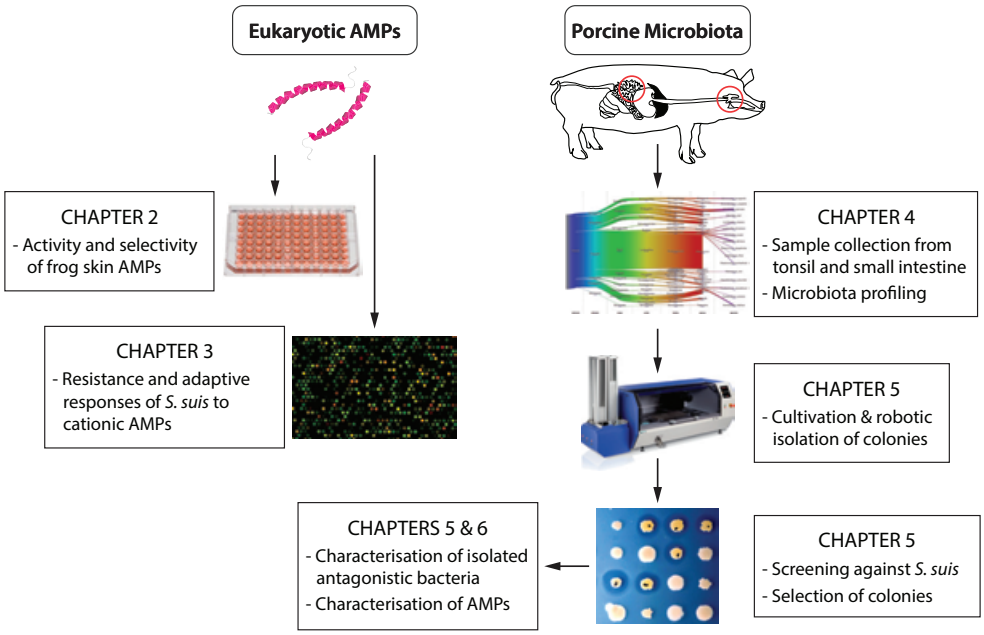

Figure 8. Schematic overview of the approach and the chapters of this thesis.

Aims and outline of this thesis

The therapeutic use of AMPs has been proposed as a promising alternative to combat multidrug resistant (MDR) pathogenic bacteria. The **first aim** of this thesis was to investigate to what extent naturally occurring AMPs could be effective for treating skin or wound infections caused by specific pathogenic bacteria. **Chapter 2** describes the testing of a selection of amphibian skin peptides and analogues thereof with different physicochemical properties in antimicrobial assays to determine their effect on a collection of Gram-positive and Gram-

negative probiotic, commensal and pathogenic bacteria. We observed a 3- to 9-fold lower MIC for five natural peptides and a 5- to 6.5-fold lower MIC for three analogues against selected pathogens than against commensal or probiotic Gram-positive bacteria. We propose that these peptides could be a good starting point for future research into use their for topical applications, based on their differential antimicrobial effect against certain skin associated multi-drug resistant pathogenic bacteria and their low cytotoxicity to mammalian cells.

Although many studies have focussed on identifying or synthesizing novel potent antimicrobial peptides, some bacteria may have evolved inducible resistance mechanisms against HDPs and bacteriocins and may even use their presence as an environmental cue to trigger a more virulent phenotype. Moreover the induced resistance mechanism may cause a degree of cross-resistance to antibiotics [84, 86, 87]. With these concepts in mind, the **second aim** of this thesis was to study the resistance and response of *S. suis* to a model cationic AMP.

Chapter 3 investigates the generic and adaptive resistance mechanisms in *S. suis* in response to the human cathelicidin LL-37 and the potential for this peptide to be recognised as a host cue for regulation of adaptive virulence mechanism. A microarray transcriptome analysis of *S. suis* S10, which is a virulent strain of serotype 2 that can be carried in pigs and humans, was performed upon short-term exposure to sub-lethal concentrations of LL-37. A significantly altered expression of genes associated with two-component signalling, membrane transport and carbohydrate uptake was identified. In addition, genes involved in pilus formation, cell surface protein anchoring and adhesion were strongly upregulated upon exposure to LL-37. Understanding the adaptive response of *S. suis* to an important host defense peptide may highlight putative novel targets for antimicrobial drug development.

The hypothesis was that the endogenous microbiota could play an important role in colonisation resistance against *S. suis* in piglets. To investigate this, a **third aim** of this thesis was to characterise the bacteria that are part of the natural microbiome of *S. suis*, which might enable us to propose commensal bacteria from the porcine microbiota with inhibitory activity against *S. suis* as well as pathogens that may promote *S. suis* virulence. **Chapter 4** describes the composition of the tonsillar and small intestinal microbiota from healthy piglets. Bacterial taxa that positively or negatively correlated with *S. suis* abundance were identified using the LEfSE pipeline, Pearson correlations and CoNET. *Lactobacillus reuteri* was found to have a strong significant negative correlation with the abundance of *S. suis* and other porcine pathogens. This chapter demonstrates that the microbiota from the same niche that is inhabited by *S. suis* contains endogenous bacteria that might display direct or indirect antagonism against *S. suis* together with a network of porcine pathogens that may promote *S. suis* virulence and its infection of piglets.

With this knowledge in mind, the goal was to find individual bacteria belonging to the porcine tonsillar and small intestinal microbiomes that displayed potent narrow-spectrum growth-inhibiting activity towards *S. suis* through the production of AMPs or secondary metabolites. **Chapter 5** describes the isolation and high-throughput screening of bacteria from the porcine tonsil and small intestine for growth inhibiting activity against clinically relevant serotypes of

S. suis. The isolation and identification of a commensal strain of *S. pasteuri* that produces two α-helical delta-lysins is reported. These peptides share high sequence similarity with and have comparable physicochemical properties to other *Staphylococcal* delta-lysins. In addition to previously attributed cytotoxic and haemolytic activity, these delta-lysins display narrow-spectrum antimicrobial activity against multiple serotypes of *S. suis*. Another example is given in **Chapter 6**, which focusses on the detailed characterization of a commensal *Rothia nasimurium* strain isolated from the porcine tonsil, producing the ionophore valinomycin. This chapter describes the molecular characterisation of the valinomycin encoding BGC, highlighting its potential recent acquisition by *R. nasimurium*, and shows that valinomycin may have antimicrobial, cytotoxic and immunomodulatory effects, thereby impacting the host as well as other microbes.

Finally, **Chapter 7** summarizes the research undertaken in this thesis and discusses its contribution to the scientific knowledge of *S. suis* occurrence in pigs and the role of the endogenous microbiota in that regard, as well as potential avenues towards alternative strategies to reduce porcine infections by this pathogenic bacterium.

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CHAPTER 2

Frog skin host–defense peptides as potential candidates for treatment of topical bacterial infections

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Abstract

Cationic, amphipathic, α-helical host-defense peptides (HDPs) that are naturally secreted by certain species of Anura possess potent broad-spectrum antimicrobial activity and show therapeutic potential as alternatives to treat infections by multi-drug resistant pathogens. Fourteen amphibian skin peptides and twelve analogues of temporin-1DRa were studied for their antimicrobial activities against clinically relevant pathogens with emphasis on opportunistic bacteria isolated from human or animal skin infections. For comparison, their potencies against a range of probiotic *Lactobacilli* were determined. The ISO-certified VITEK 2 system was used to define a profile of antibiotic susceptibility for the bacterial isolates used in this study. The minimal inhibitory concentration (MIC) values of the naturally occurring temporin-1DRa, CPF-AM1, alyteserin-1c, hymenochirin-2B, and hymenochirin-4B for pathogenic bacteria were 3- to 9-fold lower than the values for the tested probiotic strains. Similarly, temporin-1DRa and its [Lys^4], [Lys^5] and [Aib^8] analogues showed 5- to 6.5-fold greater potency against the pathogens tested compared with the probiotic bacteria. In the case of PGLa-AM1, XT-7, temporin-1DRa and its [D-Lys^8] and [Aib^{13}] analogues, no apoptosis or necrosis was detected in human peripheral blood mononuclear cells even at concentrations above the MIC. Given the differential activity against commensal bacteria and pathogenic strains, some of the tested peptides are considered promising candidates for further development into antimicrobial agents for topical treatment of human or animal skin infections with multi-drug resistant bacteria.

Introduction

The alarming increase in incidence of multidrug-resistant (MDR), pathogenic bacteria together with the decreasing discovery rates for new antibiotics represents a major societal problem and threat to human and animal health. This situation has heightened interest in naturally occurring host defense peptides (HDPs), including antimicrobial peptides (AMPs), as potential novel therapeutics [1, 2]. A widely studied class of HDPs is cationic amphipathic α-helical peptides, many of which are originally isolated from skin secretions of species belonging to the Anura order of amphibians (frogs and toads). Amphibians are, for a large part of their lifecycle, confined to warm and moist environments with high exposure to bacteria and fungi. However, frogs and toads possess excellent immunity to defend themselves against invasion by microorganisms. It is currently believed that as part of their innate immune system many species, but not all, produce and secrete a wide variety of HDPs via specialized glands in the skin [3, 4]. Amphibian peptides were amongst the first HDPs described nearly three decades ago [5, 6] and they form a highly diverse group of peptides comprising between 8 and 48 amino acid residues and generally a net charge between +2 and +6 at pH 7 [7]. Production of amphibian skin peptides seems to be evolutionarily conserved, presumably due to their role in preventing infection by pathogenic microbes although some skin peptides may also have autocrine or chemotactic functions [4, 8]. Previously, certain frog skin peptides have been proposed as candidates to treat infections on the basis of their potent and broad-range antimicrobial activity against pathogenic bacteria, fungi and protozoa [9, 10].

A disadvantage of many of these candidate HDPs in a therapeutic setting is their hemolytic activity and cytotoxicity, although this is typically observed at concentrations significantly higher than the minimal bactericidal concentration (MBC). However, it is possible to selectively reduce the cytotoxicity of HDPs through systematic amino acid substitutions to alter physiochemical properties, while retaining their potency and broad-spectrum antimicrobial activity [11]. For the above-mentioned reasons, HDPs currently show most promise as topical treatments for skin and wound infections rather than for systemic applications to treat invasive disease [2, 10, 12].

The aim of this study was to test a range of amphibian skin peptides and analogues of temporin-1DRa with different physicochemical properties in antimicrobial assays. We determined their effect on a selection of pathogens including opportunistic bacteria isolated from human or animal skin infections and MDR strains. We included several probiotic, commensal strains in the assays to investigate the spectrum of activity and selectivity of the amphibian HDPs. To benchmark the efficacy of these skin peptides, we also determined susceptibility of the selected bacteria to commonly used antibiotics using an ISO-certified assay platform.

Material and methods

Bacteria and culture conditions

Table 1 lists the bacterial strains used in this study and their source. Nine probiotic *Lactobacilli* were previously isolated from commercially available products [13]; *Lactobacillus plantarum* WCFS1 is a single colony isolated from *L. plantarum* NCIMB8826, which was originally derived from human saliva [14]. *Lactobacillus casei* Shirota (Yakult®, Japan) was originally isolated from the human intestine, *Lactobacillus reuteri* ATCC55730 was originally isolated from human breast milk [15]. *Streptococcus suis* S10 [16] was obtained from the Central Veterinary Institute (CVI, Lelystad, The Netherlands), *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* (strains 26228 and 25467) were obtained from skin infections in dogs and were obtained from University of Copenhagen (KU, Copenhagen, Denmark). The *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *P. aeruginosa* strains MDR1 and MDR2 were isolated from clinical samples were obtained from the Erasmus University Medical Centre Rotterdam (EMC, Rotterdam, The Netherlands). *Lactobacilli* were cultured and assayed in de Man, Rogosa and Sharpe (MRS) broth (VWR International) at 37°C under anaerobic conditions. All other strains were cultured in Müller-Hinton (MH) broth (Oxoid Ltd, Basingstoke, UK) at 37°C under aerobic conditions.

	Bacterial species	**strain**	**source**
commensal / probiotic	*Lactobacillus plantarum*	WCFS1	TIFN
	Lactobacillus rhamnosus	LGG	Valio
	Lactobacillus salivarius subsp. *salicinius*	DSM20554	DSMZ
	Lactobacillus salivarius	FortaFit Ls-33	Danisco
	Lactobacillus casei	R0215	Rossell
	Lactobacillus casei	Shirota	Yakult
	Lactobacillus johnsonii	LC-1	Nestle
	Lactobacillus reuteri	ATCC55730	BioGaia
	Lactobacillus acidophilus	LA5	Chr Hansen
pathogenic / opportunistic	*Streptococcus suis*	S10 3881	CVI [16]
	Staphylococcus aureus	DMS 20231	DSMZ
	Staphylococcus aureus	Sens 8325.4	EMC
	Staphylococcus aureus	MRSA B33424	EMC
	Staphylococcus pseudintermedius	E138	KU
	Staphylococcus pseudintermedius	E139	KU
	Staphylococcus pseudintermedius	E140	KU
	Staphylococcus pseudintermedius	S70E2	KU
	Staphylococcus pseudintermedius	S70E8	KU
	Staphylococcus pseudintermedius	S70F3	KU
	Pseudomonas aeruginosa	26228	KU
	Pseudomonas aeruginosa	25467	KU
	Pseudomonas aeruginosa	Sens1 PA01	EMC
	Pseudomonas aeruginosa	Sens2 ATCC27853	EMC
	Pseudomonas aeruginosa	MDR1 B38084	EMC
	Pseudomonas aeruginosa	MDR2 B31770	EMC
	Enterococcus faecium	Sens S1	EMC
	Enterococcus faecium	Sens S2	EMC
	Enterococcus faecium	VanA R39	EMC
	Enterococcus faecium	VanB R44	EMC
	Acinetobacter baumannii	MDR Bangl 027	EMC

Table 1. Bacteria used in this study.

Bacterial antibiotic susceptibility testing

The profile of antibiotic susceptibility of a panel of bacterial isolates was determined by the micro broth dilution test using the ISO-certified VITEK® 2 system (bioMérieux Benelux BV, Boxtel, The Netherlands) [17, 18]. The following antibiotic cards were used: AST-P633 (cefoxitin, benzylpenicillin, oxacillin, gentamicin, kanamycin, tobramycin, ciprofloxacin, levofloxacin, erythromycin, clindamycin, linezolid, teicoplanin, vancomycin, tetracycline, fosfomycin, fusidic acid, mupirocin, chloramphenicol, rifampicin, and trimethoprim/sulfamethoxazole), AST-N199 (piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, gentamicin, tobramycin, ciprofloxacin, colistin), and AST-P586 (ampicillin, sulbactam, cefuroxime, cefuroxime axetil, imipenem, gentamycin, streptomycin, moxifloxacin, erythromycin, clindamycin, quinupristin/dalfopristin, linezolid, teicoplanin, vancomycin, tetracycline, tigecycline, nitrofurantoin, trimethoprim/sulfamethoxazole). Bacteria were inoculated from glycerol stocks on appropriate growth medium agar plates using sterile plastic loops and incubated at 37°C overnight, after which single colonies were picked for analysis.

Peptides

The frog skin peptides (Table 2) and the temporin-1DRa analogues (Table 3) used in this study were chemically synthesized and purified as previously described [11, 19-27]. The identities of all peptides were confirmed by electrospray mass spectrometry and their purity was >98%. Lyophilized peptides were reconstituted in 20 µL 0.1% HCl and stock solutions were made at 1 or 2.5 mg/mL in sterile PBS and kept at -20°C until use.

source / peptide	length	amino acid sequence	net charge at pH 7.0	GRAVY	α-helicity
1. Pipidae					
1.1. Xenopus					
1.1.1. Xenopus amieti					
Magainin-AM1	23 aa	GIKEFAHSLGKFGKAFVGGILNQ	+2	+0.2	Non-helical
PGLa-AM1	22 aa	GMASKAGSVLGKVAKVALKAAL.NH2	+4	+0.83	9-22
CPF-AM1	17 aa	GLGSVLGKALKIGANLL.NH2	+2	+1.03	5-14
1.1.2. X. laevis x X. muelleri					
PGLa-LM1	21 aa	GMASKAGSVAGKIAKFALGAL.NH2	+4	+0.805	9-18
1.2. Silurana					
1.2.1. Silurana tropicalis					
XT-7 (CPF-ST3)	18 aa	GLLGPLLKIAAKVGSNLL.NH2	+2	+1.12	5-13
1.3. Hymenochirus					
1.3.1. Hymenochirus boettgeri					
Hymenochirin-1B	29 aa	IKLSPETKDNLKKVLKGAIKGAIAVAKMV.NH2	+6	+0.169	5-27
Hymenochirin-2B	29 aa	LKIPGFVKDTLKKVAKGIFSAVAGAMTPS	+4	+0.466	8-16
Hymenochirin-4B	28 aa	IKIPAFVKDTLKKVAKGVISAVAGALTQ	+4	+0.664	7-16
2. Alytidae					
2.1. Alytes					
2.1.1. Alytes obstetricans					
Alyteserin-1c	23 aa	GLKEIFKAGLGSLVKGIAAHVAS.NH2	+3	+0.748	2-8; 10-21
Alyteserin-2a	16 aa	ILGKLLSTAAGLLSNL.NH2	+2	+1.275	9-14
3. Ranidae					
3.1. Rana					
3.1.1. Rana draytonii					
Temporin-1DRa	14 aa	HFLGTLVNLAKKIL.NH2	+3	+0.879	5-14
[Lys^{4}]temporin-1DRa		HFL**K**TLVNLAKKIL.NH2	+4	nd	4-14
[Lys^{5}]temporin-1DRa		HFLG**K**LVNLAKKIL.NH2	+4	nd	4-14
[D-Lys^{4}]temporin-1DRa		HFL**^{D}K**TLVNLAKKIL.NH2	+4	nd	†
[D-Lys^{5}]temporin-1DRa		HFLG**^{D}K**LVNLAKKIL.NH2	+4	nd	†
[D-Lys^{8}]temporin-1DRa		HFLGTLV**^{D}K**LAKKIL.NH2	+4	nd	†
[Aib^{8}]temporin-1DRa		HFLGTLV**[Aib]**LAKKIL.NH2	+4	nd	5-14
[Aib^{9}]temporin-1DRa		HFLGTLVN**[Aib]**AKKIL.NH2	+4	nd	5-14
[Aib^{10}]temporin-1DRa		HFLGTLVNL**[Aib]**KKIL.NH2	+4	nd	5-14
[Aib^{13}]temporin-1DRa		HFLGTLVNLAKK**[Aib]**L.NH2	+4	nd	5-14
[Orn^{7}]temporin-1DRa		HFLGTL**[Orn]**NLAKKIL.NH2	+4	nd	nd
[DAB^{7}] temporin-1DRa		HFLGTL**[DAB]**NLAKKIL.NH2	+4	nd	nd
[TML^{7}] temporin-1DRa		HFLGTL**[TML]**NLAKKIL.NH2	+4	nd	nd
3.1.2. Rana boylii					
Brevinin-1BYa	24 aa	FLPILASLAAKFGPKLFCLVTKKC	+4	+1.07	4-12
3.2. Hylarana					
3.2.1. Hylarana erythraea					
B2RP-Era	19 aa	GVIKSVLKGVAKTVALGML.NH2	+3	+1.25	13-16 weak
4. Hylidae					
4.1. Pseudis					
4.1.1 Pseudis paradoxa					
Pseudin-2	24 aa	GLNALKKVFQGIHEAIKLINNHVQ.NH2	+3	-0.008	2-19; 14-19

Table 2. The naturally occurring peptides and temporin-1DRa analogues used in this study and their source species. PGLa-LM1 was found in a hybrid frog of *X. laevis* and *X. muelleri* (1.1.2)[27]. The grand average of hydropathy (GRAVY) is defined as the sum of all hydropathy values divided by the length of the sequence [28]. The α-helicity predictions were made using AGADIR [29]. Aib = α-aminoisobutyric acid, Orn = ornithine, DAB = diaminobutyric acid, TML = trimethyllysine, nd = not determined, † = decreased α-helicity.

Antimicrobial assays

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the peptides were determined by standard dilution assays in 96-well microtiter plates in two independent experiments [30]. Serial dilutions of peptide in the appropriate growth medium (25 µL) were mixed with bacterial suspension (75 µL) to obtain an inoculum of 5 x10^5 CFU/mL. Bacteria were incubated at 37°C for 18-22 hours, after which bacterial growth was measured by absorption at 600 nm using a spectrophotometer (Spectramax M5, Molecular Devices, Sunnyvale CA, USA). The MIC was determined as the lowest concentration at which no visible growth was observed. MBC was determined as the lowest concentration of peptide at which no viable bacteria could be detected, following plating of serial dilutions of suspensions from the wells on agar plates. Heatmaps were generated using the Multiple Experiment Viewer software [31], using Euclidean Distance with Average Linkage for Hierarchical Clustering of the data.

Human peripheral blood mononuclear monocyte (PBMC) cytotoxicity assay

PBMCs were isolated as previously described [32] with modifications. Buffy coats from peripheral blood of three healthy donors were obtained from the Sanquin Blood Bank, Nijmegen, The Netherlands. Isolated PBMCs were washed and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) + Glutamax (Gibco, Thermo Fischer Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Breda, The Netherlands) at a final concentration of 1×10^6 cells/mL and seeded (100 µL per well) in 96-well tissue culture plates. PBMCs were exposed to peptides at final concentrations of 1, 10 and 100 µg/mL. Exposure to LPS (1 µg/mL) was used as a positive control. After exposure for 24 h, cells were incubated with Annexin V-APC and propidium iodide (eBiosciences, Vienna, Austria) and, using flow cytometry (FACS Canto II, BD Biosciences), the proportions of live (unstained), dead (PI only), early-apoptotic (Annexin V only) and late-apoptotic (Annexin V + PI) cells were determined (BD FACSDiva). Data are presented as mean values ± SD.

Results

Antibiotic resistance of a panel of selected probiotic and pathogenic microbes

We used the bioMerieux VITEK®2 system to assay microbial resistance to commonly used antibiotics [33] for which the mode of action and bacterial target is given in Supplemental Table 1. The VITEK®2 system was chosen as it represents a widely used and well-standardized ISO-certified platform used in hospitals and medical centers to assess antibiotic resistance of clinically sampled microbes. Many Lactobacillus species were not able to grow under the incubation conditions of the VITEK®2 system; therefore, it was impossible to obtain an antibiotic susceptibility profile for these strains. The data for antibiotic resistance of each bacterium (Table 3) was used for benchmarking against each of the frog antimicrobial peptides. In the first row the number of antibiotics to which a strain was resistant is depicted by a color scheme: brighter red colors correspond to increased antibiotic resistance and brighter blue correspond to susceptibility to the tested antibiotics (Table 3). These data provide the baseline susceptibility of a selected set of bacteria to antibiotics, classifying certain strains as multi-drug resistant (MDR). The reference dataset of antibiotic resistance was then compared to a dataset of the MIC and MBC values for frog antimicrobial peptides and synthetic analogues tested against the same strains.

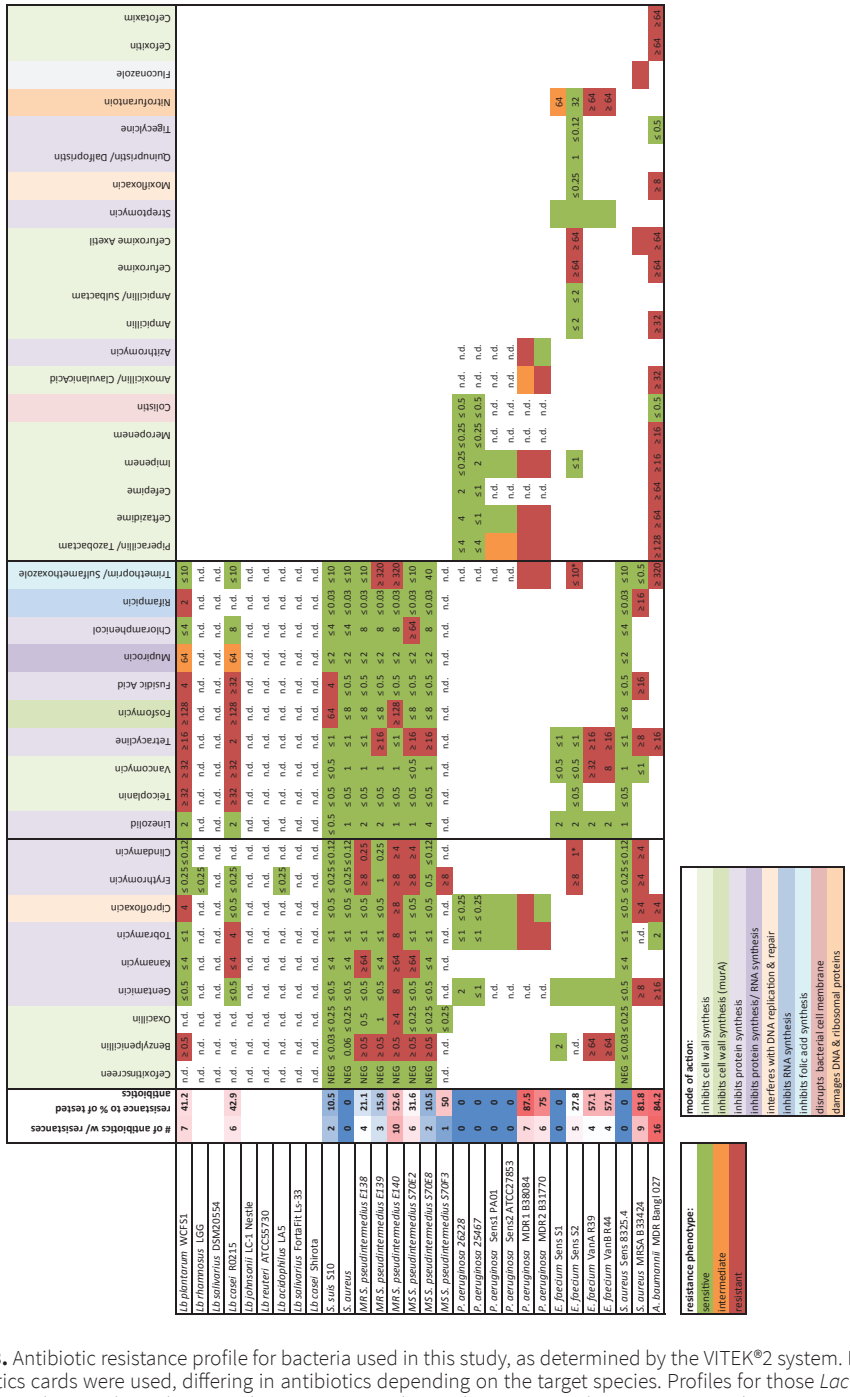

Table 3. Antibiotic resistance profile for bacteria used in this study, as determined by the VITEK®2 system. Multiple antibiotics cards were used, differing in antibiotics depending on the target species. Profiles for those *Lactobacilli* that required anaerobic culture conditions were not obtained. Resistance phenotype per antibiotic was scored as sensitive (green), intermediate (orange) or resistant (red) according to the EUCAST species specific breakoff points based on MIC values [33]. The total number of antibiotics scored as resistant is depicted in the second column, shaded from resistance to no (dark blue) to 16 (dark red) antibiotics. See Supplemental Table 1 for an overview of mode of actions of each antibiotic. nd =not determined.

Potency of frog skin peptides against probiotic and pathogenic microbes

Fourteen frog skin peptides were tested for their antimicrobial activity against a panel of bacteria. Antimicrobial activities were determined using a standard micro broth dilution assay and are presented as minimum inhibitory concentration (MIC) (Figure 1 B) and minimal bactericidal concentration (MBC) (Supplemental Table 2). Figure 1B represents the MIC values obtained for the naturally occurring frog skin peptides as a hierarchically clustered heatmap, with bright green colors corresponding to values of <8 µg/mL and bright red colors corresponding to values of >256 µg/mL. The heatmap shows that temporin-1DRa and XT-7 were effective at low concentration (<8 µg/mL) while magainin-AM1 and pseudin-2 showed no inhibition of any of the strains tested. With the exception of magainin-AM1 and pseudin-2, all the native frog peptides tested, inhibited all strains of *Staphylococcus pseudintermedius* at MIC = 8 µg/mL. The three hymenochirin peptides clustered together based on the observed MIC values, showing activity against Gram-positive MDR *S. pseudintermedius,* vancomycin-resistant *Enterococcus faecium* and the Gram-negative MDR *Acinetobacter baumanii*. The PGLa peptides also clustered together, based on their low MIC against all *S. pseudintermedius* strains. The strains of *Pseudomonas aeruginosa* were relatively insensitive to the frog peptides tested, except for the MDR2 isolate. For the Gram-positive bacteria (see Table 1) we compared the MIC of selected frog peptides against 9 different species of *Lactobacilli* (Figure 1A, green bars) and 13 pathogenic strains (excluding *S. suis*) (Figure 1A, orange bars) and measured the fold-difference in MIC value between these two bacterial groups (Figure 1A, purple bars). We found that the Gram-positive pathogens were 5.5-fold to 9-fold more susceptible to inhibition by CPF-AM1, temporin-1DRa and alyteserin-1c than the probiotic *Lactobacilli*.

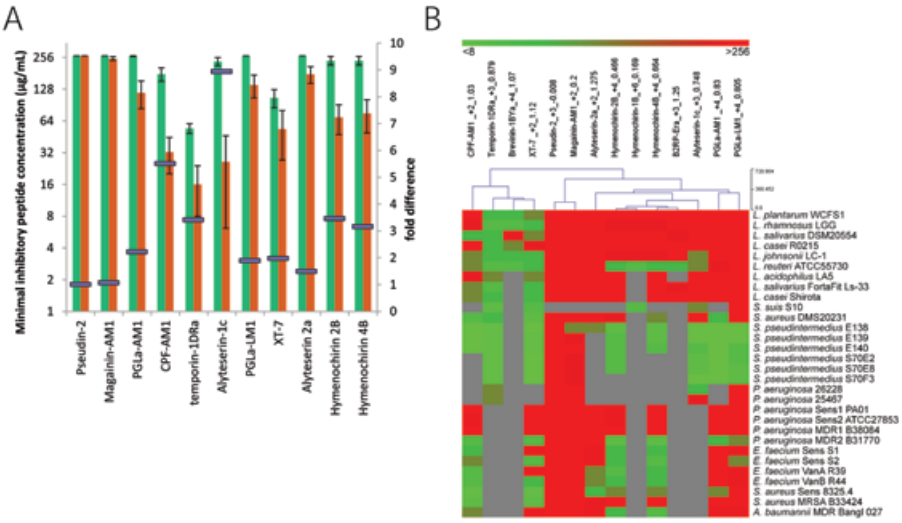

Figure 1. A) MIC values of a selection of naturally occurring frog skin peptides against 9 Gram-positive lactic acid bacteria (green) and 13 Gram-positive pathogenic bacteria (orange). The fold difference in MIC between the two groups is depicted as purple bars on the secondary axis. Average values ± SEM are shown. **B)** Heatmap representation of all MIC values of the tested frog skin peptides, including for each peptide its net charge at pH=7 and grand average of hydropathy (GRAVY). The gray color indicates MIC was not determined.

Temporin-1DRa, a peptide, first isolated from *Rana draytonii* [21], showed most promise for therapeutic activity against a range of Gram-positive pathogens including methicillin-resistant strains of *S. pseudointermedius* which are a major cause of recurring skin and wound infections in dogs [34]. Additionally, the MIC values for temporin-1DRa were approximately 3.5-fold lower for pathogens than probiotic species (Figure 1A).

Antimicrobial activities of analogues of temporin–1DRa

To investigate whether the antimicrobial activity of temporin-1DRa against pathogenic species could be increased by appropriate amino acid substitutions, we tested 14 different analogues, having single residue modifications to alter parameters such as cationicity, hydrophobicity and α–helicity [11] (Table 2). The effect of these amino acid substitutions on the physicochemical properties of α-helical peptides and the subsequent effect on cytotoxicity and antimicrobial potency has been described previously [11, 22, 35-37]. We observed MIC values that ranged from <8 to >64 µg/mL for these temporin-1Dra analogues, although multiple isolates of the same species showed similar sensitivities to a given peptide (Figure 2, Supplemental Table 3). Figure 2A shows that the analogues had altered activity against bacteria, with [Aib^{8}]temporin-1DRa having the largest fold-change (6.5) in activity between the grouped Gram-positive pathogenic and probiotic bacteria, followed by the [Lys^{4}], [Lys^{5}] and [Aib^{9}] analogues. Incorporation of α-aminoisobutyric acid (Aib) into a peptide generally promotes the formation of an α- or 3_{10}-helix or stabilizes an existing helical conformation [37].

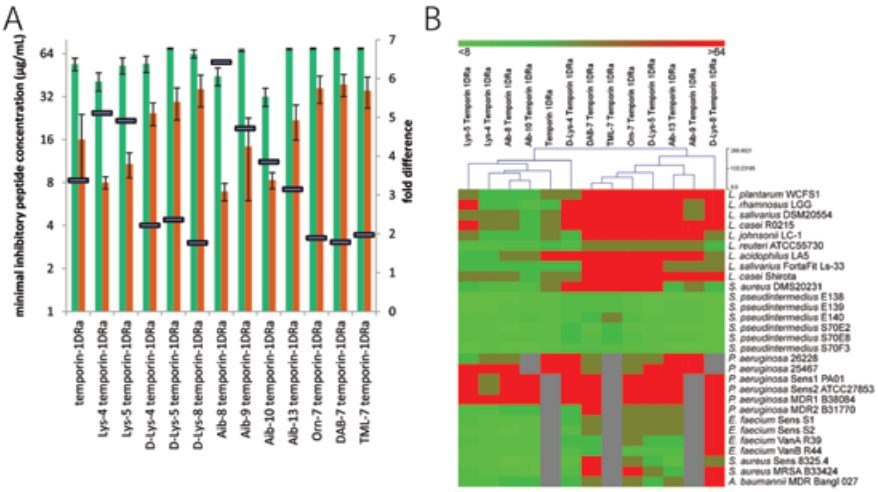

Figure 2. **A)** MIC values of temporin-1DRa analogues against 9 Gram-positive lactic acid bacteria (green) and 13 Gram-positive pathogenic bacteria (orange). The fold difference in MIC between the two bacterial groups is depicted as purple bars on the secondary axis. Average values ± SEM are shown. **B)** Heatmap representing the MIC values of temporin-1DRa analogues against the panel of tested bacteria. The gray color indicates MIC was not determined.

Cytotoxic effects of selected frog peptides and temporin–1DRa analogues

In order to determine the cytotoxic activities of those peptides that showed promising antimicrobial activity against pathogenic bacteria (PGLa-AM1, XT-7, temporin-1DRa and its [D-Lys^{8}] and [Aib^{13}] analogues, we exposed human PBMCs to the peptides and quantified apoptosis and necrosis using flow-cytometry. Figure 3 shows that none of the peptides tested had a significant effect on the viability of PBMCs during a 24 h exposure to final peptide concentrations of 1, 10 and 1000 µg/mL.

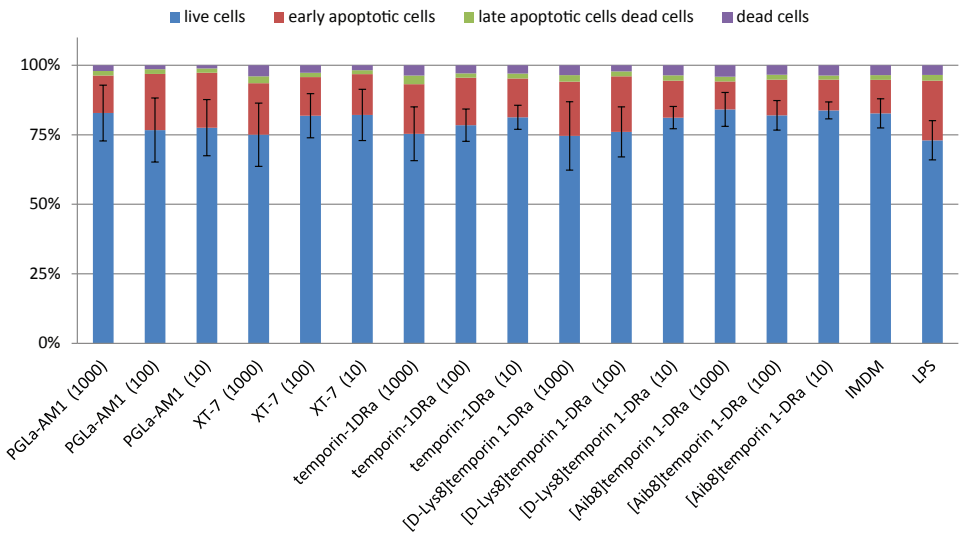

Figure 3. Human PBMCs obtained from three healthy donors were exposed for 24 h to 100, 10 or 1 µg/mL of PGLa-AM1, XT-7, temporin-1DRa, [D-Lys^{8}]temporin-1DRa, and [Aib^{13}]temporin-1DRa after which cells were stained with Annexin V and PI and analyzed using flow cytometry for the quantification of cell apoptosis and necrosis. Relative percentages of cells are shown, error bars depict SD of live cells between averaged values of all three donors.

Discussion

Many Anurans secrete host-defense peptides (HDPs) into the outer skin mucosa [3, 4], that may have potent and broad-range antimicrobial activity against bacteria, fungi and protozoa. Consequently, they are interesting candidates for antimicrobial therapeutic applications [9, 10]. The amphipathic peptides we tested can limit growth of several multidrug-resistant Gram-positive and Gram-negative pathogens. We found that the *Pseudomonas aeruginosa* strains were relatively insensitive to the action of the peptides. This is possibly due to the secretion of extracellular proteases that aid in their resilience towards peptide antimicrobials [38, 39].

The MIC and MBC values obtained for PGLa-AM1, PGLa-LM1, CPF-AM1, alyteserin-1c, hymenochirin-2B, and hymenochirin-4B and the [Aib^{8}], [Lys^{4}], [Lys^{5}] and [Aib^{9}] temporin-1DRa analogues showed promising differential activity against the pathogenic bacteria *Staphylococcus pseudintermedius*, *Enterococcus faecium*, and *Acinetobacter baumanii* compared to the probiotic strains of *Lactobacilli* (Figures 1 and 2) . In a recent study it was shown that PGLa-AM1 and CPF-AM1
have potent antimicrobial activity against a selection of oral pathogens [40]. We showed that the Gram-positive lactic acid bacteria, which are often present in probiotic supplements or food products, are not susceptible at peptide concentrations that are bactericidal to these oral pathogens [40]. This highlights the potential of these peptides for selective antimicrobial therapy against such oral pathogenic bacteria. Furthermore, the multi-drug resistant *S. pseudintermedius* and vancomycin-resistant *E. faecium* strains did not show a markedly different sensitivity from the antibiotic-sensitive strains, suggesting there is no cross-resistance mechanism to cationic amphipathic AMPs.

We also tested the potential cytotoxicity of selected peptides against human PBMCs, as it would be important not to inhibit the defensive immune response of the host if these peptides were used as topical applications to treat infections. We found that concentrations of peptides that effectively inhibited bacteria did not cause necrosis or apoptosis against human PBMCs.

Based on this study, we propose that peptides alyteserin-1c, PGLa-AM1, PGLa-LM1, CPF-AM1, temporin-1DRa and its [Lys^{4}], [Lys^{5}], [Aib^{8}], and [Aib^{9}] analogues are interesting candidates for further research into potential use as novel topical therapeutics for treatment of skin infections caused by antibiotic resistant bacteria. Based on the observed MIC values, temporin-1DRa shows great promise to be used to treat canine skin infections by *S. pseudintermedius*, a bacterium that causes high morbidity and seriously lower the quality of life of affected dogs [34]. Moreover, the hymenochirin-2B and -4B peptides displayed high potency against multi-drug resistant *A. baumannii*, pathogens that cause severe wound infections [41] and are an important cause of difficult to treat nosocomial infections [42]. The differential activity of

these antimicrobials against several pathogenic bacteria but not lactic acid bacteria might be advantageous as many commensal species of bacteria including *Lactobacilli* are considered beneficial and potentially contribute toward colonization resistance against pathogenic bacteria [43, 44].

Recently, several biotechnological tools have become available that open up avenues to develop promising applications for AMPs, including the peptides described in this study [45]. Tethering and display of AMPs on nanoparticles, fibers or polymers for localized and controlled delivery, increased stability and enhanced activity are examples of possible therapeutic applications of AMPs against MDR pathogenic bacteria in the future.

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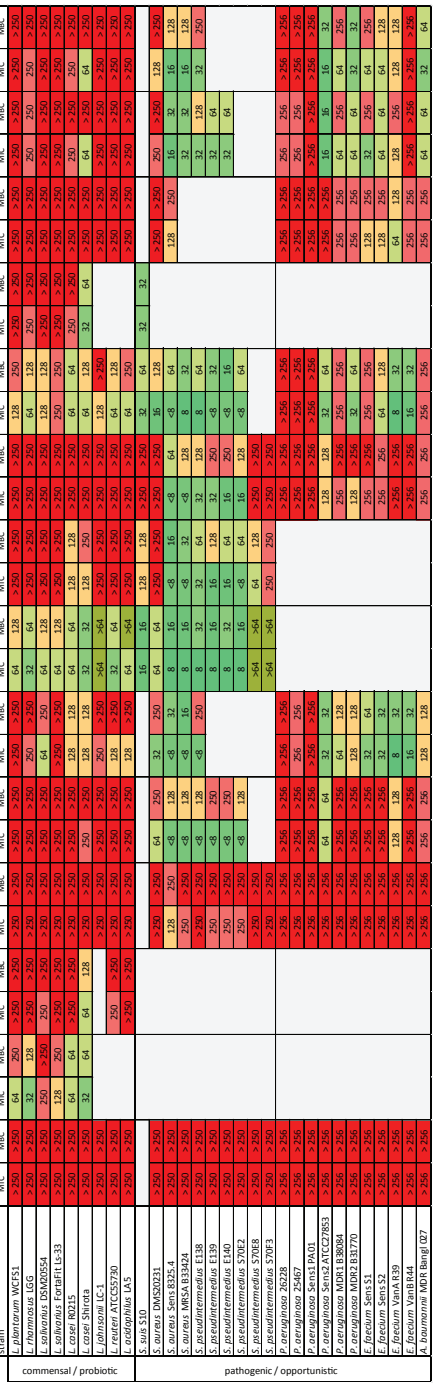
Supplemental Information

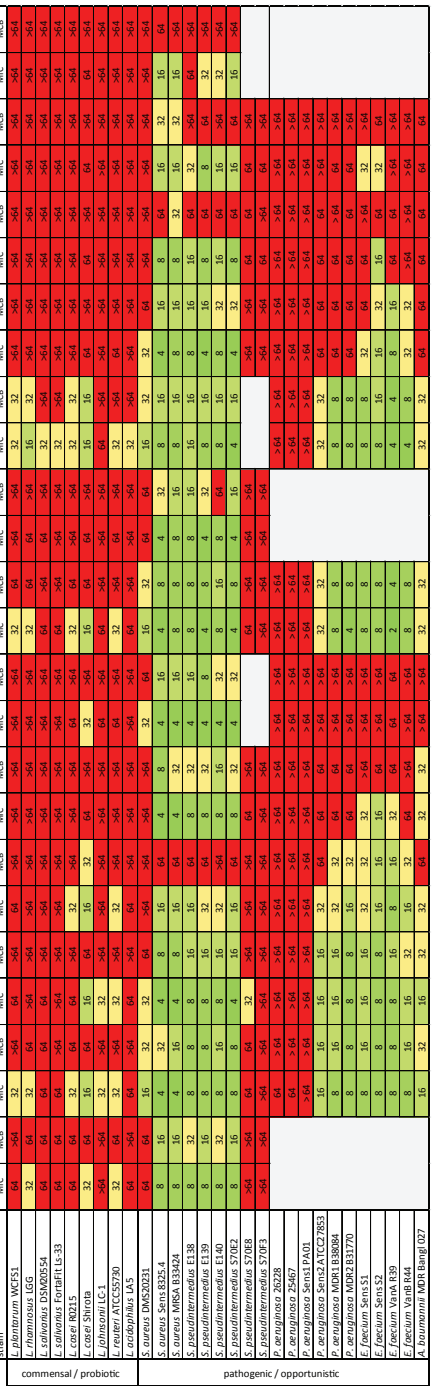
	# of antibiotics w/ resistances	resistance to % of tested antibiotics	inhibits cell wall synthesis	inhibits cell wall synthesis (murA)	inhibits protein synthesis	inhibits protein synthesis/ RNA synthesis	interferes with DNA replication and repair	inhibits RNA synthesis	inhibits folic acid synthesis	disrupts bacterial cell membrane	damages DNA, ribosomal proteins
Lb plantarum WCFS1	7	41.2	3		2		1	1			
Lb rhamnosus LGG											
Lb salivarius DSM20554											
Lb casei R0215	6	42.9	2		4						
Lb johnsonii LC-1 Nestle											
Lb reuteri ATCC55730											
Lb acidophilus LA5											
Lb salivarius FortaFit Ls-33											
Lb casei Shirota											
S. suis S10	2	10.5		1	1						
S. aureus DMS20231	0	0									
S. pseudintermedius E138	4	21.1	1		3						
S. pseudintermedius E139	3	15.8	1		1				1		
S. pseudintermedius E140	10	52.6	2	1	5		1		1		
S. pseudintermedius S70E2	6	31.6	1		5						
S. pseudintermedius S70E8	2	10.5	1		1						
S. pseudintermedius S70F3	1	50			1						
P. aeruginosa 26228	0	0									
P. aeruginosa 25467	0	0									
P. aeruginosa Sens1 PA01	0	0									
P. aeruginosa Sens2 ATCC27853	0	0									
P. aeruginosa MDR1 B38084	7	87.5	3		2		1		1		
P. aeruginosa MDR2 B31770	6	75	4		1				1		
E. faecium Sens S1	0	0									
E. faecium Sens S2	5	27.8	2		2				1		
E. faecium VanA R39	4	57.1	2		1						1
E. faecium VanB R44	4	57.1	2		2						1
S. aureus Sens 8325.4	0	0									
S. aureus MRSA B33424	9	81.8	1		5		1	1			
A. baumannii MDR Bangl 027	16	84.2	11		2		2	1			

Supplemental Table 1. VITEK antibiotic resistance profile for bacteria used in this study. The number of antibiotics for which each tested bacterial strain was classified as resistant based on the detected MIC value and clinical breakpoints are listed and colored from highest (red) to lowest (blue) number of antibiotic resistances. Additionally, the general mode of action of the corresponding antibiotics to which resistance was observed are listed.



	peptide (µg/mL)	Pseudin-2		Brevinin-1Wy		B2-AP		Magainin-AM1		PGla-AM1		CPF-AM1		Temporin-Oltu		Alyteserin-1c		PGla-AM1		YK-7		Hymenochirin-1B		Alyteserin-2a		Hymenochirin-2B		Hymenochirin-4B	
	strain	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
commensal / probiotic	*L. plantarum* WCFS1	> 250	> 250	64	250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	64	128	> 250	> 250	> 250	> 250	128	250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
	L. rhamnosus LGG	> 250	> 250	32	128	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	32	64	> 250	> 250	> 250	> 250	64	128	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
	L. salivarius DSM20554	250	> 250	250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	64	128	> 250	> 250	> 250	> 250	128	128	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
	L. salivarius FocalFit Ls-33	> 250	> 250	128	250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	64	128	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
	L. salivarius RO215	> 250	> 250	64	64	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	64	128	> 250	> 250	> 250	> 250	64	64	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
	L. coleii Strain1	> 250	> 250	32	64	64	128	> 250	> 250	250	> 250	128	128	32	64	128	> 250	> 250	> 250	64	128	32	64	> 250	> 250	64	> 250	64	> 250
	L. johnsonii LC-1	> 250	> 250					> 250	> 250	> 250	> 250	250	> 250	> 64	> 64	> 250	> 250	> 250	> 250	> 250	> 250			> 250	> 250	> 250	> 250	> 250	> 250
	L. reuteri ATCC5730	> 250	> 250			250	> 250	> 250	> 250	> 250	> 250	128	> 250	32	64	> 250	> 250	> 250	> 250	64	128			> 250	> 250	> 250	> 250	> 250	> 250
	L. acidophilus LA5	> 250	> 250			> 250	> 250	> 250	> 250	> 250	> 250	128	> 250	64	> 64	> 250	> 250	> 250	> 250	64	250			> 250	> 250	> 250	> 250	> 250	> 250
pathogenic / opportunistic	*S. suis* 510													16	16	128	128	> 250	> 250	32	64	32	32						
	S. aureus DMSZ0221	> 250	> 250					> 250	> 250	64	250	32	250	64	64	> 250	> 250	> 250	> 250	16	128	> 250	> 250	> 250	> 250	250	> 250	128	> 250
	S. aureus Sens 8325.4	> 250	> 250					128	250	<8	128	<8	32	8	16	<8	16	<8	64	<8	64	<8	<8	> 250	> 250	128	250	128	128
	S. aureus MRSA 93424	> 250	> 250					250	> 250	<8	128	<8	16	8	16	<8	32	<8	128	8	32			> 250	> 250	32	32	16	128
	S. pseudintermedius L138	> 250	> 250					> 250	> 250	<8	128	<8	250	8	16	32	32	32	64	8	64			> 250	> 250	32	128	32	250
	S. pseudintermedius L139	> 250	> 250					250	> 250	<8	250			8	16	16	128	32	250	<8	32			> 250	> 250	32	64		
	S. pseudintermedius L140	> 250	> 250					250	> 250	<8	250			8	32	16	64	16	64	<8	16			> 250	> 250	32	64		
	S. pseudintermedius S70f2	> 250	> 250					250	> 250	<8	128			8	16	<8	64	16	128	<8	64								
	S. pseudintermedius S70f6	> 250	> 250					> 250	> 250					> 64	> 64	64	128	> 250	> 250										
	S. pseudintermedius S70f3	> 250	> 250					> 250	> 250					> 64	> 64	250	250	> 250	> 250										
	P. aeruginosa 26228	> 256	> 256					> 256	> 256	> 256	> 256	> 256	> 256					> 256	> 256	> 256	> 256								
	P. aeruginosa 25467	> 256	> 256					> 256	> 256	256	256	256	256					> 256	> 256	> 256	> 256								
	P. aeruginosa Sens1 PA01	> 256	> 256					> 256	> 256	> 256	> 256	> 256	> 256					> 256	> 256	> 256	> 256								
	P. aeruginosa Sens2 ATCC27853	> 256	> 256					> 256	> 256	64	64	32	32					128	128	32	64								
	P. aeruginosa MDR1 B3084	> 256	> 256					> 256	> 256	> 256	> 256	64	128					256	> 256	256	256								
	P. aeruginosa MDR2 B3170	> 256	> 256					> 256	> 256	> 256	> 256	128	128					128	> 256	32	64								
	E. faecium Sens 31	> 256	> 256					> 256	> 256	> 256	> 256	32	64					256	> 256	256	256								
	E. faecium Sens 32	> 256	> 256					> 256	> 256	> 256	> 256	32	32					256	256	64	128								
	E. faecium VanA139	> 256	> 256					> 256	128	128	> 256	8	32					> 256	> 256	8	32								
	E. faecium VanB144	> 256	> 256					> 256	> 256	> 256	> 256	16	32					> 256	> 256	16	32								
	A. baumanii MDR Bangl 007	> 256	> 256					> 256	> 256	256	256	128	128					256	256	256	256								

Supplemental Table 2. MIC and MBC values of frog skin peptides against a panel of bacteria, as determined by micro broth dilution assay. Experiments were performed in duplicate, average values are shown.



peptide (µg/mL)		Temporin 1DRa		Lys-4 Temporin 1DRa		Lys-5 Temporin 1DRa		D-Lys-4 Temporin 1DRa		D-Lys-5 Temporin 1DRa		D-Lys-8 Temporin 1DRa		Ala-8 Temporin 1DRa		Ala-9 Temporin 1DRa		Ala-10 Temporin 1DRa		Ala-13 Temporin 1DRa		Orn-7 Temporin 1DRa		DAb-7 Temporin 1DRa		TML-7 Temporin 1DRa	
strain		MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB	MIC	MCB
commensal / probiotic	*L. plantarum* NKPS1	64	>64	32	>64	64	>64	64	>64	>64	>64	>64	>64	32	64	>64	>64	32	>64	>64	>64	>64	>64	>64	>64	>64	>64
	L. rhamnosus LGG	32	64	32	64	>64	>64	>64	>64	>64	>64	>64	>64	32	64	64	>64	16	32	>64	>64	>64	>64	>64	>64	>64	>64
	L. salivarius DSM20554	64	64	64	64	>64	>64	>64	>64	>64	>64	>64	>64	64	>64	64	>64	32	>64	>64	>64	>64	>64	>64	>64	>64	>64
	L. salivarius FerdiFLs-33	64	64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
	L. casei A025	64	64	64	64	>64	>64	32	>64	>64	>64	64	>64	32	64	>64	>64	32	>64	>64	>64	>64	>64	>64	>64	>64	>64
	L. casei Shirota	32	64	16	64	16	64	16	32	64	>64	32	>64	16	64	64	>64	16	64	>64	>64	>64	>64	>64	>64	64	>64
	L. johnsonii LC-1	>64	>64	32	>64	32	>64	>64	>64	>64	>64	64	>64	64	>64	>64	>64	64	>64	>64	>64	>64	>64	>64	>64	>64	>64
	L. reuteri ATCC55730	32	64	32	>64	32	>64	32	>64	>64	>64	64	>64	32	>64	64	>64	32	>64	>64	>64	>64	>64	>64	>64	>64	>64
	L. acidophilus LA5	64	>64	64	>64	64	>64	64	>64	>64	>64	>64	>64	64	>64	>64	>64	32	>64	>64	>64	>64	>64	>64	>64	>64	>64
pathogenic / opportunistic	*S. aureus* DM620231	64	64	16	32	32	64	>64	>64	>64	>64	32	64	16	32	64	64	16	32	32	64	>64	>64	>64	>64	>64	>64
	S. aureus Sens 8325.4	8	16	4	32	4	8	16	64	4	8	4	16	4	8	4	32	8	16	4	16	8	64	16	32	16	64
	S. aureus MRSA 633424	8	16	4	16	4	8	16	64	4	32	4	16	8	8	8	16	8	16	8	16	8	32	16	32	16	>64
	S. pseudintermedius E138	8	32	8	8	8	16	16	64	8	32	4	16	8	8	8	16	16	16	8	16	16	64	32	>64	64	>64
	S. pseudintermedius E139	8	16	8	8	8	16	32	64	8	32	4	8	4	8	4	32	8	16	4	16	8	64	8	64	32	>64
	S. pseudintermedius E140	8	32	8	16	8	16	32	>64	8	16	4	32	8	16	8	64	8	16	8	32	16	64	16	>64	32	>64
	S. pseudintermedius S70E2	8	16	8	8	4	16	16	64	8	32	4	32	4	8	4	16	4	16	4	32	8	64	16	64	16	>64
	S. pseudintermedius S70E8	>64	>64	64	64	32	>64	>64	>64	64	>64	64	>64	64	>64	>64	>64	>64	>64	>64	>64	64	64	64	64	>64	>64
	S. pseudintermedius S70F3	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	64	64	>64	>64	>64	>64
	P. aeruginosa 26228	64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
	P. aeruginosa 25467	64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
	P. aeruginosa Sens1 PA01	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
	P. aeruginosa Sens2 ATCC27853	16	16	16	16	16	16	64	64	64	64	>64	>64	32	32	32	32	32	64	64	64	64	64	64	64	64	>64
	P. aeruginosa MDR1 B38084	8	16	16	16	32	32	64	64	64	64	>64	>64	8	8	8	8	8	64	64	64	64	64	64	>64	64	>64
	P. aeruginosa MDR2 B31770	8	8	8	8	16	32	64	64	>64	>64	>64	>64	4	8	8	8	8	64	64	64	64	64	64	>64	64	>64
	E. faecium Sens S1	8	16	16	16	32	32	32	32	>64	>64	>64	>64	8	8	8	8	8	32	64	64	64	64	>64	32	>64	>64
	E. faecium Sens S2	8	8	8	8	16	16	16	32	64	64	>64	>64	8	8	8	8	16	16	32	64	16	64	64	64	64	64
	E. faecium VanA B39	8	8	8	8	16	16	8	16	32	64	64	64	2	4	4	4	4	8	16	64	64	64	64	>64	>64	>64
	E. faecium VanB R44	8	16	16	16	32	16	32	64	64	>64	>64	>64	8	8	4	8	4	32	32	>64	>64	>64	>64	>64	>64	>64
	A. baumannii MDR Bangl 027	16	32	16	32	64	64	32	32	>64	>64	32	32	32	32	32	32	32	64	64	64	>64	64	64	64	64	64

Supplemental Table 3. MIC and MBC values of temporin-1DRa analogues against a panel of bacteria, as determined by micro broth dilution assay. Experiments were performed in duplicate, average values are shown.

CHAPTER 3

Transcriptome analysis of the generic and adaptive responses of *Streptococcus suis* to sub–lethal concentrations of LL–37

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Abstract

Many bacteria have evolved inducible resistance mechanisms to resist the action of antimicrobial peptides (AMPs) and may even use their presence as environmental cues to regulate virulence-associated genes. The aim of this study was to investigate if generic and adaptive resistance could be induced in *S. suis* upon exposure to sub-lethal dosages of a model cationic AMP, the human cathelicidin LL-37. A flow cytometric assay for live and dead bacteria was used to determine that the lethal concentration of LL-37 for *S. suis* was 2.5 μM, which is similar to that determined for *Bacillus subtilis*. The susceptibility of *S. suis* to LL-37 was not affected by the presence of a capsule as shown by the use of a capsule-negative mutant J28. However, *S. suis* strain 8039 (serotype 7) appeared to be slightly less susceptible to LL-37 killing at concentrations below the MBC than strains S10 (serotype 2) or 8067 (serotype 9). Microarray gene expression profiling of *S. suis* exposed to 1 and 0.25 uM LL-37 for 10 and 30 min revealed a complex stress response consisting of a general stress response, including increased expression of the heat shock operons. Putative peptide antibiotic sensing and transporter systems found to play a role in other Gram-positive bacteria in export of specific cell-envelope acting peptide antibiotics were also highly induced upon exposure to LL-37. The *S. suis* deletion mutant of TCS SSU0827/28, which is located downstream of the induced putative peptide transporter gene cluster, was equally sensitive to LL-37. Although the *dlt* operon has been shown in other bacteria increase resistance to cationic AMPs, a deletion mutant of *dltA* was not more susceptible than the wt strain to LL-37. Additionally, we found that exposure to LL-37 substantially increased expression of 11 genes encoding (putative) surface proteins or exported proteins, some of which are proposed to be involved in virulence. This work shows that *S. suis* appears to possess a transcriptional response program that regulates tolerance to sub-lethal concentrations of the cationic AMP LL-37. Understanding these key processes in bacterial defense responses to host immune mediators may provide leads to novel strategies to combat *S. suis* in pig husbandry.

Introduction

Streptococcus suis is an encapsulated Gram-positive facultative anaerobe and opportunist pathogen, frequently present as a member of the porcine microbiota on the mucosal surfaces of the oro- and nasopharynx, and the intestinal and genital tracts [1-4]. Carriage of *S. suis* in adult pigs is usually asymptomatic whereas young pigs post-weaning (weeks 4 to 12) are most at risk from invasive disease [5, 6], with symptoms such as acute sepsis and meningitis, endocarditis, pneumonia and arthritis often being reported [7, 8]. *S. suis* is also a zoonotic pathogen and human infections worldwide have increased in the past years, with most cases originating in Southeast Asia [9] [10]. Currently, no efficacious, cross-protective vaccines exist to protect animals against infection by *S. suis* [11, 12]. It is worrisome that resistance of *S. suis* to macrolides, lincosamides, tetracyclines, and sulphonamides has been reported for up to 85% of isolates in many countries throughout the world [13]. Of concern are the recent emergence of highly virulent strains causing more rapid and severe disease [14] and the risk that the burden of both zoonotic and porcine disease will increase due to European Union policies on reducing veterinary antibiotic use [15]. Consequently several alternatives are being considered for disease prevention in livestock animals [16] including applications of eukaryotic antimicrobial peptides (AMPs) and their analogues which have been been proposed as new treatment strategies for multi-drug resistant pathogens [17, 18]. For *S. suis* and many other pathogens, successful colonization of the host mucosa is a prerequisite to invasive disease. Host AMPs, (also known as host-defense peptides (HDPs)) are an important component of the innate defense against pathogens and are expressed at mucosal sites either constitutively or in an induced manner by pattern recognition receptor-mediated recognition of microbes [19, 20]. One important family of HDPs are the cathelicidins, comprising a wide variety of peptides of varying length and structure, but all containing a C-terminal cationic antimicrobial domain that becomes active after being freed from the conserved N-terminal cathelin domain of the holoprotein [21, 22]. In humans cathelicidin hCAP-18 is produced as an inactive 18-kDa pre-pro-protein that is proteolytically cleaved to generate the active carboxy-terminal 37 amino acid □–helical peptide LL-37 [21, 23, 24].

Most AMPs are cationic and amphipathic and mediate bacterial killing through the aggregation of peptide monomers to form transmembrane channels or upon insertion of the peptides into the cell membrane to disrupt the native integrity of cell membrane [17, 25, 26]. This leads to dissipation of the ion gradient, transmembrane potential and cell death. AMPs have also been identified with other modes of action including inhibition of cell replication or transcriptional processes [27-30].

LL-37 is produced by epithelial cells and neutrophils at several body sites and has been shown to possess a plethora of functions, such as antimicrobial, cytotoxic, immunomodulatory, wound-healing and endotoxin-binding activities [22, 31, 32]. Human LL-37 and the porcine cathelicidin PR-39 are produced in the oral cavity and respiratory tract [33-37], two body sites which can be colonized by *S. suis* [2]. The alimentary and respiratory tracts are also inhabited by other microbes, many of which are capable of producing AMPs, including ribosomally

synthesized bacteriocins and non-ribosomally synthesised secondary small molecule metabolites, to improve their competitiveness [38, 39]. As a result, the mucosal surfaces that can be colonised by *S. suis* may contain multiple host- or microbe-derived AMPs.

Given the role of cationic AMPs, such as LL-37, in innate immunity, many bacteria have evolved to develop redundant strategies to counter and survive regular exposure to such peptides [40, 41]. Such bacterial mechanisms for detection, electrostatic repulsion, enzymatic degradation, and active efflux of cationic AMPs were recently reviewed elsewhere [40, 41]. We hypothesize that *S. suis* strains that are commonly carried in pigs might possess mechanisms of resistance towards specific cationic AMPs [40], and these mechanisms could be cross-protective against other host- or microbe-derived AMPs considering their comparative mode of membrane-disruptive action [42].

Interestingly, host AMPs have been proposed to serve as environmental cues to elicit virulence gene expression in pathogenic bacteria colonising host mucosal surfaces, leading to bacteria gaining a more "invasive phenotype" [43-47]. Additionally, AMP-induced changes in gene expression could lead to increased adaptive resistance towards conventional antibiotics, such as macrolides, fluoroquinolones, or aminoglycosides [46, 48]. Surprisingly, acquisition of adaptive DNA mutations leading to increased bacterial mucoidy and rifampin resistance were observed upon exposure to sub-inhibitory concentrations of LL-37 [49].

The aim of this study was to investigate if generic and adaptive resistance could be induced in *S. suis* upon exposure to sub-lethal dosages of LL-37, and the potential for this cationic peptide to be recognised as a host cue for regulation of virulence gene expression in *S. suis*. We performed a transcriptome analysis of *S. suis* S10, a virulent strain of serotype 2 that can be carried in pigs and humans and is the major cause of invasive disease [9], upon short-term exposure to sub-inhibitory concentrations of LL-37. The aims of this study were to search for inducible mechanisms of resistance to AMPs as well as induction of virulence gene expression upon exposure to AMPs, in order to gain insights into host-derived factors that might mediate *S. suis* pathogenesis. If such resistance- or virulence-mediating mechanisms would exist, interfering with them may provide leads to novel strategies to combat *S. suis* in pig husbandry.

Materials and Methods

Bacteria and culture conditions

The virulent *S. suis* serotype 2 strain S10, originally isolated from the tonsil of a healthy pig [50], and *S. suis* strains 8039 (SS7) [51], and 8067 (SS9) [52], were obtained from Central Veterinary Institute (Lelystad, Netherlands). The genome of *S. suis* S10 is genetically highly similar (>99%) to the sequenced *S. suis* serotype 2 reference strain p1/7 [53] (unpublished Genome Announcement). All *Streptococcus* strains were cultured without agitation at 37°C with 5% atmospheric CO_2 in Todd-Hewitt Broth (THB) (Oxoid, Basingstoke, UK), supplemented with 100 µg mL^{-1} spectinomycin (Invitrogen) when required.

Generation of *S. suis* mutants

Insertional deletion mutants of the TCS genes SSU0827/0828 and SSU1930/1931, were made in *S. suis* strain S10 using Gene Splicing Overlap Extension PCR and allelic replacement as described previously [54]. A mutant lacking the *dltA* gene (SSU0596) was made similarly, by transformation of *S. suis* with linear DNA containing a spectinomycin resistance cassette (*aad9*) between upstream and downstream regions of *dltA*. The plasmid p5ΔdltA [55] was used as a template for the amplification of the linear DNA fragment using the oligonucleotide primer pair dlt_fw (5'-GGTGCATGCTGAAGAGAAAG-3') and dlt_rev (5'-GACCGACCGAAACGTTAAATAAG-3'). *S. suis* strains were grown overnight in THB broth at 37°C under 5% CO_2. The overnight culture was diluted 1:40 into pre-warmed media, and grown at 37°C until $OD_{600\ nm}$ of 0.035 (Spectramax M5, Molecular Devices, Sunnyvale CA, USA). The bacterial suspension (100 µL) was incubated with 200 ng donor DNA together with 250 µM competence inducing peptide [56] for 2 hours at 37°C in 1.5 ml Eppendorf Safe Lock Tubes™, after which the samples were diluted and plated onto THB agar media containing 100 µg mL^{-1} spectinomycin.

Bactericidal effect of LL–37 determined by Live/Dead staining and flow cytometry

All media and buffers were filtered prior to use with 0.2 µm syringe filters (Merck Millipore, Darmstadt, Germany). THB was inoculated 1:1000 from a frozen glycerol stock of *S. suis*, and incubated at 37°C with 5% CO_2 for 4 h to obtain a culture with an $OD_{600\ nm}$ of ~0.2. Bacteria were collected by centrifugation at 6000 x *g* for 5 min, and the pellet was resuspended in PBS (supplemented with 0, 1, 5, or 10% v/v THB) to obtain an inoculum containing ~5 x 10^5 CFU ml^{-1}. In 96-well microtiter plates, bacterial suspension (90 µL) was added to each well containing peptide dilutions in PBS (10 µL). Bacteria were exposed to peptide for 30 or 60 min at 37°C with 5% CO_2, and then stained using the LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (Molecular Probes, Leiden, The Netherlands) according to manufacturer's instructions. Briefly, 150 µL 0.85% NaCl was added to each well, and the total volume was transferred to a flow-cytometry tube containing 0.75 µL of dye A + B mix. Tubes were vortexed shortly and after 15 min incubation at RT in the dark samples were analysed using flow cytometry (FACS Canto II, BD Biosciences). The proportions of live (SYTO

9 positive) and dead (propidium iodide positive) bacteria were determined (BD FACSDiva) and data were represented as percentage live bacteria (# live/(# live + # dead)) x 100).

Experimental design and RNA extraction

A volume of 5 mL THB was inoculated from a frozen glycerol stock of *S. suis* S10 and was incubated overnight at 37°C with 5% CO_2. This culture was diluted 1:40 in pre-warmed THB and grown for approximately 3 h until an $OD_{600\ nm}$ of 0.25 was reached. Cells were collected by centrifugation for 5 min at 5000 x g, and the pellet resuspended in an equal volume of fresh, pre-warmed THB. In a 50 mL Falcon tube, 2.5 mL of this cell suspension was added to 47.5 mL of pre-warmed sterile PBS, immediately followed by addition of LL-37 to a final concentration of 0.1 µM or 0.25 µM. Bacteria were incubated at 37°C for 5 or 25 min and collected by centrifugation for 5 min at 8000 x g at 30°C. Supernatant was carefully discarded and the pellets were resuspended in 2.5 mL PBS plus 5 mL RNAprotect Bacteria Reagent (Promega) by vortexing and incubated for 5 min at RT. The bacteria were collected by centrifugation and the pellet immediately frozen in liquid nitrogen and stored at -80°C until further handling. The frozen pellet was redissolved in 110 µL of TE containing protease K (1.25 µg mL^{-1}) and lysozyme (15 µg mL^{-1}) by thorough vortexing and incubated at RT for 10 min. Then, 700 µL RLT buffer (Promega) containing 7 µL of freshly added β-mercaptoethanol was added and the whole volume was transferred to FastPrep matrix B tubes and run for 40 sec at 6.0 m sec^{-1} on a FastPrep-24 (MP Biomedicals, Solon, OH) for mechanical disruption of the bacteria. Total RNA was purified from the obtained bacterial lysate using the RNeasy Mini Kit (Qiagen) following manufacturer's instructions. The quality, concentration and integrity of RNA were assessed using the Experion System (Bio-Rad) and by spectrometric analysis of the A260/A280 ratio (NanoDrop 8000 UV-Vis). cDNA was synthesized with a SuperScript III Reverse Transcriptase Kit (Invitrogen) using Aminoallyl-UTP and purified with the Illustra CyScribe GFX Purification Kit (GE Healthcare). The aminoallyl-cDNA was labelled using the CyDye Post-Labelling Reactive Dye Pack (GE Healthcare).

Microarray transcriptome analysis

An *S. suis* oligonucleotide array (Array Express Accession: A-MEXP-1916) containing *in situ* synthesized 60-mers (8 x 15K) was produced by Agilent Technologies (Santa Clara, USA), based on the genome sequence of *S. suis* p1/7 [53]. A total of 7651 unique 60-mers having a theoretical melting temperature of approximately 81°C and representing 1960 ORFs were selected as described [57]. Genes were represented by 4 (91%), 3(4%), 2(2%), or 1(3%) probe(s). Twenty-five putative genes were not represented because no unique probes could be selected that met the selection criteria. Co-hybridization with Cy3- and Cy5-labelled cDNA probes was performed on these oligonucleotide arrays for 16 h at 65°C in GEx HI-RPM Hybridisation buffer (Agilent). The slide was washed in Gene Expression Wash Buffer (Agilent) 1 (2x) and then in Wash Buffer 2 (1x) and air-dried in an ozone-free environment. The slide was scanned in a ScanArray Express 4000 (Perkin Elmer, Wellesley, MA) subsequently at three

different photomultiplier (PMT) values. Data were normalized using Lowess normalization [58] as available in MicroPrep [59] and corrected for inter-slide differences on the basis of total signal intensity per slide using PostPrep [59]. Significance of differential gene expression was based on false discovery rate (FDR) values lower than 0.05. (Data will be deposited in the NCBI GEO database, accession #)

Transcriptome data mining

Short Time-series Expression Miner (STEM) [60] was used for clustering and comparing gene expression intensities across different peptide concentrations. The Bonferroni correction method for multiple hypothesis testing of model profiles was used, with a significance level of 0.05. Significant models that showed a correlation of more than 0.6 were clustered.

Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) was used to filter the data for the most statistically significant differentially expressed genes with the following parameters: standard deviation (gene vector): 300; ≥ 4 observations with absolute value higher than 20; subtraction between maximum and minimum value: ≥ 200.

Heatmaps displaying normalised expression values or log-transformed intensity ratios were generated using the Multiple Experiment Viewer (MeV) version 4.9.0 (http://www.tm4.org/mev.html) [61].

Results

Assays to determine the minimum inhibitory and bactericidal concentrations of LL–37

As an initial step towards the investigation of adaptive responses to LL-37 we determined the minimal inhibitory concentrations (MIC) of LL-37 for different strains and serotypes of *S. suis*. Initially we performed the MIC assay for LL-37 in Todd-Hewitt Broth (THB), a rich medium commonly used for laboratory growth of *S. suis,* or a complex medium (CM) supplemented with 1% (v/v) glucose or maltotriose [62]. However, the peptide concentrations that were required to achieve inhibition of growth (25 – 50 μM) resulted in peptide precipitation with media components, which interfered with optical assessment of bacterial growth (data not shown). To overcome the problem of components in THB broth interfering with LL-37 or promoting its precipitation we used a less rich medium and a rapid fluorescence-based flow cytometry assay for enumeration of live or dead bacteria and the determination of the minimal bactericidal concentration (MBC) of LL-37. In PBS supplemented with 0, 1, 5 or 10% (v/v) of THB, almost complete killing of *S. suis* was observed after 1 h in the presence of 2.5 μM LL-37 (Figure 1 A) which was 10-fold lower than for the peptide in undiluted THB (data not shown). These less rich growth conditions probably reflect better the nutrient availability *in vivo*, which will typically be more growth restrictive than rich laboratory medium due to competition between other microorganisms and rapid absorption of digested food by the host. *S. suis* S10 appeared to be more susceptible to killing by LL-37 in PBS alone compared to exposure in PBS supplemented with 1, 5 or 10 % (v/v) THB, although this difference was not statistically significant (Figure 1A).

Recovery experiments

After 1 h exposure to 0, 0.25, 1.0 or 2.5 μM LL-37 the bacteria were resuspended in fresh, pre-warmed THB medium and cultured for a further 6 hours, while growth was monitored by measuring the optical density of the culture (Figure 1B). The goal here was to determine the extent to which cells exposed to LL-37 (Figure 1A) can recover and grow normally. No growth of *S. suis* was observed when the bacteria had been previously incubated in PBS or in PBS plus 1% (v/v) THB with 2.5 μM LL-37, whereas slight increase in turbidity was seen between 5 and 7 hours in bacteria exposed to 2.5 μM LL-37 in PBS supplemented with 5 or 10 % (v/v) THB. This may be due to the effect of interfering compounds in the rich medium as mentioned above. Prior incubation of S. suis with 1.0 or 0.25 μM LL-37 allowed growth in fresh THB medium, although there was a LL-37 concentration-dependent effect on the length of the lag phase (Figure 1 B).

Influence of capsule on inhibition by LL–37

As the *S. suis* capsule is an important virulence factor and a stereotypic marker of strains associated more commonly with invasive disease, we investigated the sensitivity of strains 8039 (serotype 7) and 8067 (serotype 9), S10 (serotype 2) and its un-encapsulated isogenic mutant J28, to LL-37 in PBS containing 5% THB. Overall the sensitivity of the strains to LL-

37 was similar with viability decreasing to zero between 1 and 2.5 µM (Figure 1C). However strain 8039 (SS7) appeared to be more resistant than S10 and 8067 to 1 µM LL37, with 40% of bacteria remaining viable after 1 hour. After 1 hour exposure to1 µM LL-37, only about 10% of strains S10, J28 (unencapsulated), and 8067 (SS9) were viable, suggesting that the capsule itself does not confer significant protection against this AMP.

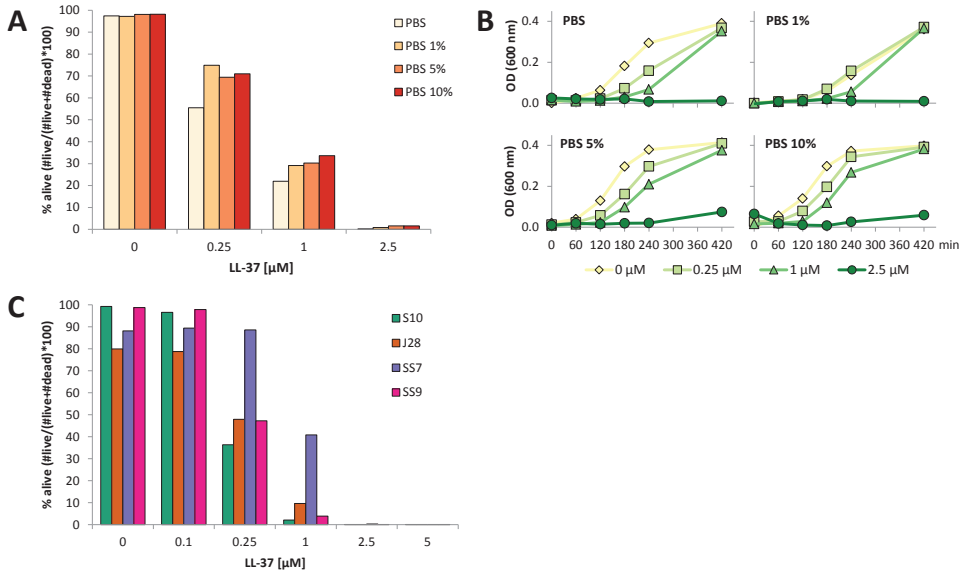

Figure 1. Bacterial viability as measured by Live/Dead staining and flow cytometry analysis of **A)** *S. suis* S10 exposed for 60 min to LL-37 in PBS or PBS supplemented with 1%, 5%, or 10% THB. **B)** Recovery (growth measured as optical density at 600 nm) of *S. suis* S10 after resuspension in fresh THB following exposure to LL-37 for 60 min in either PBS or PBS supplemented with 1%, 5%, or 10% **C)** Viability of *S. suis* strains S10, J28, 8039 (SS7), and 8067 (SS9) after 60 min exposure to LL-37 in PBS with 5% THB, determined by Live/Dead staining and flow cytometry analysis.

Effect of D–alanylation of teichoic acids and two–component systems on sensitivity to LL–37

The D-alanylation of teichoic acids has previously been shown to contribute to the resistance of Gram-positive bacteria to cationic antimicrobial peptides by electrostatic repulsion [63]. The gene products of the *dltABCD* operon incorporate D-alanyl esters in lipoteichoic acid (LTA) and wall-teichoic acid (WTA), which is considered to decrease the negative charge of the bacterial surface and to increase the cell wall density due to altered conformation of LTA which sterically hinders AMP access to the membrane [64]. We generated a deletion mutant of *dltA* in *S. suis* S10 and tested its sensitivity to LL-37 (Figure 2AB). Additionally, we investigated the role of two previously uncharacterised two-component systems (TCS) mutants on sensitivity to LL-37 as these mutants were less virulent *in vivo* and more sensitive to neutrophil killing [54]. Using the flow cytometry assay for live/dead staining described above we found that the wt S10 was not markedly more resistant to LL-37 than the *dltA* mutant when exposed for 60 min in PBS plus 5% (v/v) THB. Also after 30 min exposure to 2.5 µM LL-37 S10 and the *dltA* mutant were completely killed (Figure 2AB).

Surprisingly, the strain with a deletion of TCS SSU1930/31 was less susceptible to killing by LL-37 compared to the wild-type S10. However, this difference in susceptibility was only observed at LL-37 concentrations lower than the minimal bactericidal concentration (2.5 µM) at which complete killing of the bacteria was observed. For example, at 0.25 µM 90% of TCS mutant SSU1930/31 were viable after 60 min whereas only 60% bacteria of S10 remained alive (Figure 2B). In contrast the sensitivity of the second TCS mutant SSU0827/28 was not significantly different to S10 (Figure 2B).

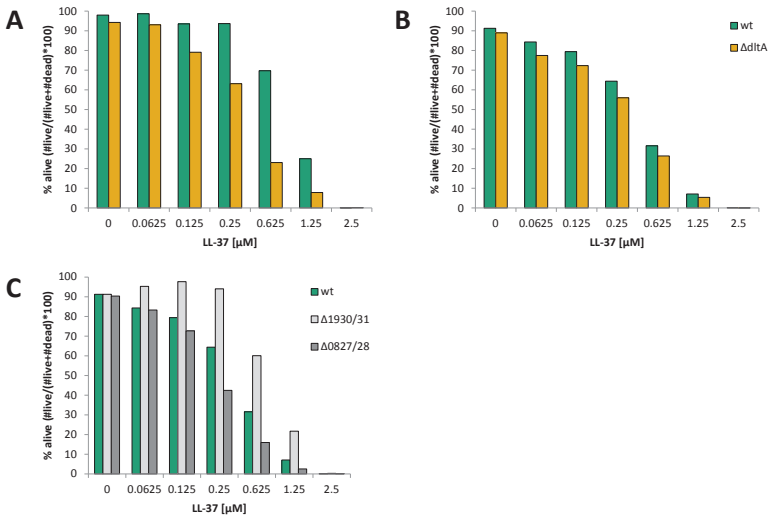

Figure 2. Bacterial viability after LL-37 exposure in PBS with 5% THB as measured by Live/Dead staining and FACS. **A)** 30 min or **B)** 60 min exposure of *S. suis* S10 (wt) and isogenic mutant Δ*dltA*. **C)** 60 min exposure of *S. suis* S10 (wt) and TCS mutants Δ1930/31 and Δ0827/28.

Transcriptome responses to sub–lethal concentrations of LL–37 – STEM analysis

To investigate the stress response and potential induction of defence mechanisms in *S. suis* exposed to LL-37, we prepared RNA from *S. suis* incubated with sub-lethal (below the MIC) concentrations of 0.1 or 0.25 µM LL-37 in PBS + 5% THB for 10 or 30 min (see Figure 1). As a control RNA was also prepared from *S. suis* incubated without LL-37. To gain insights in the altered global biological processes associated with exposure to sub-lethal concentrations of LL-37, we used STEM [60] to categorise specific concentration dependent profiles of gene expression. Figure 3 shows the STEM profiles of genes that were significantly differentially expressed with increasing LL-37 concentrations after 10 min or 30 min exposure.

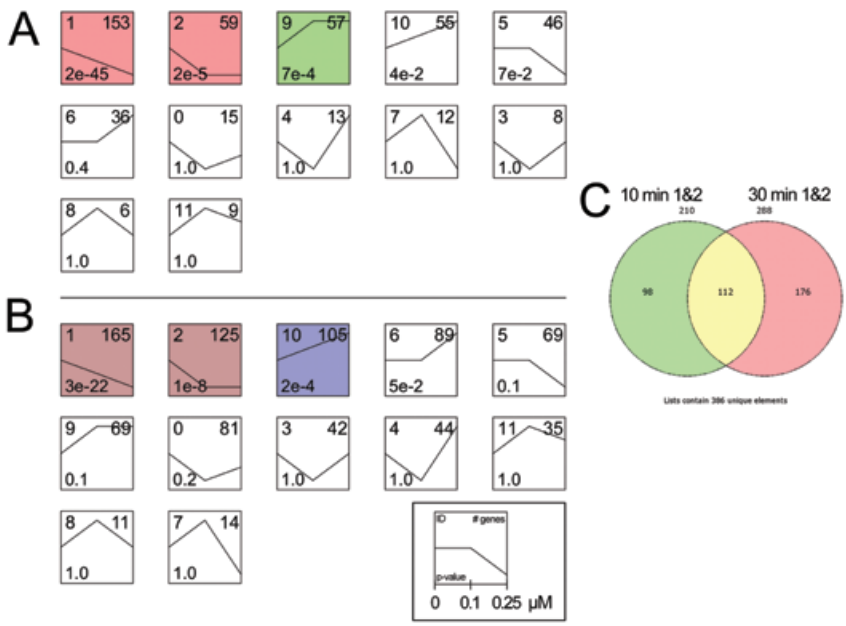

Figure 3. Differentially expressed genes with increasing LL-37 concentration were assigned to model profiles by STEM, similar profiles are clustered and have the same colour shading. All profiles for exposure time of **A)** 10 min (top) and **B)** 30 min (bottom) are shown. (profile number in top left corner, p-value of enrichment in bottom left corner and number of genes assigned in top right corner). **C)** Venn diagram representing the number of differentially genes present within profiles 1 and 2 of both time points.

STEM profiles 1 and 2 for both the 10 min and 30 min time point indicate gene transcripts that were significantly decreased with increasing concentrations of LL-37. STEM also provides the gene ontology (GO) terms associated with the genes within each profile. The GO terms that were enriched, i.e. statistically significantly overrepresented, in the clustered profile 1 and 2 for time point 10 min and 30 min are listed in Table 1 and Table 2, respectively. These results indicate a concentration-dependent decrease in transcripts of genes related to biological processes involved in transmembrane transport of ions and/or carbohydrates.

Profile 10 contains genes that were continuously being induced. GO term enrichment calculation of the profile 10 genes after 30 min exposure to LL-37 (Figure 3B) showed that these genes were enriched for GO terms associated with signal transduction, suggesting that exposure of *S. suis* to increasing concentrations of LL-37 increases signal transduction.

Profile 1 and 2 GO terms – 10 min								
Category ID	Category Name	#Genes Category	#Genes Assigned	#Genes Expected	#Genes Enriched	p-value	Corrected p-value	Fold
GO:0006810	**transport**	194	50	20.9	29.1	2.90E-10	<0.001	2.4
GO:0051234	**establishment of localization**	196	50	21.1	28.9	4.30E-10	<0.001	2.4
GO:0071702	**organic substance transport**	75	27	8.1	18.9	2.80E-09	<0.001	3.3
GO:0044765	**single-organism transport**	139	38	15	23	1.10E-08	<0.001	2.5
GO:0022891	**substrate-specific transmembrane transporter act.**	90	29	9.7	19.3	1.30E-08	<0.001	3
GO:1902578	**single-organism localization**	141	38	15.2	22.8	1.60E-08	<0.001	2.5
GO:0022892	**substrate-specific transporter activity**	94	29	10.1	18.9	4.00E-08	<0.001	2.9
GO:0022857	**transmembrane transporter activity**	123	33	13.3	19.7	1.90E-07	<0.001	2.5
GO:0008643	**carbohydrate transport**	53	19	5.7	13.3	8.10E-07	<0.001	3.3
GO:0008982	**protein-N(PI)-phosphohistidine-sugar PTS activity**	31	14	3.3	10.7	9.40E-07	<0.001	4.2
GO:0009401	**phosphoenolpyruvate-dependent sugar PTS system**	36	15	3.9	11.1	1.30E-06	<0.001	3.9
GO:1901476	**carbohydrate transporter activity**	38	15	4.1	10.9	3.10E-06	<0.001	3.7
GO:0015144	**carbohydrate transmembrane transporter activity**	38	15	4.1	10.9	3.10E-06	<0.001	3.7
GO:0016021	**integral component of membrane**	165	36	17.8	18.2	1.10E-05	0.002	2
GO:0031224	**intrinsic component of membrane**	165	36	17.8	18.2	1.10E-05	0.002	2
GO:0044425	**membrane part**	171	36	18.4	17.6	2.70E-05	0.004	2
GO:0022804	**active transmembrane transporter activity**	95	24	10.2	13.8	3.20E-05	0.004	2.3
GO:0015075	**ion transmembrane transporter activity**	72	19	7.8	11.2	1.20E-04	0.03	2.4
GO:0016773	**phosphotransferase act., alcohol group as acceptor**	74	19	8	11	1.80E-04	0.04	2.4
GO:0015294	**solute:cation symporter activity**	26	10	2.8	7.2	1.90E-04	0.04	3.6
GO:0015293	**symporter activity**	26	10	2.8	7.2	1.90E-04	0.04	3.6
GO:0015171	amino acid transmembrane transporter activity	7	5	0.8	4.2	2.40E-04	0.054	6.6
GO:0015291	secondary active transmembrane transporter activity	34	11	3.7	7.3	5.30E-04	0.078	3
GO:0005342	organic acid transmembrane transporter activity	8	5	0.9	4.1	5.90E-04	0.086	5.8
GO:0046943	carboxylic acid transmembrane transporter activity	8	5	0.9	4.1	5.90E-04	0.086	5.8
GO:0008324	**cation transmembrane transporter activity**	59	15	6.4	8.6	9.80E-04	0.122	2.4
GO:0051119	**sugar transmembrane transporter activity**	26	9	2.8	6.2	1.00E-03	0.126	3.2
GO:0008514	organic anion transmembrane transporter activity	9	5	1	4	1.20E-03	0.154	5.2
GO:0071705	nitrogen compound transport	9	5	1	4	1.20E-03	0.154	5.2
GO:0005402	**cation:sugar symporter activity**	24	8	2.6	5.4	2.50E-03	0.26	3.1
GO:0005351	**sugar:proton symporter activity**	24	8	2.6	5.4	2.50E-03	0.26	3.1
GO:0015295	**solute:proton symporter activity**	24	8	2.6	5.4	2.50E-03	0.26	3.1
GO:0016772	**transferase activity**	158	28	17	11	4.10E-03	0.336	1.6
GO:0005886	**plasma membrane**	106	20	11.4	8.6	7.40E-03	0.488	1.8

Table 1. Gene Ontology (GO) terms enriched in STEM profile 1 and 2, after 10 min exposure to LL-37. GO categories depicted in bold font were also enriched for the 30 min timepoint profiles (Table 2).

Profile 1 and 2 GO terms – 30 min								
Category ID	Category Name	#Genes Category	#Genes Assigned	#Genes Expected	#Genes Enriched	p-value	Corrected p-value	Fold
GO:0006810	**transport**	194	67	28.6	38.4	1.90E-13	<0.001	2.3
GO:0051234	**establishment of localization**	196	67	28.9	38.1	3.30E-13	<0.001	2.3
GO:0008643	**carbohydrate transport**	53	27	7.8	19.2	3.10E-10	<0.001	3.5
GO:0044765	**single-organism transport**	139	48	20.5	27.5	9.70E-10	<0.001	2.3
GO:1902578	**single-organism localization**	141	48	20.8	27.2	1.70E-09	<0.001	2.3
GO:0022891	**substrate-specific transmembrane transporter act.**	90	35	13.3	21.7	7.00E-09	<0.001	2.6
GO:0071702	**organic substance transport**	75	31	11.1	19.9	9.80E-09	<0.001	2.8
GO:0009401	**phosphoenolpyruvate-dependent sugar PTS system**	36	20	5.3	14.7	1.00E-08	<0.001	3.8
GO:0008982	**protein-N(PI)-phosphohistidine-sugar PTS activity**	31	18	4.6	13.4	2.20E-08	<0.001	3.9
GO:0022892	**substrate-specific transporter activity**	94	35	13.9	21.1	2.70E-08	<0.001	2.5
GO:1901476	**carbohydrate transporter activity**	38	20	5.6	14.4	3.50E-08	<0.001	3.6
GO:0015144	**carbohydrate transmembrane transporter activity**	38	20	5.6	14.4	3.50E-08	<0.001	3.6
GO:0022857	**transmembrane transporter activity**	123	41	18.1	22.9	6.30E-08	<0.001	2.3
GO:0016021	**integral component of membrane**	165	46	24.3	21.7	3.50E-06	0.002	1.9
GO:0031224	**intrinsic component of membrane**	165	46	24.3	21.7	3.50E-06	0.002	1.9
GO:0044425	**membrane part**	171	46	25.2	20.8	1.00E-05	0.002	1.8
GO:0015075	**ion transmembrane transporter activity**	72	25	10.6	14.4	1.30E-05	0.002	2.4
GO:0022804	**active transmembrane transporter activity**	95	30	14	16	1.50E-05	0.004	2.1
GO:0051119	**sugar transmembrane transporter activity**	26	13	3.8	9.2	2.10E-05	0.004	3.4
GO:0005886	**plasma membrane**	106	31	15.6	15.4	6.00E-05	0.012	2
GO:0016773	**phosphotransferase act., alcohol group as acceptor**	74	24	10.9	13.1	7.10E-05	0.018	2.2
GO:0071944	cell periphery	121	33	17.8	15.2	1.60E-04	0.026	1.8
GO:0005402	**cation:sugar symporter activity**	24	11	3.5	7.5	2.50E-04	0.036	3.1
GO:0005351	**sugar:proton symporter activity**	24	11	3.5	7.5	2.50E-04	0.036	3.1
GO:0015295	**solute:proton symporter activity**	24	11	3.5	7.5	2.50E-04	0.036	3.1
GO:0015294	**solute:cation symporter activity**	26	11	3.8	7.2	5.80E-04	0.1	2.9
GO:0015293	**symporter activity**	26	11	3.8	7.2	5.80E-04	0.1	2.9
GO:0019200	carbohydrate kinase activity	7	5	1	4	1.10E-03	0.182	4.8
GO:0008324	**cation transmembrane transporter activity**	59	18	8.7	9.3	1.30E-03	0.198	2.1
GO:0016772	**transferase activity**	158	37	23.3	13.7	1.70E-03	0.216	1.6

Table 2. Gene Ontology (GO) terms enriched in STEM profile 1 and 2, after 30 min exposure to LL-37. Bold GO categories depicted in bold were also enriched for the 10 min timepoint profiles (Table 1).

Although the STEM analysis gave insight into global biological processes that were altered in *S. suis* upon exposure to sub-lethal concentrations of LL-37, we were also interested in the individual genes that were differentially expressed. The most differentially expressed genes were identified by pre-filtering of the gene expression intensity data using Cluster 3.0 (see Methods). A total of 97 genes remained after filtering and manual removal of genes encoding ribosomal proteins. These genes were clustered using Multiple Experiment Viewer (MeV) using Euclidean distance and average linkage, and are displayed in Figure 4.

Eight genes associated with bacterial general stress response were upregulated with increasing LL-37 concentrations: these genes included the heat shock response transcriptional regulator HrcA, heat shock protein GrpE, chaperone protein DnaK and DnaJ, heat shock protein 60 family chaperone GroEL (60 kDa chaperonin) and co-chaperone GroES (10 kDa chaperonin) (Figure 4). The chaperones encoded by these genes associate with proteins to prevent misfolding and to assist in proper folding [65, 66].
One cluster of genes that were strongly upregulated after exposure to LL-37, especially 0.1 µM for 30 minutes, included the genes SSU0831 to SSU0836 which form a putative operon that is predicted to be involved in lantibiotic transport or immunity. The genes SSU0832

and SSU0835 are annotated as ABC transporter ATPase, and SSU0831 and SSU0834 are annotated as ABC transporter permease. The downstream genes SSU0827/28 encoding a TCS were also slightly, but not significantly, upregulated upon exposure to LL-37. This resembles the organisation of the antibiotic peptide sensing and transporter systems that have been described for some Gram-positive bacteria [67-70], where sensing of the antimicrobial peptide through a TCS histidine kinase induces expression of upstream ABC transporter genes which actively remove the peptide.

This cluster of upregulated genes also contains 11 putative membrane, surface-associated, or exported proteins with unknown function (Figure 4).

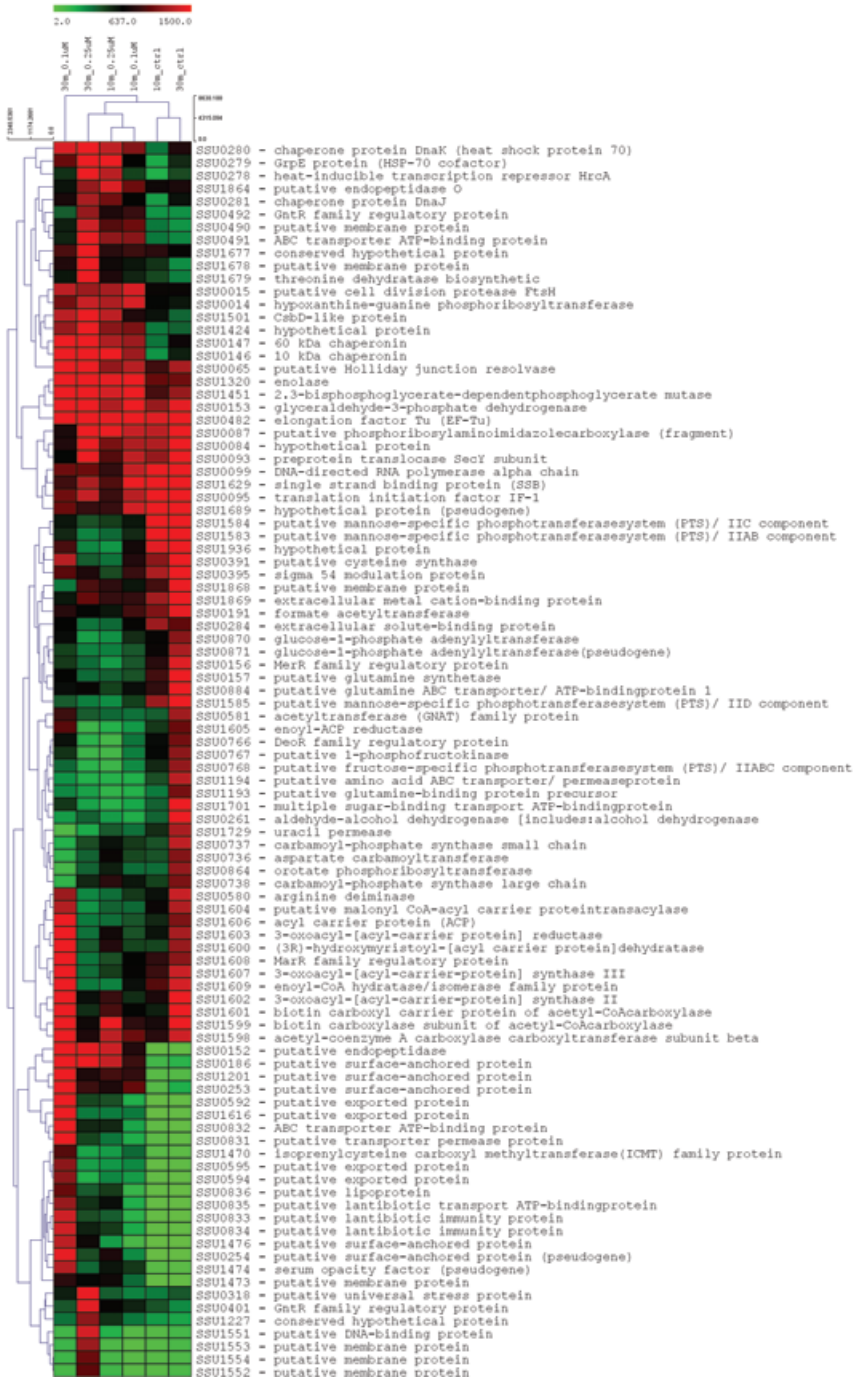

Figure 4. Heatmap displaying the 96 most differentially expressed genes after filtering with Cluster 3.0 (see Methods), clustered using average linkage and Euclidean distance.

Effect of exposure to LL–37 on the expression of known or putative virulence–associated genes

We examined the effect of LL-37 exposure on the transcription of genes known to be associated with virulence and pathogenesis of *S. suis* [71]. Exposure to LL-37 at concentrations below the MIC for 10 and 30 minutes, moderately increased relative transcription of 23 virulence-associated genes including, neuraminidase, fibronectin-binding protein, suilysin, subtilisin-like protease, amylopullulanase, and heparinase (Figure 5). Highly upregulated in response to LL-37 were transcripts of genes encoding serum opacity factor (9.3- to 31-fold), adhesin SadP (4.3- to 16.5-fold), a pilus-encoding operon (SSU1885-89) (1.9- to 102.8-fold), and an unknown cell wall anchored protein (SSU1201) (11.9- to 35-fold). The fact that these genes, located at different loci dispersed across the chromosome, were expressed in such a similar manner suggested that LL-37 might induce a regulatory mechanism for the expression of these genes. Transcription of several regulatory genes was increased in response to LL-37, including the TCS-like regulator *revSC21* which is thought to control expression of virulence-associated genes [72].

Multiple TCS are reported to be important for sensing and responding to antibiotic- or AMP-induced envelope stress [69]. The TCS response regulator CovR, belonging to the CovRS (CsrRS) TCS that plays a role in resistance to exposure of LL-37 in streptococci [43, 47], was induced significantly ($p<0.01$) upon LL-37 exposure to 0.25 µM LL-37 for 10 and 30 min and 0.1 µM for 30 min. Similarly, the gene encoding response regulator CiaR, belonging to the CiaHR TCS which is involved in responses to cell-envelope associated stress [73, 74], showed modest (but not significant) increased transcript levels at 10 min exposure to LL-37, and significant ($p<0.01$) upregulation at the 30 min timepoint. TCS WalKR has been shown to be involved in response to antibiotic induced stress in *Bacillus anthracis* [75], and the orthologous genes (SSU1190/91) were also upregulated significantly ($p<0.01$) in *S. suis*. Genes encoding three other TCS (SSU0387/88 (LiaSR), SSU0827/28, SSU1471/72) were also upregulated in a LL-37 concentration-dependent manner at both time-points, although this was only statistically significant ($p<0.01$) for SSU0387/88 across all timepoints and LL-37 concentrations, for SSU0827/28 at 30 min with 0.1 µM LL-37 and for SSU1471/72 30 min with 0.25 µM LL-37 (data not shown).

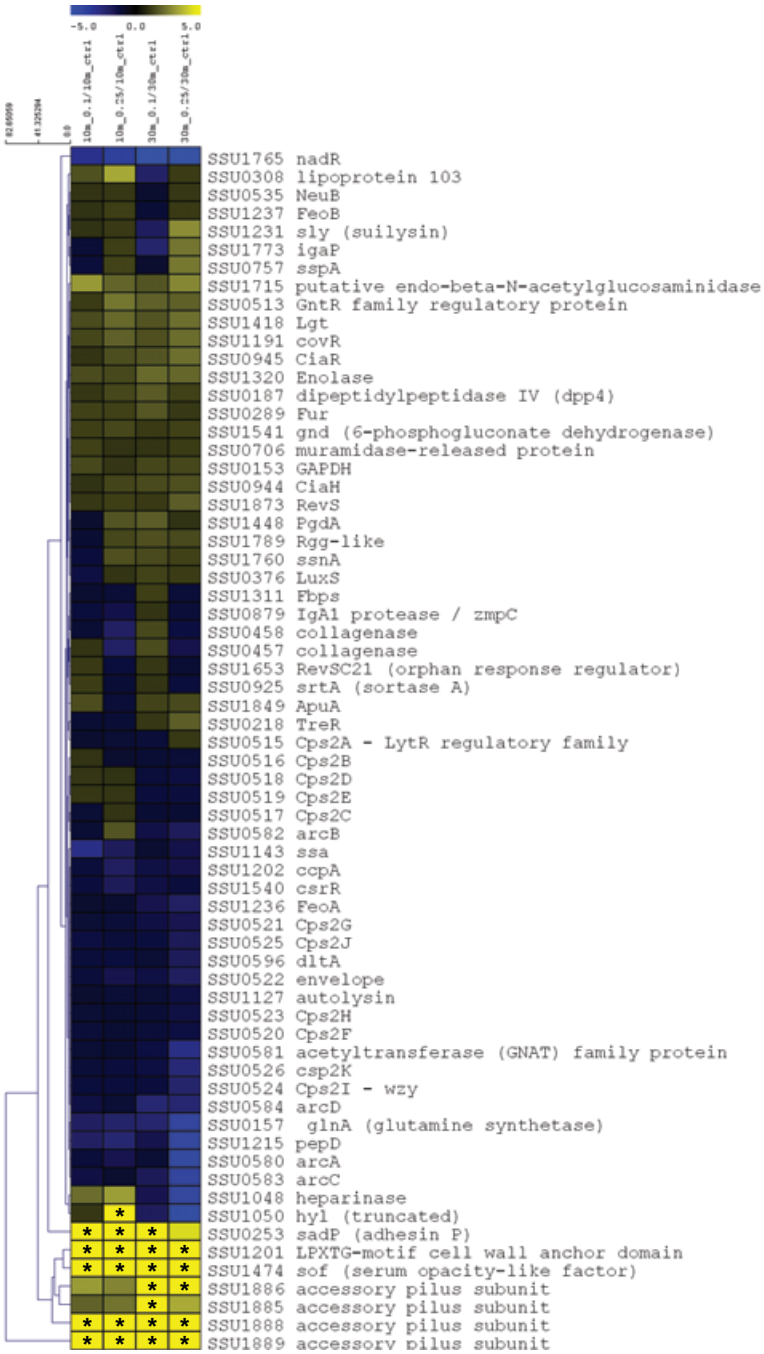

Figure 5. Clustered heatmap showing fold-change in expression of genes that are potentially associated with virulence, adhesion and invasion. Colours indicate fold-change compared to the control (dark blue -5 and yellow +5), with 23 values (marked with *) being off-scale above the upper limit.

Discussion

Antimicrobial peptides (AMPs) are attractive alternatives to antibiotic therapy for the control of pathogenic microbes. Unfortunately, several pathogenic bacteria have been shown to activate cellular pathways mediating tolerance to AMPs, or even, induce microbial virulence mechanisms together with AMP survival pathways. In this study, we investigated if, and to what extent and through which pathways, exposure of *S. suis* to the human cationic AMP LL-37 induced tolerance in *S. suis* In the MIC assay we found that LL-37 precipitated at concentrations at which no continued *S. suis* growth occurred when using standard THB culture medium. This precipitation interfered with optical assessment of bacterial growth and likely affected the actual concentration of bioactive peptide. However, in PBS supplemented with up to 10% (v/v) of THB the observed MIC was approximately 10-fold lower and using flow cytometry we were able to measure rapid killing of the entire inoculum of *S. suis* at a LL-37 concentration of 2.5 μM. Recovery experiments in fresh medium after 1 h incubation with LL-37 confirmed complete killing of *S. suis* at 2.5 μM and continued growth at concentrations of 1 and 0.25 μM, although the length of the lag phase was influenced by initial concentration of LL-37. The MBC of 2.5 μM is similar to that determined for *Bacillus subtilis* (between 2 and 4 μM), which has a cationic peptide sensing and detoxification mechanism consisting of a two-component system and an ABC transporter [76].

The polysaccharide capsule forms the outermost surface of many Gram-positive bacteria, and is the first structural bacterial component that cationic AMPs encounter. Unencapsulated streptococci were found to have increased susceptibility to cationic AMPs [77-79]. In our flow cytometry killing assay, the susceptibility of *S. suis* to LL-37 was not affected by the presence of a capsule as shown by the use of a capsule-negative mutant J28. We also evaluated differential sensitivity of different *S. suis* serotypes and did not observe a difference in MBC. However, *S. suis* strain 8039 (serotype 7) appeared to be slightly more tolerant to the bactericidal effects of LL-37 at concentrations below the MBC than strains belonging to serotype 2 or serotype 9. Serotype 7 strains are more often found in the lungs compared to other serotypes, whereas serotype 2 and serotype 9 are mostly found brains, hearts and joints, among others [51]. It would be of interest to investigate if different cationic AMPs in the different organs may correlate with differential sensitivity *S. suis* serotypes to the different AMPs and thus, differential organ colonisation.

We also tested if mutants of genes involved in cell wall composition and charge showed differential sensitivity to LL-37. Although the *dlt* operon has been shown in some Gram-positive bacteria to confer increased resistance to cationic peptides like LL-37 [63, 80-82] we found the *dltA* mutant of strain S10 to be not more sensitive to LL-37 than the wt strain (Figure 2 AB). Previously, upregulation of transcription of all four genes of the *dlt* operon was described in *Streptococcus pneumoniae* upon exposure to ~1 μM LL-37 [44], but in *S. suis* we did not observe a significant change in expression of genes belonging to the *dlt* operon.

LL-37 permeabilizes the cytoplasmic membrane, which at effective concentrations causes loss of membrane integrity and leakage of small molecules and ions from the cytoplasm into

the surrounding medium. In previous studies with *Bacillus subtilis*, LL-37 was shown to lead to membrane permeabilisation that could be measured by Sytox Green uptake within 5 min. This implies that the membrane will also be permeable to protons resulting in a dissipation of the proton-motive force (PMF) [83]. As ATP synthesis is linked to the generation of a PMF, LL-37 was predicted to inhibit energy requiring processes including protein synthesis. Loss of membrane integrity, even at sub-lethal concentrations of LL-37, may be one of the factors underlying the general stress responses that resulted in increased transcription of the heat shock response regulons including HrcA, heat shock protein GrpE, chaperone protein DnaK and DnaJ, heat shock protein 60 family chaperone GroEL (60 kDa chaperonin) and co-chaperone GroES (10 kDa chaperonin).

In other streptococci, TCS have been shown to regulate the transcriptional programs that mediate a bacterial stress response to AMPs. We tested two TCS deletion mutants for their sensitivity to LL-37 because these strains were more susceptible to *in vitro* killing by neutrophils and less virulent than the parent strain in a zebrafish larvae infection model [54]. Surprisingly a mutant of the TCS-encoding genes SSU1930/31 was less susceptible to sub-lethal concentrations of LL-37 (Figure 2C) and expression of the genes encoding this TCS are slightly (not statistically significant, $p>0.01$) downregulated upon peptide exposure, in contrast to many upregulated other TCS genes (data not shown). The regulon of the *S. suis* TCS SSU1930/31 is presently unknown, as are the pathways regulated by this TCS. Follow-up transcriptomics studies could be performed to determine if exposure of this mutant to the used concentrations of LL-37 induces lower induction of genes involved in bacterial stress pathways.

The second TCS mutant SSU0827/28 lies downstream of a cluster of genes that are strongly upregulated after exposure to LL-37. These genes SSU0832 and SSU0835 are both annotated as ABC transporter ATPases, and SSU0831 and SSU0834 are both annotated as ABC transporter permeases. The locus organization resembles that of the peptide antibiotic sensing and transporter systems found in some other Gram-positive bacteria [69, 76], which are involved in sensing cell envelope stress (e.g. exerted by cell wall active antibiotics or AMPs) and regulate genes important for cell envelope integrity, detoxification, and virulence. Although a deletion mutant of TCS encoding genes SSU0827/28 was not more susceptible to killing by LL-37, we could not rule out a role for the induced transporter in removing specific classes of AMPs affecting the cell envelope integrity as as shown for the BceSR system of *B. subtilis* which regulates expression of an ABC transporter to remove bacitracin [84, 85]. The investigation of the role of these transporters and the other TCS differentially expressed upon exposure to LL-37 will be the subject of future genetic and biochemical studies.

This work has shown that in *S. suis*, there appears to be a transcriptional response program that regulates tolerance of these bacteria to sub-lethal concentrations of the cationic AMP LL-37. Among the genes that showed differential expression upon exposure to LL-37 were transcriptional regulators that mediate general bacterial stress responses including the heat-shock response, suggesting that *S. suis* may indeed use conserved pathways to endure sub-

lethal concentrations of AMPs. Of course, at later time-points that were not measured here, more specific stress response and damage control pathways may be induced, for instance DNA damage pathways that include the induced Holliday junction resolvase or cell wall integrity pathways that include the induced enolase. Importantly, the *S. suis* response to sub-lethal concentrations of LL-37 also appears to modulate its lifestyle, altering the expression 9 of the 17 virulence genes that can be upregulated by carbohydrate catabolite control [86] were significantly induced upon exposure to LL-37 in at least two tested conditions. From this, it can be hypothesised that *in vivo* exposure of *S. suis* bacteria that are colonising mucosal surfaces to increasing concentrations of cationic AMPs, such as LL-37, might induce virulence genes in these bacteria. Induction of these virulence genes might promote host invasion. It is therefore of interest to investigate effects of increasing accumulations of human or porcine host- or microbiota-derived AMPs at mucosal surfaces on *S. suis* adhering to the same mucosal surfaces, using *in vitro* invasion models. Paradoxically, it might be that a stronger innate immune response, including secretion of AMPs at mucosal surfaces, is correlating with higher virulence of colonising *S. suis* bacteria.

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CHAPTER 4

Compositional Profiling of Porcine Microbiota to Identify Bacteria with Strong Positive or Negative Associations with *Streptococcus suis* Abundance

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Abstract

Streptococcus suis is an opportunistic porcine pathogen, mainly causing invasive disease in post-weaned piglets. The microbial communities that colonise piglets are thought to play an important role in abundance of *S. suis* and the development of invasive disease. This study aimed to gain insight into which bacterial taxa positively or negatively correlated with *S. suis* abundance, in order to identify putative antagonists of *S. suis*. Microbiological samples from the tonsil and small intestine of healthy piglets were collected and 454 pyrosequencing was applied for compositional profiling of the microbiota. Several bacterial species and taxa with strong co- and anti-occurrences with *S. suis* were identified within these microbial communities: pathogenic species from the genera *Actinobacillus*, *Actinomyces*, *Fusobacterium* and *Streptococcus* showed strong co-occurrence with *S. suis*, whereas *Lactobacillus* spp., and *Lactobacillus reuteri* in particular, were found to be strongly negatively associated with *S. suis* abundance. This study supports the ideas that a network of other pathobionts may support the occurrence and pathogenicity of *S. suis*, and that specific groups or species of bacteria that display microbial antagonism against *S. suis* (and its co-occurring pathogens) can be found in the microbiota of niches that are colonised by this pathogen.

Introduction

Streptococcus suis is a pathogen of swine and one of the most important causes of bacterial mortality in piglets worldwide. Clinical infections are seen mainly in piglets 2-5 weeks after weaning and in growing pigs, but less frequently in suckling piglets and adult animals. Healthy pigs can carry multiple serotypes of *S. suis*, many of which are considered a low risk for causing invasive disease [1]. The porcine niche where *S. suis* occurs at highest densities is the upper respiratory tract of pigs, more particularly the nasal cavities and the tonsils [1, 2]. *S. suis* is also found in the small intestine of asymptomatic pigs [2, 3] albeit at lower relative abundances than in the tonsils. Due to its prevalence in domestic pigs, *S. suis* is considered a normal inhabitant of the microbiota of these niches [1, 4].

The porcine palatine tonsils, secondary lymphoid organs in the oral cavity, are part of the mucosa-associated lymphoid tissue and are the site of microbial sampling by the pig and of colonisation, replication and invasion of bacterial pathogens [5-7]. Piglets can also become colonized with *S. suis* from vaginal secretions during parturition and while nursing or by contact with colonized sows. *S. suis* is usually transmitted nasally or orally; transmission between herds occurs by the movement and mixing of healthy carrier pigs and may lead to the introduction of a highly virulent strain into a herd resulting in invasive disease in weaning and growing pigs [5].

Some herds may suddenly develop serious *S. suis* clinical disease, which is considered to be due to predisposing factors such as overcrowding, poor ventilation, excessive temperature fluctuations, and coinfections with other pathogens such as porcine influenza or reproductive and respiratory syndrome virus [5, 8, 9]. Still, other environmental factors may promote outgrowth of *S. suis* as well. Previously, we have shown that the carbohydrate source determines expression of *S. suis* virulence genes [10], suggesting that a change in nutrient composition towards more complex carbohydrates, including starch from the pig's feed, might promote the transition from a commensal to a pathogenic mode of *S. suis*. The microbial community is likely to strongly influence disease occurrence, for example by the antagonism or promotion of *S. suis* by other bacteria. Respiratory disease in pigs is often polymicrobial in nature, and although one pathogen may dominate, other species in the microbial community may contribute to pathogen colonization and pathological processes in the host [11]. These interactions are complex and understanding them requires quantitative information about the organization and principles that govern the associations of the different species with *S. suis*. Approaches using Pearson's correlation and other correlation measures have been recently used to reconstruct molecular ecological networks in soil [12] marine [13] and gut [14] microbial communities. The aim of this study was to gain insights into the correlative population structure of *S. suis* with other species in the tonsil and small intestine.

In this microbiota profiling study, we wanted to achieve: (1) gain insight into the bacterial taxa that show positive (possible symbionts of *S. suis*) and negative (possible antagonists) correlations with *S. suis* abundance; (2) find out if some of the taxa showing anti-correlation with *S. suis* abundance could be considered candidate natural antagonists of *S. suis*; and (3) find out if bacteria that co-occur with *S. suis* could promote *S. suis* virulence. In other words, we wanted to explore the idea that *S. suis* is part of a consortium of opportunistic porcine pathogens that co-occur in the intestine and/or the palatine tonsils. Since *S. suis* is also considered a respiratory pathogen that is commonly sampled in the upper respiratory tract, we hypothesised that taxa co-occurring with *S. suis* in the tonsillar areas might also be respiratory pathogens. In two separate groups of healthy piglets around the age of weaning, we have sampled the microbiota from the palatine tonsils and/or small intestine. We applied barcoded 454 FLX pyrosequencing of the 16S rRNA genes to describe the composition and diversity of the microbial community of these sampled niches.

Material and Methods

Animals and sample collection

Two separate groups of piglets were used for the collection of biological samples from the tonsil and small intestine.

Tonsil samples: Piglets (breed TEEZN, Table S1, Figure S1) were bred and housed under specific pathogen free (SPF) conditions at Varkens Innovatie Centrum (Sterksel, The Netherlands). Animals were weaned between 21 and 29 days of age, with an average of 24 days for the sampled pigs. Tonsil swabs were obtained from 20 piglets at days 9 and 30 post-weaning, by brushing both palatine tonsils thoroughly for 20s with a sterile toothbrush. The top of the toothbrush was cut off and transferred to a tube containing 4 mL sterile, cold phosphate-buffered saline (PBS) and kept at 4°C. The samples were homogenized by thorough vortexing and divided into 1 mL aliquots, either with or without 20% (v/v) glycerol. Samples were stored at -80°C within 4 hours of collection.

Intestinal content and tonsil tissue: Piglets (breed Topigs20 F1, all boars, Table S2) were raised on a SPF farm (Van Beek Varkens BV, Lelystad, The Netherlands). Animals were weaned at 28 days of age. When aged 3 – 5 weeks, piglets were anaesthetized and killed, after which the abdominal cavity was opened and ~20 cm sections of three different locations of the small intestine (duodenum, jejunum, ileum) were clamp-closed and removed. The luminal content (~2 mL) from the intestinal sections was collected by gentle squeezing and split into two cryotubes, either with or without 20% glycerol. All samples were directly frozen in liquid nitrogen and stored at -80°C.

DNA extraction, and 16S rRNA gene sequence–based microbiota composition profiling

For the oropharyngeal samples 1 mL aliquots from tonsil-brushed material were freeze-dried prior to DNA extraction. For the small intestinal content samples, approximately 0.2 gram of material was used. Total DNA was extracted from these samples using the Repeated Bead Beating method as described in [15] using a FastPrep-24 instrument (MP-Biomedicals, France), purified using the QIAmp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) and stored at -20°C.

Amplicons from the V3 to V6 region of the 16S rRNA genes were generated by PCR using forward primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3') 5'-extended with a 'NNNNNN' barcode sequence and the titanium sequencing adapter A (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3') at the 5' end. The 'NNNNNN' barcode is a unique six nucleotide sequence used for each separate sample. Each reaction was performed using the reverse primer 1061R (5'-crrcacgagctgacgac-3') 5'-extended with the titanium sequencing adapter B (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3') at the 5' end.

PCR reactions were performed using a thermocycler in a total reaction volume of 50 µL containing 1x buffer, 5 µL dNTP mix, 3 µL $MgSO_4$, 1 unit of KOD Hot Start DNA polymerase (Novagen, Toyobo, Japan), 200 nM of forward and reverse primer, and 2 µL of undiluted or 10x diluted template DNA. The amplification program comprised an initial denaturation

step at 95°C for 2 min, and 35 cycles of: denaturation at 95°C for 20 s, annealing at 55°C for 10 s, elongation at 70°C for 15 s. The size of the amplicons (789 bp) was confirmed by gel electrophoresis. Both negative and positive control PCR reactions were run alongside the amplification, and yielded no amplicon and an amplicon of the expected size, respectively.
PCR products were purified using MSB® Spin PCRapace columns (Stratec Molecular GmbH, Berlin, Germany) and the PureLink® PCR Purification kit (Invitrogen) with high cut-off buffer B3 to remove fragments <300 bp. Yield and purity of the DNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).
All PCR amplicons were mixed in equimolar concentrations to 31 ng μL^{-1} and 5 internal standard controls were included. The pooled PCR amplicons were subjected to pyrosequencing, using half a picotiterplate on the Genome Sequencer FLX in combination with titanium chemistry (GATC-Biotech, Konstanz, Germany).
Pyrosequencing data were analysed with a workflow based on QIIME v1.2 [16], using settings as recommended in the QIIME 1.2 tutorial, with the following exceptions: reads were filtered for chimeric sequences using Chimera Slayer [17] and OTU clustering was performed with settings as recommended in the QIIME newsletter of 17 December 2010 [18] using an identity threshold of 97%. Diversity metrics were calculated as implemented in QIIME 1.2. Hierarchical clustering of samples was performed using UPGMA with weighted UniFrac as a distance measure as implemented in QIIME 1.2. The Ribosomal Database Project classifier version 2.2 was performed for taxonomic classification [19]. Additional data handling, including manual examination of proper species assignment, was done using in-house developed Python and Perl scripts (NIZO Food Research, Ede, The Netherlands).

Microbiome data mining: searching for microbial biomarkers using LEfSE

To investigate if bacterial taxa were enriched in a specific microbiota sample group, Linear Discriminant Analysis (LDA) effect sizes were determined and statistically compared between groups by LEfSE [20]. Briefly, bacterial abundance data were normalized by dividing each single abundance in each sample by the sum of (total) abundances in the sample and multiplying the obtained fraction by 1 million. The non-parametric factorial Kruskal-Wallis (KW) sum-rank test was used to detect bacterial taxa with significant differential abundance with respect to location (small intestine vs. tonsil) or *S. suis* load (binned into “high”, “low” or “none”, based on the median of 16S abundance) of individual piglets. Significant statistical interactions between location (main classifier) and piglet *S. suis* load (subclassifier) were subsequently determined by pairwise tests using the unpaired Wilcoxon rank-sum test. Finally, LEfSE used LDA to estimate the effect size of each differentially abundant microbial taxon, whereby statistical significance is coupled to biological relevance (here, LDA measures the strength of the enrichment of a given bacterial taxon by only selecting those taxa that are most contrasting between the two locations or the three *S. suis* load bins).

Microbiome data mining: co–occurrence analysis using Pearson correlations and CoNET

We used two different methods to obtain bacterial co-occurrence networks. The first method used the Pearson correlations based on the 16S abundance data of all microbial taxa from all locations and animals. We chose to combine animals and locations in order to achieve highest statistical support, with the knowledge that we could annotate the resulting network when needed, e.g. to plot microbes enriched in animals with higher or lower *S. suis* load. Although frequently used in microbiome analysis, using Pearson correlations has some disadvantages including sensitivity to outliers. To have the possibility to evaluate microbial co-occurrences in different, stricter ways, CoNET was used in addition. CoNet [14] is a microbiome-data-handling tool that offers ensemble-based network construction, offering the advantage to combine multiple similarity measures into a single network calculation.

Rare taxa were discarded by lumping all taxa that were present in < 20 samples into a garbage taxon and converting counts into relative abundances. Five methods: Pearson, Spearman, Mutual Information, Bray Curtis and Kullback-Leibler dissimilarity, were selected for ensemble inference. Instead of specifying their thresholds manually, for each method, the top 300 positive correlations (edges) were calculated together with the top 300 negative correlations (edges).

The permutations output to compute p-values included randomisation, shuffling of rows to resample initial results of ensemble inference, and renormalisation to compensate for compositionality biases were stored in a file prior to method- and edge-specific permutation. This permutation file was used to obtain bootstrap score distributions to compute final p-values, merging all five method-specific p-values of an edge into one single p-value. The resulting network data were transformed into a network using Cytoscape 2.8.3 [21], showing edges that corresponded to p-values < 0.05.

Antimicrobial assay

S. suis strains S10 (SS2), 8039 (SS7), 8067 (SS9), or 13730 (SS14) were grown in Todd-Hewitt Broth (THB) (Oxoid Ltd, Basingstoke, UK) at 37°C with 5% CO_2. Reuterin (3-hydroxypropanal) was purchased (Matrix Scientific, Columbia SC, USA) as a 1 M solution in water. Standard dilution assays in 96-well microtiter plates were used to determine the minimal inhibitory concentration (MIC) of reuterin in two independent experiments. Ten-fold serial dilutions of reuterin in PBS (20 µL) were mixed with bacterial suspension in THB (180 µL) to obtain an inoculum of 5 x10^5 CF mL^{-1}. Plates were incubated at 37°C with 5% CO_2 for 22 hours, after which bacterial growth was measured by absorption at 600 nm using a spectrophotometer (Spectramax M5, Molecular Devices, Sunnyvale CA, USA). The MIC was determined as the lowest concentration at which no visible growth was observed. Bacteria were exposed to 100 µg/mL streptomycin (Invitrogen) or PBS as controls. All conditions were assayed in duplicate.

Ethical statement

All animal experiments were approved by the ethical committee of Wageningen UR, The Netherlands, in accordance with the Dutch law on animal experiments.

Results

Bacterial abundance enrichment analysis using LEfSE

The first objective was to gain insight into differential abundance of bacterial taxa in the piglets' tonsils and small intestine. Using the statistical LEfSE pipeline (see Methods), we found that out of a total of 313 identified taxa, 142(45%) were statistically more abundant in the tonsil (49 samples), and 20 (6%) in the small intestine (32 samples, of which 10 from duodenum, 11 from jejunum, and 11 from ileum). To make biological interpretations more pertinent, the enriched taxa are shown at the taxonomically terminal species level in Table 1; the enriched taxonomically "deeper" taxa (from genus to phylum level) are not shown. QIIME taxonomic classifications of OTUs to species level were evaluated by manual inspection using in-house scripts (NIZO Food Research, Ede, The Netherlands), graphics calling and expert evaluation.

Taxon	region	LDA score	p-value (Kruskal-Wallis)
Actinobacillus equuli	tonsil	3.889323	0.006833
Actinobacillus indolicus	tonsil	4.774283	5.88E-13
Actinobacillus minor	tonsil	3.721275	7.65E-06
Actinobacillus porcinus	tonsil	4.935675	2.79E-11
Actinobacillus rossii	tonsil	3.545464	1.39E-07
Actinomyces denticolens	tonsil	3.585802	1.41E-08
Actinomyces hyovaginalis	tonsil	4.550342	1.02E-12
Arcanobacterium pyogenes	tonsil	4.445029	3.10E-11
Arcobacter cryaerophilus	tonsil	3.485532	0.041107
Atopobium minutum	tonsil	3.58961	0.000128
Bacteroides coprocola	tonsil	4.794031	2.13E-14
Bacteroides denticanoris	tonsil	3.32526	0.014113
Campylobacter mucosalis	tonsil	3.611075	9.13E-12
Catenibacterium mitsuokai	tonsil	3.611318	1.93E-09
Collinsella aerofaciens	tonsil	3.276762	0.002941
Coprococcus catus	tonsil	3.440239	0.005338
Corynebacterium diphtheriae	tonsil	3.333376	9.52E-06
Deinococcus xinjiangensis	tonsil	3.451583	0.041107
Dorea formicigenerans	tonsil	3.645955	0.001153
Dorea longicatena	tonsil	3.404981	0.000235
Faecalibacterium prausnitzii	tonsil	3.728934	1.54E-08
Filifactor alocis	tonsil	3.96707	0.016915
Filifactor villosus	tonsil	3.447888	6.53E-07
Fusobacterium necrophorum	tonsil	4.248722	1.44E-14
Fusobacterium nucleatum	tonsil	3.594978	9.77E-10
Fusobacterium ulcerans	tonsil	4.058233	2.66E-14
Granulicatella adiacens	tonsil	3.699811	4.10E-11
Klebsiella pneumoniae	tonsil	3.476566	0.005181
Kurthia gibsonii	tonsil	3.310304	0.002686
Megasphaera elsdenii	tonsil	3.467616	0.007516
Mycoplasma hyorhinis	tonsil	3.072899	0.002686
Pasteurella aerogenes	tonsil	3.672429	1.54E-11
Prevotella copri	tonsil	4.036657	1.44E-11
Prevotella ruminicola	tonsil	3.59596	0.041107
Ruminococcus bromii	tonsil	3.582797	0.000132
Selenomonas sputigena	tonsil	3.778899	0.0043
Streptococcus dysgalactiae	tonsil	4.208334	4.05E-12
Streptococcus ferus	tonsil	3.73773	4.09E-07
Streptococcus hyointestinalis	tonsil	3.987304	3.49E-05
Streptococcus orisratti	tonsil	3.480155	0.00022
Streptococcus porcinus	tonsil	3.734663	1.24E-06
Streptococcus ratti	tonsil	3.285496	0.006833
Streptococcus suis	tonsil	3.654945	6.71E-11
Subdoligranulum variabile	tonsil	3.651386	7.40E-08
Tannerella forsythensis	tonsil	3.624986	1.15E-05
Treponema pedis	tonsil	3.673888	4.28E-08
Treponema vincentii	tonsil	3.900779	0.001664
Veillonella parvula	tonsil	3.551059	2.12E-08
Clostridium butyricum	intestine	3.784249	0.006223
Lactobacillus acidophilus	intestine	3.301214	2.64E-05
Lactobacillus coleohominis	intestine	3.318533	2.10E-05
Lactobacillus crispatus	intestine	3.468845	0.013017
Lactobacillus gasseri	intestine	3.674634	4.30E-06
Lactobacillus johnsonii	intestine	5.134426	4.63E-12
Lactobacillus pontis	intestine	3.42111	9.45E-05
Lactobacillus reuteri	intestine	5.338082	7.04E-14
Lactobacillus ultunensis	intestine	4.799174	0.010893
Lactobacillus vaginalis	intestine	3.29626	1.44E-09
Lactococcus raffinolactis	intestine	3.873204	0.004554
Streptococcus parasanguinis	intestine	3.950966	0.026922

Table 1. Enrichment of bacterial taxa in porcine tonsils and small intestine. The LDA score is displayed as logarithmic LDA score. Taxa are only shown at species level, and taxa are alphabetically ordered for each location.

Table 1 shows that *S. suis* and other streptococci are significantly more abundant in the tonsil, together with other porcine respiratory pathogens including *Actinobacillus* spp., *Arcobacter cryaerophilus*, *Klebsiella pneumoniae* and *Pasteurella aerogenes*. In the small intestine, *Lactobacillus* spp. are significantly more abundant, together with butyrate-producing commensal *Clostridium butyricum* and the symbiont *Streptococcus parasanguinis* [22].

Because it is unknown if piglet infection by *S. suis* is a consequence of its abundance, it is of interest to look at the distribution of *S. suis* across individual piglets (Figure 1A). Two piglets were positive for *S. suis* in the intestine, of which one showed substantial abundance. Although *S. suis* was clearly more abundant on the tonsils, three piglets were negative for *S. suis* and 5 piglets showed relatively low abundance. The 16S rRNA gene abundance data of *S. suis* (Figure 1B) showed that piglets could, relative to the median, be divided into three groups or "bins", characterised by "high", "low" or "none" *S. suis* 16S sequence, interpreted as the "*S. suis* load" of a piglet. Figure 1B shows the abundance of *S. suis* per bin for the individual piglet samples of all the intestinal and tonsil samples combined.

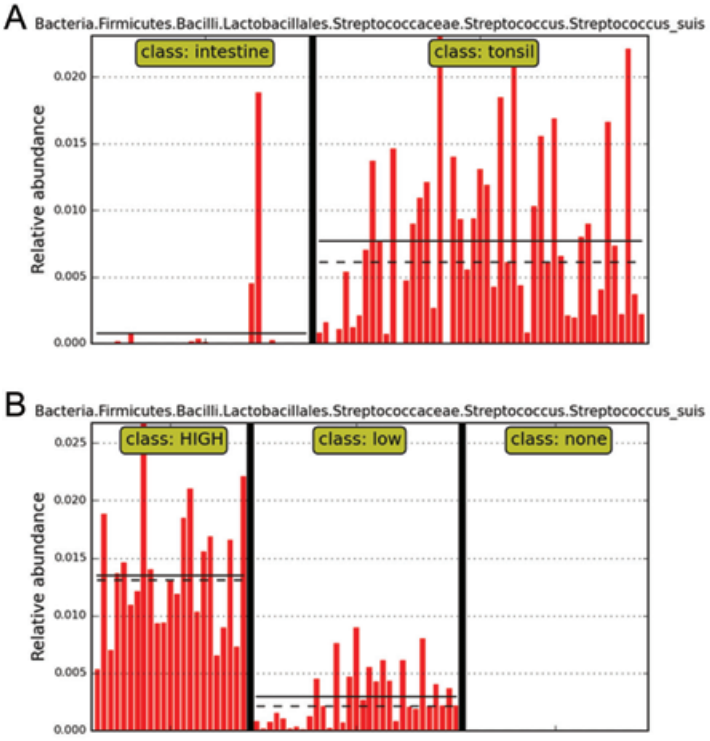

Figure 1. A) Abundance of *S. suis* in the small intestine and tonsil per individual piglet sample. **B)** Abundance of *S. suis* in each of three bins (groups) based on high, low or no abundance of 16S rRNA gene sequences of *S. suis*. Bins were generated across the small intestine and the tonsil samples. Horizontal lines represent the mean, and the dotted lines the median abundance.

We were interested to see if specific bacterial taxa were enriched in each bin, since co-occurrence and anti-occurrence of bacteria with *S. suis* is a first step to suggest which bacteria might be symbionts (bacteria enriched when *S. suis* counts are high) or antagonists (bacteria enriched when no *S. suis* was present). Table 2 shows the enrichment of bacteria in the three bins across all piglets. LEfSE analysis found that out of the 313 identified taxa, 34 (11%) were enriched in piglets with high *S. suis* load, 3 (1%) were enriched in piglets with low *S. suis* load, and 10 (3%) were enriched when no *S. suis* was present. LEfSE analysis shows that on the tonsils and in the small intestine, piglets with a high *S. suis* load also showed relatively high abundance of other potentially pathogenic bacteria including *Actinobacillus* spp., *Corynebacterium diphteriae*, *Pasteurella aerogenes*, *Fusobacterium ulcerans*, and several *Streptococcus* species. In contrast, *Lactobacillus* spp. and *Treponema pedis* were enriched in piglets with no *S. suis*.

Taxon	*S. suis* load	LDA score	p-value (Kruskal-Wallis)
Lactobacillus johnsonii	none	5.084389	2.44E-05
Lactobacillus reuteri	none	5.270496	2.72E-06
Treponema pedis	none	3.516628	8.13E-06
Prevotella copri	low	3.989732	1.68E-06
Actinobacillus indolicus	HIGH	4.849962	4.38E-10
Actinobacillus minor	HIGH	3.041014	0.000108
Actinobacillus porcinus	HIGH	4.949623	8.24E-09
Actinomyces denticolens	HIGH	3.530911	3.82E-07
Corynebacterium diphtheriae	HIGH	3.265174	2.52E-06
Fusobacterium ulcerans	HIGH	4.026356	5.09E-09
Granulicatella adiacens	HIGH	4.386953	8.72E-08
Pasteurella aerogenes	HIGH	3.780639	1.78E-09
Streptococcus ferus	HIGH	3.869253	5.63E-07
Streptococcus orisratti	HIGH	3.118125	1.63E-05
Streptococcus porcinus	HIGH	3.920725	6.94E-07
Streptococcus suis	HIGH	3.830303	2.37E-16
Veillonella parvula	HIGH	3.93556	2.35E-06

Table 2. Enrichment of bacterial taxa in tonsils and small intestine of piglets with high, low or no *S. suis* load. The LDA score is displayed as logarithmic LDA score. Taxa are only shown at species level, and taxa are alphabetically ordered per bin.

The data in Table 2 suggest some negative correlation or anti-occurrence between *Streptococci* including *S. suis* and *Lactobacillus* spp.. Although many *Lactobacilli* can occur at multiple sites of the upper gastro-intestinal (GI) tract, from the oral cavity to the small intestine [23], it is also possible that anti-occurrence of *Lactobacilli* and *Streptococci* is caused by them occupying different niches. It was therefore of interest to inspect enrichment of *S. suis* per GI region. We first checked for differential abundance of bacteria across the three sampled regions of the small intestine: duodenum (10 samples), jejunum (11 samples) and ileum (11 samples). LEfSE analysis did not find any taxa that were enriched in any region (not shown) so the 32 intestinal samples from the three regions were pooled in further analyses. We also checked if there was a differential abundance of taxa across the small intestine with

the three *S. suis* bins as first classifier and intestinal region as subclassifier; that did show a significant result for the bins, but the effect of intestinal region of subclass did not contribute substantially as *S. suis* was only marginally present in 2 piglets (not shown).

In the small intestine, LEfSE analysis found that out of the total of 313 identified taxa, 32 (10%) were enriched in the intestine of piglets with high *S. suis* load, 28 (9%) were enriched in the intestine of piglets with low *S. suis* load, and 6 (3%) were enriched in the intestine of piglets with no *S. suis*. Piglets with a high *S. suis* load also showed relatively high abundance of other potentially pathogenic taxa including *Actinobacillus* spp., *Corynebacterium diphteriae, Pasteurella aerogenes, Fusobacterium ulcerans* and *Streptococcus* spp.. The species *Lactobacillus coleohominis*, isolated from healthy humans [24], was enriched in piglets with no *S. suis* in the intestine.

Taxon	*S. suis* load	LDA score	p-value (Kruskal-Wallis)
Lactobacillus coleohominis	none	3.402267	0.0286
Bacteroides coprocola	low	4.609421	0.000826
Bifidobacterium animalis	low	4.061458	0.039291
Campylobacter mucosalis	low	3.503331	0.039523
Fusobacterium necrophorum	low	3.974706	0.037646
Actinobacillus indolicus	HIGH	4.550149	3.42E-05
Actinobacillus porcinus	HIGH	5.121409	4.24E-05
Actinobacillus rossii	HIGH	4.45799	0.005552
Actinomyces denticolens	HIGH	4.222156	0.001707
Actinomyces hyovaginalis	HIGH	4.97434	0.004376
Corynebacterium diphtheriae	HIGH	3.624362	0.001038
Fusobacterium ulcerans	HIGH	4.169602	0.00095
Howardella ureilytica	HIGH	3.777632	0.018069
Pasteurella aerogenes	HIGH	3.966449	0.001575
Streptococcus dysgalactiae	HIGH	4.514429	0.000623
Streptococcus orisratti	HIGH	4.524303	0.012721
Streptococcus pasteurianus	HIGH	4.959211	0.027218
Streptococcus suis	HIGH	4.217685	4.72E-09

Table 3. Enrichment of bacterial taxa in small intestine of piglets with "high", "low" or "none" *S. suis* load. The LDA score is displayed as logarithmic LDA score. Taxa are only shown at species level, and taxa are alphabetically ordered per bin.

We were also interested to inspect which bacteria were co-occurring with *S. suis* in the tonsil areas of piglets with high, low or no *S. suis* load. LEfSE analysis found that out of the total of 313 identified taxa, 6 (2%) taxa were enriched in the tonsil areas of piglets with a high *S. suis* load, and 3 (1 %) taxa were enriched in the tonsil areas of piglets with no *S. suis*. LEfSE analysis showed that the porcine pathogens *Arcobacter cryaerophilus*, *Actinobacillus indolicus* and *S. porcinus* [25-27] were enriched in the tonsil areas of piglets with high *S. suis* load.

Taxon	*S. suis* load	LDA score	p-value (Kruskal-Wallis)
Arcobacter cryaerophilus	HIGH	3.5882	0.03641
Actinobacillus indolicus	HIGH	4.5851	0.0356
Streptococcus porcinus	HIGH	3.9163	0.01206
Streptococcus suis	HIGH	3.8617	2.85E-08

Table 4. Enrichment of bacterial taxa in tonsils of piglets with high, low or no *S. suis* load. The LDA score is displayed as logarithmic LDA score. Taxa are only shown at species level, and taxa are alphabetically ordered per bin.

Network co–occurrence analysis of bacteria that show positive or negative correlation with *S. suis* abundance

The LEfSE analyses showed that several microbial pathogens are positively correlated with higher *S. suis* abundance, whereas symbiotic *Lactobacilli* showed anti-correlation with higher *S. suis* abundance. To further explore correlations between presence or absence data of bacterial taxa with *S. suis* load, a network view was constructed where nodes represent bacterial taxa and edges represent positive or negative Pearson correlations. In order to obtain highest statistical support, Pearson correlations representing the microbiome co-occurrence data of all piglets were pooled and only edges representing p-values lower than 0.001 were used; positive p-values representing positive correlations or co-occurrence are displayed in black, and negative p-values representing anti-correlation are displayed in red. To annotate the network, we coloured the nodes representing bacterial taxa enriched in piglets with high, low or no *S. suis* load (Figure 2).

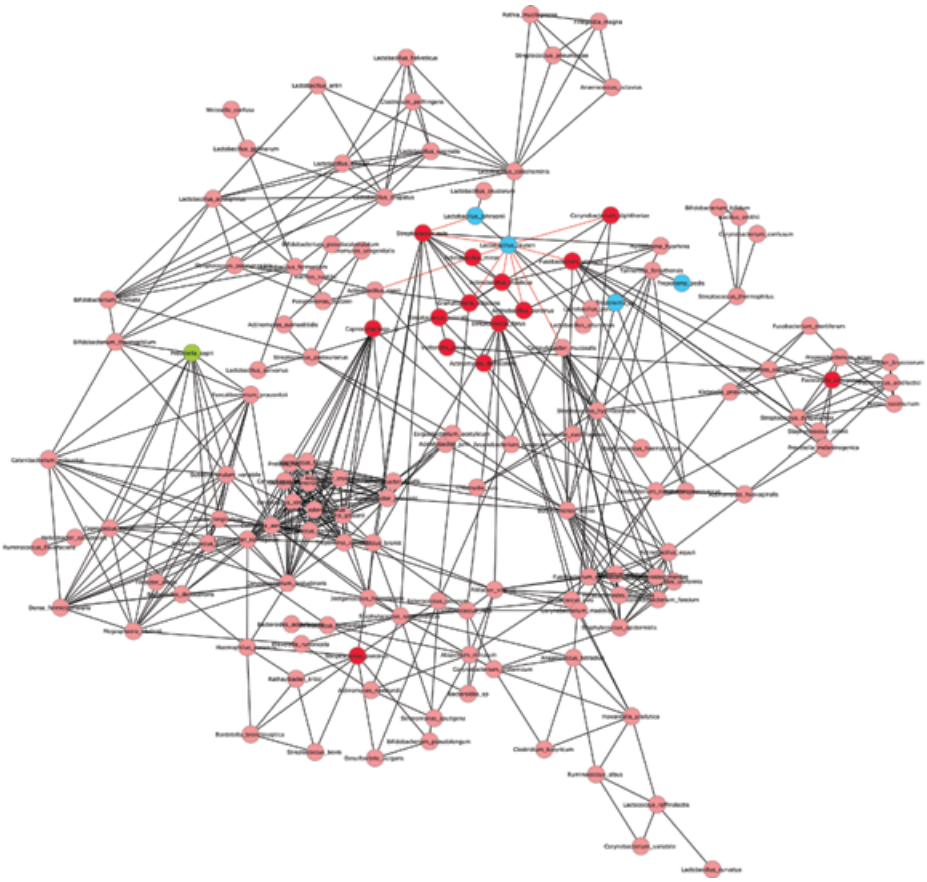

Figure 2. Co-occurrence network representing positive (edges displayed in black) and negative (edges displayed in red) Pearson correlations between abundance data of bacterial taxa. The edges represent p-values lower than 0.001. Where possible, nodes are coloured based on enrichment of the respective taxa in piglets with no (blue), low (green) or high (red) *S. suis* load (see Table 2).

From this network in Figure 2, it is clear that *S. suis* appears to be part of a consortium of opportunist pathogens that include species belonging to the genera *Actinobacillus*, *Actinomyces*, *Pasteurella*, *Streptococcus*, and *Fusobacterium* that were also observed in LEfSE enrichment analysis. Several *Lactobacillus* species show negative correlations with this pathogen consortium. The network analysis thus supports the notion that *S. suis* may be part of a consortium of pathogens that are also relevant respiratory pathogens.

Although Pearson correlations are commonly used in ecological research to infer co-occcurrence of taxa, it is useful to explore additional correlation metrics that are less sensitive to outliers. The CoNET software tool has been developed to this goal and was used here to combine 5 different similarity measures including the Pearson correlation (see Methods). Exporting the CoNET correlation data to Cytoscape resulted in a co-occurrence

network where nodes represent microbial taxa and edges represent positive and negative correlations. (Figure 3).

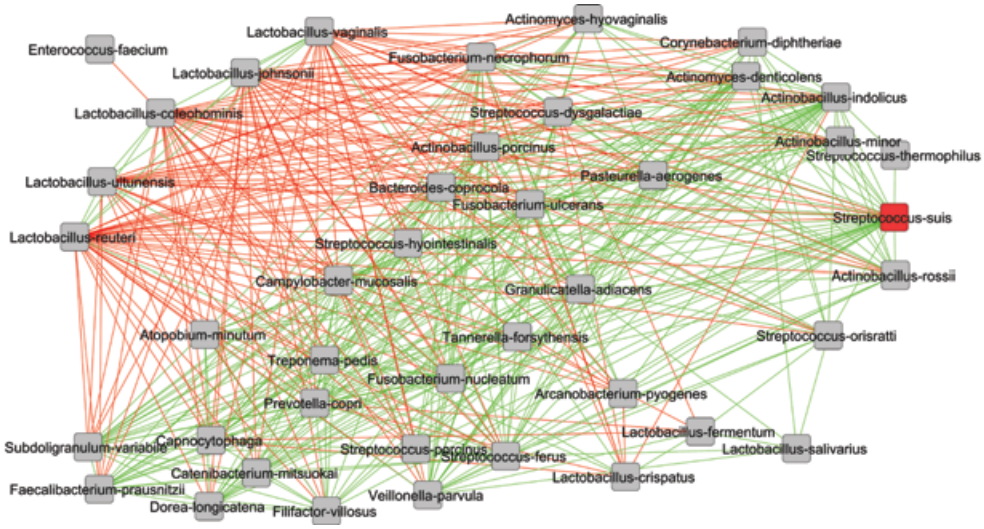

Figure 3. Co-occurrence network representing positive (edges displayed in green) and negative (edges displayed in red) correlations between abundance data of bacterial taxa, calculated using CoNET. The node representing *S. suis* is coloured red.

Inspection of the above network shows that the strongest positive correlations exist between *S. suis* and pathogenic species belonging to the genera *Actinobacillus*, *Actinomyces*, *Fusobacterium* and *Streptococcus*; the strongest negative correlations exist between *S. suis* and the positively correlated pathogenic taxa on the one hand, and species belonging to the genera *Lactobacillus* spp. (especially *Lactobacillus reuteri*) and *Enterococcus faecium* on the other hand. When considering only the data from the tonsil samples, the CoNET co-occurrence network looks slightly different (Figure 4). Using only the tonsil data to generate the species co-occurrence network that is displayed in Figure 4, it can be seen that *S. suis* was strongly directly positively correlated with *Streptococcus porcinus* and *Granulicatella adiacens,* two pathogenic microbes, and more distantly with pathogenic *Actinobacillus* species and with *Corynebacterium diphtheriae* and several other pathogens.

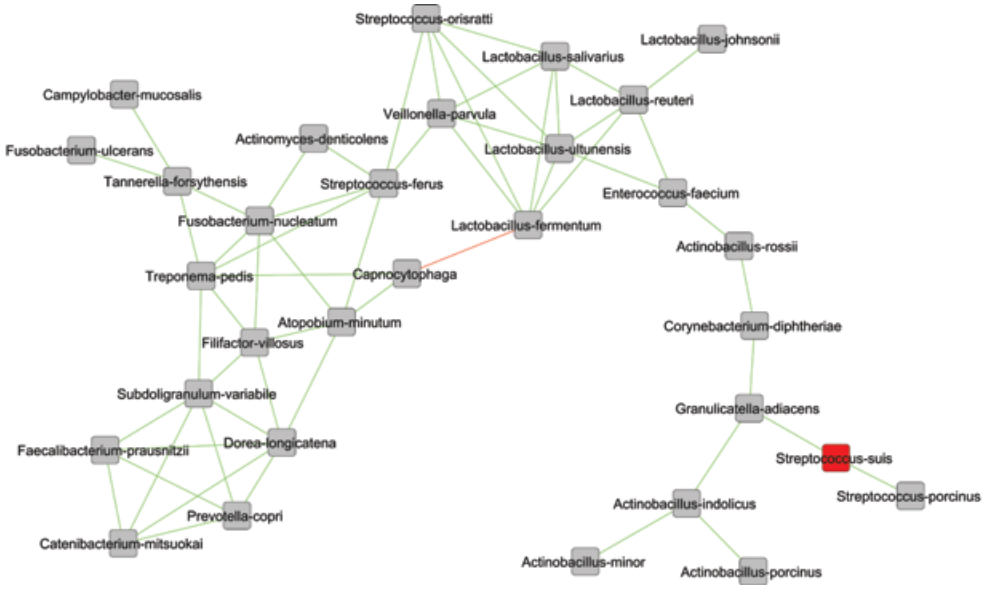

Figure 4. Co-occurrence network representing the strongest correlations between abundance values of bacterial taxa, calculated using CoNET for the tonsil samples dataset only. The edges represent positive correlation (green) or negative correlation (red). The node representing *Streptococcus suis* is coloured red.

Theoretically, negative correlation between *S. suis* and its co-occurring pathogens and *Lactobacillus* spp. could hint at bacterial antagonism, but also at different niches. Although the species *L. crispatus*, *L. fermentum* and *L. vaginalis* have been found in the intestine and oral cavity, the species *L. reuteri* is most often retrieved from the intestine but not from the oral cavity [23] although *L. reuteri* is a substantial component of the biofilms that line parts of the pig upper digestive tract and can adhere to cells of the stratified squamous epithelium [28]. In these biofilms and during planktonic growth in liquid media, *L. reuteri* produces the antimicrobial compound reuterin [29] that has a broad-spectrum antimicrobial activity against different Gram-negative and Gram-positive bacteria, including *Streptococcus salivarius* [30]. To directly test if *L. reuteri* might display antagonism towards *S. suis* via the production of reuterin, four *S. suis* strains representing the serotypes 2, 7, 9 and 14 were incubated with reuterin concentrations ranging from 0.25 to 64 mM, a range during which most sensitive bacteria are killed by reuterin [30]. We found that none of the *S. suis* strains were killed or inhibited by reuterin under the conditions used in our micro broth dilution assays (data not shown).

Discussion

Streptococcus suis bacteria are among the most predominant bacteria associated with pigs and are economically relevant bacteria that cause serious porcine diseases and increased mortality especially a few weeks after weaning. On the other hand, also asymptomatic pigs are colonised by *S. suis*, in the upper airways and upper gastrointestinal tract including the tonsils and small intestine [1]. We were interested to find out if the tonsillar and small intestinal colonies of *S. suis* could be part of a consortium of porcine pathogens and if interactions between bacteria could be involved in suppressing (negative correlations, antagonism) or promoting (positive correlation, symbiosis) virulence of *S. suis*.

To be able to assess the relative abundance of *S. suis* and other members of the porcine microbiota, pyrosequencing was used to profile the bacterial communities using abundance of the 16S rRNA gene. The microbial diversity in terms of most abundant identified taxa were similar to previous data where 16S profiling of the porcine tonsil [7, 31] and intestine [32] had been carried out. One important insight of our analysis was that, although *S. suis* was enriched in the tonsil, its abundance on the tonsils of individual piglets was highly variable, and two piglets showed high intestinal colonisation by *S. suis*. Unfortunately, we did not possess sufficiently detailed metadata to state if these piglets showed clinical symptoms of intestinal infection by *S. suis*.

Perhaps surprisingly, a comparison of enrichment of specific bacterial taxa in the tonsillar and intestinal areas showed that the tonsil, a lymphoid tissue organ, harboured a higher proportion of enriched bacteria. A similar trend was observed when microbiomes of the human GI tract were compared; this study included tonsil samples and stool samples [33] instead of the small intestinal samples that we included in our study. It therefore appears that our data fall well into the diversity spectra that have been reported by other studies, and therefore appear to be of general value.

LEfSE analyses of bacterial taxa enriched in piglets with high, low or no *S. suis* load and network-based analyses suggested that *Lactobacillus* species, most notably *L. reuteri*, could be antagonists of *S. suis*. Alternatively, the observed anti-correlation between abundance of *S. suis* versus *Lactobacillus* spp. could be based on differential niche occupation in the studied piglets. LEfSE analysis revealed a strong *L. reuteri*-*S. suis* anti-correlation when all microbiota samples from the tonsillar and small intestine areas were analysed together. However, LEfSE analysis did not show this anti-correlation when only the tonsillar samples or intestinal samples were analysed. This absence of statistical anti-correlation was less likely to be caused by differences in sample sizes (32 intestinal vs. 49 tonsillar samples) but might have been a result of low abundance of *S. suis* in the intestine and low abudance of *L. reuteri* in the tonsil areas. *S. suis* was observed at low frequencies in the intestine of three piglets, and in different regions, whereas *L. reuteri* was most abundant in the small intestine.

Additionally, the differential intestinal abundances may have been influenced by differential abundance of microbes per intestinal region. Unfortunately, the sample sizes per region were low (duodenum 11 samples, jejunum 10 samples, and ileum 11 samples) and it was not possible to obtain statistically significant enrichment results by LEfSE analysis for any taxon across the three regions.

We thus assayed for direct antagonism between *L. reuteri* and *S. suis* in a bioassay using reuterin, the broad-spectrum antibiotic compound produced by *L. reuteri*, and found that *S. suis* is not sensitive to reuterin at concentrations 3-4 times higher than those shown to kill other bacteria including *S. salivarius*. We therefore assumed that the observed anti-correlation between *L. reuteri* and thus possibly also other lactobacilli, and *S. suis* was probably not due to direct bactericidal effect of reuterin against *S. suis*. It could be that the observed anti-correlation was at least substantially influenced by differential niche occupation of *S. suis* and *L. reuteri*. Alternatively, *L. reuteri* might, through the production of reuterin, directly affect abundances of other bacteria that are positively correlated with *S. suis*, thereby indirectly mediating anti-correlations. And there may be other (in)direct factors that we are not aware of, including differential impact on microbial taxa via host AMPs and other molecules.

Both the LEfSE and network analyses supported the notion that *S. suis* may be part of a consortium of opportunist pathogens, of which several of the species belonging to the genera *Actinobacillus*, *Actinomyces*, *Pasteurella* and *Streptococcus* are known to cause respiratory disease [34, 35]. The pathogenicity of intranasally or orally administered *Actinobacillus minor*, *A. porcinus*, and *A. indolicus* has been studied in colostrum-deprived gnotobiotic piglets, and under these conditions with a limited natural microbiota disease was only observed sporadically [36]. It is therefore possible that these pathogens promote the virulence and infection capacity of *S. suis* and/or other members of this consortium. This finding was very relevant to us since it answered one of our main research questions: Can we find evidence that the pathogenicity of *S. suis* can be promoted by other microbes? This finding has practical applications, for instance, it suggests that *in vivo S. suis* pathogenicity may be better studied in experiments where the endogenous microbiome is taken into account. The influence of additional opportunistic pathogens on the outcome of *S. suis* infections also offers an explanation why controlled infection bioassays and natural infections of pigs by *S. suis* show variable results [37]. We consider that the impact of polymicrobial infections and virus-*S. suis* co-infections in pig populations warrants more attention. Our data may contribute to a better understanding of the composition of bacterial consortia potentially involved in polymicrobial disease, and may help to select bacterial candidates to explore this further in a context of *S. suis* virulence and pig infection.
The LEfSE tonsil- and intestine- specific analyses and co-occurrence data still suggested that some bacterial groups might be natural antagonists of *S. suis* (and the pathogenic bacteria it associates with). To test this in direct assays, following up on the reuterin bioassays, we

decided to use culture-based analysis of porcine microbial communities, employing a robotic system, in order to screen thousands of porcine microbiota members for the production of compounds that kill or substantially inhibit the growth of *S. suis*. The results of this approach are reported in the next chapters of this thesis.

Acknowledgements

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Supplemental Information

#	animal	sow	pen	sex	feed prior to weaning	weaning age (d)	weaning weight (kg)	pool t1	pool t2
1	6714	2987	1	m	n	25	5,8	A1	A2
2	7094	3078	1	f	n	22	6,7	A1	A2
3	7009	3080	1	m	n	24	6,6	A1	A2
4	6884	2985	1	f	n	24	6,7	A1	A2
5	7084	3078	2	m	y	22	7	B1	B2
6	6961	2799	2	f	y	24	6,7	B1	B2
7	6877	2985	2	m	y	24	6,8	B1	B2
8	6662	2996	3	m	n	25	6,1	A1	A2
9	6511	2829	3	f	n	27	6,2	A1	A2
10	6804	2814	3	f	n	24	5,6	A1	A2
11	7173	3075	3	m	n	21	6,2	A1	A2
12	7086	3078	4	m	y	22	6,5	B1	B2
13	6498	2829	4	m	y	27	7	B1	B2
14	6962	2799	4	f	y	24	6,2	B1	B2
15	6718	2987	4	m	y	25	6,8	B1	B2
16	7152	3004	5	m	y	21	7,1	B1	B2
17	6825	2824	5	f	y	24	6,9	B1	B2
18	6397	2915	5	m	y	27	7,1	B1	
19	6721	2987	5	f	y	25	6,7	B1	B2
20	6383	3074	6	m	n	29	5,1	A1	A2
21	6952	2799	6	m	n	24	5,7		B2

Table S1. Animals sampled using tonsil swabs for collection of oropharyngeal microbiota. For subsequent screening, samples were pooled (A1, A2, B1, B2) based on their animals' intake of feed prior to weaning and the sampling time point (9d or 30d post-weaning). Animal 6397 had died due to a streptococcal infection between day 9 and 30 post-weaning, 6952 served as a replacement but was allocated incorrectly to the B2 pool.

Pig #	Age (d)	Weight (kg)	Sex	Sow / pen	Weaned	Ileum sample pool
2	34	6.8	m	1109 / 25	yes	A
3	20	7.1	m	333 / 15	no	B
4	27	9.4	m	333 / 15	no	B
5	34	8.5	m	333 / 28	yes	A
6	21	6.1	m	98 / 17	no	B
7	28	7.6	m	98 / 17	no	B
8	35	7.8	m	98 / 25	yes	A
9	15	4.9	m	33 / 15	no	B
10	25	6	m	33 / 15	no	B
11	32	7.6	m	33 / 28	yes	A

Table S2. Animals sampled for collection of small intestinal and oropharyngeal microbiota. Colours are used to group animals that belong to same litter from one sow. For the creation of individual intestinal microbiota isolate collections, two pools of ileum content from either weaned (pool A) or not-weaned (pool B) animals were used.

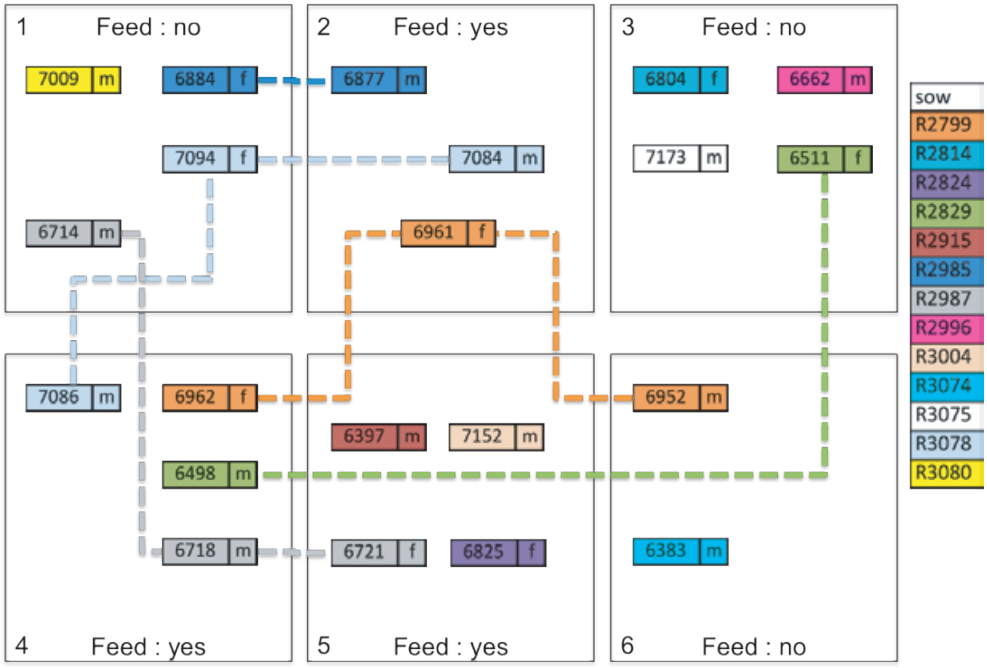

Figure S1: Graphical representation of relatedness, pen of housing and sex of the animals that were sampled using tonsil swabbing for the collection of oropharyngeal microbiota (see Table S1). Boxes numbered 1 to 6 represent the sampled pens, each in which a total of 10 animals were grouped based on their intake of solid feed prior to weaning. Each sampled animal is represented by its number and sex, while colours and dashed lines indicate and link piglets born to the same sow.

CHAPTER 5

Narrow–spectrum Inhibition of *Streptococcus suis* by Delta–Lysins of Commensal *Staphylococcus pasteuri* Isolated from Porcine Tonsil Microbiota

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Importance

Streptococcus suis is an important porcine and zoonotic pathogen for which there is no effective cross-protective vaccine. *Str. suis* is the cause of significant economic losses to the pig production industry worldwide, and there is a need for alternative strategies to prevent colonisation and invasive disease, especially in young pigs. We have explored the microbiota of healthy piglets with the aim to find commensal bacteria that can specifically kill or inhibit the growth of *Str. suis*. Here we describe the identification and characterization of two short antimicrobial peptides secreted by a commensal *Staphylococcus pasteuri* strain that effectively inhibit a wide range of *Str. suis* strains. Until recently, this family of Staphylococcal peptides was considered only to be cytolytic against eukaryotic cells. We demonstrate that the biological activity of these peptides could affect both microbe-host and microbe-microbe interactions, although the antibacterial activity appears to be narrow-spectrum.

Abstract

Given that microbes within communities fiercely compete for resources we expected to identify bacteria in the microbiota from porcine tonsil or small intestine that could kill or inhibit growth of the porcine pathogen *Str. suis*. A library of 10,150 individually picked colonies of tonsil and ileum microbiota was screened for growth-inhibiting activity against clinically relevant *Str. suis* serotypes. One isolate with a narrow spectrum of activity against seven serotypes of *Str. suis* was identified as *Staphylococcus pasteuri*. The *S. pasteuri* genome sequence revealed two genes located within RNAIII of the staphylococcal *agr* locus. These genes encode 25-amino acid, α-helical amphipathic peptides with high sequence identity to staphylococcal delta-lysins. The two delta-lysins were purified from *S. pasteuri* cultures and their antimicrobial and cytotoxic activities were compared with their chemically synthesised peptide equivalents. Both *S. pasteuri* delta-lysins show rapid, narrow-spectrum bactericidal activity with an MIC and MBC of 2 μM; there was no significant loss of porcine epithelial cell viability at this concentration. The fact that bacteria producing narrow-spectrum AMPs against *Str. suis* can be isolated from the same host niche highlights the potential to harness intrinsic competing capacities within the microbiota to enhance colonisation resistance against *Str. suis*. The delta-lysins were cytotoxic to human epithelial cells and PBMCs, and caused haemolysis of erythrocytes. Notably, the highly similar delta-lysin from *S. aureus* showed a different spectrum of antimicrobial activity against *Str. suis*. This study shows that delta-lysins may display both antimicrobial activities against competing microbes as well as interactions with the host.

Introduction

Mammalian mucosal surfaces in the gastrointestinal tract and the oropharynx are home to a rich community of microorganisms known as the microbiota. One of the well-established mutualistic aspects of mammalian host-microbiota relationships is colonization resistance against pathogens [1, 2]. Colonization resistance refers to the competition between pathogenic microbes and the resident microbiota for niches and nutrients as well as the need for pathogens to evade innate immunity elicited by the indigenous microbial communities [1]. Direct microbe-microbe competition within the microbiota may involve the production of antimicrobial peptides (AMPs), which can prevent colonization of the host by pathogenic bacteria or reduce their ability to establish an infection [3-5]. Loss of microbiota diversity following antibiotic treatment can increase susceptibility of the host to infections by pathogenic, pathobiont or opportunistic bacteria [6-8]. Potential use of the endogenous microbiome to protect the host against harmful bacteria has fuelled great interest in identifying commensal bacteria that can produce AMPs *in situ* that effectively inhibit specific pathogens [5, 9]. In addition, purified natural or modified AMPs hold great potential as therapeutics to combat potentially harmful bacteria [10].

Streptococcus suis is an encapsulated Gram-positive facultative anaerobic microbe, frequently present as a member of the porcine microbiota of the oro- and nasopharynx, and the intestinal and genital tracts [11-14]. Carriage of *Str. suis* in adult pigs is usually asymptomatic whereas young pigs (weeks 4 to 12) are most at risk from invasive disease [15, 16], with symptoms such as acute sepsis, meningitis, endocarditis, pneumonia and arthritis often being reported [17, 18]. *Str. suis* is also a zoonotic pathogen and human infections worldwide have increased significantly in the past years, with most cases originating in Southeast Asia [19]. Currently, no efficacious, cross-protective vaccines exist for *Str. suis* [20, 21] and resistance to macrolides, lincosamides, tetracyclines, and sulphonamides has been reported for up to 85% of *Str. suis* isolates in many countries [22]. In some countries *Str. suis* is the primary cause of mortality and morbidity in young pigs and the most frequent reason to prescribe antibiotics of the amino-penicillin group as a preventative measure.

Of major concern is the emergence of strains causing more rapid and severe disease and the risk that the burden of both zoonotic and porcine disease will increase as plans for continued reduction of antibiotic use in livestock are implemented across Europe [23]. Thus *Str. suis* problems on farms indirectly contribute to the spread of antibiotic resistance and alternative strategies for controlling this disease are needed [24].

Since adult pigs are usually asymptomatic carriers of *Str. suis*, we reasoned that healthy pigs might harbour members of the microbiota that could suppress outgrowth of *Str. suis* and reduce risk of invasive disease. To identify AMP-producing members of the microbiota that inhibit the growth of *Str. suis* but not of other commensals inhabiting the same niche, we used

a high-throughput screening assay on isolated bacteria from the porcine tonsil and small intestine. Here we describe the detailed characterisation of a *Staphylococcus pasteuri* isolate producing two α–helical delta-lysins with high sequence identify to other staphylococcal delta-lysins that display narrow-spectrum antimicrobial activity against *Str. suis*.

Material and Methods

Animals and sample collection

We used two separate groups of piglets for the collection of biological samples from the tonsil and small intestine (see Chapter 4).

Tonsil samples: Piglets (breed TEEZN) (see Chapter 4: Table S1, Figure S1) were bred and housed under specific pathogen free (SPF) conditions at Varkens Innovatie Centrum (Sterksel, The Netherlands). Animals were weaned between 21 and 29 days of age, with an average of 24 days for the sampled pigs. Tonsil swabs were obtained from 20 piglets at days 9 and 30 post-weaning, by brushing both palatine tonsils thoroughly for 20s with a sterile toothbrush. The top of the toothbrush was cut off and transferred to a tube containing 4 mL sterile, cold phosphate-buffered saline (PBS) and kept at 4°C. The samples were homogenized by thorough vortexing and divided into 1 mL aliquots, either with or without 20% (v/v) glycerol. Samples were stored at -80°C within 4 hours of collection.

Intestinal content and tonsil tissue: Piglets (breed Topigs20 F1, all boars) (see Chapter 4: Table S2) were raised on a SPF farm (Van Beek Varkens BV, Lelystad, The Netherlands). Animals were weaned at 28 days of age. When aged 3 – 5 weeks, piglets were anaesthetized and killed, after which the abdominal cavity was opened and ~20 cm sections of three different locations of the small intestine (duodenum, jejunum, and ileum) were clamp-closed and removed. The luminal content (~2 mL) from the intestinal sections was collected by gentle squeezing and split into two cryotubes, either with or without 20% glycerol. All samples were directly frozen in liquid nitrogen and stored at -80°C.

Bacterial strains and culture conditions

Isolates of *Streptococcus suis* comprising eight different serotypes, including the un-encapsulated strain J28 and its parental strain S10, were obtained from Central Veterinary Institute (Lelystad, Netherlands) and are listed in Table 1. All *Streptococcus* strains were cultured without agitation at 37°C with 5% CO_2 in Todd-Hewitt Broth (THB) (Oxoid, Basingstoke, UK), broth with vigorous shaking at 37°C. Bacterial isolates from porcine microbiota were grown in THB, in THB supplemented with K_2HPO_4 (36 mM), KH_2PO_4 (13.2 mM), sodium citrate (1.7 mM), $MgSO_4$ (0.4 mM), $(NH_4)_2SO_4$ (6.8 mM) and 4.4% glycerol (THB freezing medium), or in BHI. The following culture media were used for other tested organisms; MRS (VWR International, Leuven, Belgium) for *Lactobacillus* strains, BHI for all *Staphylococcus* strains and *Listeria monocytogenes*, Luria-Bertani (LB) (Merck, Darmstadt, Germany) for *Salmonella enteriditis*, and trypticase soy yeast extract medium (BD, Erembodegem-Dorp, Belgium) for *Enterococcus faecalis*. All bacteria were cultured at 37°C and agar (1.5% w/v) was added to culture media for bacterial growth on plates.

Isolate library creation and inhibition screening assay

Tonsil samples were pooled based on the animal's feeding behaviour prior to weaning (has eaten solid feed or not yet) and the time point of sample collection (9 days or 30 days post-

weaning), resulting in 4 separate pools. Only the ileum content was pooled from all the intestinal samples. Pooled microbiota samples were each plated directly onto THB agar Q trays, which were incubated overnight (~18 h) aerobically at 37°C. Single colonies were isolated from THB agar Q trays using a Genetix QPIX II-XT (Molecular Devices, Berkshire, UK) colony-picking robot and inoculated into 96-well microtiter plates containing THB freezing medium. The plates were incubated overnight at 37°C and subsequently stored at -80°C.
Individual colonies were replicated from these 96-well microtiter plates onto THB agar plates using a 96-pin microplate replicator (Boekel Scientific) and grown overnight at 37°C. Plates were exposed to UV light (λ=312 nm) for 30 min to kill the bacterial cells. Soft THB agar (0.75% w/v agar) was cooled to 50°C and seeded with a *Str. suis* target strain (approximately 1 x 10^6 CFU mL^{-1}) from an overnight culture and a 35 mL aliquot was rapidly transferred to overlay each plate. The seeded soft-THB agar was allowed to solidify and dry, and growth inhibition zones were detected following overnight incubation at 37°C. Protease sensitivity of the antimicrobial compound was evaluated to ensure activity was due to a peptide and not due to non-specific fermentative metabolites such as lactate or acetate. A selection of colonies were grown overnight and exposed to UV light, a 1 µL solution (>600 mAU mL^{-1}) of proteinase K (Qiagen) or trypsin (Sigma) was spotted next to each colony and plates were incubated for 1 h at 37°C prior to applying the *Str. suis* seeded agar overlay.

Crude peptide extraction and agar diffusion assay

Selected isolates from porcine microbiota that showed activity in the screening overlay assay against specific *Str. suis* strains, were further characterized using a modification of the agar diffusion assay [25]. Glycerol stocks of isolates that had shown growth-inhibitory activity to *Str. suis* were used to inoculate 5 mL THB or BHI media, which was incubated overnight at 37°C. From this culture, THB or BHI agar plates were inoculated with an appropriate dilution of bacteria to obtain a lawn of growth following overnight incubation at 37°C. A volume of 5 mL sterile NaCl 0.9% solution was added and bacteria were detached from the agar surface by 5 min incubation at room temperature on a horizontal shaker followed by gentle scraping with a sterile plastic T-shaped spreader (VWR International, Amsterdam, The Netherlands). The cell suspension was collected in sterile 15 mL Falcon tubes and centrifuged for 15 min at 3200 x g at 4°C. Supernatant was filtered using a 0.2 µm sterile syringe filter (Merck Millipore, Darmstadt, Germany) to obtain cell-free culture supernatant (CFS). Bacterial cell pellets were resuspended in 70% 2-propanol (IPA), 0.1% trifluoracetic acid (TFA) and stirred for 4 hours at 4°C. The IPA-TFA cell extract (CE) was obtained by centrifugation for 15 min at 10.000 x *g* at 4°C, and filtered using a 0.2 µm sterile syringe filter. To partially purify the antimicrobial peptide from the CE, the IPA-TFA was removed by rotary evaporation, and the remaining sample applied to a 2g, 12 mL, Strata C18-E solid phase extraction column (Phenomenex, Cheshire, UK) pre-conditioned with methanol and equilibrated with water. The column was washed with 40% ethanol and the antimicrobial activity eluted in stepwise concentrations of IPA with 0.1% TFA, and each elution fraction was collected. Appropriate nutrient agar (0.75%

w/v agar) was cooled to 50°C and seeded with a selected target bacterium (approximately 1 x 10^6 CFU mL^{-1}). A volume of 25 mL was rapidly transferred to sterile Petri dishes and allowed to solidify and dry, after which wells (4.6 mm diameter) were made using a sterile glass Pasteur pipette. A volume of 50 µL or 100 µL of the CFS, CE or reverse-phase HPLC fractions (obtained as described below) was dispensed into the wells and the plates were incubated at 37°C overnight. Antimicrobial activity was determined by the presence and size of clearly visible zones of growth inhibition around the wells.

Whole–genome sequencing and identification of the strain inhibiting growth of *Str. suis*

Total DNA was extracted from isolate #26 and whole-genome sequencing was performed as described elsewhere (refer to Genome Announcement) (NCBI WGS submission ID: SUB1512943). To identify the isolate, the complete 16S rRNA gene sequence was derived from the whole genome sequence using RNAmmer 1.2 [26]. The *tuf* and *gap* gene sequences [27, 28] were retrieved after annotation of the genome using RAST [29, 30]. Phylogenetic neighbours and their corresponding 16S rRNA gene sequences were found using the EzTaxon server (http://www.ezbiocloud.net/eztaxon) [31]. Using the Phylogeny.fr web server software suite (http://www.phylogeny.fr/) [32, 33], gene sequences were aligned (T-coffee), the alignments were trimmed and curated (Gblocks), and a phylogenetic tree was estimated using the Maximum Likelihood criterion (PhyML v3.0) with 500 times bootstrapping.

Genome mining for antimicrobial peptide encoding genes

The web-based tool BAGEL3 (http://bagel.molgenrug.nl/) [34] was used to mine the *S. pasteuri* whole genome sequence for putative bacteriocin or antimicrobial peptide encoding genes. Amino acid sequences of two putative antimicrobial peptides were used as input for helical wheel representation using the tool available on http://rzlab.ucr.edu/. PEPFOLD 1.5 [35, 36] was used for *de novo* three-dimensional conformation prediction of the peptides.

***Staphylococcus pasteuri* peptide purification**

Five mL of BHI medium was inoculated with 25 µl of isolate #26 (*Staphylococcus pasteuri*) from a -20°C glycerol stock and incubated overnight at 37°C with agitation at 125 rpm, and was used to inoculate 1 litre of BHI medium which was then grown overnight. The culture was harvested by centrifugation (8000 rpm, 20 minutes, 4°C), and the cell-pellet was resuspended in 150 mL of 70% IPA 0.1% TFA and stirred for 3-4 hours at room temperature. The antimicrobial peptides were first partially purified from cells using a Strata C18-E solid phase extraction column eluted with 70% IPA 0.1% TFA as described above and the IPA-TFA was removed by rotary evaporation. The eluent was applied in 249 µl aliquots to an analytical Proteo Jupiter RP-HPLC column (4.6 x 250 mm, 4µ, 90Å, Phenomenex, Cheshire, UK) running a 30-62% acetonitrile 0.1% TFA gradient for 5 minutes followed by a 62-75% gradient over 35 minutes. Eluent was monitored at 214 nm and fractions were collected at 1 minute intervals.

Synthetic peptides

Peptides were synthesised using Microwave-assisted Solid Phase Peptide Synthesis (MW-SPPS) performed on a LibertyBlue™ CEM microwave peptide synthesiser, according to the amino-acid sequences predicted from the *hld* genes. Both peptides were synthesised on an Fmoc-L-Lys(BOC) HMBP-Chemmatrix® resin (PCAS BioMatrixInc, Quebec, Canada).

Peptides were purified using RP-HPLC on a C4 Vydac (10 x 250 mm, 10 µ, 300Å) column (Vydac, California, USA) and eluted using a gradient of 30% acetonitrile, 0.1% TFA for 5 minutes, 30-40% acetonitrile, 0.1% TFA over 5 minutes, followed by 40-60% acetonitrile, 0.1% TFA over 40 minutes. The flow rate was 2.5 mL min^{-1} and eluent was monitored at 214 nm. Fractions were collected at 1 minute intervals and fractions containing the desired molecular mass were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF-MS) performed on an Axima TOF^2 mass spectrometer (Shimadzu Biotech, Manchester UK). Fractions deemed pure were pooled and lyophilised on a Genevac HT 4X (Genevac Ltd., Ipswitch, UK).

Time–course bactericidal assay

A culture of *Str. suis* S10 was grown overnight in THB at 37°C 5% CO_2, diluted 100-fold into pre-warmed THB and incubated for 6 h. For the assay, the bacteria were suspended in fresh, pre-warmed THB at a density of $5x10^5$ CFU mL^{-1} and exposed at 37°C to 1 µM (½x MIC) or 4 µM (2x MIC) of synthetic peptide diluted in THB, or an equal volume of THB as control. At 30 min intervals, 20 µL aliquots were removed and 10-fold serial dilutions in PBS were plated onto THB agar plates, and *Str. suis* survival was enumerated following overnight incubation at 37°C 5% CO_2. The experiment was performed in duplicate and average colony forming units (CFU) values are presented.

Transmission electron microscopy

For visual assessment of peptide-induced membrane damage, bacteria were collected in exponential growth phase as described above, were exposed to 1, 2 or 8 µM peptide and were fixed 30 or 120 min after exposure according to the lysine-acetate-based formaldehyde/glutaraldehyde ruthenium red-osmium (LRR) fixation procedure [37]. After dehydration using ethanol, samples were embedded in Epon and ultrathin sections were cut and placed on copper grids. Fixed samples were imaged using a JEOL JEM 1011 transmission electron microscope.

Haemolysis assay

To assess haemolytic activity of the delta-lysins towards human cells, human erythrocytes from 3 different donors (Sanquin Blood Bank, Nijmegen, The Netherlands) were assayed. Erythrocytes were isolated by centrifugation (750 x *g*, 5 min), washed three times in sterile 0.9% NaCl solution and resuspended at a density of $2x10^7$ cells mL^{-1} in Dulbecco's PBS (dPBS) without calcium or magnesium (AppliChem, Darmstadt, Germany). After 1h incubation at

37°C with 5% CO_2 in the presence of different concentrations of peptide in dPBS , the cells were centrifuged (12,000 x *g*, 15s) and haemoglobin release, indicating cellular damage, was measured as absorbance of the supernatant at 405 nm and 600 nm (background) (Spectramax M5, Molecular Devices, Sunnyvale CA, USA). Parallel exposure to 0.5% (v/v) Tween-20 and dPBS was used to determine 100% and 0% (background) haemolysis, respectively. Experiments were performed in duplicate, and represented as mean values with SD of the three donors. The LD_{50} was calculated as the concentration resulting in 50% of haemolysis based on these averaged values.

Neutral red uptake assay

IPEC-J2 cells, originating from porcine jejunal epithelium [38], were used to determine cytotoxicity of the two delta-lysins for pigs, by assessing porcine cell viability using the neutral red uptake assay [39]. Briefly, cells were seeded in a flat-bottom 96-well microtiter plate at a density of ~$1x10^5$ cells $well^{-1}$ in 89% Dulbecco's Modified Eagle's Medium (DMEM) + GlutaMAX™ (Gibco, Life Technologies), containing 10% heat-inactivated Fetal Bovine Serum (FBS) and 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Invitrogen, Breda, The Netherlands). After incubation at 37°C with 5% CO_2 for 24 hours cells had reached confluency and culture medium was removed and replaced with 200 µL treatment medium (Keratinocyte Serum Free Medium (Gibco, Life Technologies)) containing a concentration range of synthetic peptide. Following 20 h incubation at 37°C with 5% CO_2, the treatment medium was removed and 100 µL neutral red solution (40 mg mL^{-1}) was added. After 3 h incubation, neutral red solution was removed, cells were washed with 150 µL PBS and neutral red was extracted from the cells by adding 150 µL 50% ethanol, 1% glacial acetic acid. The released neutral red content, indicative of cell viability, was measured spectrophotometrically at 540 nm (Spectramax M5, Molecular Devices, Sunnyvale CA, USA), values from duplicates conditions were averaged and normalized to cells treated with PBS only and expressed with the SD as percentage viable cells.

Human peripheral blood mononuclear cell (PBMC) viability assay

PBMCs were isolated and prepared as previously described [40] with modifications. Buffy coats of peripheral blood of 3 healthy donors were obtained from the Sanquin Blood Bank, Nijmegen, The Netherlands. Isolated PBMCs were washed and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) + GlutaMAX™ (Gibco, Life Technologies) supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Invitrogen, Breda, The Netherlands) to a final concentration of 1×10^6 cells mL^{-1} and seeded (100 µL per well) in 96-well tissue culture plates. Two-fold serial dilutions of active CE fractions were prepared in sterile PBS and 11 µL was suspended per well. After 3 hours of incubation at 37°C 5% CO_2, cells were harvested and stained using the commercially available Annexin V: Propidium Iodide (PI) Apoptosis Detection Kit APC (eBiosciences, Vienna, Austria) according to the manufacturer's

instructions. Viability was assessed by flow-cytometry and data were analysed using FACS Diva software.

DNA extraction and 16S rRNA gene sequence–based microbiota composition profiling

For the oropharyngeal samples, 1 mL aliquots from tonsil-brushed material were freeze-dried prior to DNA extraction. For the small intestinal content samples, approximately 0.2 gram of material was used. Total DNA was extracted from these samples using the Repeated Bead Beating method as described in [41] using a FastPrep-24 instrument (MP-Biomedicals, France), purified using the QIAmp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) and stored at -20°C.

Amplicons from the V3 to V6 region of the 16S rRNA genes were generated by PCR using forward primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3') 5'-extended with a 'NNNNNN' barcode sequence and the titanium sequencing adapter A (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3') at the 5' end. The 'NNNNNN' barcode is a unique six nucleotide sequence used for each separate sample. Each reaction was performed using the reverse primer 1061R (5'-crrcacgagctgacgac-3') 5'-extended with the titanium sequencing adapter B (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3') at the 5' end.

PCR reactions were performed using a thermocycler in a total reaction volume of 50 µL containing 1x buffer, 5 µL dNTP mix, 3 µL $MgSO_4$, 1 unit of KOD Hot Start DNA polymerase (Novagen, Toyobo, Japan), 200 nM of forward and reverse primer, and 2 µL of undiluted or 10x diluted template DNA. The amplification program comprised an initial denaturation step at 95°C for 2 min, and 35 cycles of: denaturation at 95°C for 20 s, annealing at 55°C for 10 s, elongation at 70°C for 15 s. The size of the amplicons (789 bp) was confirmed by gel electrophoresis. Both negative and positive control PCR reactions were run alongside the amplification, and yielded no amplicon and an amplicon of the expected size, respectively.

PCR products were purified using MSB® Spin PCRapace columns (Stratec Molecular GmbH, Berlin, Germany) and the PureLink® PCR Purification kit (Invitrogen) with high cut-off buffer B3 to remove fragments <300 bp. Yield and purity of the DNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

All PCR amplicons were mixed in equimolar concentrations to 31 ng μL^{-1} and 5 internal standard controls were included. The pooled PCR amplicons were subjected to pyrosequencing, using half a picotiterplate on the Genome Sequencer FLX in combination with titanium chemistry (GATC-Biotech, Konstanz, Germany).

Pyrosequencing data were analysed with a workflow based on QIIME v1.2 [42], using settings as recommended in the QIIME 1.2 tutorial, with the following exceptions: reads were filtered for chimeric sequences using Chimera Slayer [43] and OTU clustering was performed with settings as recommended in the QIIME newsletter of 17 December 2010 [44] using an identity threshold of 97%. Diversity metrics were calculated as implemented in QIIME 1.2. Hierarchical clustering of samples was performed using UPGMA with weighted UniFrac as a distance measure as implemented in QIIME 1.2. The Ribosomal Database Project classifier version 2.2

was performed for taxonomic classification [45]. Additional data handling was done using in-house developed Python and Perl scripts (NIZO Food Research, Ede, The Netherlands).

Ethical statement

All animal experiments were approved by the ethical committee of Wageningen UR, The Netherlands, in accordance with the Dutch law on animal experiments. The human PBMC and haemolysis assays were approved by Wageningen University Ethical Committee and performed according to the principles of the Declaration of Helsinki. Written consent was provided by the donors.

Results

Collections of porcine microbiota isolates

We used two separate groups of piglets for the collection of microbiological samples from the tonsil and small intestine, respectively. From one group of 20 piglets tonsil swabs were obtained from each animal at days 9 and 30 post-weaning (Chapter 4, Table S1), and from 9 different piglets the intestinal content and tonsil tissue were collected 1 wk prior to weaning (n=3), at weaning (n=3) or 1 wk post-weaning (n=3) (Chapter 4, Table S2). Tonsil samples harvested from the first group of animals at the same sampling time point were pooled and ileal content from all animals from the second group were pooled. We plated the pooled microbiota samples, picked individual colonies using a colony-picking robot, and cultured these in 96-well plates, creating two separate isolate libraries originating from the tonsil and the ileum. These isolate libraries of individually picked bacterial colonies from tonsillar and ileal microbiota were used for antimicrobial activity screening against *Str. suis*.

Identification of porcine microbiota isolates inhibiting growth of *Str. suis*

In total 10,150 colonies were screened for their capacity to inhibit growth of Str. *suis* serotype 2 (un-encapsulated mutant; J28), serotype 7 (strain 8039), and serotype 9 (strain 8067) using an overlay assay. We selected 86 colonies from the tonsil library and 18 colonies originating from the ileum library that displayed inhibition against one or more serotypes of *Str. suis* in the overlay assays, and these were tested further in overlay assays against other strains of *Str. suis* (Table 1).

Serotype	Strain	Virulence	Screening
1/2	15964	ND	
1	6388	HV	
1	6555 / NCTC 428	V	
2	T15	AV	
2	S735 / NCTC 10234	WV	
2	p 1/7	V	
2	3881 / S10	V	
2	J28 (S10 cpsΔEF)	AV	y
3	15965	ND	
4	5213	ND	
7	8039	ND	y
9	7997	AV	
9	8067	AV	y
9	8017	AV	
9	7709	ND	
9	C132	ND	
9	5973	AV	
9	22083 R	ND	
9	7998	ND	
9	8186	ND	
14	13730	ND	

Table 1: *Streptococcus suis* isolates with different serotypes used in this study. Strain 8039, 8067, and J28 were used for screening of the isolate banks, encompassing the most prevalent serotypes 2 and 9 as well as an unencapsulated mutant. AV = avirulent, V = virulent, HV = highly virulent, ND = not determined.

We selected 32 tonsil isolates out of the original 86 isolates based on their different colony morphologies and consistent inhibitory activity *in vitro* against a panel of *Str. suis* strains. In total 28 isolates lost *Str. suis* inhibiting activity after culturing under laboratory conditions and 23 isolates were only active against three or less serotypes of *Str. suis* (Table S1). Similarly, from the original 18 ileum isolates only a single isolate showed inhibition of multiple serotypes of *Str. suis*. From these tonsil and ileum libraries we selected 7 isolates with the largest zones of inhibition, and of which the inhibitory effect was abrogated after protease treatment. We identified these 7 isolates by 16S rRNA gene sequencing and characterised them further.
Here we focus on the detailed characterisation of one bacterial isolate (#26) originating from the tonsil, as in overlay assays it showed consistent growth inhibition of multiple serotypes of *Str. suis* with large, clear inhibition zones (Figure 1A, Table S1). Isolate #26 was identified as *Staphylococcus pasteuri* [46] based on the genome sequence. In the pyrosequencing reads obtained from all tonsil samples, the 16S rRNA gene sequence of this isolate was found in one tonsil swab sample with 100% identity and in tonsil swabs from 17 out of 21 sampled animals with >99% identity (data not shown). Although the high degree of 16S sequence

similarity between closely related Staphylococcal species [28] and the possible presence of sequencing errors in the ~510 bp reads does not allow for reliable species assignment, this confirms the presence of *S. pasteuri* isolate #26 and closely related isolates in the sampled porcine tonsils.

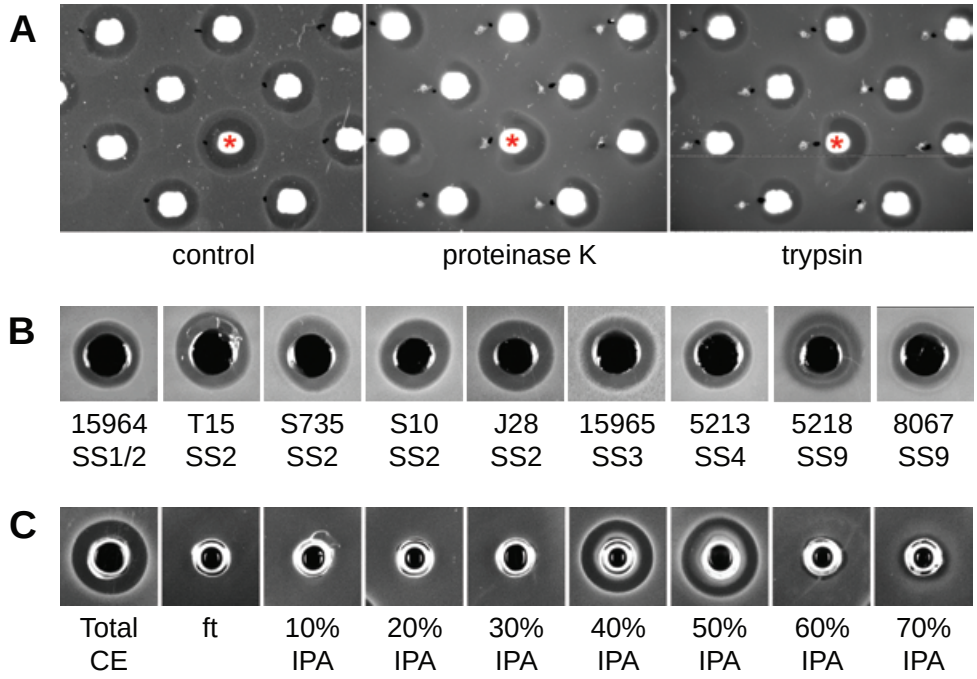

Figure 1. A) Overlay assay showing a selection of tonsil isolates (Table S1) with inhibition zones against *Str. suis* SS2 S10. Spotting of proteinase K or trypsin (black dots) next to the bacterial colonies locally abolishes this inhibitory effect, indicating production of a proteinaceous inhibiting substance. *S. pasteuri* #26 is indicated with a red asterisk. **B)** Well-diffusion assay with CFS of a high optical density culture of *S. pasteuri* #26 against nine *Str. suis* strains belonging to 5 different serotypes. Clear zones around the wells indicate inhibition of growth. **C)** Well diffusion assay against *Str. suis* S10 of CE fractions (50 µL per well) eluted with stepwise increased concentration of IPA from a SPE column. Activity is present in the 40% and 50% IPA elution fraction, and in total cell extract (as was applied onto the column) but not in other fractions or the flow-through (ft). Whitish halo around the 50% IPA well presumably is caused by protein precipitation in absence of IPA. Protein content of these fractions was analysed by Tricine-SDS-PAGE (Figure S1B).

Antimicrobial activity detection in cell–free supernatant and 70% 2–propanol cell extract

The antimicrobial activity of *S. pasteuri* #26 was abolished by proteinase K and trypsin in overlay assays (Figure 1A), indicating the production of a proteinaceous inhibitory substance. Antimicrobial activity against multiple serotypes of *Str. suis* was found in cell-free supernatant (CFS) of liquid cultures grown overnight to high optical density ($OD_{600\ nm}$ >10) at

37°C with aeration by agitation at 225 rpm (Figure 1B), whereas this activity was absent in the CFS of cultures grown without aeration by agitation that reached a much lower optical density. When the bacteria were grown aerobically overnight on BHI agar plates, and cells were removed and washed in saline solution, this supernatant also contained antimicrobial activity (data not shown). Most antimicrobial activity was extracted in 70% 2-propanol (IPA), 0.1% trifluoracetic acid (TFA) crude cells extracts (CE) of bacteria grown either to high OD in liquid culture or on agar plates. This suggests that, although some of the antimicrobial activity is released into the culture medium, a large part of this activity remains attached to the bacterial cell envelope and can effectively be released by extraction with an organic solvent such as IPA. The inhibitory substance present in the CFS and CE was likely to be a small peptide as shown by Tricine-SDS-PAGE (Figure S1A). To purify the antimicrobial fraction, the crude CE was applied to a C18-E solid phase extraction (SPE) column and elution of bound material by stepwise addition of increasing concentrations of IPA was performed. IPA was removed by evaporation and the eluent fractions were subsequently subjected to antimicrobial testing. We only found antimicrobial activity in elution fractions with 40% and 50% IPA (Figure 1C and Figure S1B) and we used these fractions, after evaporation of IPA, for testing the antimicrobial activity against different strains of *Str. suis* and other bacterial species. Fractionation by HPLC on a C12 column of the 40% and 50% IPA samples, and subsequent testing of these collected fractions for antimicrobial activity against *Str. suis* S10, showed the presence of different bioactive components (Figure S3A).

Identification of two *hld* genes encoding delta–lysins by bioinformatic prediction

To identify genes of *S. pasteuri* #26 that may encode antimicrobial peptides, total DNA was purified and sequenced on an Illumina HiSeq 2500 system (reference to Genome Announcement). Two genes encoding putative bacteriocins that could be responsible for the antimicrobial activity were identified using BAGEL3, a web-based genome-mining tool for the identification of bacteriocins [34]. Further analysis of the genomic context revealed that the two putative bacteriocin-encoding ORFs were located within the regulatory RNAIII coding region that is part of the conserved staphylococcal accessory gene regulatory (*agr*) locus (reviewed in [47]) (Figure 2A). The *agr* locus comprises two divergent transcripts called RNAII and RNAIII [48, 49], the former being an operon encompassing the genes *agrBDCA* that encode the entire staphylococcal peptide-induced quorum sensing module, and the latter playing a complex post-transcriptional regulatory role in virulence. In most staphylococcal species the RNAIII also contains a single small ORF annotated as *hld* [48], encoding the delta-lysin peptide known for its haemolytic and cytotoxic activity [50, 51]. However, in *S. pasteuri* the RNAIII is predicted to encode two non-identical copies of delta-lysins of 25 amino acid residue length; delta-lysin I [MAADIISTIGDLVKLIINTVKKFQK] and delta-lysin II [MTADIISTIGDFVKWILDTVKKFAK]. Delta-lysin I and II resemble each other with 19 identical residues and 6 amino acid residue substitutions (A2T, L12F, L15W, I17L, N18D, and Q24A). Both peptides contain a large proportion of hydrophobic amino acid residues (52%) and

secondary structure prediction using PEPFOLD1.5 [35, 36] and helical wheel representations (see Methods) predicts that both peptides are likely to form amphiphilic α-helices (Figure 2B,C). At physiological pH the net charge of the predicted delta-lysin peptides is +1 for delta-lysin I, 0 for delta-lysin II, which is similar to other staphylococcal delta-lysins [52]. Sequence alignment of the two delta-lysins from *S. pasteuri* #26 with delta-lysins from other staphylococcal species revealed high similarity (Figure 3). Notably, *S. pasteuri* #26 delta-lysin I is identical to *S. warneri* delta-lysin I, but their delta-lysin II peptides differ by one residue on position 24 (A24T). The delta-lysin II peptide in *S. pasteuri* #26 is identical to the delta-lysin found in *S. epidermidis* E229 DL [53].

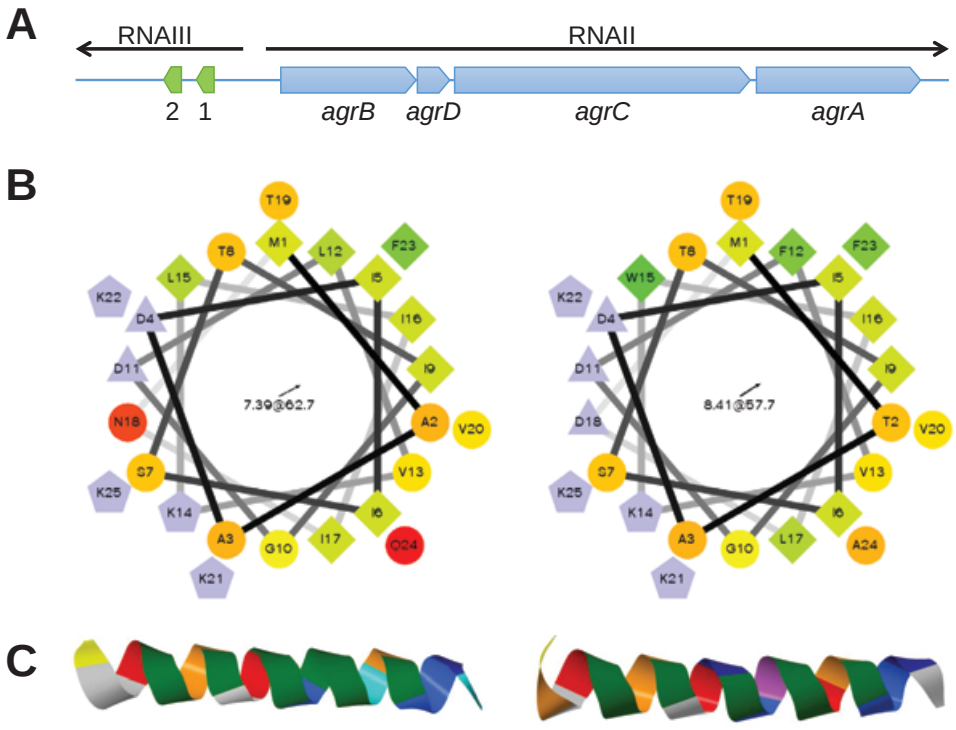

Figure 2. **A)** Graphical representation of the molecular arrangement of the *agr* locus in *S. pasteuri* #26, with the two BAGEL3 predicted genes encoding delta-lysin I and II (1 and 2, highlighted in green) located within RNAIII **B)** Helical wheel representation of delta-lysin I (left) delta-lysin II (right) showing peptide amphipathicity. Hydrophobic amino acids (diamond shape) are colour coded from highest (green) to lowest (yellow) hydrophobicity. Hydrophilic amino acids (round shape) are shaded from highest (red) to lowest (yellow) hydrophilicity, positively charged residues (pentagons) and negatively charged residues (triangles) are shown in light purple. Values depicted are hydrophobic moment (HM) and angle of HM, the arrow indicates the HM direction. **C)** 3D conformation prediction by PEPFOLD1.5 of delta-lysin I (left) and delta-lysin II (right) , coloured by residue, showing the α-helical peptide structures.

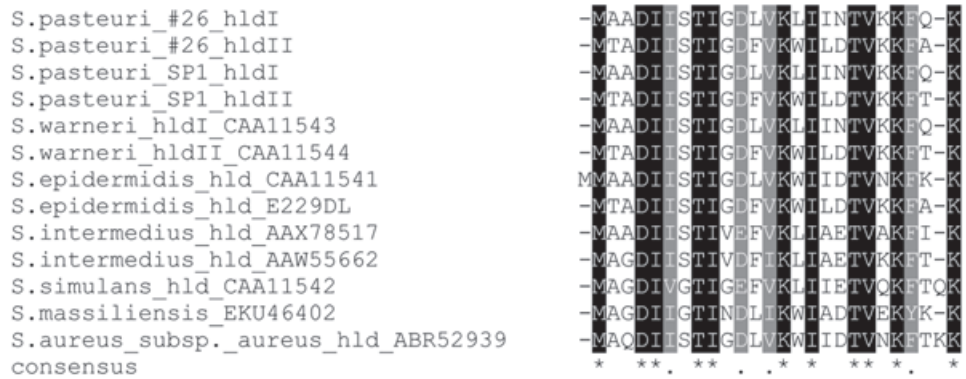

Figure 3. Alignment of peptide sequences of delta-lysins from various staphylococcal species, including delta-lysin I and II from *S. pasteuri* #26. Alignment was performed using T-Coffee and visualized with BOXSHADE, with the fraction identity threshold for shading set at 1.

S. pasteuri secretes two highly similar delta–lysins

To confirm whether the two predicted *hld* encoded delta-lysins were responsible for the antimicrobial properties of *S. pasteuri* #26, we employed MALDI-ToF-MS to detect and characterize these peptides. In colonies of *S. pasteuri* #26 grown on BHI agar, in CFS, and in CE the main peaks identified by MALDI-ToF-MS were identical, and corresponded to a mass-to-charge ratio (*m/z*) between 2300 and 2950 (Figure S2). The *S. pasteuri hld* gene sequences were predicted to encode delta-lysin I with a mass of 2760.34 Da and delta-lysin II with mass of 2841.37 Da. MALDI-ToF-MS on active CE shows the presence of 2759.52 Da and 2787.56 Da masses corresponding to delta-lysin I and 2840.50 Da and 2868.47 Da masses corresponding to delta-lysin II (Figure S2). The two masses obtained for each peptide differed by 28 Da, which corresponds to absence or presence of a formyl group (HCO-) on the N-terminal methionine, respectively.

In order to establish the proposed antimicrobial activity and mass of the two delta-lysins, we purified the natural individual peptides from the 70% IPA 0.1% TFA CE using C4 and C18 HPLC columns. Due to the high similarity in physiochemical characteristics of the two peptides, analytical columns were required to obtain good separation (Figure 4A). After purification, the yield from the CE obtained from 1 L of *S. pasteuri* #26 culture was approximately 0.8 mg/L for delta-lysin I and 4.4 mg/L for delta-lysin II. MALDI-ToF-MS showed that the masses of the two dominant HPLC peaks purified from the CE matched the theoretical masses of the N-terminal formylated delta-lysins [54-56] and matched the peptides predicted from the *hld* gene sequences (Figure 4B,C and Figure S2). We compared the antimicrobial activity of these purified peptides with that of chemically synthesised peptides (see section below).

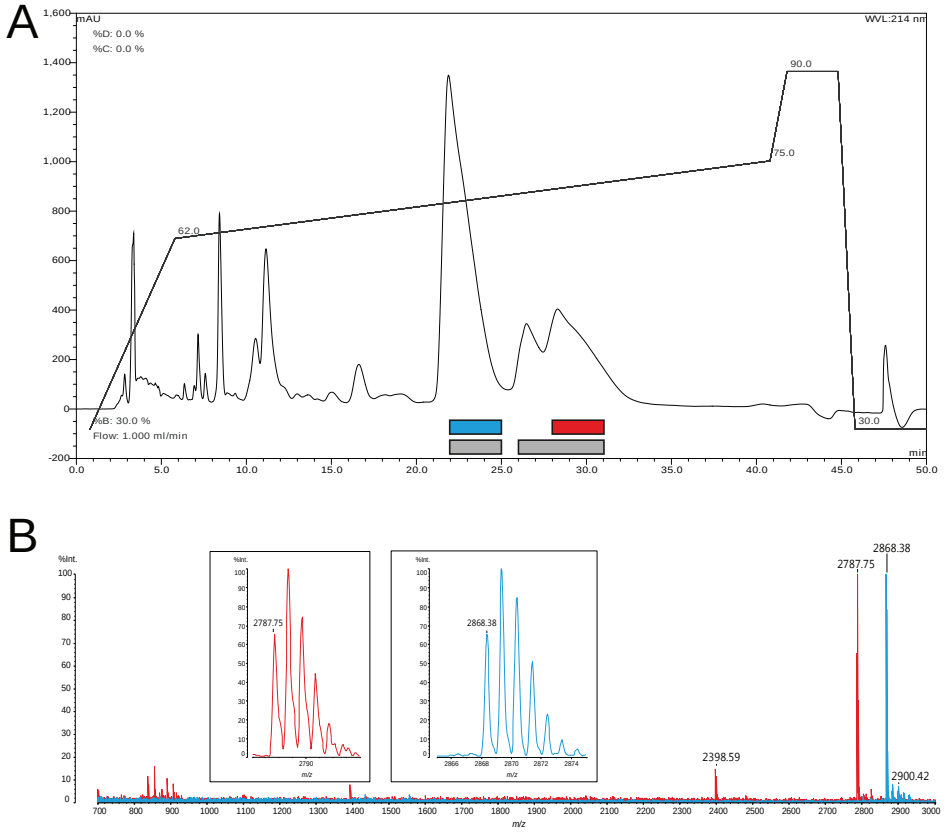

Figure 4. A) Chromatogram of RP-HPLC purification of delta-lysins from 70% IPA 0.1% TFA *S. pasteuri* cell extract. The dashed line represents the elution gradient with acetonitrile 0.1% TFA. Fractions were collected every minute and assayed for antimicrobial activity on *Str. suis* S10 indicator plates, showing activity eluting in fractions 23-25 and 27-32 (grey boxes). Delta-lysins were purified from two pooled fractions of interest (red and blue boxes). **B)** Overlaid reflectron mode MALDI-ToF-MS spectrum of purified delta-lysin I from fraction pool 1 (red) and delta-lysin II from fraction pool 2 (blue). Boxed enlargements show protonated masses of formylated delta-lysin I and delta-lysin II, respectively.

Trypsin cleavage of purified formylated peptides yielded masses of 1474.7 Da (pos. 1-14) and 800.4 Da (pos. 15-21) for delta-lysin I and 1538.8 Da (pos 1-14) and 874.5 Da (pos 15-21) for delta-lysin II (data not shown), which are identical to theoretical masses predicted by *in silico* digestion. This confirms the trypsin cleavage site in the purified peptides as predicted from the *hld* gene encoded peptides.

S. pasteuri delta–lysins have specific antimicrobial activity against seven serotypes *of Str. suis*

In the initial antimicrobial activity assays, we used the SPE column-purified CE; because the exact concentrations of the delta-lysins were unknown, the CE dilution factor that still showed antimicrobial activity was taken as a relative measure of inhibitory activity. Interestingly, among the 13 different bacterial species tested only *Str. suis* strains were susceptible (Figure 5), including strains of serotype 2 and 9 that are most frequently associated with clinical infections. Only serotype 7, represented by a single strain originally isolated from a pig with streptococcal pneumonia, appeared insensitive to the *S. pasteuri* CE. *S. mutants, S. mitis, S. salivarius, S. aureus, S. epidermidis, Lb. plantarum, Lb. rhamnosus, Lb. salivarius, Lb. reuteri, E. faecalis, S. enteriditis, L. monocytogenes* were not susceptible to the lowest dilution of CE tested (data not shown).

To investigate whether any of the *Str. suis* genes considered to be involved in resistance to antimicrobial peptides [57] could influence sensitivity to the delta-lysins we tested several isogenic mutants generated in *Str. suis* S10. These included mutants of the two-component systems (TCS) Δ1930, Δ0827 [58], and Δ*CiaHR,* as well as a mutant of *dltA,* which can provide some resistance against positively charged antimicrobial peptides via alanylation of teichoic acid. However, none of these mutants were more sensitive to the antimicrobial peptides present in the *S. pasteuri* CE. Also, the un-encapsulated mutant J28 did not show a difference in susceptibility compared to its parental strain S10 (Figure 5).

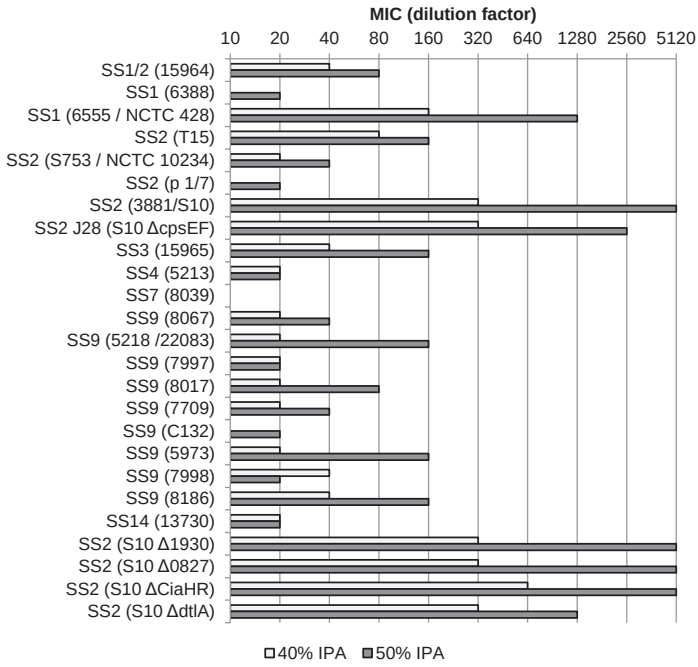

Figure 5. Minimal Inhibitory Concentrations (MIC) of *S. pasteuri* cell extracts (CE) against several strains of *Str. suis* and a panel of other bacteria. The MIC is represented as a dilution factor of extract, with higher values indicating higher bacterial susceptibility. CE applied on a SPE column was eluted subsequently with 40% and 50% IPA and both collected fractions were tested for antimicrobial potency.

Synthetic delta–lysins confirm antimicrobial activity of purified peptides

To further confirm that the antimicrobial activity displayed by *S. pasteuri* was caused by the two predicted delta-lysins, we chemically synthesized these peptides. Both synthetic peptides had a minimal inhibitory concentration (MIC) of 2 μM, which is also the minimal bactericidal concentration (MBC) against *Str. suis* S10, whereas purified (N-formylated) delta-lysin I and delta-lysin II have a MIC of 2 and 4 μM, respectively. The difference in MIC of synthetic and purified delta-lysin II could be due to the presence of the N-terminal formyl group that confers an overall reduction in net-charge of 1. Combinations of equimolar concentrations of the two peptides showed no synergistic activity (data not shown); other ratios of peptide were not tested. Microbroth dilution assays with the synthetic peptides confirmed the spectrum of activity as determined using the CE (data not shown).

S. pasteuri delta–lysins display immediate bactericidal activity by disrupting the membrane

A time-course experiment performed with *Str. suis* S10 and each of the synthetic delta-lysins showed that their bactericidal activity was immediate, from the moment of exposure

(Figure 6A). Peptide concentrations of 4 μM (2 x MIC) resulted in complete loss (5 log fold reduction) of viable bacteria after 90 min for delta-lysin I and after 150 min for delta-lysin II. Additionally, both peptides inhibited growth of *Str. suis* at a concentration of 1 μM (½ x MIC). Bacteria treated for 30 min with a concentration of 8 μM (4 x MIC) delta-lysin show minor plasma membrane disturbance, but complete disruption of the membrane was not evident (Figure 6B,C,D). Remarkably, delta-lysin appeared to cause partial delocalization of plasma membrane, resulting in cytoplasmic accumulation of membrane-like structures (Figure 6E). Bacteria exposed to peptides appeared to have areas of increased granulation with higher density in the cytoplasmic space compared to untreated bacteria.

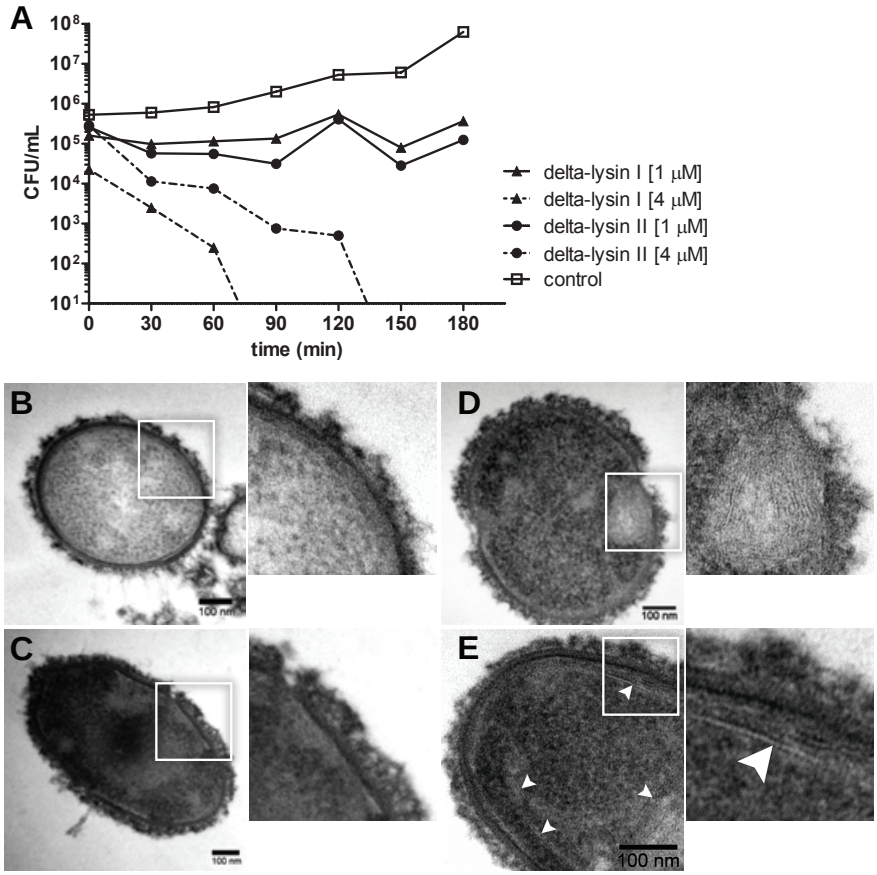

Figure 6. **A)** Time-kill curve of synthetic delta-lysin 1 and 2 at concentrations of ½ x MIC (1 μM) and 2 x MIC (4 μM) against an inoculum of 5 x 10^5 CFU mL^{-1} *Str. suis* S10 in exponential growth phase. Aliquots were collected at regular time intervals and serial dilutions in PBS were plated on THB agar for CFU enumeration. All conditions were tested in duplicate, average values are depicted. Representative transmission electron microscopy images of *Str. suis* S10 exposed to **B)** PBS (negative control) for 120 min, **C)** 8 μM delta-lysin I for 30 min, **D,E)** 8 μM delta-lysin II for 30 min. Boxes indicate areas of shown enlargements. Arrows highlight possible membrane-like structure delocalization.

Haemolytic and cytotoxic activity of *S. pasteuri* delta–lysins

Because delta-lysins from *S. aureus* are known to display haemolytic activity, we tested both *S. pasteuri* delta-lysin I and delta-lysin II for their potency to lyse human erythrocytes. Exposure for 1 hour of human erythrocytes in PBS reveals a clear dose-dependent haemolytic activity that is very similar for both delta-lysins, although purified (N-formylated) peptides show slightly lower maximum haemolysis (Figure 7A,C). The crude cell extract (40% IPA CE) against porcine erythrocytes showed a similar dose dependent haemolytic effect (data not shown). As staphylococci inhabit the oral-, nasal- and gastrointestinal tract, we tested the effects of the delta-lysins on a porcine intestinal epithelial cell line, IPEC-J2. A dose-dependent effect on epithelial cell viability was observed after 20 h exposure to peptide and no significant impact on viability was observed at the concentration of 2 μM (Figure 7B), which was sufficient to rapidly kill *Str. suis* S10.

To be able to directly relate the spectrum of antimicrobial activity results to cytotoxicity, we also used SPE column-purified CE to assay cytotoxicity against human peripheral blood mononuclear cells (hPBMCs). 40% IPA and 50% IPA CE dilutions ranging from 1/10 to1/2560 were incubated for 3 hours with freshly isolated hPBMCs. Cells were stained for viability with Annexin V, which binds to phosphatidylserine on early apoptotic cells, and propidium iodide (PI), which stains permeable, dead cells. This allows for the distinction of live (unstained), necrotic (PI only), early apoptotic (Annexin V only) and late apoptotic (both Annexin V and PI) cell populations by flow-cytometry. The 50% IPA CE dilutions of 1/10 and 1/40, which were inhibiting growth of 95% and 62% of tested *Str. suis* strains, respectively, have a clear cytotoxic effect on hPBMCs, and a 40% IPA CE dilution of 1/10, inhibiting 81% of tested *Str. suis* strains, resulted in an increase of necrotic cells. At lower concentrations of both CE fractions no significant cytotoxic effect can be observed.

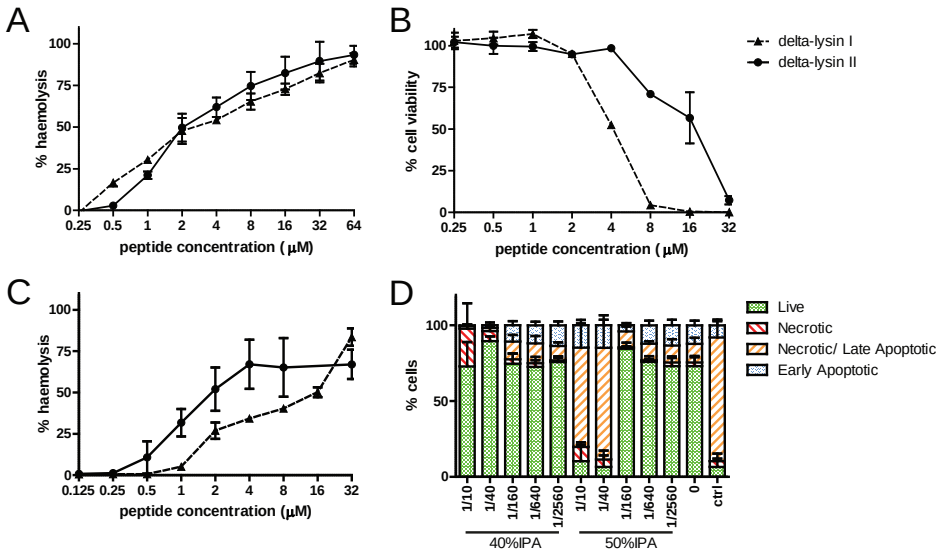

Figure 7. **A)** Haemolytic activity of both synthetic peptides against human erythrocytes from 3 different donors, after 1 h exposure. Delta-lysin I LC_{50} = 2.83 μM and delta-lysin II LC_{50} = 3.98 μM. Error bars represent SD between averaged, normalized haemolysis values per donor. **B)** Cytotoxic activity of both synthetic peptides against porcine IPEC-J2 cells, as measured by neutral red uptake after 20h exposure to a range of peptide concentrations. Error bars represent SD of averaged, normalized technical duplicates. **C)** Haemolytic activity of purified delta-lysins against human erythrocytes from 3 different donors, after 1 h exposure. Error bars represent SD between averaged, normalized haemolysis values per donor. **D)** hPBMCs obtained from 3 healthy donors were exposed for 3 h to dilutions of 40%IPA and 50%IPA CE, after which cells were stained with Annexin V and PI and analysed using FACS for the quantification of cell apoptosis and necrosis. PBS was used as a negative control, DMSO (1% v/v) was used as a positve control (ctrl). Each sample was performed in duplicate, error bars depict SD between averaged values per donor.

S. aureus delta-lysins possess a different spectrum of antimicrobial activity against *Str. suis*

Considering the high sequence similarity between delta-lysins from several S*taphylococcus* species, we hypothesized that it is unlikely that only *S. pasteuri* peptides exert an antimicrobial effect on *Str. suis*. Therefore, we tested the known delta-lysin producing *S. aureus* laboratory strain 8325-4 [59] and clinical isolate FPR3757 (USA300) [60], and delta-lysin lacking mutant RN6911 (8325–4 Δ*agr*) [61]. An overlay assay and agar diffusion assay with CFS showed *agr*-dependent antimicrobial activity of *S. aureus* against *Str. suis* S10, but no activity against other *S. aureus* strains (Figure S4). Testing of antimicrobial activity against 12 *Str. suis* strains using an overlay assay showed activity of both *S. aureus* 8325-4 and FPR3757 against *Str. suis* S10, but the *agr* mutant RN6911, lacking both RNAII and RNAIII, did not show a zone of growth inhibition against this strain (Figure 8). Strikingly, no other tested *Str. suis* strains were susceptible to *S. aureus*, whereas we observed large zones of inhibition around *S.*

pasteuri #26 (Figure 8). Because *agr* is known to regulate other toxins beside the RNAIII encoded delta-lysin in *S. aureus*, this result does not exclusively imply that lack of delta-lysin causes loss of antimicrobial activity. Therefore, we also tested synthetic *S. aureus* delta-lysin [MAQDIISTIGDLVKWIIDTVNKFTKK] in parallel with synthetic *S. pasteuri* delta-lysins against nine strains of *Str. suis*. Delta-lysins from *S. aureus* and *S. pasteuri* #26 had comparable activity against *Str. suis* strain S10 and 6555, whereas delta-lysin from *S. aureus* was not active against other *Str. suis* strains (MICs of 64 μM or higher) (Table 2). This susceptibility of *Str. suis* S10 to synthetic delta-lysin is in accordance with the results seen in the overlay assay, while the size of the growth inhibition zones does not necessarily correspond to the MIC. We conclude that despite the high sequence similarity between these Staphylococcal delta-lysins, their spectrum of antimicrobial activity is differential.

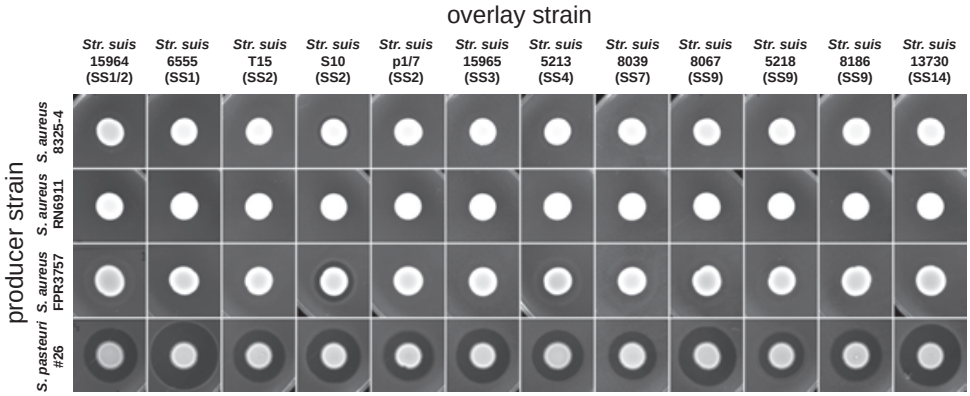

Figure 8. Overlay assay with producer strains *S. aureus* 8325-4 [59], RN6911 (8325–4 Δ*agr*) [61], FPR3757 (USA300 JE2)[60] and *S. pasteuri* #26 spotted on the same BHI agar plate, overlaid with 12 different strains of *Str. suis*.

	S. pasteuri hld I	*S. pasteuri* hld II	*S. aureus* hld
Str. suis 15964 (SS1/2)	16	64	64
Str. suis 6555 / NCTC 428 (SS1)	2	16	8
Str. suis S10 (SS2)	2	4	2
Str. suis p1/7 (SS2)	32	>64	>64
Str. suis 15965 (SS3)	4	>64	>64
Str. suis 8039 (SS7)	32	>64	>64
Str. suis 8067 (SS9)	16	16	>64
Str. suis 5218/22083 (SS9)	16	16	64
Str. suis 13730 (SS14)	16	n.d.	>64

Table 2. Minimal Inhibitory Concentration values of synthetic delta-lysin from *S. pasteuri* #26 and *S. aureus* against nine strains of *Str. suis*, as determined by microbroth dilution assay. Experiment was performed in duplicate, with technical duplicates per condition, averaged values are represented.

Discussion

Given the lack of an effective vaccine against multiple serotypes of *Streptococcus suis* [20, 21], in combination with the presence of multi-drug resistant strains in pigs [22] and the potential of these bacteria to cause zoonotic disease [62], alternative strategies to control this porcine pathogen are needed. Bacteriophages, phage lysins [63, 64] and plant extracts [65] are among the alternatives that have been investigated to date. Another promising approach could be via administration of bacteria producing antimicrobial peptides (AMPs) to suppress or prevent the colonization by *Str. suis* [4]. Such a strategy may be very suitable in young pigs, which are most susceptible to *Str. suis* infections around the time of weaning. The feasibility of such an approach was shown for a mixture of five candidate probiotic strains from the porcine intestine which inhibited *Salmonella enterica in vitro* and ameliorated diarrhoea when administered as a mixture to *S. enterica* infected pigs. In this case, the protective effect was hypothesized to be attributed to the production of a bacteriocin by one probiotic isolate [66-68]. Recently, the production of bacteriocins by selected avirulent strains of *Str. suis* that can inhibit virulent *Str. suis* of different sequence types was described as a similar strategy [69-71]. We performed a high-throughput screen for AMP-producing bacteria from the microbiota of the tonsil and small intestine of piglets and screened for microbes displaying antimicrobial activity against *Str. suis*.

In healthy piglets we sampled the tonsil and small intestine, niches which can be colonized by *Str. suis* [11, 12, 14]. Microbiota analysis by 454 pyrosequencing of all individual samples confirmed the presence of *Str. suis* in 38 of 40 tonsil swabs, but in only 2 of 10 ileal content samples, with varying abundance (see CHAPTER 4). Bacteria were isolated from the samples using culture medium and conditions that supported the growth of *Str. suis*, in anticipation of increasing the likelihood of recovering competing, antagonistic bacteria. We screened bacterial isolates from tonsil and small intestine of piglets for growth inhibitory activity against different *Str. suis* strains, including a capsule-free *cps* deletion mutant of *Str. suis* S10.

The majority of the initially selected 104 isolates exhibiting an inhibitory effect against *Str. suis* originated from the tonsil samples. This might be due to the apparent higher diversity of the microbiota in the tonsils samples compared to the ileum samples (see CHAPTER 4). We can not exclude that tonsil samples appeared more diverse because their sampling procedure included brushing which is likely to disrupt the tonsil biofilm and release associated bacteria, whereas the small intestinal luminal content collection did not involve scraping of mucosally adhered microbial populations. Many of the initially selected bacteria lost their *Str. suis*-inhibiting activity during culturing under laboratory conditions, possibly due to regulation of AMP production in response to environmental conditions and/or quorum sensing regulatory mechanisms. Therefore, we carried out further tests with 7 promising candidate isolates that showed consistent *in vitro* antibacterial activity against *Str. suis*.

One isolate (#26) showed consistent growth inhibiting activity against 17 of the 21 tested strains of all major clinical serotypes of *Str. suis* [62] (Table S1). Additionally, this isolate had a narrow spectrum of activity, only inhibiting *Str. suis* and none of the other 13 species tested. The specificity may be an advantage when using the inhibitory factor or organism to inhibit *Str. suis in vivo* as it is expected not to have a major effect on the resident microbiota, which is considered to have an important role in colonization resistance against pathogens [4, 5]. Presence of activity in the cell-free supernatant (CFS) hinted at secretion of this factor, while local loss of antimicrobial activity in overlay assays upon protease treatment indicated that the inhibiting substance was of a proteinaceous nature.

We identified isolate #26 as *Staphylococcus pasteuri*, a commensal coagulase-negative bacterium in humans and animals and a common colonizer of the intestine and oral cavity [46, 72]. The mining of the genome of *S. pasteuri* strain #26 revealed the presence of two putative antimicrobial peptide or haemolysin (*hld*) encoding genes located within a region homologous to the *S. aureus* regulatory RNAIII. The *S. aureus* RNAIII has been shown to be regulated by the *agr* peptide-induced quorum sensing system [73], possibly explaining why antimicrobial activity was only detected in high-density cultures of *S. pasteuri*. The *agr* locus is conserved in many staphylococcal species and has also been found in other taxa [47, 74]. In *S. pasteuri* #26 the divergent *agr* transcripts RNAIII and RNAII and the genes they comprise are similar to those of other staphylococci, although the *S. pasteuri* RNAIII encodes two non-identical copies of *hld* genes (Figure 2A), whereas most staphylococcal species encode a single *hld* gene within their RNAIII [48]. Notably, the phylogenetically close relative *S. warneri* (99.52% 16S rRNA gene sequence identity to *S. pasteuri* #26) also produces two non-identical delta-lysins [56, 75].

Staphylococcal delta-lysins have been described to possess haemolytic or cytotoxic activity [50-52, 76, 77]. The haemolytic activity we observed for the two *S. pasteuri* delta-lysins was in the same range as that described for *S. aureus* and *S. warneri* delta-lysins [52, 56, 76]. Although the delta-lysins of *S. pasteuri* also exerted dose-dependent cytotoxic effects on porcine epithelial cells (Figure 7B) there was a prominent difference between the kinetics of the antimicrobial and the cytotoxic effects, with all *Str. suis* bacteria being rapidly killed within 90 to 150 minutes, and the cytotoxic effects taking several hours (Figure 7B). Moreover, concentrations of CE required to cause necrosis in PBMCs appeared also 2- to 4-fold higher than the concentrations required to kill all *Str. suis* cells.

Our results support the conclusion from other studies that staphylococcal delta-lysins have narrow-spectrum antimicrobial activity. Previously, antimicrobial activity of staphylococcal delta-lysin was demonstrated against several strains of *Legionella*, *Bacillus megatherium*, *S. aureus,* and *Streptococcus pyogenes* [53, 56, 78]. Membrane disruption of *S. aureus* delta-lysin is, in part, a result of the high degree of peptide α-helicity [77], which is comparable to that of the

two *S. pasteuri* peptides. Delta-lysins were originally considered to lack antimicrobial activity [76] and be directed towards the host, most likely due to their narrow-spectrum antimicrobial activity and the limited panels of target bacteria that were tested. Sequence alignment of delta-lysins from several staphylococcal species shows a high level of conservation (Figure 3), and taking into account the similarity in structural and physicochemical properties, one can speculate that all these delta-lysins could possess antimicrobial activity against certain target bacteria. This is in agreement with our observation that two strains of *S. aureus* and chemically synthesised *S. aureus* delta-lysin peptide can effectively inhibit growth of *Str. suis* S10 but not of other tested *Str. suis* strains.

Delta-lysin is a member of the group of staphylococcal phenol-soluble modulin (PSM) peptides, and is sometimes referred to as delta-toxin or PSMγ [55, 79]. PSMs are involved in virulence and inflammation, but also have a role in the commensal lifestyle of staphylococci by facilitating growth and bacterial spreading on epithelial surfaces [80, 81]. Activation of *agr* is important in biofilm growth and regulation of genes associated with the switch from planktonic to biofilm lifestyle. Indeed, PSMs have important roles in biofilm formation and structuring [82]. Staphylococcal delta-lysins can kill *Streptococcus pyogenes* via synergistic interactions with host-derived antimicrobial peptides [83], and similarly, staphylococcal PSMα can give the producer strain a competitive advantage against other (pathogenic) bacteria, such as *Streptococcus pyogenes* [84]. Both N-formylated as well as deformylated PSMs are recognized by the human formyl peptide receptor 2 (FPR2/ALX), leading to host immune activation [85, 86]. Thus PSMs appear to have multiple roles in (in)direct bacterial competition and in host colonization [81].

To investigate whether *S. pasteuri* also can produce other PSMs, we searched for peptide sequence homology and *AgrA* binding motifs using MEME (http://meme-suite.org/). This revealed the presence of genes encoding PSMβ1 and PSMβ2 orthologues in the whole genome sequence of *S. pasteuri* #26, but we did not find homologs of other PSMs. Exactly corresponding masses to these PSMβs, as predicted from the gene sequence, were not detected by MALDI-ToF-MS in BHI agar grown colonies or in CE (Figure S2), nor in Tricine-SDS-PAGE analysis of active CFS and CE (Figure S1). As the majority of masses detected exactly match delta-lysins I and delta-lysin II, we propose that they are responsible for the observed antimicrobial and cytotoxic effects of the CFS and CE.
The level of PSM production and FPR2/ALX activation by a given staphylococcal strain correlates closely with its virulence potential [86, 87]. Of all PSMs, PSMα peptides have the strongest leukolytic, pro-inflammatory, chemotactic, and surfactant activities. The apparent absence of genes encoding PSMα and some well-known *S. aureus* cytotoxic molecules associated with virulence, such as alpha-toxin, might at least partially explain why *S. pasteuri* is not reported as a cause of infection or disease in pigs.

Theoretically, delta-lysins might be toxic to the producer strain but the genes *pmtABCD*, encoding the dedicated ATP transporter required for peptide export and immunity, are found in all PSM-producing staphylococci [88]. Homology searches revealed that *S. pasteuri* also contains *pmtABCD* homologs, likely explaining why *S. pasteuri* delta-lysins have no effect on *S. aureus* or *vice versa* (Figure S4).

Our results demonstrate that bacterial isolates producing (narrow-spectrum) antimicrobial compounds against *Str. suis* can be found by screening isolates of the microbiota that inhabit the same niche. We hypothesized that, for instance in the case of weaning-associated complications with *Str. suis* in piglets, such bacteria could be used to, temporarily, shift the microbiota towards a more antagonistic composition, thereby repressing the colonization and outgrowth of *Str. suis* in the oropharynx and small intestine. *S. aureus*, which produces other toxins capable of damaging the host, is commonly found as a harmless commensal of the nasal cavity suggesting that the *S. pasteuri* strain described here is a promising candidate for follow-up experiments *in vivo*. However, the safety of using these AMP-producing bacteria to antagonise *Str. suis* colonization *in vivo* still needs to be established. Additionally, this study shows that the biological activity of these peptides is not exclusively restricted to competing microbes but extends to interactions with the host epithelium and immune cells, making their ecological function difficult to predict.

Acknowledgements

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Supplemental Information

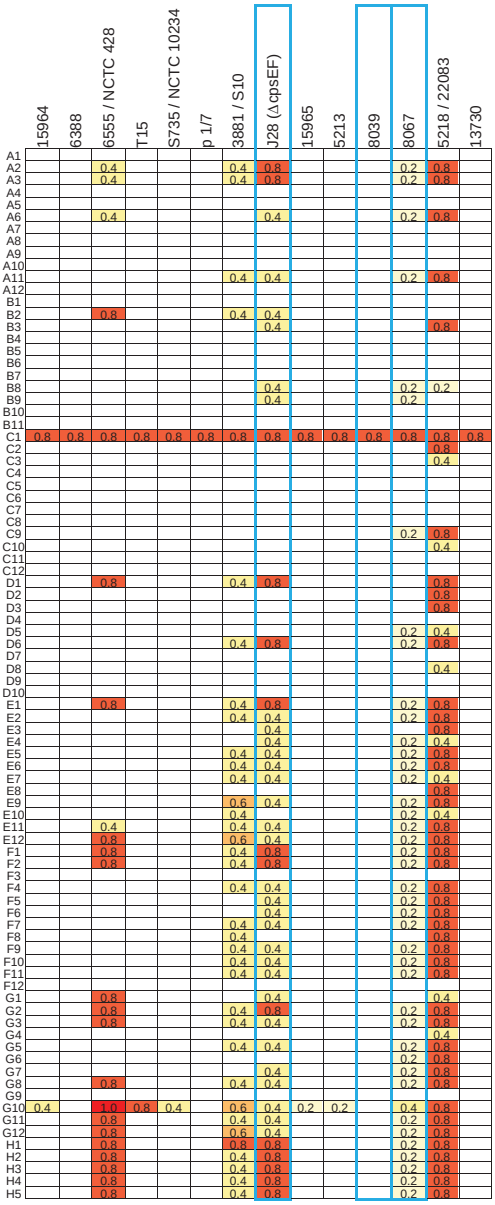

Table S1. Growth inhibition of *Str. suis* strains (see Table 1, strains used in screening boxed in blue) by the initially selected 86 tonsil microbiota isolates, scored according to relative size of the inhibition zone on a relative scale from 0 (no inhibition) to 1 (maximum inhibition as seen in this overlay assay). Many isolates lost their original antimicrobial activity after culturing under laboraty conditions. Data were obtained from duplicate experiments. The isolate in well G10, identified as *S. pasteuri* (#26), showed growth inhibition against multiple serotypes of *Str. suis* (see also Figure 1)

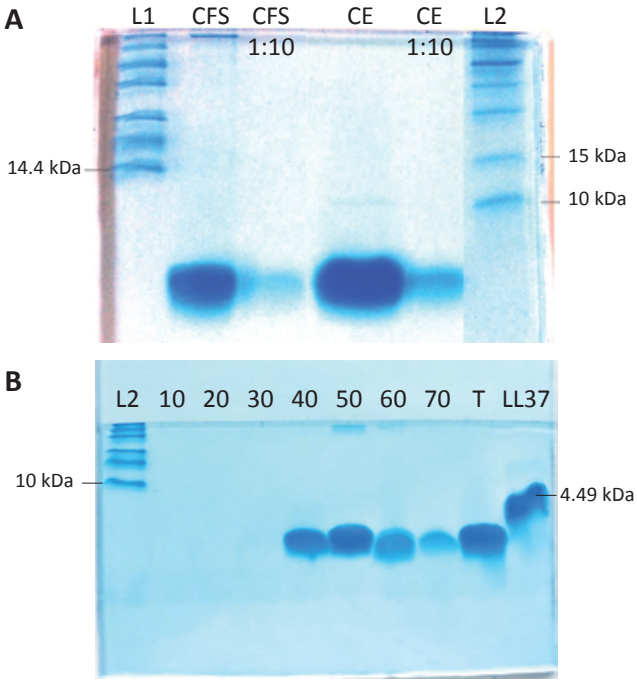

Figure S1. Tricine-SDS-PAGE [89] of *S. pasteuri* #26 **A)** CFS or total CE, indicating the abundance of mainly small peptide(s). **B)** *S. pasteuri* #26 CE was applied to SPE columns and elution fractions (stepwise increased IPA concentration from 10% to 70%) and total CE (T) were analysed using Tricine-SDS-PAGE. Antimicrobial activity against *Str. suis* was detected in 40% and 50% IPA fractions (Figure 1C). Human LL-37 was included as a reference for molecular weight of small peptides.

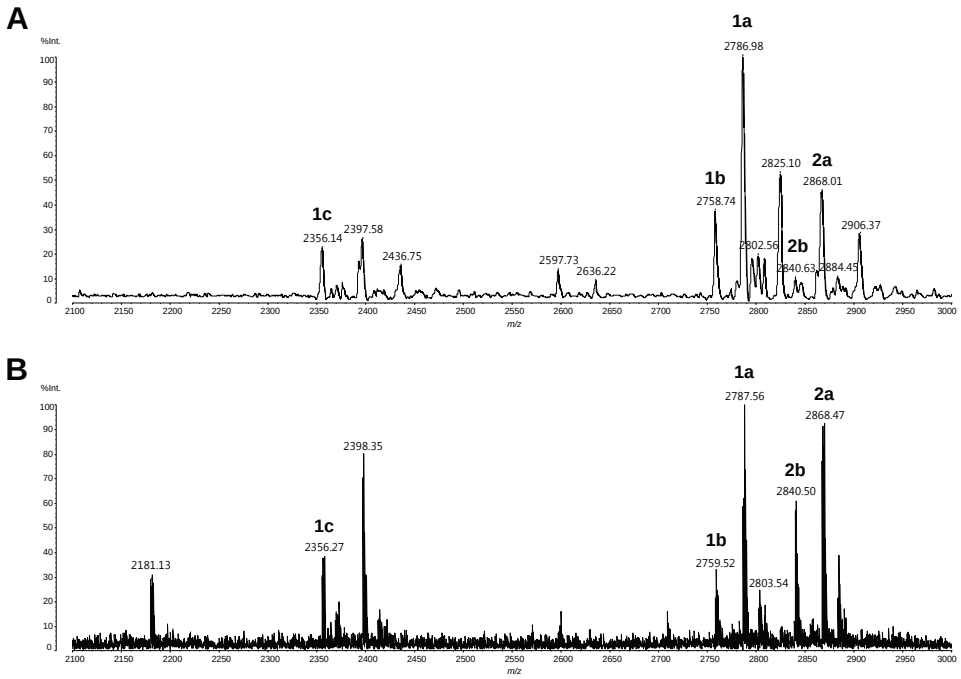

Figure S2. MALDI-ToF-MS spectra obtained from **A)** multiple colonies of *S. pasteuri* #26 grown for 24 h on BHI agar and **B)** 70% IPA 0.1% TFA bacterial cell extract (CE) of a *S. pasteuri* BHI culture. The following masses are of interest: 1a (2787.56) delta-lysin I with formylated N-terminal methionine, 1b (2759.52) delta-lysin I with deformylated N-terminal methionine, 1c (2356.27) delta-lysin I fragment (residue 1-22), 2a (2868.47) delta-lysin II with formylated N-terminal methionine, 2b (2840.50) delta-lysin II with deformylated N-terminal methionine. In addition to these masses for singly protonated molecules, corresponding peaks for $[M+Na^+]$ (+22) and $[M+K^+]$ (+39) adducts can be seen. Predicted peptides were identified according to the experimental masses using FindPept (http://web.expasy.org/findpept/)

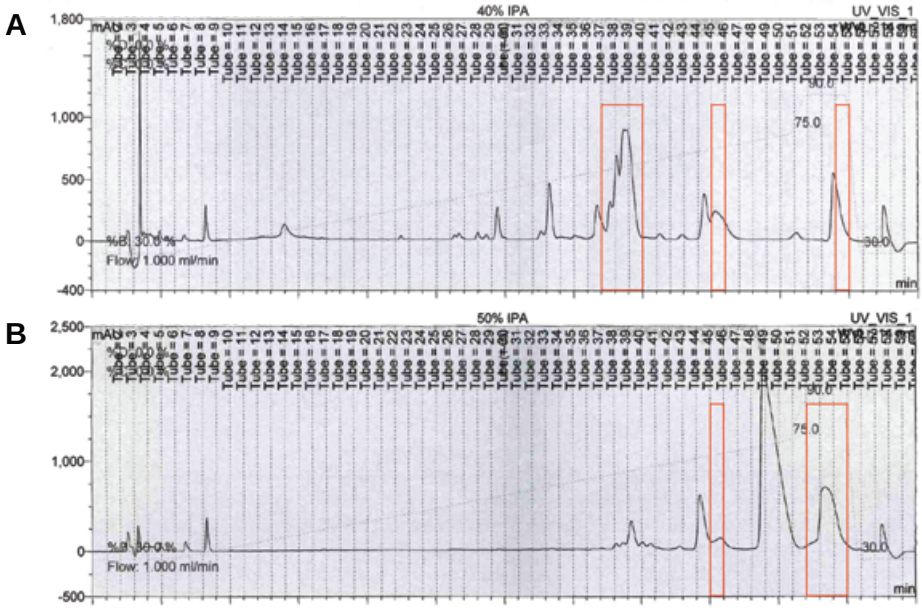

Figure S3. Chromatograms of RP-HPLC fractionation of 40% and 50% IPA SPE column fractions of *S. pasteuri* #26 CE. Each collected fraction was tested for antimicrobial activity against *Str. suis* S10, active fractions are indicated by red boxes.

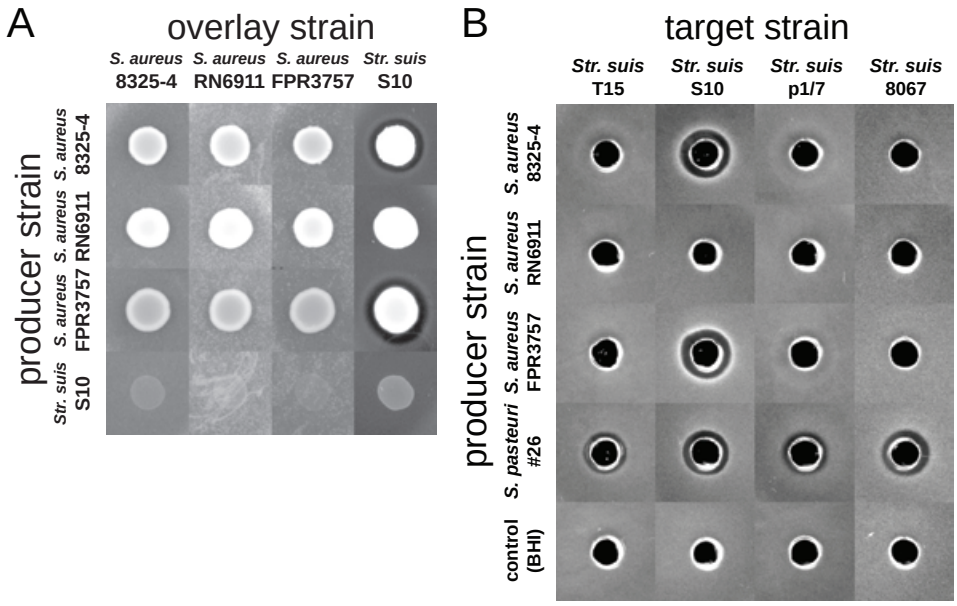

Figure S4. A) Overlay assay with producer strains *S. aureus* 8325-4 [59], RN6911 (8325–4 Δ*agr*) [61], FPR3757 (USA300 JE2)[60] and *Str. suis* S10 against each other. A volume of 10 µL overnight culture was spotted onto BHI agar plates and incubated overnight at 37 °C with 5% CO_2, after which plates were exposed to UV light for 30 min and overlaid with soft nutrient agar (0.75% w/v) at 45°C inoculated with approximately 10^6 CFU/mL target strain. Growth inhibition zones were imaged after 20 h incubation at 37 °C with 5% CO_2. Experiment was performed in duplicate and representative results are shown. **B)** Agar diffusion assay with CFS from overnight cultures in BHI broth of producer strains *S. aureus* 8325-4 [59], RN6911 (8325–4 Δ*agr*) [61], FPR3757 (USA300 JE2)[60] and *S. pasteuri* #26. Soft THB agar (0.75% w/v) at 45°C was inoculated with with approximately 10^6 CFU/mL from an overnight culture of the target strain. After solidification of the agar, wells (4.6 mm diameter) were punched and 50 µL CFS was dispensed per well. Growth inhibition zones were imaged after 22 h incubation at 37 °C with 5% CO_2.

CHAPTER 6

Modulation of mucosal barrier function and immunity by valinomycin, an antibiotic ionophore produced by the mammalian commensal *Rothia nasimurium*

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Abstract

Many members of the mammalian microbiota may produce non-ribosomally synthesized secondary metabolites that have the potential to modulate microbe-microbe and microbe-host interactions. Such small molecules may be responsible for direct microbial antagonism and as such could play a role in colonisation resistance by commensal species against pathogenic microbes. Previously, we screened the microbiota from healthy piglets for isolates that displayed direct antagonism against *Streptococcus suis*, an important swine pathogen that causes economic losses worldwide. Here a common *Rothia nasimurium* isolate from the porcine tonsil that was able to inhibit the growth of *S. suis* and other bacteria is described. Genome mining revealed the presence of a putative non-ribosomal peptide synthetase (NRPS) gene cluster that showed identical organization of modules and domains to the *Streptomyces vlm* cluster involved in the biosynthesis of valinomycin, a cyclododecadepsipeptide ionophore antibiotic. *In silico* analysis hinted at recent acquisition of this biosynthetic gene cluster. The antimicrobial compound was purified from *R. nasimurium* cultures and identified using MALDI-ToF MS, LC-MS/MS, and NMR as valinomycin. Antimicrobial, cytotoxic and immunomodulatory effects of valinomycin were investigated. This study shows that commensal bacteria from mammalian microbiota may produce NRPS-derived molecules that impact other microbes as well as the host, and could help further elucidate the exact biological role of valinomycin production by *R. nasimurium*.

Introduction

Mammalian mucosal surfaces in the gastrointestinal tract and in the oropharynx are home to a rich and diverse community of microorganisms known as the microbiota. Microbe-host and microbe-microbe interactions within the microbiota can increase susceptibility for infection, for example by influencing host immunity and physiology or by creating a synergistic habitat for pathogens [1, 2]. Conversely, microbial communities may also provide direct colonisation resistance against pathogenic species through a variety of mechanisms including the production of small molecules, such as antimicrobial peptides or secondary metabolites, thereby conferring a benefit to the host [1, 3, 4]. Non-ribosomal peptides (NRPs) are common products of biosynthetic gene clusters (BGCs) among microbes found in soil and aquatic environments and were the foundation for most of the currently licensed classes of antibiotics [5]. Recently, a remarkable number of BGCs was discovered in the genomes and metagenome sequences of human-associated bacteria and the small molecules they produce were proposed to have an important role as mediators of complex interactions within and between the enteric microbiota and the host [6, 7]. However, relatively few NRPs from human-associated bacteria have been characterized in detail. Further efforts to characterise the products of such BGCs is likely to provide valuable insights into the interactions that shape microbial communities, as well as contribute to a better understanding of the establishment of polymicrobial infections and the pathogenesis of diseases [6, 7].

We were interested to characterise commensal bacteria of the microbiota from healthy piglets as we reasoned that these could potentially suppress excessive blooms of the porcine opportunistic pathogen *Streptococcus suis*, a common coloniser of the porcine oral cavity and small intestine [8, 9]. Here we describe the isolation of a *Rothia nasimurium* strain from porcine tonsil microbiota which is capable of inhibiting the growth of *S. suis* as well as other species of bacteria. The inhibitory activity was protease-, pH- and heat-stable, suggesting that the active compound might be the product of a small molecule BGC. A cyclic dodecadepsipeptide responsible for the antimicrobial activity was purified and shown to be structurally equivalent to the ionophore valinomycin. The non-ribosomal peptide synthetase (NRPS) gene cluster encoding the biosynthesis of this antibiotic was identified in the genome of the *R. nasimurium* isolate; *in silico* analysis revealed signs of recent acquisition of the BGC by horizontal gene transfer, while it surprisingly showed only distant evolutionarily relation with well-characterized valinomycin BGC from *Streptomyces*. We tested the effects of valinomycin on barrier permeability in a model of the porcine epithelium. To gauge the potential effects of valinomycin on inflamed mucosa, we tested the capacity of valinomycin to induce apoptosis in mononuclear cells and promote IL-1β secretion in primed macrophages by triggering the inflammasome. Decreased barrier permeability, mononuclear cell apoptosis and macrophage inflammasome activation all occurred at valinomycin concentrations that were tolerated by the producing *R. nasimurium* strain, while inhibiting other bacteria.

Material and Methods

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. Bacterial isolates from porcine microbiota were grown under aerobic conditions at 37°C on Todd-Hewitt Broth (THB) (Oxoid, Basingstoke, UK) agar plates or in THB supplemented with K_2HPO_4 (36 mM), KH_2PO_4 (13.2 mM), sodium citrate (1.7 mM), $MgSO_4$ (0.4 mM), $(NH_4)_2SO_4$ (6.8 mM) and 4.4% glycerol (THB freezing medium). *Rothia nasimurium* was cultured aerobically at 37°C in Brain-Heart Infusion (Oxoid, Basingstoke, UK). Isolates of *Streptococcus suis* of different serotypes and an un-encapsulated mutant J28 were obtained from the Central Veterinary Institute (Lelystad, The Netherlands). All *Streptococcus* strains were cultured in THB aerobically without agitation at 37°C with 5% CO_2. The following culture media were used for other tested organisms; MRS (VWR International, Leuven, Belgium) for *Lactobacillus reuteri*, BHI for *Staphylococcus aureus, Staphylococcus pasteuri* and *Listeria monocytogenes*, and Luria-Bertani (LB) (Merck, Darmstadt, Germany) for *Salmonella enteriditis* and *Escherichia coli*.

Species	Strain	Serotype	Virulence	Screening
Streptococcus suis	15964	1/2	ND	
	6388	1	HV	
	6555 / NCTC 428	1	V	
	T15	2	AV	
	S735 / NCTC 10234	2	WV	
	p 1/7	2	V	
	3881 / S10	2	V	
	J28 (S10 ΔcpsEF)	2	AV	x
	15965	3	ND	
	5213	4	ND	
	8039	7	ND	x
	7997	9	AV	
	8067	9	V	x
	8017	9	AV	
	7709	9	ND	
	C132	9	ND	
	5973	9	AV	
	22083 R	9	ND	
	7998	9	ND	
	8186	9	ND	
	13730	14	ND	
Streptococcus mutans	DSM 20523			
Streptococcus mitis	Is. MS VID			
Streptococcus salivarius	Is. Ms Oral D6			
Lactobacillus reuteri	ATCC 55730			
Staphylococcus aureus	8325-4 WT			
Staphylococcus pasteuri	T#26			
Enterococcus faecalis	DMS 20478			
Salmonella enteriditis	NIZO1241			
Listeria monocytogenes	EGD-e			
Escherichia coli	Nissle 1917			
Escherichia coli	Pro 083			

Table 1. Bacteria used in this study. *Streptococcus suis* strains J28, 8039 and 8067 were used for the initial antimicrobial screening assay of the libraries of porcine commensal isolates. AV = avirulent, V = virulent, HV = highly virulent, ND = not determined.

Overlay inhibition assay

BHI medium was inoculated from a -80°C glycerol stock of *Rothia nasimurium* isolate #4 or #32 and incubated overnight at 37°C without agitation. Ten microliters of this culture were spotted onto BHI agar plates and these were incubated overnight at 37°C to allow growth of bacterial colonies. Plates were then exposed to UV light for 30 min to kill the bacterial cells. Proteinase K (Qiagen; 1 ul, >600 mAU/ml) was spotted next to each colony, and incubated at 37°C for 30 min. Then each plate was overlaid with 20 ml of soft THB agar (0.75% w/v agar) at 48°C, inoculated with approximately 1 x 10^6 CFU/mL of the indicator *S. suis* strain from an overnight culture in THB. Plates were then allowed to solidify and dry, and inhibition zones measured after overnight incubation at 37°C.

Well diffusion assay

Appropriate nutrient agar (0.75% w/v agar) was cooled to 50°C and seeded with a selected target bacterium (approximately 1 x 10^6 CFU mL^{-1}). A volume of 25 mL was rapidly transferred to sterile Petri dishes and allowed to solidify and dry, after which wells (4.6 mm diameter) were made using a sterile glass Pasteur pipette. A volume of 50 µL or 100 µL of the 70% IPA, 0.1% TFA bacterial extract after evaporation of the IPA (obtained as described below) was dispensed into the wells and the plates were incubated at 37°C overnight. Antimicrobial activity was determined by the presence and size of clearly visible zones of growth inhibition around the wells.

Genome–mining for secondary metabolite synthesis genes

Genomic DNA was extracted from two strains that inhibited growth of *S. suis*, first designated as isolates #4 or #32, and sequenced as described elsewhere (Genome Announcement manuscript in preparation) (WGS project deposited at DDBJ/ENA/GenBank under the accession LXWF00000000). The web-tool antiSMASH 3.0 [10, 11] was used for identification and annotation of secondary metabolite biosynthesis gene clusters, and MultiGeneBlast [12] was used for comparative analysis with known valinomycin and cereulide BGCs. The valinomycin BGC was submitted to the Minimum Information about a Biosynthetic Gene cluster (MIBiG) repository (http://mibig.secondarymetabolites.org/) [13], under accession BGC0001341.

Bacterial identification

The complete 16S rRNA gene sequences were predicted from the draft genome sequences using RNAmmer 1.2 [14]. The 16S rRNA gene sequences of the nearest phylogenetic neighbours were found using the EzTaxon server (http://www.ezbiocloud.net/eztaxon) [15]. With Phylogeny.fr (http://www.phylogeny.fr/)[16, 17], the 16S rRNA gene sequences were aligned (T-coffee), the alignments were trimmed and curated (Gblocks), and a phylogenetic tree was constructed using the Maximum Likelihood method and 500 times bootstrapping in PhyML v3.0.

PCR detection of *vlm* cluster

Total DNA was isolated and purified from forty 1 mL aliquots of tonsil-brushed material that was used for the isolation of porcine microbiota (**Chapter 4**) following the Repeated Bead Beating method as described in [18] using a FastPrep-24 instrument (MP-Biomedicals, France) and the QIAmp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). Based on the draft genome sequence of *Rothia nasimurium*, PCR primers were designed for the detection of the *vlm* gene cluster. Primers fl_F (5'-CTTTCCCGTATGGCTGAGAG-3') and fl_R (5'- CGCGTACCACGTCCATTAAG-3') generated an amplicon that spanned the genetic linkage between the proposed insertion site of the *vlm* cluster and the genome, primers int_F (5'-CTACGTCCCAGTCGGATTATTC-3') and int_R (5'- AGCACAGAGGATAGCCATCTAC-3') generated an amplicon within the *vlm1* gene. PCR reactions were performed using a thermocycler in a total reaction volume of 50 µL containing 1x GoTaq Flexi buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1.25 u GoTaq G2 Hot Start DNA polymerase (Promega, Madison (WI), USA), 1 µM of forward and reverse primer, and 1 µL of undiluted template DNA (0.5 – 1.3 ng/50 µL). The amplification program comprised an initial denaturation step at 95°C for 2 min, and 30 cycles of: denaturation at 95°C for 1 min, annealing at 59.5°C for 30 s, elongation at 72°C for 30 s. The size of the amplicons was confirmed by gel electrophoresis.

Epithelial Barrier Function

NPTr cells, originating from newborn pig trachea [19] were cultured in Dulbecco's Modified Eagle's Medium (DMEM) plus GlutaMAX™ (Gibco, Life Technologies), supplemented with 10% fetal calf serum (FCS) (PAA) and 100 U mL^{-1} penicillin/ 100 µg mL^{-1} streptomycin (Sigma, Zwijndrecht, The Netherlands) in a humidified atmosphere with 5% CO_2 at 37°C. A volume of 500 µL (2 x 10^4 cells) was seeded in each 24-well filter (0.4 µm) inserts (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and cultured for 1 week with medium replacement every 3 days. At t = 0 the media was replaced with DMEM without serum or antibiotics (800 µL basolateral, 200 µL apical), and then stimulus (20 µL valinomycin in MeOH + 180 µL DMEM) was added apically, reaching a final MeOH concentration of 5% in all wells. Trans-epithelial resistance (TER in Ω x cm^2; indicates barrier to passive ion flux) was continuously measured over time using the cellZscope® (Nanoanalytics, Münster, Germany) across the filter-grown monolayers of NPTr cells. TER values were normalized per well to 100% at t = 0. Each condition was measured in triplicate and data represented as mean ± SEM.

Cytotoxicity assay

To assess cytotoxicity of valinomycin against human peripheral blood mononuclear cells (hPBMCs), hPBMCs were isolated and prepared as previously described [20], with modifications. Buffy coats from peripheral blood of 3 healthy donors were obtained from the Sanquin Blood Bank, Nijmegen, The Netherlands. Isolated hPBMCs were washed and resuspended at 1*10^5 cells per well in 96-well tissue culture plates in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Life Technologies) with 5% FCS and penicillin/

streptomycin and exposed to valinomycin concentrations ranging from 0.1 to 1000 nM. Following treatment for 18 h in a humidified atmosphere with 5% CO_2 at 37°C, cells were harvested and then stained using the commercially available Annexin V: Propidium Iodide (PI) Apoptosis Detection Kit APC (eBiosciences, Vienna, Austria) according to the manufacturer's instructions. Using flow cytometry (FACS CantoII, BD Biosciences) the proportions of viable, dead and early- and late-apoptotic cells were determined (BD FACSDiva software). Values from each donor, with each condition measured in duplicate, were normalized to total cells in the monocyte gate, averaged for all donors and are represented as mean ± SD.

IL–1β quantification

Wild type C57BL/6 mice were obtained from Charles River, UK. *Nlrp3*$^{-/-}$ mice on a C57BL/6 background were produced by Millennium Pharmaceuticals. Primary bone-marrow derived macrophages (BMDM) were prepared and cultured for 6-9 days as previously described [21]. BMDMs were plated in 96-wells plates (200.000 cells per well), primed with 200 ng mL^{-1} ultrapure LPS from *Escherichia coli* O111:B4 (InvivoGen) for 3 hours at 37 °C and stimulated with Valinomycin for 4 or 18 hours.

Secreted IL-1β was measured by ELISA in the culture supernatants according to the manufacturer's instructions (OptEIA Mouse IL-1β Set, BD Biosciences).

Immunofluorescence microscopy

WT and *Nlrp3*$^{-/-}$ LPS-primed primary macrophages were stimulated with valinomycin 10 μM or nigericin 5 μM (Sigma) for 18 hours, then fixed in -20°C methanol for 5 minutes, after washing with PBS, non-specific labeling was blocked by incubation at 37°C for one hour in 5% BSA (Sigma) containing 0.1% saponin (Sigma). ASC was labeled with anti-ASC primary (1:500, AL177; Enzo) and Alexa-fluor 568 secondary antibody (1:1000, anti-rabbit Alexa-fluor 568 (Invitrogen). Cells were counterstained using DAPI mounting medium (Vecta Labs) and ASC-speck quantification was performed by counting the ASC specks from around 1000 cells per treatment, from randomly selected fields.

Results

Rothia nasimurium isolated from porcine tonsils inhibits growth of multiple strains of *S. suis*

Previously, we described a high-throughput screening assay to detect antagonist bacteria against *Streptococcus suis* from the porcine microbiota (see Chapter 5). Two bacterial isolates (#4 and #32) originating from porcine tonsil biofilm strongly inhibited growth of *S. suis* strains that represented the serotypes 1/2, 1, 2, 3, 4, 7, 9 and 14 (Figure 1 A). Phylogenetic analysis of the full-length 16S rRNA gene sequence, which was identical for isolates #4 and #32, identified them as members of the genus *Rothia* with the closest related species at 97.9% sequence identity being *Rothia nasimurium* [22] (Table S1 and Figure S1). From here on, we refer to isolates #4 and #32 as *Rothia nasimurium*.

Isopropanol (70% v/v), TFA (0.1% v/v)-extracted and solubilised components from bacterial pellets of isolates #4 and #32 showed dose-dependent antimicrobial activity against *S. suis* S10 (Figure 1 B). Agar well diffusion assays with these extracts showed variability in the susceptibility of tested *S. suis* strains, with absence of the polysaccharide capsule (mutant J28) leading to a more sensitive phenotype (Figure 1 C). Of the other bacteria tested *Streptococcus mutans*, *Streptococcus salivarius*, *Lactobacillus reuteri*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Salmonella enteriditis*, *Listeria monocytogenes* and *Escherichia coli* were not susceptible in agar well diffusion assays, whereas growth of *Streptococcus mitis* was inhibited up to a dilution of 1/32 (data not shown). Treatment of the extract with proteases confirmed that the antimicrobial activity was resistant to proteinase K and trypsin. Furthermore, heating at 100°C for 20 min did not diminish the antimicrobial activity of the extract (data not shown). MALDI-ToF mass spectrometry on the bacterial colonies indicated that the majority of molecules present have a mass to charge ratio (m/z) smaller than 2500 Da (data not shown).

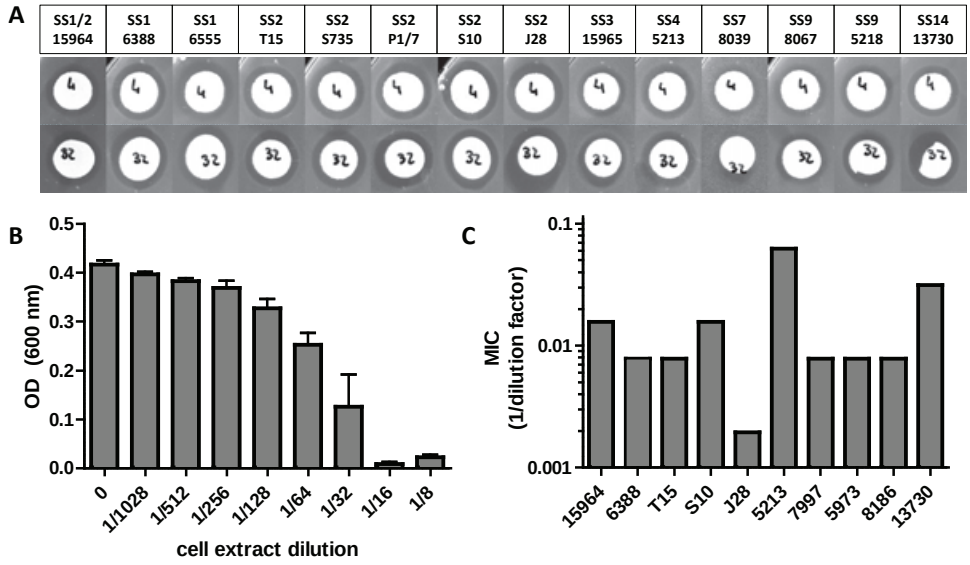

Figure 1. A) Overlay assay showing consistent inhibitory activity around the colonies of *Rothia nasimurium* isolate #4 and #32 against multiple serotypes and strains of *S. suis*. Spotted proteinase K above (#4) or below (#32) the colonies did not abolish growth inhibition of *S. suis* around the colonies of *R. nasimurium*. Growth inhibiting effect of *Rothia nasimurium* cell extracts prepared in 70% IPA 0.1% TFA, determined using **B)** a micro-well dilution assay against *S. suis* S10. $OD_{600\,nm}$ was measured after 18 incubation at 37°C, 5% CO_2, showing a concentration dependent reduction of bacterial growth, represented as mean values of four replicates with SD, and **C)** an agar well diffusion assay against nine *S. suis* strains including the capsule-deficient mutant J28. The Minimal Inhibitory Concentration (MIC) is represented as 1 divided by the highest extract dilution factor that still showed a zone of growth inhibition, and ranges from 1/16 to 1/512.

R. nasimurium isolates possess non–ribosomal peptide synthetase gene clusters with similarity to *vlm* and *ces*

To gain insight in the molecular and genetic basis underlying the ability of the two *R. nasimurium* strains to inhibit growth of *S. suis*, genomic DNA was purified and sequenced (Genome Announcement manuscript in preparation). The web tool antiSMASH 3.0 [10, 11] was used for identification and annotation of secondary metabolite biosynthesis gene clusters in the draft genome sequence of the two *Rothia nasimurium* strains. In both strains, two putative non-ribosomal peptide synthetase (NRPS) gene clusters were detected, of which one was predicted to be involved in the production of a siderophore. Bioinformatic analysis of the second NRPS cluster revealed that its two encoded NRPS proteins comprise four modules in total, with the domains A_1-KR_1-T_1-C_2-A_2-T_2-E_2-C_3 and A_3-KR_3-T_3-C_4-A_4-T_4-TE (Figure 2). The organization of the modules and domains of these two NRPS genes is identical to those found in the *vlm* cluster involved in the biosynthesis of valinomycin, a cyclododecadepsipeptide ionophore antibiotic produced by several soil-dwelling *Streptomyces* species [23-27] and

the cereulide biosynthetic genes (*ces*) found in *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates [28].

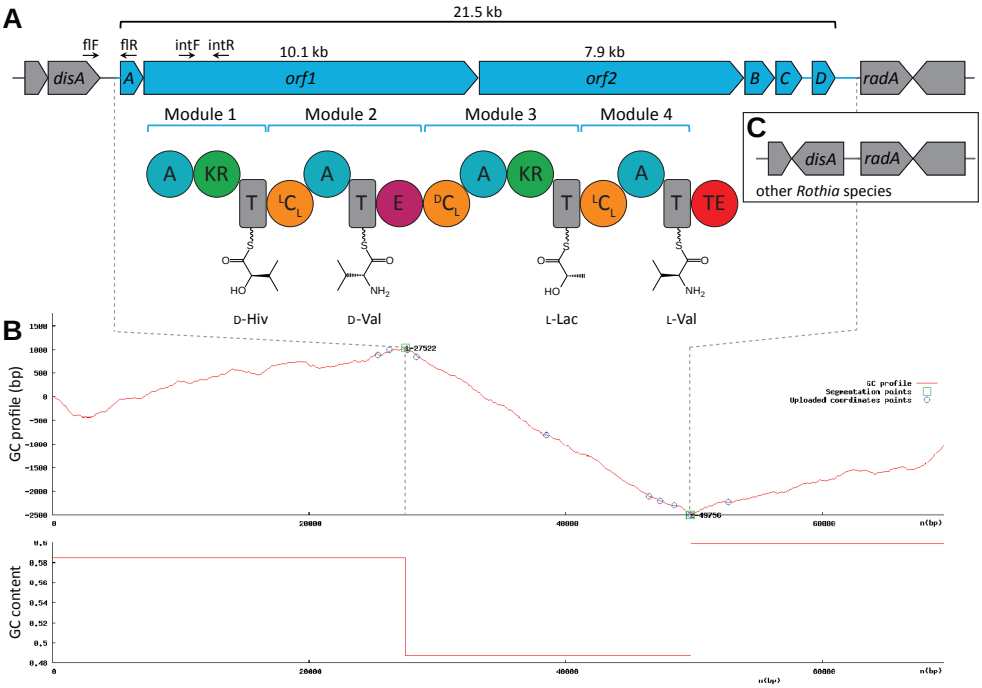

Figure 2. A) Graphical representation of the NRPS cluster identified by *in silico* analysis of the draft genome sequence of *Rothia nasimurium,* including the approximate size of the detected cluster and the predicted NRPS structural genes. The cluster (in blue) consists of *orfA* encoding a discrete type II thioesterase (TEII), two structural synthetase encoding genes *orf1* and *orf2*, ABC transporter encoding genes *orfB* and *orfC* and a 4'-phosphopantetheinyl transferase (PPTase) encoding gene *orfD*. Underneath is depicted the organization of the four modules, their corresponding enzymatic domains and the predicted incorporated monomers attached to the thiolation domains. The wavy line represents the phosphopantetheine arm of the primed T domain. Predictions were made using AntiSmash [10, 11]. Binding sites of primer pairs used for detection of the cluster are indicated (flF and flR spanning the junction between chromosomal DNA and NRPS cluster, intF and intR amplifying within the NRPS cluster). Genes *orfA12BCD* were later renamed to *vlmA12BCD* (see main text) **B)** GC profile (top) and GC content (bottom) of the *Rothia nasimurium* genomic scaffold containing the NRPS cluster, as generated by the tool on http://tubic.tju.edu.cn/GC-Profile [29]. The segmentation points in GC profile and shift in GC content are 142 bp upstream of gene *orfA* (TTE) and 592 bp downstream of gene *orfD*. **C)** Orientation of disA and *radA* in other *Rothia* species.

The substrate specificity of the adenylation (A) domains was predicted *in silico* from the amino acid sequence encoded by *orf1* and *orf2* [30, 31]. This prediction was supplemented by a phylogenetic comparison of the A domains from *R. nasimurium*, known valinomycin producers *Streptomyces tsusimaensis* and *Streptomyces fulvissimus*, as well as the cereulide

producing bacterium *Bacillus cereus* [26, 32, 33]. The *R. nasimurium* A domains were most similar to the *Streptomyces* A domains from the *vlm* NRPS (see Figures S2 and S3) and, based on the conserved features of the biosynthesis of valinomycin and cereulide [26, 32, 33], are predicted to recognize and activate the substrates α-ketoisovalerate (Kiv), L-valine (L-Val), pyruvate, and L-Val.

Modules 1 and 3 encoded by the biosynthetic cluster contain A domains that preferentially activate and load α-keto acids onto the T domain [26]. Using NADPH as a redox cofactor, the ketoreductase (KR) domains in these modules then catalyse the stereospecific reduction of the T-domain-tethered α-keto acyl group to an α-hydroxyacyl group. The KR domain in module 1 would therefore reduce α-Kiv to D-α-hydroxyisovalerate (D-Hiv), and the KR domain in module 3 would reduce pyruvate to L-lactate (L-Lac), as proposed by Li *et al*. [34].

The condensation (C) domains catalyse the condensation reaction between the peptidyl chain tethered to the phosphopantetheinyl arm of the upstream T domain to the amino acid bound to the downstream T domain, thereby elongating the peptidyl chain [35]. The C domains in module 2 and 4 (Figure 2, C_2 and C_4) were classified by the bioinformatic tool NaPDoS [36] as the $^{L}C_{L}$ subtype, with L-specificity for the peptidyl donor and L-specific for the aminoacyl acceptor, catalyzing the peptide bond formation between two L-amino acids. The C domain in module 3 is of the $^{D}C_{L}$ subtype, showing D-specificity for the peptidyl donor and L-specificity for the aminoacyl acceptor [37]. Domain C_3 lies immediately downstream of the E_2 domain, and thus catalyses chain elongation onto D-Val which results from the epimerization of L-Val by E_2 in module 2 [38].

Assuming that this synthetase follows the general linearity principles of known *vlm* NRPS clusters, it would form three tetradepsipeptides with the subunits D-Hiv, D-Val, L-Lac, L-Val, as monomers or as dipeptides, which then undergo cyclization by macrolactonization by the terminal thioesterase (TE) domain [25, 26, 32, 39].

Gene *orfA* lies upstream of *orf1* and *orf2* and encodes a discrete type II thioesterase (TEII) that displays 49% identity to *S. tsusimaensis* TE protein sequence [25], and may have a similar function in optimizing NRP assembly by hydrolysing misprimed substrates from T domains [40, 41]. We predicted that *orfD* encodes a PPTase that is involved in catalysing the transfer of the 4'-phosphopantetheine group of coenzyme-A to the T domain by ester bond formation, thus priming the T domains for subsequent tethering of activated amino acids during peptide assembly [42, 43].

The proteins encoded by downstream genes *orfB* and *orfC* are predicted to function as an ATP-binding protein and ABC transporter permease, respectively. Amino acid sequences of OrfB and OrfC share highest identity with proteins CesC and CesD, respectively, from *Bacillus* as best BLAST hits against a manually curated bacterial ABC transporter database [44] and against all protein sequences in the MIBiG database [13]. CesC and CesD are annotated as putative ABC transporters associated with the plasmid-borne cereulide synthetase cluster from *Bacillus cereus*, and have been found to be essential for export and biosynthesis of cereulide [45, 46]. Interestingly, the encoded protein OrfB shows 44.4% identity and 65.5%

similarity to CesC protein sequence (YP_009080546.1) and OrfC shows 29.7% identity and 65.8% similarity to CesD protein sequence (YP_009080547.1); OrfC also bears the same predicted six transmembrane helical regions typical for an ABC transporter permease (Figure S4) [47, 48].

No genetic elements involved with regulation of expression of the NRPS cluster could be detected in the immediate vicinity of the operon.

The NRPS cluster in *R. nasimurium* has a GC content of 48.7%, which is markedly lower than the overall GC content of the genome of this isolate (57.98%), as can be seen from the GC profile (Figure 4B)[29]. The NRPS cluster appears to have inserted between chromosomal genes *disA* and *radA*, and compared to the genomes of *R. mucilaginosa*, *R. dentocariosa* and *R. aeria,* the orientation of *disA* has inverted (Figure 4C).

Purification and characterization the antimicrobial compound from *R. nasimurium* identifies it as valinomycin

Antimicrobial activity was detected in *R. nasimurium* bacterial cells extracts made in 70% IPA, 0.1% TFA and to a greater extent in extracts with 100% MeOH (data not shown). We purified the antimicrobial compounds from MeOH extracts using the solvents Et_2O and MeCN followed by reverse-phase HPLC, and characterized the purified molecules by MALDI-ToF-MS and ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) (Supplemental Information, Figures S5-S8). We found three compounds that were also detected in commercial valinomycin with an *m/z* [$M+H^+$] of 1111, 1125 and 1139 (Figure S6 and S7). Based on the identity in HPLC retention times, and detected masses and principally identical ^{1}H- and ^{13}C-NMR spectra between purified commercial valinomycin fractions and our purified compounds (Figure S8), we concluded that this *R. nasimurium* isolate produces the dodecadepsipeptide valinomycin.

Rothia nasimurium and its associated *vlm* cluster is commonly present in the sampled tonsils

Considering the similarities of the NRPS gene cluster with *vlm* from *Streptomyces* and the detection of valinomycin produced by *R. nasimurium*, we decided to rename the responsible gene cluster *vlm.* PCR was used to confirm that the *vlm* cluster was present in the total DNA purified from porcine tonsil swab samples from which valinomycin-producing strains *R. nasimurium* #4 and #32 were originally isolated (See Chapter 4). Although *Rothia* species are frequently identified in porcine tonsillar microbiota [49, 50], we wondered if the valinomycin-producing *R. nasimurium* could also be found in other pigs. To investigate this, we designed one PCR primer set (FLANK) to amplify the region spanning the intersection (i.e. GC content segmentation point) upstream of the *vlm* cluster with the *Rothia* genomic DNA, and a second set of primers (INT) to amplicon to generate an amplicon within the *vlm* cluster (Figure 2). The *Rothia nasimurium vlm* amplicons were detected in the DNA of tonsil swab samples from 17 of 21 sampled pigs (Table 2). In some samples, only the INT amplicon was detected. The microbiota composition of these tonsil samples was also determined using

454 pyrosequencing, and a high percentage of 16S reads with >99% and >99.4% identity to the 16S sequence of *R. nasimurium* were identified in the *vlm*-positive samples (Table 2).

	piglet	6714		7094		7009		6884		7084		6961		6877		6662		6511		6804	
	sample #	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62
pyro seq.	% reads >99% id.	0.18	0.19	0.06	0.12	0.19	0.09	0.13	0.07	0.08	0.10	0.02	0.18	0.26	0.05	0.06	0.04	0.32	0.02	0.56	0.16
	% reads >99.4% id.	0.03	0.00	0.00	0.02	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.02	0.09	0.02	0.02	0.02	0.02	0.00	0.03	0.02
PCR	vlm FLANK	++	+	+/-	+/-	+	++	+	+	-	+/-	-	-	++	+/-	+	+/-	+	-	+	-
	vlm INT	++	+	+	+/-	+	+	+	+	+	+	+	+	++	+	+	+	+	+/-	+	+

	piglet	7173		7086		6498		6962		6718		7152		6825		6397	6952	6721		6383	
	sample #	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82
pyro seq.	% reads >99% id.	0.56	0.10	0.06	0.03	0.14	0.18	0.29	0.18	0.18	0.07	0.28	0.12	0.18	0.09	0.10	0.00	0.07	0.10	0.08	0.53
	% reads >99.4% id.	0.05	0.00	0.00	0.00	0.00	0.00	0.04	0.03	0.02	0.00	0.02	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
PCR	vlm FLANK	++	+/-	+/-	-	+/-	+/-	+	+	+	+/-	+	-	-	+	-	-	++	+/-	+	+
	vlm INT	++	+	+	+/-	+	+	+	+	+	+	+	+	+/-	+	+/-	+/-	++	+	+	+

Table 2. Presence of *vlm* cluster in the *R. nasimurium* genome detected in sampled porcine tonsils determined by PCR, semi-quantified from high (++, dark red), medium (+, orange) and low (+/-, light yellow) to absent (-, light blue) by intensity of the band corresponding to the expected amplicon on an agarose gel. The percentage of 454 pyrosequencing reads with >99% and >99.4% identity to *R. nasimurium* 16S sequence are indicated for each sample, coloured from largest (dark green) to smallest value (white).

The valinomycin-producing strains of *Rothia* are less susceptible to its antibacterial activity than non-producing *Rothia* species and *S. suis*

Antibacterial activity of valinomycin, especially against Gram-positive bacteria, has been described quite extensively, although the exact biological function of this ionophore has not been elucidated [51] [52]. We hypothesized that the production of valinomycin could provide *Rothia nasimurium* a competitive advantage by inhibiting the growth of competing microbes, for instance other species within the *Rothia* genus. First, we performed a microbroth dilution assay with *R. nasimurium* isolate #4 and #32, and reference strains *R. nasimurium* CCUG35957, *R. dentocariosa* DSM43762 and *R. mucilaginosa* DSM20746 to assess the valinomycin concentrations that are tolerated by the producer strains relative to those tolerated by other *Rothia* strains. These reference *Rothia* strains do not inhibit each other's growth, nor do they inhibit the growth of *S. suis* S10 (data not shown), and mining of the available genomes of *R. dentocariosa* and *R. mucilaginosa* did not reveal the presence of a *vlm* NRPS cluster, nor were we able to detect the cluster by PCR in DNA from strain CCUG35957 (data not shown). We observed a higher than 100-fold higher minimal inhibitory concentration (MIC) for valinomycin in *R. nasimurium* strains #4 and #32 (>100 µM) as compared to the reference strain CCUG35957 (1 µM) (Figure 3A). Furthermore, *R. dentocariosa* appeared to be quite resistant to valinomycin, with a MIC of >100 µM, whereas *R. mucilaginosa* was found to be more susceptible (MIC 1 µM) (data not shown). Interestingly, the optical density of the culture of *R. nasimurium* #4 and #32 after 24 h incubation is reduced significantly to about 40% at concentrations of 1 µM and above. Concentrations of valinomycin of 1 µM and above also completely inhibited growth of *S. suis* (Figure 3B), while growth of *S. suis* S10 was significantly reduced at 100 nM valinomycin. Additionally, bacterial colony morphology of *R. nasimurium*

#4 and #32 was altered in the presence of valinomycin concentrations that inhibit the growth of the reference strain CCUG35957 (Figure 3C).

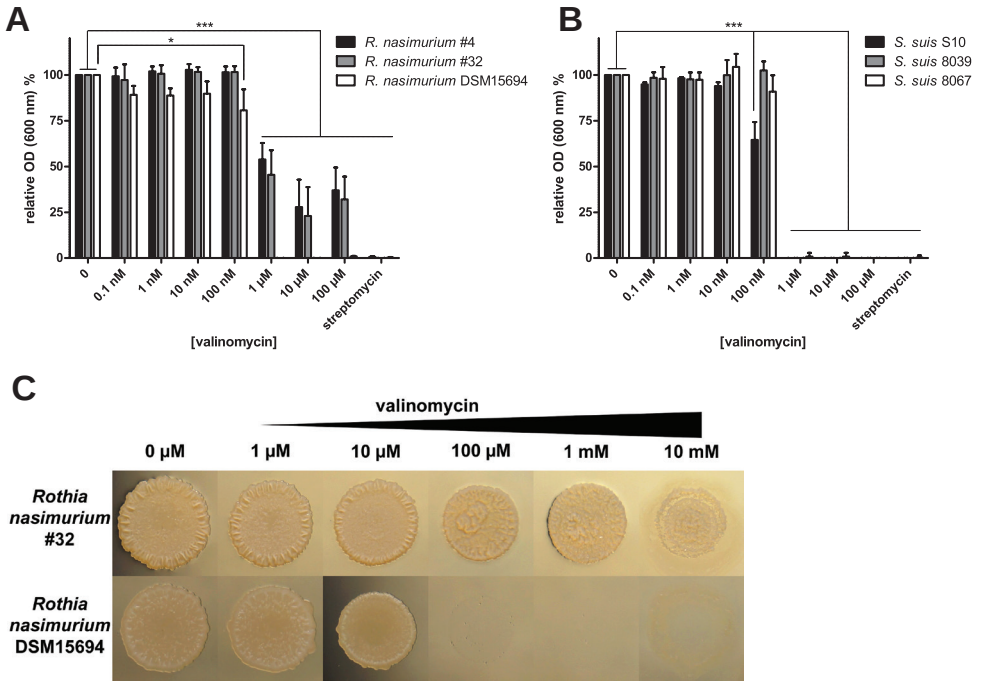

Figure 3. A) *R. nasimurium* and **B)** *S. suis* microbroth dilution assay with valinomycin. Optical density at 600 nm was measured after 24 h incubation at 37 °C in a humidified atmosphere with 5% CO_2, data was normalized to the control and is represented as average values from three independent experiments with technical duplicates ± SD, *** indicates P < 0.0001 and * indicates P < 0.05 compared to control treatment, as determined by one-way ANOVA and Tukey's Multiple Comparison Test). 500 µg mL^{-1} streptomycin was used as a positive control. **C)** Effect of valinomycin on growth and plaque morphology of valinomycin producer *Rothia nasimurium* isolate #32 and reference strain *R. nasimurium* DSM15694. Ten microliter of valinomycin solution (concentration ranging from 10 mM to 1 µM) in MeOH were spotted on a BHI agar plate, and after solvent evaporation these spots were overlaid with 7.5 uL droplets of overnight bacterial culture. Plates were incubated for 48 h at 37°C in a humidified atmosphere with 5% CO_2.

Valinomycin increases permeability of epithelial monolayers and induces dose–dependent apoptosis in PBMCs

It is known that in epithelial cells Na^{2+}/K^{+}-ATPase enzyme activity and intracellular K^{+} homeostasis are required for tight junction formation [53]. We hypothesized that the K^{+} ionophore valinomycin produced by mucosal-associated *Rothia nasimurium* might affect the barrier function of the epithelium through altered tight junction formation or cell apoptosis. When 10 µM valinomycin was added to a confluent monolayer of porcine tracheal epithelial cells, the trans-epithelial resistance (TER) gradually decreased over 12 to 24 h, indicating a

loss of barrier integrity (Figure 4A). Incubation of NPTr cells with 10 µM valinomycin for 18 hours led to modest but significant decrease of 15% of neutral red uptake indicating that the viability or transport capacity of epithelial cells was reduced (Figure S8).

As *Rothia* species inhabit the same niche as oral and respiratory pathogens which can trigger inflammation and recruitment of monocytes and neutrophils to the sites of infection, we investigated whether valinomycin was cytotoxic for peripheral blood mononuclear cells. Perturbation of K^+ homeostasis has been shown to be associated with apoptosis [54], with valinomycin being an important effector of this pathway of induced cell-death [55, 56]. After 18 h exposure of freshly isolated human PBMC to valinomycin we observed a significant dose-dependent effect on cell viability and apoptotic cells at valinomycin concentrations of 1 nM and higher (Figure 4B), indicating that PBMCs are substantially more sensitive to the cytotoxic effects of valinomycin than epithelial cells.

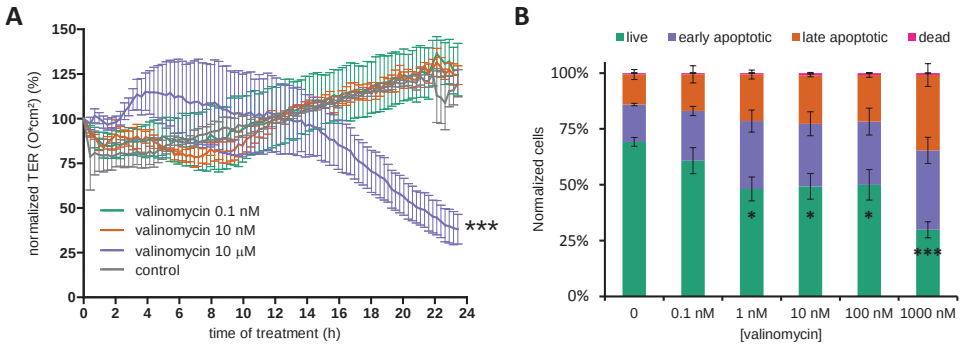

Figure 4. A) Trans Epithelial Resistance (TER), a key parameter of epithelial tightness, of NPTr cells treated with different concentrations of valinomycin, represented as mean values of technical triplicates ± SEM, normalized per replicate to t=0 (*** indicates $P < 0.0001$ compared to control treatment, as determined by one-way ANOVA and Dunnett's Multiple Comparison Test). **B)** Cytotoxicity of 18h exposure to valinomycin on human PBMC's, using fluorescent staining with Annexin-V and propidium iodide and measurement by flow-cytometry (n = 3, mean ± SD, * indicates $P < 0.05$ *** indicates $P < 0.0001$ of viable cells compared to treatment control, as determined by one-way ANOVA and Tukey's Multiple Comparison Test).

Valinomycin induces NLRP3–independent secretion of IL–1β in LPS–primed *ex vivo* bone marrow–derived macrophages

Potassium efflux has been suggested as a common signal leading to NLRP3 inflammasome activated maturation of Il-1b. To test whether valinomycin would promote Il-1b release and inflammasome assembly, we tested its effects in LPS-primed WT and *Nlrp3*$^{-/-}$ bone-marrow derived macrophages (BMDM). Valinomycin stimulation triggered NLRP3-dependent IL-1β secretion at 4 and 18 hours (Figure 5A-B). Accordingly, ASC specks, indicative of inflammasome assembly, were observed in Valinomycin-stimulated WT BMDMs, whereas *Nlrp3*$^{-/-}$ BMDMs rarely displayed ASC specking (Figure 5C-D). This finding indicates that

valinomycin is capable of stimulating the production of the pro-inflammatory cytokine IL-1β in a NLRP3-dependent manner, possibly due to its effects on potassium efflux.

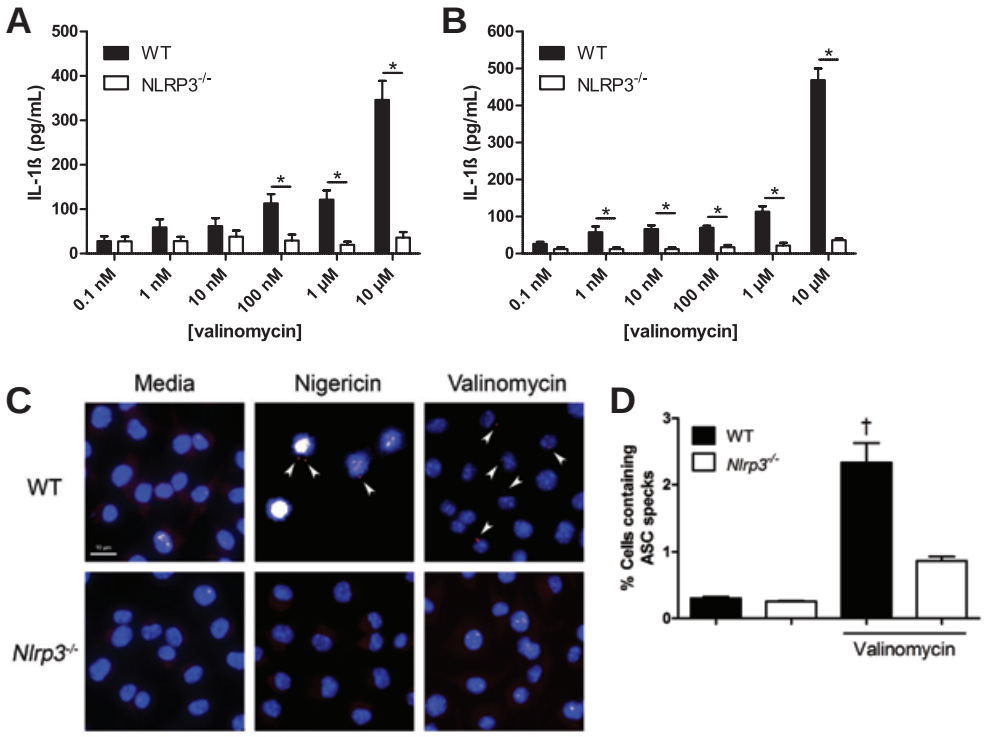

Figure 5. A,B) IL-1β secretion by WT and *Nlrp3*$^{-/-}$ BMDMs treated with different concentrations of valinomycin. **A)** 4 hours incubation and **B)** 18 hours incubation. **C)** ASC-speck formation after BMDM stimulation with 10 µM valinomycin for 18 hours. **D)** Quantification of speck formation. (For **A**, **C** and **D** n = 3, for **B** n = 2, mean ± SEM, * indicates $P < 0.05$ as determined by student's t-test, † indicates $P < 0.05$ as determined by one-way ANOVA and Tukey's Multiple Comparison Test).

Discussion

Reducing usage of antibiotics in animal husbandry is highly relevant. One potential strategy to suppress outgrowth of porcine pathogens such as *Streptococcus suis* is by introducing or stimulating outgrowth of porcine commensals that efficiently antagonise *S. suis* but not other commensals. Here we show that two *Rothia* isolates of the porcine tonsil microbiota, with *R. nasimurium* as the closest relative, inhibit *in vitro* growth of *S. suis*. We purified the *Rothia*-derived compound that inhibited growth of *S. suis* and showed it to be the antibiotic valinomycin. Sequence analysis of the draft genome of this *R. nasimurium* isolate showed presence of two NRPSs. Products of functional NRPS in *Rothia* have not been described before, although biosynthetic gene clusters tentatively originating from this genus were found in human metagenomic data [6]. To our knowledge, this is the first time a commensal bacterium from mammalian microbiota has been shown to produce the ionophore antibiotic valinomycin. Previously, the presence of *vlm* NRPS gene clusters and the production of valinomycin has only been attributed to members of the *Streptomyces* genus [23-27] and isolates belonging to the *Bacillus pumilus* group [28].

The small size of the growth inhibition zones around the colonies of *Rothia nasimurium* seen in the overlay assay or around the wells in the agar diffusion assay are likely to be caused by slow diffusion of valinomycin into the surrounding agar due to its highly hydrophobic nature [57]. Therefore, the different growth rates and media compositions of the panel of target bacteria that were assayed using the agar diffusion method make it difficult to compare inhibitory activities. We tested a panel of twelve bacterial species in agar well diffusion assays, in which only the growth of Gram-positive species *S. suis* and *S. mitis* was clearly inhibited by *R. nasimurium* cell extracts (data not shown). We showed different susceptibility to valinomycin of species within the *Rothia* genus. It was previously reported that valinomycin has a wider spectrum of antibacterial activity against Gram-positive bacteria than Gram-negative bacteria [51, 52]. Therefore, our findings do not completely support the assumed broad-spectrum activity of valinomycin.

Despite the fact that the *R. nasimurium* and *Streptomyces fulvissimus* NRPS's are not very similar in protein sequence, the purified compounds produced by *R. nasimurium* are characterised by identical HPLC retention time, matching MALDI-ToF-MS spectra, and highly similar NMR spectra with commercial valinomycin from *S. fulvissimus*. In addition to valinomycin (*m/z* $[M+H^+]$=1111), we detected a smaller amount of structural variants of valinomycin with one (+14 Da) or two (+28 Da) additional CH_3 groups in purified fractions of the *R. nasimurium* isolates described here and the commercial valinomycin derived from *S. fulvissimus* (Figure S6 & S7). Recently, the same variants of valinomycin were also identified by automated search algorithms in the extract of a *S. fulvissimus* strain [58]. It is not uncommon to find very similar structural variants of cyclododecadepsipeptides that differ in one of the three four-residue comprising units, as is described for bacillistatin, where one of the 3S-methylvaleric acid moieties being replaced by a 4-methylvaleric acid [59, 60]. Similarly, such chemo-diversity of cereulide from *B. cereus* has recently been described and was proposed to be attributed

to relaxed proof-reading of the NRPS complex [61]. To further elucidate the composition and identity of molecules produced by the *R. nasimurium vlm* cluster, we collaborated with Pieter Dorrestein at University of California San Diego to employ LC-MS/MS and mass spectrometry imaging (MSI) (see Supplemental Information for methods). Although this work is still in progress, initial results confirmed the production of valinomycin by *R. nasimurium* isolate #4 and #32 when cultured on BHI agar plates (Figure 6 AB). Additionally, we observed masses corresponding to montanastatin, a cyclooctadepsipeptide with the structure, cyclo-(D-Hiv-D-Val-L-Lac-L-Val)$_2$ [62] (Figure 6 A). Montanastatin and valinomycin were both found to be produced by a soil-dwelling *Streptomyces anulatus*, while the former did not show any antimicrobial activity [62].

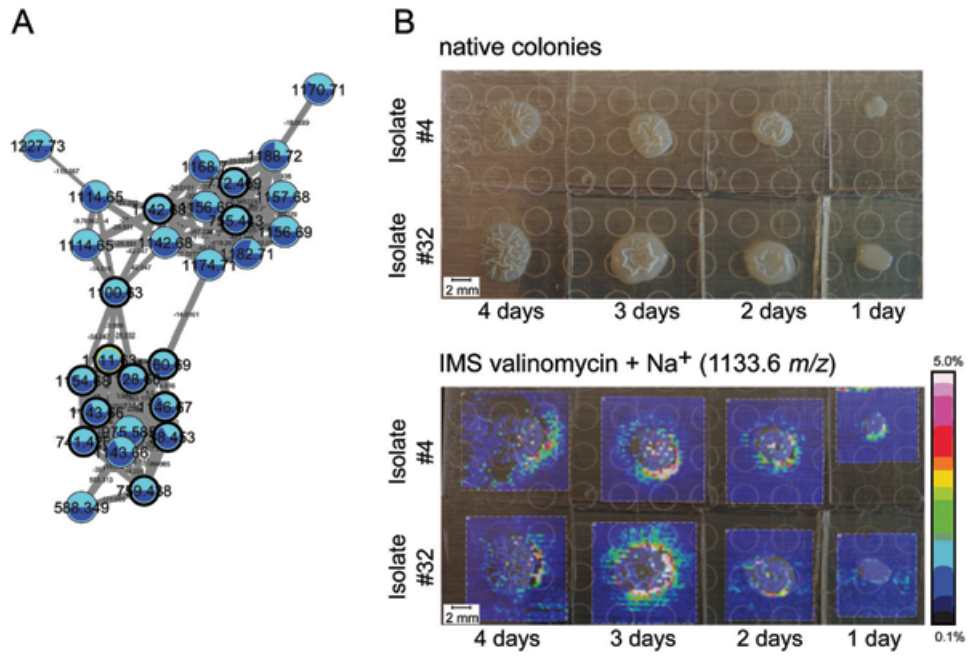

Figure 6. A) Network of masses predicted to belong to structurally related molecules detected by LC-MS/MS in *R. nasimurium* cultures, including valinomycin (yellow circle) with *m/z* [M+H$^+$]=1111.63 **B)** Temporal and spatial visualization of the production of valinomycin by *R. nasimurium* #4 and #32 colonies cultured for 1 to 4 days on BHI agar plates (top), as measured by IMS (bottom). (see Supplemental Information for detailed methods)

The *vlm* cluster in *R. nasimurium* has a much lower GC content than the average for the genome of this isolate (Figure 4B). The median GC content of valinomycin-producing *Streptomyces griseus* (17 assemblies) is 72.6% and the GC content from combined *vlm* sequences of multiple Streptomyces is 70% [27], while *Bacillus cereus* (208 assemblies) has a median GC content of 35.2%, with the plasmid encoded *ces* gene cluster (ref|NG_036207.1) having a median GC content of 35.6%. This may imply that *vlm* cluster in *R. nasimurium,*

having an average GC content of 48.7%, may have been acquired from a Firmicute origin. In several *Bacillus* species, the *vlm*-like *ces* operon is located on large plasmids [45] which may contribute to the horizontal gene transfer of the *ces* operon to other species. Analogously, the *vlm* clusters in *Streptomyces tsusimaensis* (ATCC 15141) and in *S. fulvissimus* (DSM 40593) are flanked by putative transposase encoding genes, which could also facilitate horizontal gene transfer [25, 63]. The *R. nasimurium* appears to lack such flanking transposase gene, but with the limited availability of *ces*- and *vlm*-like clusters from different species the assignment of the origin of the vlm cluster in *R. nasimurium* remains speculative. It has been proposed that the *vlm* gene cluster and the *ces* gene cluster may share a relatively distant common ancestor, and that these two gene clusters have since evolved independently [27]. However, the low GC content of the *R. nasimurium vlm* cluster in combination with the genetically linked *ces*-like transporter genes could hint at yet a different evolutionary path, which may involve combinatorial recombination events to generate novel mosaic-like recombinant clusters. Compared to other NRPS gene clusters, which tend to evolve very rapidly towards the production of different chemistry [64], the product of the *Rothia vlm* cluster has remained evolutionarily stable over long periods of time, which is remarkable and indicates that valinomycin could be seen as a 'living fossil' among natural products.

We detected the *Rothia* genome-encoded *vlm* NRPS cluster in 19 out of 21 sampled pigs, suggesting valinomycin-producing *Rothia nasimurium* was a common member of the tonsil microbiota, at least on the farm where these pigs were sampled. Because the ionophore activity of valinomycin is reported to be antimicrobial and cytotoxic, we further explored its possible effects at mucosal sites where *Rothia* is colonising the host in mixed biofilm communities. Although valinomycin is highly hydrophobic, limiting its diffusion *in vivo*, it is quite resistant to proteolysis and might aggregate in bacterial biofilms, potentially leading to substantial local concentrations. Experiments employing LC-MS/MS to detect and quantify valinomycin or variants thereof in duplicate porcine tonsil swab samples (See Table 2) have been planned, but at the time of printing these results were not yet available. Presence of valinomycin in these samples could then possibly be correlated with the abundance of 16S rRNA gene reads belonging to *Rothia nasimurium* and/or the PCR-based detection of the *vlm* cluster in the same sampled animals.

Cytotoxicity of valinomycin to eukaryotic cells is considered to be due to the efflux of K^+ from the cell leading to a drop in the transmembrane inner membrane potential, thereby uncoupling electron transfer from oxidative phosphorylation in mitochondria [65-67]. Interestingly, the chemical stressor dinitrophenol (which uncouples oxidative phosphorylation) has been shown to increased gut permeability and immune cell recruitment [68], suggesting that valinomycin might have similar effects at mucosal sites, and potentially may have a role in releasing nutrients that could aid *R. nasimurium* colonization. Indeed, we observed an increased permeability of porcine epithelial monolayers at valinomycin concentration of 10 μM, which may be attainable *in vivo* given its insensitivity to proteases and potential to accumulate in biofilms. At this concentration neutral red uptake by epithelial cells was only

reduced by 15% over 18 h, suggesting they are much more resistant to apoptosis than other cell types reported in the literature. We did find that PBMC's were substantially more sensitive to valinomycin than epithelial cells, suggesting valinomycin producing *Rothia* species could play a role in polymicrobial infections by inducing apoptosis in immune cells recruited to inflamed mucosa. The reason for the differential cytotoxicity to epithelial and immune cells is not known but may be due to epithelial cells generating high potassium gradients *via* Na^+/K^+ ATPases in the basolateral membrane, which export 3 sodium ions from the cell in exchange for 2 potassium ions.

Efflux of K^+ from immune cells such as macrophages is responsible for triggering the assembly of the NLRP3 inflammasome in the cytoplasm, a macromolecular complex responsible for the maturation of IL-1β, a potent pro-inflammatory cytokine [69]. Indeed, our observations indicate that increasing valinomycin concentrations stimulates the assembly of the inflammasome and production of IL-1β in a process mediated by the NOD-like receptor NLRP3.

The mammalian microbiota has great potential to produce NRPS-derived small molecules with a variety of biological activities that could possibly modulate microbe-host and microbe-microbe interactions [7]. However, relatively few microbiota-derived NRPS products have currently been described, and the focus is mainly on harmful effects on the host. For example, colibactin is a DNA-damaging molecule produced by certain enterobacteria including *E. coli* phylotype B2, which is increased in abundance in inflammatory bowel disease patients [70, 71]. Tilivalline, a NRP toxin causing apoptosis in human cells, is produced by pathobiont strains of *Klebsiella oxytoca* associated with antibiotic-induced colitis. It is notable that colibactin and tilivalline are produced in dysbiotic intestinal environments and tilivalline is implicated in the pathogenesis of disease [72].

To our knowledge this is the first time a commensal bacterium from mammalian microbiota has been shown to produce the ionophore antibiotic valinomycin. We showed that a *Rothia nasimurium* strain from the porcine tonsil biofilm can produce valinomycin, which could limit the growth other bacteria, such as the pathogen *S. suis* and other *Rothia* species. Previously, a competitive advantage of K^+ ionophore producers over (mainly Gram-positive) bacteria in alkaline (pH >8) and potassium rich environments (such as insect gut, decaying plant material or manure) has been proposed as one ecological function of this ionophore [52]. Additionally, we demonstrated that valinomycin may cause increased barrier permeability and trigger inflammation and IL-1β release. We hypothesize that valinomycin production could be a niche factor [73] that aids *R. nasimurium* survival in the environment and colonization of epithelial surfaces by interacting with other microbes as well as with the host. This competitive colonization advantage might explain the maintenance of the large *vlm* cluster while this causes inevitable toxicity of higher concentrations of valinomycin to *R. nasimurium* itself. However, assessing the effects on colonisation and competitiveness with other microbes within the tonsil biofilm, the implications on host physiology and immunity,

and *in vivo* could help elucidate the exact biological role of valinomycin production by *R. nasimurium*.

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Supplemental figures

Rank	Name	Strain	Accession	Pairwise Similarity(%)	Diff/Total nt	Completeness(%)
1	***Rothia nasimurium***	CCUG 35957(T)	AJ131121	97.89	31/1467	100
2	***Rothia endophytica***	YIM 67072(T)	KC806052	97.78	33/1488	100
3	***Rothia marina***	JSM 078151(T)	FJ425908	97.64	34/1441	99.04
4	***Rothia amarae***	JCM 11375(T)	AY043359	97.09	42/1442	98.26
5	***Rothia terrae***	L-143(T)	DQ822568	97.00	43/1432	98.29
6	***Kocuria polaris***	CMS 76or(T)	JSUH01000031	96.56	51/1484	100
7	***Kocuria rosea***	DSM 20447(T)	X87756	96.48	52/1476	100
8	***Rothia mucilaginosa***	ATCC 25296(T)	ACVO01000020	96.43	53/1484	100
9	***Kocuria halotolerans***	YIM 90716(T)	DQ979377	96.30	55/1485	100
10	***Kocuria koreensis***	P31(T)	FJ607312	96.29	55/1481	100
11	*Kocuria atrinae*	P30(T)	FJ607311	96.25	55/1468	100
12	***Rothia dentocariosa***	ATCC 17931(T)	CP002280	96.09	58/1485	100
13	*Kocuria sediminis*	FCS-11(T)	JF896464	95.94	58/1428	97.54
14	*Kocuria palustris*	DSM 11925(T)	Y16263	95.86	60/1451	99.32
15	*Micrococcus terreus*	V3M1(T)	FJ423763	95.81	62/1479	100
16	***Kocuria kristinae***	DSM 20032(T)	X80749	95.79	62/1471	100
17	*Arthrobacter crystallopoietes*	DSM 20117(T)	X80738	95.78	62/1470	100
18	*Kocuria turfanensis*	HO-9042(T)	DQ531634	95.76	61/1440	98.63
19	*Arthrobacter pascens*	DSM 20545(T)	X80740	95.72	63/1473	100
20	*Kocuria flava*	HO-9041(T)	EF602041	95.69	62/1440	98.63
21	*Kocuria himachalensis*	K07-05(T)	AY987383	95.67	63/1456	100
22	*Arthrobacter woluwensis*	DSM 10495(T)	X93353	95.56	65/1463	100
23	*Micrococcus aloeverae*	AE-6(T)	KF524364	95.46	64/1410	97.04
24	***Rothia aeria***	GTC 867(T)	AB071952	95.44	61/1338	92.55
25	*Arthrobacter phenanthrenivorans*	Sphe3(T)	CP002379	95.41	68/1480	100
26	*Arthrobacter cumminsii*	DMMZ 445(T)	X93354	95.34	68/1460	100
27	*Kocuria salsicia*	104(T)	GQ352404	95.32	67/1432	98.09
28	*Arthrobacter methylotrophus*	TGA(T)	AF235090	95.31	68/1451	99.32
29	*Nesterenkonia flava*	CAAS 251(T)	EF680886	95.31	66/1408	97.39
30	*Arthrobacter globiformis*	NBRC 12137(T)	BAEG01000072	95.21	71/1482	100
31	*Arthrobacter mysorens*	LMG 16219(T)	AJ639831	95.21	70/1461	100
32	*Micrococcus lactis*	DW152(T)	FN673681	95.21	68/1419	97.06
33	***Micrococcus luteus***	NCTC 2665(T)	CP001628	95.21	71/1481	100
34	*Arthrobacter siccitolerans*	4J27(T)	CAQI01000001	95.14	72/1483	100
35	*Nesterenkonia lutea*	YIM 70081(T)	AY588278	95.11	72/1473	100

Table S1. Based on the complete 16S rRNA gene sequence of the *Rothia nasimurium* isolate, the top 35 phylogenetic neighbours were identified using the EzTaxon server. (http://www.ezbiocloud.net/eztaxon) [15]. The species marked in bold font were included for the building of a phylogenetic tree (Figure S1).

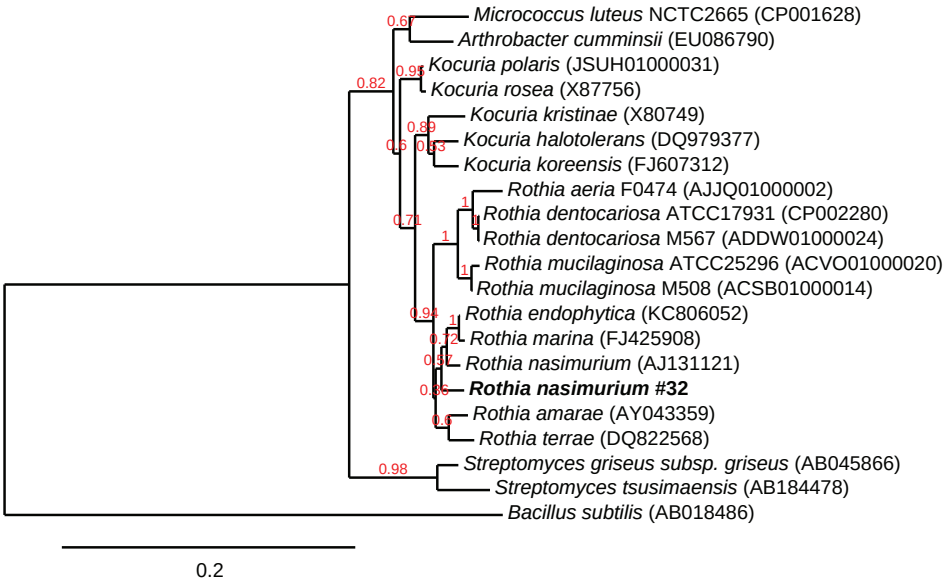

Figure S1. Bootstrapped phylogenetic tree based on curated 16S rRNA gene sequence comparison (see methods section), showing that isolate #32 is closely related to *R. nasimurium*. However, members of this group include *R. endohytica*, *R. marina*, *R. amarae*, and *R. terrae*, which cannot reliably be distinguished by phylogenetic approaches using only the 16S rRNA gene sequence.

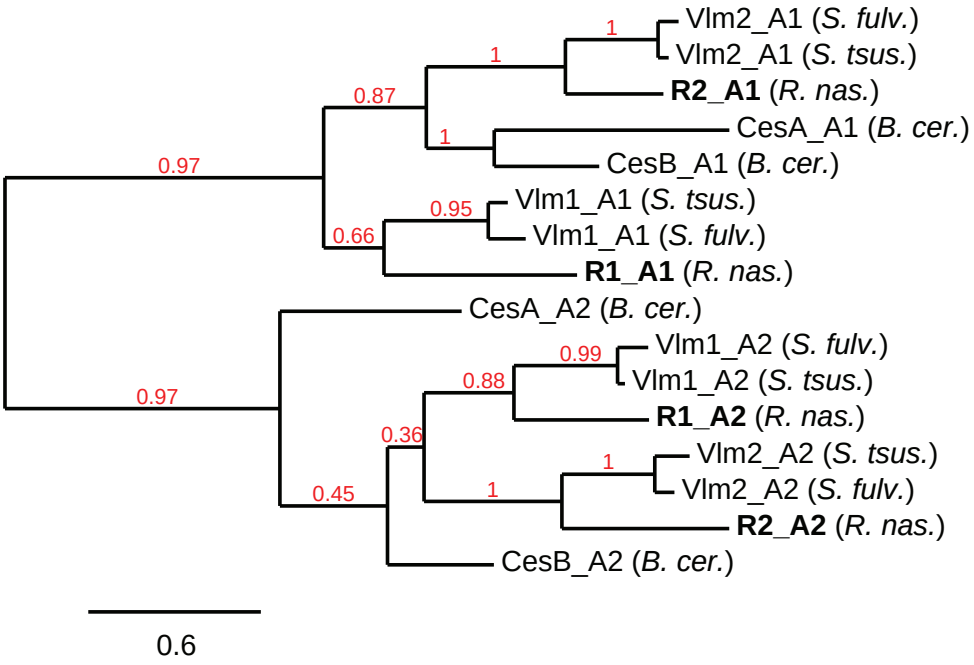

Figure S2. Protein sequences of the adenylation domains encoded by *vlm* (*S. fulvissimus*, *S. tsusimaensis*), *ces* (*B. cereus*), and *vlm* (*R. nasimurium* #32) were trimmed, aligned and a phylogenetic tree was constructed using Phylogeny.fr (http://www.phylogeny.fr/) [16, 17] with 500 times bootstrapping. *Rothia* A domains are more similar to *Streptomyces vlm* than to *B. cereus ces* A domains, which can partly be explained by the different substrates recognised by these domains. Accession numbers of proteins that were used are AGK81693 and AGK81692 (*S. fulvissimus*), ABA59547 and ABA59548 (*S. tsusimaensis*), ABD14711 and ABD14712 (*B. cereus*).

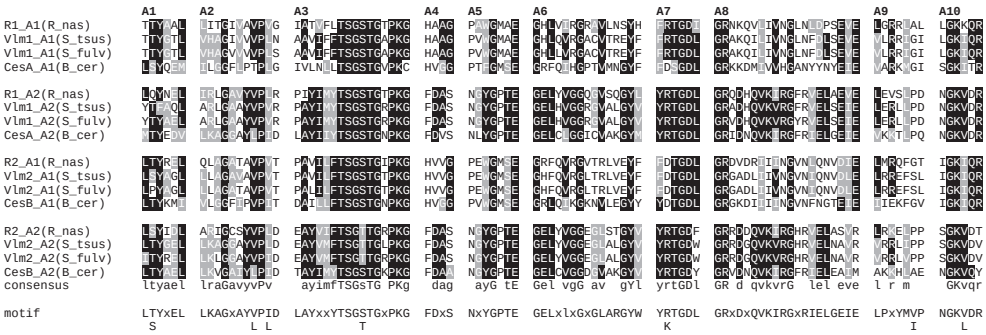

Figure S3. Conserved core domains from aligned protein sequences of the adenylation domains from Vlm1 and Vlm2 (*S. fulvissimus*, *S. tsusimaensis*), CesA and CesB (*B. cereus*), and Vlm1 and Vlm2 (designated R1 and R2) (*R. nasimurium*) and consensus motifs for these core as described by [74] and [75]. *Rothia* A domains are more similar to *Streptomyces* Vlm than to *B. cereus* Ces A domains, even if the A domain is involved in recognition and activation of the same l-Val residue (R2_A2).

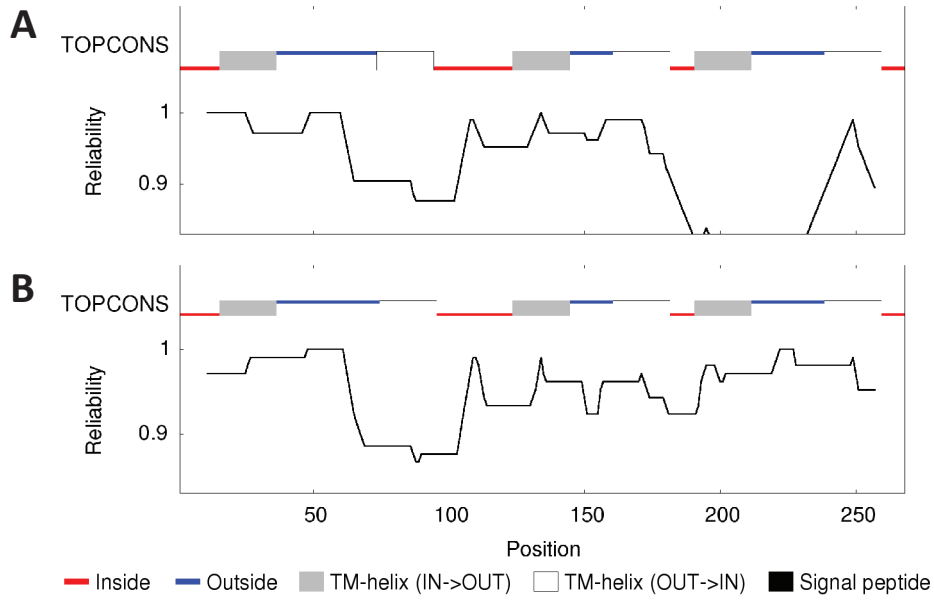

Figure S4. Consensus topology of the putative ABC transporter permeases **A)** CesD (YP_001967167.1) from *B. cereus* and **B)** gene *vlmC* product from *R. nasimurium* as predicted by TOPCONS [48]

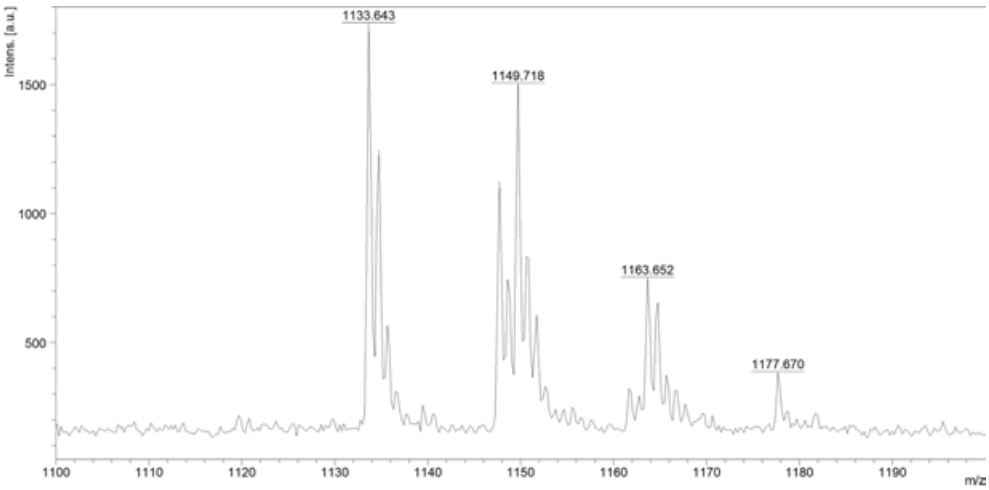

Figure S5. MALDI-ToF-MS spectrum of crudely purified valinomycin and structural variants from MeOH *R. nasimurium* extract, using sequential Et_2O and MeCN solvent steps. This spectrum is representative for multiple, independent extractions.

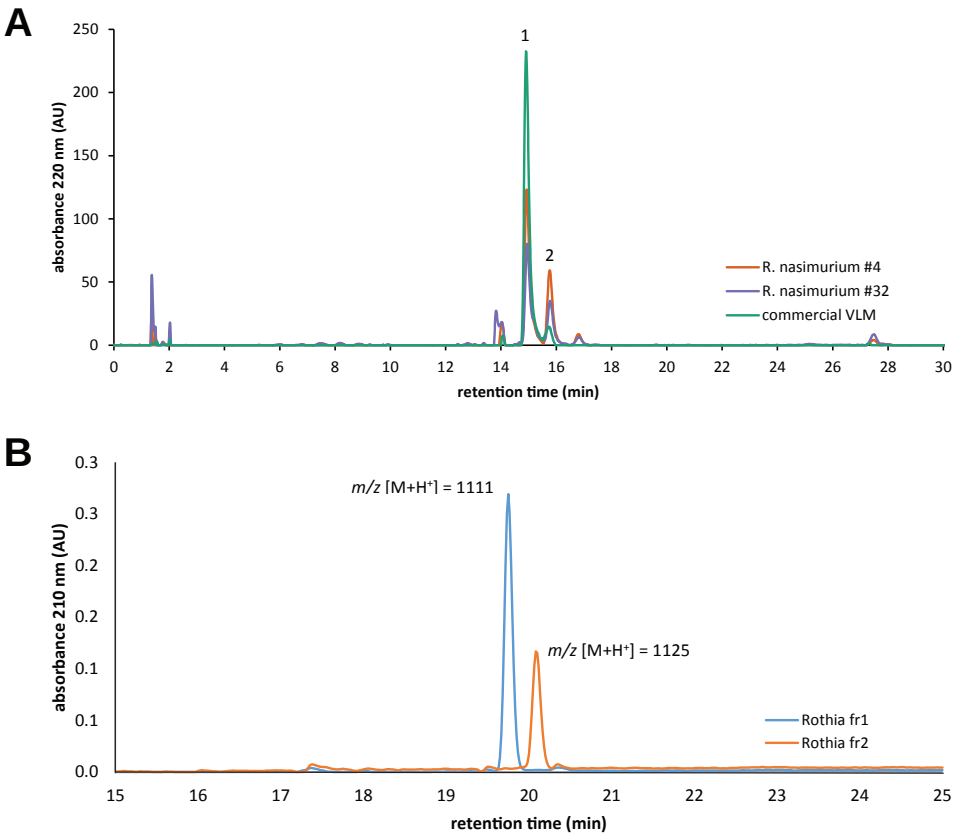

Figure S6. A) Chromatogram of analytical HPLC of purified bacterial extracts from *R. nasimurium* #4 and #32. Fraction 1 (retention time = 14.7 min) and fraction 2 (retention time = 15.7 min) were further purified. Commercial valinomycin from *Streptomyces fulvissimus* (VLM) was used as a reference. **B)** Chromatogram of HPLC purified *R. nasimurium* fractions 1 and 2, with the MALDI-ToF-MS mass of the purified molecule assigned to each peak (see Figure S7 for the MALDI-ToF-MS spectra for these fractions).

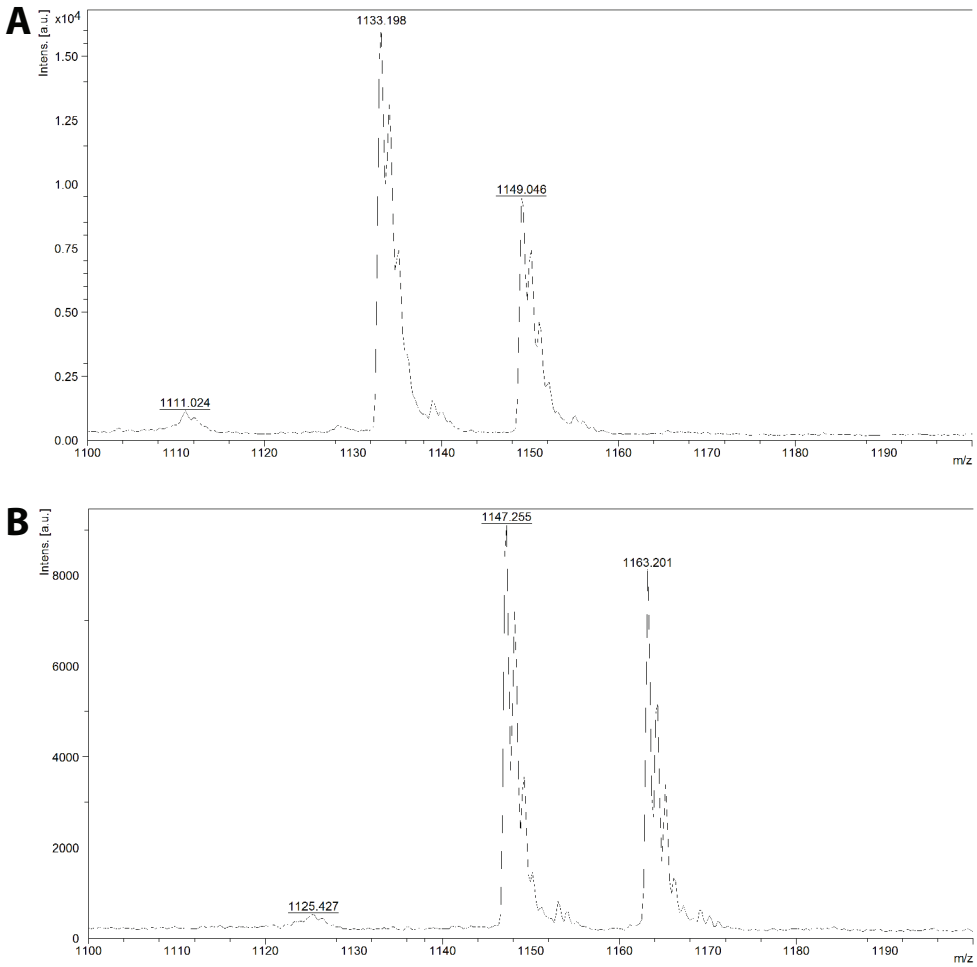

Figure S7. MALDI-ToF-MS spectrum of **A)** *R. nasimurium* purified valinomycin (molecular formula $C_{54}H_{90}N_6O_{18}$) (fraction 1, see Figure 6) with the most prominent ion adducts *m/z* [M+H$^+$]: 1111, *m/z* [M+Na$^+$]: 1133 and *m/z* [M+K$^+$]: 1149, **B)** *R. nasimurium* purified valinomycin variant (fraction 2, see Figure 6) with the most prominent ion adducts *m/z* [M+H$^+$]: 1125, *m/z* [M+Na$^+$]: 1147 and *m/z* [M+K$^+$]: 1163. The shift of +14 could be indicative of an additional CH_2 group.

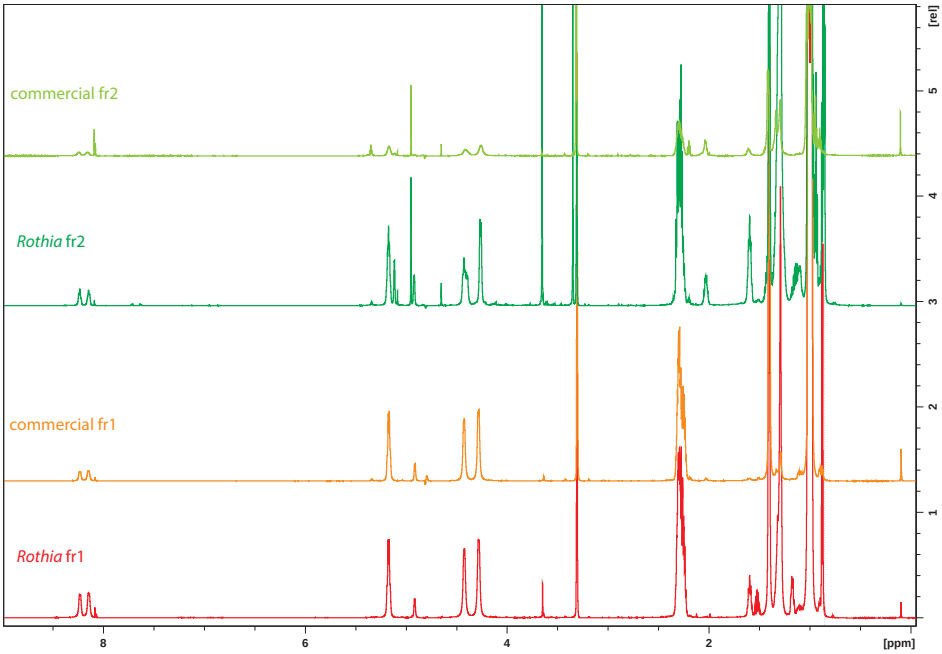

Figure S8. NMR spectra of HPLC purified fractions 1 and 2 from *R. nasimurium* extracts (red and dark green) and commercial valinomycin from *S. fulvissimus* (orange and bright green).

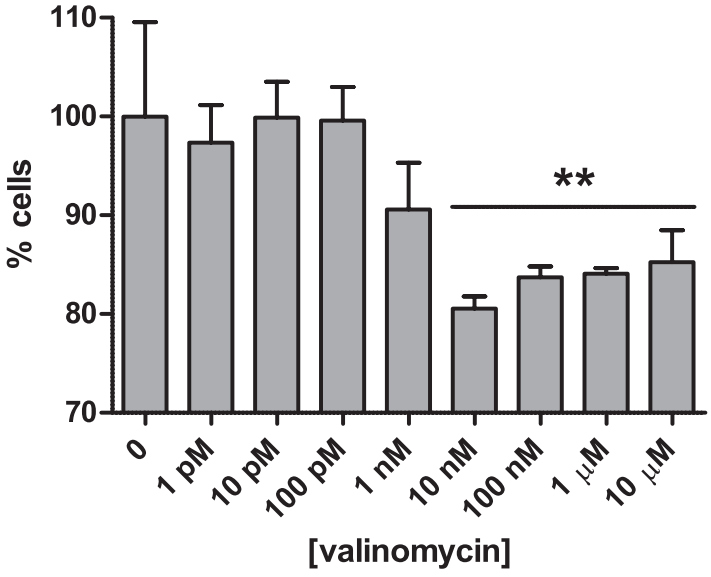

Figure S9. Effect of valinomycin on NPTr cell viability, measured by the neutral red uptake assay. (n=3, mean ± SD, ** indicates $P < 0.01$ compared to control as determined one-way ANOVA and Dunnett's Multiple Comparison Test).

Supplemental information

Extraction and crude purification of peptide

BHI medium was inoculated from a -80°C glycerol stock of *R. nasimurium* and incubated overnight at 37°C without agitation. BHI plates containing 1.25% agar were inoculated with a volume of 200 µL of the overnight bacterial culture and incubated for 48 h at 37 °C to create a lawn of bacterial growth. A volume of 15 mL cold 70% v/v isopropanol (IPA) 0.1% v/v trifluoracetic acid (TFA) or 100% methanol (MeOH) was then added to each agar plate and the bacteria were removed by gentle scraping with a sterile T-spreader (VWR International, Amsterdam, The Netherlands). The bacterial suspensions were pooled and collected in sterile 50 mL Falcon tubes (Eppendorf, Hamburg, Germany), vortexed thoroughly and incubated on a rotary shaker for 6 h at 4°C. To remove insoluble matter the suspension was centrifuged for 25 min at 12.000 x g at 4 °C and the supernatant was filtered using a 0.2 µm cellulose acetate sterile syringe filter (Whatman, GE Healthcare). After removal of IPA by rotary evaporation, the solution was used for agar well diffusion assays as described above. For the purification, the cell-free solution was evaporated to dryness by rotary evaporation and 1 mL MeOH and 1 mL Milli-Q water were added to the residue, followed by sonication to aid re-solubilisation. Approximately 5 volumes of diethyl ether (Et_2O) were added and the suspension was sonicated thoroughly. The Et_2O solution was filtrated (589[3] Blauband, Schleicher & Schuell) to remove precipitates and the solvent removed by rotary evaporation. The precipitate was subsequently resuspended by thorough sonication in acetonitrile (MeCN) and MeOH with elimination of insoluble precipitates by filtration at each step and solvent removal by rotary evaporation. The residue was dissolved in 1 mL MeOH and loaded onto a C18-E solid phase extraction column (Strata, Phenomenex), which had been conditioned with Milli-Q water. The column-bound molecules were eluted with stepwise increasing concentrations from 10% to 100% of MeOH and the collected 10 mL fractions were analysed using MALDI-ToF-MS (Bruker). The elution fractions containing peptides of interest without impurities were pooled and solvent was removed by evaporation. These crudely purified peptide preparations were stored at -20°C.

Peptide purification and characterization

Crudely purified peptides were further purified via preparative high pressure liquid chromatography (HPLC). Analytical HPLC and MALDI-ToF-MS were employed to assess purity of the separated peptide fractions. Commercial valinomycin from *Streptomyces fulvissimus* (abcam, Cambridge, UK) was used as a reference molecule for MALDI-ToF-MS and NMR analysis and for establishing optimal HPLC conditions.

Preparative reverse–phase HPLC

Column: Waters™ XBridge™ BEH130 C18, 10 x 250 mm, 5 µm
Pump: Waters™ 600 Pump
Detector: Waters™ 996 Photodiode Array Detector

Buffer A: 99.9% H_2O, 0.1% TFA
Buffer B: 99.9% MeOH, 0.1% TFA

Purified fractions were collected manually. Spectra were processed as follows: the raw data were exported using the Waters Empower 3 software and imported in Microsoft Excel. The baseline (blank) was subtracted and negative absorbance values were corrected to 0 (zero).

Analytical reverse–phase HPLC
Column: Phenomenex Luna® C18, 4.6 x 150 mm, 5 µm, 100Å
Agilent 1100 Series
Pump: G1310A Isocratic Pump
Detector: G1314A Variable Wavelength Detector
Buffer A: 99.9% H_2O, 0.1% formic acid
Buffer B: 99.9% MeOH, 0.1% formic acid

Gradient:

Time (min)	Flow (mL min^{-1})	%B
0	1.2	30
10	1.2	100
25	1.2	100
26	1.2	30
32	1.2	30

Spectra were processed as follows: the raw data were exported using the Agilent ChemStation software and imported in Microsoft Excel. The baseline (blank) was subtracted and negative absorbance values were corrected to 0 (zero).

MALDI–ToF–MS
Instrument: Bruker Microflex™
Matrix: α-cyano-4-hydroxycinammic acid in MeCN:H2O:TFA (50:47.5:2.5) (10 mg mL^{-1})
Target: MSP AnchorChip 600/96 microScout (Bruker Daltonics)

NMR
HPLC purified fractions 1 and 2 from commercial (abcam, Cambridge, UK) and *R. nasimurium* extracted valinomycin were dissolved in a minimal volume of CD_3OD and analysed by ^{1}H- and ^{13}C-NMR on a Bruker Avance III HD 600 MHz instrument equipped with a 1.7-mm cryogenically cooled $^{13}C/^{1}H$ DCH probe head.

LC–MS/MS
LC-MS/MS experiments were performed on an Vanquish ultra-high performance liquid chromatography (UHPLC) system coupled to a Q-Exactive mass spectrometer (Thermo

Fisher Scientific, Bremen, Germany). A C18 core-shell column (Kinetex, 50 x 2 mm, 1.8 um particle size, 100 A pore size, Phenomenex, Torrance, USA) with a flowrate of 0.5 mL/min (Solvent A: H_2O + 0.1 % formic acid (FA), Solvent B: Acetonitrile (ACN) + 0.1 % FA) was used for LC separation. Herby a linear gradient from 0-0.5 min, 5 % B, 0.5-4 min 5-50 % B, 4-5 min 50-99 % B, flowed by a 2 min washout phase at 99% B and a 2 min re-equilibration phase at 5 % B was used. For MS and MS/MS measurements the electrospray ionization (ESI) parameters were set to 35 L/min sheath gas flow, 10 L/min auxiliary gas flow, 2 L/min sweep gas flow and 400 °C auxiliary gas temperature. The spray voltage was set to 3.5 kV, the inlet capillary was set to 250 °C and a 50 V S-lens radio frequency (RF) level was applied. MS/MS spectra were recorded in data dependent acquisition (DDA) mode. Both MS^1 survey scans (150-1500 *m/z*) and up to 5 MS/MS scans of the most abundant ions per duty cycle were measured with a resolution (R) of 17,500 with 1 micro-scan in positive mode. The maximum ion injection time was set to 100 ms and a MS/MS precursor selection windows was set to 3 *m/z* with 0.5 *m/z* offset. Normalized collision energy was stepwise increased from 20 to 30 to 40 % with z = 2 as default charge state. MS/MS experiments were automatically triggered at the apex of a peak within 2 to 15 s from their first occurrence. A dynamic exclusion was set to 5s. Ion species with unassigned charge states as well as isotope peaks were excluded. Thermo raw data files were converted to mzXML in centroid mode using MSConvert (part of proteowizard) for the downstream data analysis.

Molecular networking

A Molecular network of all LC-MS/MS runs was created with the online workflow at GNPS (http://gnps.ucsd.edu) [76]. Herby similar MS/MS from the same precursor mass were clustered with MS-Cluster [77] with a precursor mass tolerance of 0.02 Da and a MS/MS fragment ion tolerance of 0.02 m/z. Consensus spectra that contained less than 2 spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. The edges between two nodes were kept in the network only when each of the nodes appeared in each other's respective top 10 most similar spectra. The spectra in the network were searched against GNPS's spectral libraries. The library spectra were filtered in the same manner as the input data. All spectra library matches were required to have a score above 0.6 and at least 6 matched peaks and were manually validated.

MALDI–ToF MS Imaging (MSI)

After the cultivation of Rothia #4 and #32 isolates on thinlayer BHI agar, colonies were transferred to a stainless steel MALDI target plate and a 1:1 mixtures of CHCA and DHB was sieved onto the target as described previously [78]. After trying the sample over night at 37° C the target plate was transferred to a Autoflex MALDI-TOF mass spectrometer equipped with a Nd:YAG smartbeam2 laser (Bruker Daltonics, Bremen, Germany). For MALDI imaging experiments, the laser width was set to "wide". For each raster spot of 300×300 µm, 1000 laser

shots (200 shots random walk) with a relative laser energy of 50% at a frequency of 1000 Hz were acquired. TOF measurements were herby performed in positive reflectron mode. The mass spectrometer was herby set to a suppression of matrix ions below 300 m/z. The TOF mass range was set to 300-2800 m/z with a detector gain multiply of 12. The mass spectra were recorded and further visualized using FlexImaging 4.1 (Bruker, Daltonics, Bremen, Germany).

Neutral red uptake assay

Viability of NPTr cells [19] was quantified using the neutral red uptake assay to determine cytotoxicity of valinomycin to epithelium [79]. Briefly, cells were seeded in a flat-bottom 96-well microtiter plate at a density of ~$1x10^5$ cells $well^{-1}$ in 89% Dulbecco's Modified Eagle's Medium (DMEM) + GlutaMAX™ (Gibco, Life Technologies), containing 10% heat-inactivated Fetal Bovine Serum (FBS) and 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Invitrogen, Breda, The Netherlands). After incubation at 37°C with 5% CO_2 for 24 hours cells had reached confluency and culture medium was removed and replaced with 200 µL treatment medium (Keratinocyte Serum Free Medium (Gibco, Life Technologies)) containing a concentration range of valinomycin. Following 18 h incubation at 37°C with 5% CO_2, the treatment medium was removed and 100 µL neutral red solution (40 mg mL^{-1}) was added. After 3 h incubation, neutral red solution was removed, cells were washed with 150 µL PBS and neutral red was extracted from the cells by adding 150 µL 50% ethanol, 1% glacial acetic acid. The released neutral red content, indicative of cell viability, was measured spectrophotometrically at 540 nm (Spectramax M5, Molecular Devices, Sunnyvale CA, USA), values from triplicate conditions were averaged and normalized to cells treated with PBS only and expressed with the SD as percentage viable cells.

CHAPTER 7

General Discussion

Host–Defense Peptides

The increase and spreading of antibiotic resistant bacteria is an urgent global health problem [1]. As a consequence, existing antibiotics are becoming increasingly ineffective against multi-drug resistant (MDR) pathogenic bacteria leading to increased morbidity, mortality and overall healthcare costs in both human and veterinary medicine. Several alternatives to conventional antibiotics have been proposed for therapeutic applications to reduce the risk of infections by pathogenic bacteria. Antimicrobial peptides (AMPs), also referred to as host-defense peptides (HDPs) due to their other biological defense functions, are among the more promising broad-spectrum antimicrobial compounds and have received substantial scientific interest [2-4]. Although naturally occurring peptides differ widely in potency and spectrum of activity, their direct antibacterial activity is typically modest compared to most conventional antibiotics. Several approaches to optimize naturally occurring AMPs or to design novel AMPs have been explored with the aim to deliver potent, broad-spectrum antibiotics in a more cost-effective manner [5]. However, many AMPs suffer from poor absorption into host blood or tissue, low *in vivo* peptide stability and proteolytic degradation, and systemic cytotoxicity to the host. Given these limitations AMPs currently hold most promise as topical treatments for skin and wound infections caused by pathogenic bacteria [6, 7]. **Chapter 2** aimed to investigate whether frog skin AMPs could be effective for treating skin infections caused by MDR pathogenic bacteria, while minimizing the impact on commensal bacteria. We showed that several naturally occurring peptides and some amino-acid substituted analogues of temporin-1DRa displayed promising differential activity against pathogenic bacteria compared to commensal or probiotic lactic acid bacteria. These peptides, or modifications thereof, could be interesting candidates for further development as novel therapeutics for skin infections. Several bionanotechnological tools have been described that could open up avenues towards innovative therapeutic applications of AMPs, such as nanofibers, nanoparticles, and biofilms [8]. The combination of these nanostructures with potent AMPs, might be further developed into a formulation to be used as successful topical treatment of skin infections by pathogenic bacteria. One example would be treatment of recurrent skin and wound infections by the opportunistic pathogen *Staphylococcus pseudintermedius* in dogs. Infections caused by methicillin-resistant strains of *S. pseudintermedius* (MRSP) have proven very difficult to treat as they are resistant to essentially all antimicrobial agents available in veterinary medicine [9, 10]. In these situations, the topical application of narrow-spectrum AMPs, for example formulated in a polymeric hydrogel, as a complement to conventional antibiotic therapy may provide a more effective way to reduce *S. pseudintermedius* infections in dogs.

Despite initial enthusiasm about using AMPs to treat infections with antibiotic resistant pathogens, it is now recognised that a broad range of diverse resistance mechanisms can be present in bacteria [11, 12]. One well-described resistance mechanism involves electrostatic repulsion of the generally cationic AMPs, for instance by the incorporation of positively charged D-alanyl esters into teichoic acids by gene products of the *dlt* operon [13, 14]. In

Chapter 3, we studied the susceptibility of *S. suis* to the human cathelicidin LL-37, and also included an isogenic *dltA* mutant in our flow cytometry-based antimicrobial assays. To our surprise, no altered susceptibility to LL-37 of the *dltA* mutant was observed after 30 min or 1 hour exposure when compared to the wild-type strain S10. Additionally, we evaluated if the polysaccharide capsule that forms the outermost surface of many Gram-positive bacteria that AMPs have to cross in order to reach the target cytoplasmic membrane, was involved in susceptibility to AMPs. Our hypothesis was that capsule composition or lack of a capsule might shield the membrane and thus impact on susceptibility to AMPs. However, the capsule did not influence the susceptibility of *S. suis* to LL-37, as determined by testing serotype 2 and a capsule-negative mutant J28, and strains belonging to serotype 7 and 9 (**Chapter 3**).

To confirm that certain genetic factors responsible for (modification of) surface molecules may influence the susceptibility of bacteria to the cationic AMP LL-37, we used an available selection of isogenic deletion mutants of the Gram-positive bacterium *Lactobacillus plantarum* WCFS1 obtained from NIZO Food Research (Ede, The Netherlands). This selection included a *dltX-D* mutant and several mutants lacking individual or multiple capsular polysaccharide (cps) encoding gene clusters [15-17]. We found that the mutants lacking all capsular polysaccharide gene clusters (cps1-4), the *gtcA3* mutant, and the *dltX-D* mutant were more susceptible to killing by LL-37 in a microbroth dilution assay (data not shown). The gene product of *gtcA3* is involved in the glycosylation of teichoic acids, and altered expression of this protein was previously proposed to be implicated with altered immunomodulatory properties of *L. plantarum* WCFS1 *in vitro* [18]. However, mutants of individual cps clusters did not show an altered susceptibility to LL-37. These data suggest that bacterial resistance towards cationic AMPs, such as LL-37, is a complex phenomenon involving multiple different processes and/or molecules.

Bacterial resistance towards AMPs is an important consideration to make when developing AMPs into therapeutic applications, as it has been shown to cause cross-resistance in the target bacterium to other antibiotics [19-21]. Furthermore, AMPs such as LL-37 could be used by certain bacteria as environmental cues to become more virulent or invasive [22-24]. In **Chapter 3** we performed microarray gene expression profiling to describe the generic and adaptive resistance mechanisms that could be activated in the important porcine pathogen *Streptococcus suis* in response to the human cathelicidin LL-37. Because of the global problems imposed by *S. suis* on pig husbandry (see next section), and the increasing pressure to reduce antibiotic chemotherapy to control *S. suis*, there is an interest in using alternatives approached including the application of AMPs. We observed that exposure of *S. suis* to sub-lethal concentrations of LL-37 induces a complex response comprising general stress responses (including heat shock proteins) as well as what seems to be increased expression of a potential AMP transporter that might be regulated by a two-component system; an homologous system present in *Bacillus* species mediates resistance to AMPs [25,

26]. Additionally, expression of 3 surface proteins were increased substantially, one of which plays a role in adhesion to epithelial cells. These findings raise the possibility that sub-lethal concentrations of LL-37 function as an environmental cue for *S. suis* leading to induction of virulence-associated genes inside the host. If such a response would indeed occur under natural *in vivo* conditions, it would suggest that the interplay between pig immune response and shifts of *S. suis* from a commensal to a pathobiont lifestyle might be mediated by external factors including porcine infections by different pathogens. It also shows that applying exogenous AMPs to avoid *S. suis* infections will not be straightforward.

In practice, the use of pure AMPs alone will probably be insufficient for controlling *S. suis* in the swine industry, aside from the fact that it would be prohibitively expensive due to the large amounts of AMPs required and the associated high costs of peptide synthesis. We reasoned that the modest antimicrobial activity of many naturally occurring AMPs to some bacteria results in a balance between the host and different bacterial taxa within the host-colonizing microbiota. From the literature information, and taking report biases into account, we hypothesized that at least some pathogens appear to be more sensitive to common AMPs than commensals. Under this hypothesis, AMPs contribute to lowering bacterial numbers and modulating numbers of more (pathogenic) or less (commensal) sensitive species, and negative interactions between commensal and pathogenic species would then avoid outgrowth of pathogenic bacteria. *S. suis* is a natural occupant of the upper respiratory tract and the proximal intestine in pigs. Therefore, we also carried out microbiota analysis of porcine tonsils and small intestine to identify bacteria that would directly antagonise *S. suis*. This research forms the second part of the thesis (**Chapters 4-6**).

Streptococcus suis infection and colonisation resistance

The second part of this thesis focussed on an alternative strategy involving bacterially produced AMPs to control *Streptococcus suis* infections in piglets. As mentioned above, *S. suis* is an important swine pathogen associated with morbidity and mortality in weaning piglets worldwide, causing large economic losses to the pig production industry [27, 28]. In adult pigs *S. suis* is usually carried asymptomatic and it is frequently found as a member of the microbiota of the oro- and nasopharynx, and at relatively lower abundances of the small-intestinal tract microbiota [28-33]. Clinical infections are mainly seen in piglets 2–5 weeks post-weaning and in growing pigs, less frequently in suckling piglets and adult animals [34, 35]. Different mechanisms for pathogenesis have been proposed, but it is generally accepted that in pigs the palatine tonsils are a primary site where *S. suis* colonizes and replicates, which may lead to invasion of the mucosal tissues and dissemination into the lymphatic system or blood [27, 36]. Translocation of *S. suis* across the gastro-intestinal mucosal barrier has also experimentally been shown to be a possible route of infection [37, 38].

Several stress-related factors are known to correlate with the development of *S. suis* clinical disease, such as crowding, sudden weather change, mixing or moving of animals,

and vaccination [27]. Additionally, co-infection with other pathogenic microbes (e.g. swine influenza virus or porcine reproductive and respiratory syndrome virus (PRRSV)) has been described as important predisposing factor for increased susceptibility to *S. suis* [39-42]. Moreover, a change of diet towards more complex carbohydrates, such as starch, has been hypothesized to contribute to outgrowth of and infection with *S. suis*; it was previously shown that the expression of virulence genes is determined by the available carbohydrate source [43]. This, together with our observation that increasing concentrations of LL-37 AMPs also induced expression of *S. suis* virulence genes, shows that the interplay between microbiota, host immunity and *S. suis* infections is very much dependent on external, intertwined factors.

Microbiota profiling analysis suggests that tonsil populations of *S. suis* are part of a pathogen complex

Microbial colonisation of a mammalian host starts directly after birth, initially forming an compositionally unstable microbiota that develops into a more stable adult-like microbiota over time. Environmental factors and nutrition are an important determinant in shaping the microbiota during early life, with weaning associated changes having significant effects [44-46]. As little was known about which specific bacterial taxa might have an impact on *S. suis* abundance, we have investigated co- and anti-occurrence of bacterial taxa with *S. suis* in the porcine small intestine and tonsil (**Chapter 4**). The 16S profiling study provided our first glimpse of bacteria that were enriched or underrepresented in correlation with *S. suis* abundance. All the piglets that were sampled for the collection of tonsillar and small intestinal microbiota, formed a relatively heterogeneous group in terms of sex, sow, bodyweight, feed intake, housing pen and age at weaning. In addition, we sampled piglets from two different breeds raised on two separate specific-pathogen free (SPF) farms with low incidence of *S. suis* related morbidity (**Chapter 4**, Supplemental Information). Accordingly, the collected microbiota samples are unbiased for a particular phenotype but all originate from healthy piglets around the time of weaning.

According to our microbiota profiling results, *S. suis* abundance was among others positively correlated with the relative abundance of well-known respiratory pathogens, and negatively with multiple *Lactobacillus* species. The positive correlations between *S. suis* and porcine pathogens belonging to different species from multiple different genera confirmed our hypothesis that the occurrence of *S. suis* invasive disease in colonised pigs is dependent on the commensal microbial community structure and most likely the presence of a complex of other pathogens. The existence of a microbial complex, responsible for polymicrobial infections in pigs, had been suggested previously by Brockmeier *et al.* [47] and Hansen *et al.* [48]. This idea and our findings are supported by the apparent difficulty to experimentally induce invasive infections in pigs after intranasal inoculation or aerosol exposure to *S. suis* without predisposing animals by inducing mucosal damage [49, 50]. Studies of colonisation and transmission of *S. suis* have been performed without inducing mucosal damage but

then systemic infection occurred only sporadically [51]. Also in porcine gastro-intestinal challenge models, translocation and systemic infection of *S. suis* was only observed at relatively low frequencies (13-27% of animals) [37, 38]. Our finding is of scientific interest but also of applied relevance, because it provides a possible explanation for the lack of a good and robust animal model for experimental *S. suis* infection. For future strategies to control *S. suis*, it is of interest to study *S. suis* infections in the context of a pathogenic complex, where the different pathogens may differentially impact on an animal's health status and immune system [52]. It is noteworthy to consider that, if under natural conditions, accumulations of AMPs do indeed induce *S. suis* virulence genes, AMPs induced by pathogens that co-occur with *S. suis* may indirectly promote expression of *S. suis* virulence.

The LEfSE and network analyses of the microbial taxa co-occurring with *S. suis* showed a strong negative correlation with multiple *Lactobacillus* species, with the strongest negative correlations observed between *L. reuteri* and the pathogen complex including *S. suis*, *Actinobacillus*, *Actinomyces,* and *Pasteurella* species. Exploration of where *L. reuteri* occurs most often (based on its 16S rRNA gene sequence abundance) suggested that this anti-correlation could be caused by it being relatively highly abundant in the small intestine, whereas *S. suis* and the associated pathogens were most abundant in the palatine tonsils. The main antimicrobial compound produced by *L. reuteri* is reuterin, a mixture of the hydrated and nonhydrated form of 3-hydroxypropionaldehyde and its dimer [53]. Sensitive species are killed by reuterin in the micromolar range, including *Streptococcus salivarius* which is killed at dosages between 10-20 μM [54]. However, we observed no decreased growth of *S. suis* at dosages ranging from 1 to 64 μM of reuterin, lending support to the notion that the negative co-occurrence of *L. reuteri* and *S. suis* was mainly determined by differential niche occupation or indirect effects rather than direct antagonism *via* the production of reuterin (**Chapter 4**). However, at least three of the other *Lactobacillus* species that displayed anti-correlation with three "bins" of *S. suis* load (high, low or no *S. suis*) and bacteria that could be part of the presumptive pathogen-complex, are also known to colonise the oral cavity. It is also possible that the observed negative correlation of lactobacilli with *S. suis* abundance is caused by one or more immune-mediated colonisation resistance mechanisms [55, 56]. Of course, there may be other presently unknown factors that directly or indirectly cause the negative correlation we observed between abundance of *S. suis* and *Lactobacillus* species.

The research described in **Chapter 4** demonstrated that the porcine microbiome contains bacteria that might display direct or indirect microbial antagonism against *S. suis*, thereby playing an important role in colonisation resistance against this and possibly other (respiratory) porcine pathogens. To further validate this hypothesis, we applied high-throughput culture-based approaches to find individual bacteria belonging to the porcine tonsillar and small intestinal microbiomes that displayed potent narrow-spectrum growth-inhibiting activity towards *S. suis* through the production of AMPs or secondary metabolites.

We believed that this strategy would be complimentary to the culturing-independent microbiota-profiling approach for understanding the interplay between members of the endogenous microbiota and *S. suis* (see Figure 1).

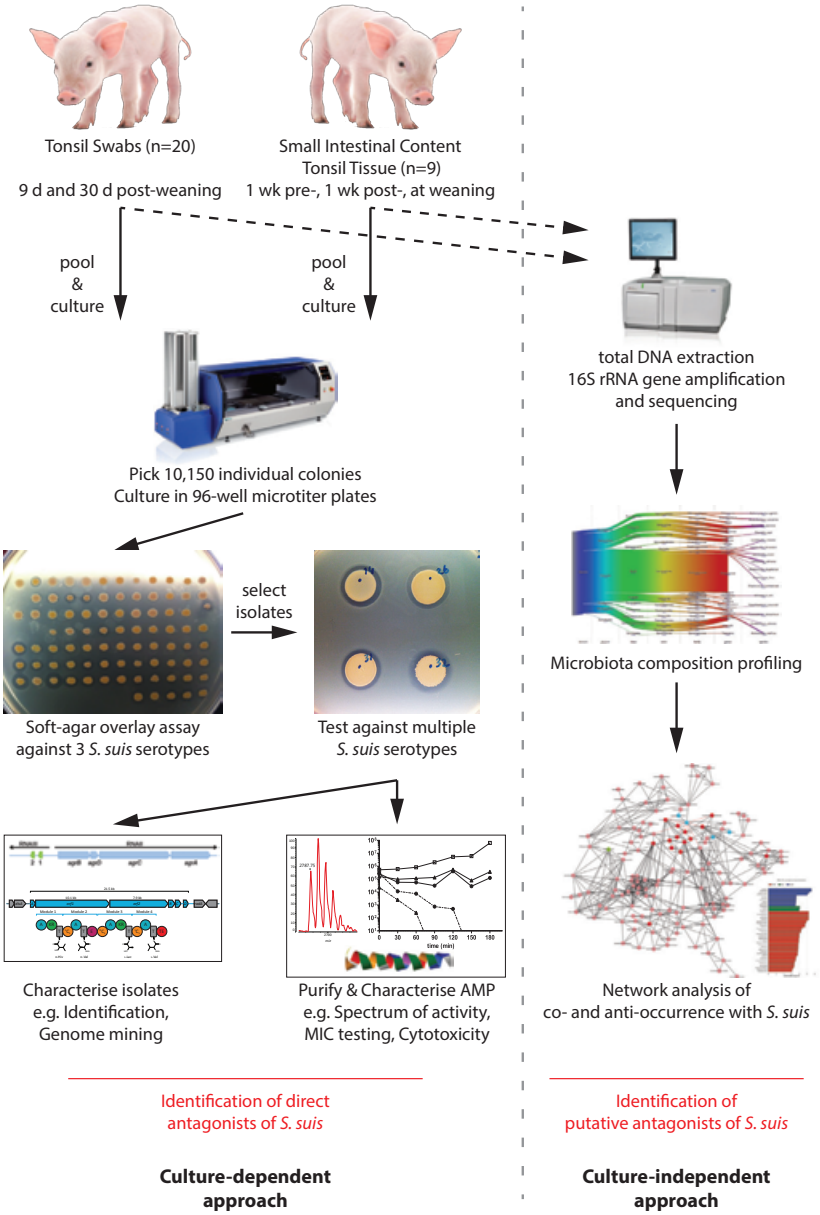

Figure 1. Schematic overview of the culture-dependent approach described in **Chapters 5** and **Chapter 6** (left) and the culture-independent approach described in **Chapter 4** (right) to identify antagonistic bacteria against *S. suis* within the porcine microbiome.

Culture–dependent approach led to the identification of seven isolates that inhibit *S. suis*

Together with the group of Colin Hill at University College Cork, Ireland, a high-throughput approach using a robotic colony picking system was employed to isolate individual bacterial colonies originating from the microbiota of the palatine tonsils and small intestinal content of healthy piglets. The culturing conditions, like nutrient agar and aerobic atmosphere, that we used favoured the growth of *S. suis*. We isolated a total of 10,150 individual bacterial colonies from two pooled libraries of microbiota originating from the tonsils and small intestinal content samples, which were subsequently screened for growth inhibiting activity against three serotypes of S. suis using soft-agar overlay assays (see Figure 1). Isolates that showed clear zones of inhibition were selected for further characterisation, such as protease sensitivity to check if the antimicrobial compound was proteinaceous or due to the production of non-specific fermentative metabolites, such as acetate or lactate (Chapter 5). From the tonsil and small intestinal isolate libraries, 7 colonies with consistent *in vitro* activity displaying the largest zone of inhibition against more than one *S. suis* serotype were selected. These isolates were identified by alignments of their 16S rRNA gene sequence and/or MALDI-ToF MS Biotyping (data not shown) (Table 1).

Isolate #	Location	Closest Species	16S rRNA Identity	MALDI-ToF MS Biotyping
4	Tonsil	*Rothia nasimurium*	97.89 %	(-) / (+)
5	Tonsil	*Staphylococcus aureus* *	99.20 % #	(++)
26	Tonsil	*Staphylococcus pasteuri*	99.86 %	(++)
31	Tonsil	*Streptococcus gallolyticus* subsp. *pasteurianus*	99.73 %	(++)
32	Tonsil	*Rothia nasimurium*	97.89 %	(-) / (+)
14 †	Ileum	*Streptococcus gallolyticus* subsp. *pasteurianus*	99.73 %	(++)
G	Tonsil	*Streptococcus ursoris*	98.11 % #	n.d.

Table 1. Identification of isolates that displayed consistent growth inhibition of *S. suis* in overlay assays. Closest phylogenetic match based on the 16S rRNA gene sequence was identified using the EzTaxon server [57]. MALDI-ToF MS Biotyping identification probability score: (++) = secure genus identification, probable species identification, (+) = probable genus identification, (-) = unreliable identification. n.d. = not determined; # = partial 16S rRNA gene sequence (~500 bp) used for alignment identification; * = this *Staphylococcus aureus* group includes species that are not distinguishable based on 16S rRNA gene sequence alone; † = impure colony.

Three candidate antagonistic isolates (belonging to two species) were described in detail in this thesis. **Chapter 5** described the characterisation of isolate #26, identified as *Staphylococcus pasteuri*, of which BAGEL3, a web-based tool for genome mining for putative bacteriocin genes [58], predicted the presence in the draft genome of two small ORFs encoding small antimicrobial peptides with high sequence similarity to *Staphylococcal* delta-lysins. We purified these two delta-lysins from bacterial cultures, and compared their antimicrobial and cytotoxic activities with their chemically synthesized peptide equivalents.

Our results support the conclusion from previous studies that Staphylococcal delta-lysins may have narrow-spectrum antimicrobial activity against several strains of *Legionella*, *Bacillus megatherium*, *Staphylococcus aureus,* and *Streptococcus pyogenes* [59-61]. Interestingly, the highly similar synthetic delta-lysin from *S. aureus* displayed a different spectrum of activity against a panel of *S. suis* strains when compared to the *S. pasteuri* synthetic peptides. The delta-lysin from *S. aureus* was initially considered to possess only haemolytic and cytotoxic activity [62-64]. However, based on current knowledge, delta-lysins, which are part of the group of staphylococcal phenol-soluble modulin (PSM) peptides [65, 66], appear to have roles in both host colonization and direct competition with a narrow spectrum of bacterial species [67].

Chapter 6 focussed on the characterisation of porcine tonsil isolates #4 and #32, for which *Rothia nasimurium* (Actinobacteria) was identified as the closest phylogenetic neighbour. This species is distantly related to *Streptomyces*, soil bacteria that are well-known producers of antibiotics [68]. Genome mining for biosynthetic gene clusters using AntiSmash 3 [69, 70] showed the presence of a non-ribosomal peptide synthetase (NRPS) gene cluster that codes for an NRPS assembly line organization identical to *vlm* from Streptomyces [71-75] and *ces* from *Bacillus* [76, 77]. Based on the sequences and substrate specificity predictions of its adenylation domains, this NRPS cluster was predicted to synthesize a cyclic dodecadepsipeptide consisting of the amino acids L-valine and D-valine, D-hydroxyvaleric acid and L-lactic acid. Mass spectrometry and nuclear magnetic resonance (NMR) analyses identified the purified peptide as valinomycin, a potassium (K^+) ionophore or high-affinity K^+ transporter that facilitates the movement of K+ ions through phospholipid membranes [71, 72, 78, 79].

To our knowledge, NRPS gene clusters or NRPS-derived compounds have not been described in the genus *Rothia*. Moreover, valinomycin was, until now, only known to be produced by soil-dwelling *Streptomyces* species [71-75] and possibly also isolates belonging to the *Bacillus pumilus* group, although in the latter case the employed detection method was not suitable for reliable secondary metabolite identification [76]. Based on different G+C content of the NRPS gene cluster (48.7%) compared to the rest of the *R. nasimurium* genome (57.98%), we assumed it was relatively recently acquired from a Firmicute origin. Analysis of the genome sequence did not reveal any obvious features of a mobile genetic element that might explain how this *R. nasimurium* isolate acquired the *vlm* gene cluster. However the NRPS gene cluster in *R. nasimurium* is located between the genes *disA* and *radA* (**Chapter 6**, Figure 4), and compared to genomes of *R. mucilaginosa*, *R. dentocariosa* and *R. aeria*, the orientation of *disA* has been inverted in the valinomycin producing *R. nasimurium* isolate. DisA (DNA integrity-scanning protein) is a non-specific DNA binding protein associated with DNA repair [80] and production of cyclic diadenosine monophosphate (c-di-AMP) [81]. The second messenger c-di-AMP has been linked to DNA repair [82] and regulation of the *ktrAB* operon that encodes a potassium transporter [83]. RadA (Radiation sensitive gene A), a highly

conserved protein in bacteria, is a member of the RecA/RadA/Rad51 protein superfamily and plays a critical role in homologous DNA recombination and DNA repair [84]. Furthermore, RadA can act as an antagonist of DisA by physical interaction to inhibit its c-di-AMP synthesis [85]. It is remarkable that these genes involved in DNA integrity and DNA recombination are in such close proximity to the inserted *vlm* gene cluster, and it is tempting to speculate that their reorientation could be related to a past recombination event.

Species belonging to the genus *Rothia* are found in diverse habitats ranging from soil and sediments to the respiratory and intestinal tracts of humans and other mammals [86-91]. *Rothia* spp. are not considered to be obligate pathogens, but *R. dentocariosa* has been associated with dental and periodontal ailments, and rare cases of systemic infection such as peritonitis [92] or endocarditis [93, 94]. *Rothia mucilaginosa* is often present in high abundance in the lung of cystic fibrosis patients [95] and usually treated as part of the normal oral microbiota, but its significance in pulmonary disease may be underestimated due to possible misidentification of the infecting microbe [96]. Mining the available genomes of *R. dentocariosa* (8) and *R. mucilaginosa* (20) did not reveal the presence of a putative NRPS cluster predicted to produce valinomycin. This was confirmed by the absence of *S. suis* S10 inhibition in overlay assays by type strains *R. nasimurium* CCUG 35957, *R. dentocariosa* DSM 43762, and *R. mucilaginosa* DSM 20746 (data not shown). Based on biochemical, phenotypic and phylogenetic evidence on an unknown murine bacterial isolate, Collins *et al.* [86] proposed the novel species *Rothia nasimurium*, setting it apart from other members of the genus *Rothia.* There are, however, differences between the type strain *R. nasimurium* CCUG 35957T referred to by Collins *et al.* and the *R. nasimurium* isolates described in this thesis. When considering the 97.89% 16S rRNA gene sequence similarity, the difference in GC content (56% for *R. nasimurium* CCUG 35957T as determined by [86] vs. 57.98% for *R. nasimurium* #32) and the distinctive property of valinomycin production, one could argue that the strain we describe here could form a new species within the *Rothia* genus. Additional experimental evidence, such as biochemical characterization and cellular fatty acid analysis, would be required to make such a taxonomic decision and is outside the scope of this thesis.

Not all of the identified active isolates (see Table 1) were described in detail in this thesis. Isolate #31, for example, was identified as *Streptococcus gallolyticus* subsp. *pasteurianus* by 16S rRNA gene sequence analysis and MALDI-ToF MS Biotyping, and its observed growth-inhibiting activity against *S. suis* may be due to several factors. We ran BAGEL3, a web-based tool for genome mining for putative bacteriocin genes [58], on the draft genome sequence of isolate #31. BAGEL3 identified three putative bacteriocin encoding gene clusters: two were predicted to encode for linear azol(in)e-containing peptide (LAP) and one for an unmodified class II bacteriocin (data not shown). Additionally, initial analysis of the draft genome sequence using PHAST [97] identified 1 intact, and 4 incomplete or questionable phage DNA sequences. We did not experimentally attempt to isolate lytic phages from this isolate, but phages that

selectively target *S. suis* could be interesting candidates for therapeutic applications [98, 99]. Most antimicrobial activity against *S. suis* was found in extracts of bacterial cells made in 70% 2-propanol 0.1% TFA, and not in cell-free culture supernatant of overnight liquid cultures or agar plate-grown bacterial cultures (data not shown). Tricine SDS-PAGE of this active fraction revealed the prominent presence of a peptide with a mass of ~5 kDa, which is in accordance with one of the BAGEL3 predicted LAPs. These initial results might suggest the presence of a small hydrophobic antimicrobial compound, such as a bacteriocin, rather than a lytic phage that is responsible for the observed antimicrobial effect of isolate #31 against *S. suis*. Further characterisation of this *Streptococcus gallolyticus subsp. pasteurianus* isolate is required to determine the exact nature of its antimicrobial effect against *S. suis* and the potential effects on host cells. This isolate might prove to be an interesting candidate, alone or in combination with the other isolates described in this thesis, for *in vivo* inhibition and reduction of *S. suis* in piglets.

The findings described in **Chapter 5**, **Chapter 6**, and in the section above, show that very diverse molecules, produced by phylogenetically unrelated bacteria that are part of the natural porcine microbiome, may display antimicrobial activity towards *S. suis*. Inspection of 16S rRNA gene sequence abundance showed that *S. pasteuri* was present in the tonsil samples of 18 out of 21 assayed piglets although at different abundances. Reads of 16S sequences with >99.4% identity to the valinomycin-producing *R. nasimurium* isolate were present in tonsil samples of 13 out of 21 sampled piglets. The *S. pasteuri* and *R. nasimurium* isolates identified in the culture-dependent approach as direct antagonists of *S. suis* did not show clear anti-correlation with *S. suis* abundance as described in **Chapter 4**.

Also, our culture-based screens did not identify any *Lactobacillus* antagonists of *S. suis*. This could strengthen the differential niche occupation theory, that where *S. suis* nearly exclusively occurs in the tonsillar areas, potentially antagonistic lactobacilli such as *L. reuteri*, a species that antagonises diverse bacteria through secretion of the bacteriocidal compound reuterin, hardly occur in the tonsillar areas. However, at least three other lactobacilli that displayed anti-correlation with *S. suis* abundance do occur in the oral cavity. It is likely that the growth conditions that were used during the culturing of microbiota isolates and agar overlay assays, in particular the media optimised for *S. suis* growth, were unsuitable for proper growth of *Lactobacillus* species; these species generally require different media for growth under laboratory conditions and are less tolerant to oxygen. For future studies, it would be of interest to obtain a combination of culture media favouring *Lactobacillus* growth and overlay agars that favour simultaneous growth of *S. suis*. Isolation and characterisation of *Lactobacillus* strains from the porcine tonsil or small intestine may lead to novel insights about the molecular mechanisms behind the observed anti-correlation with *S. suis*.

Effects of AMPs on host cells

It is difficult to know at which concentrations the delta-lysins and valinomycin are produced by *S. pasteuri* and *R. nasimurium*, respectively, in the porcine oropharynx and/ or small intestinal tract and if such concentrations are high enough to actually inhibit *S. suis*. Detection and quantification of these molecules in tonsil or intestinal content samples by LC-MS/MS would be a suitable approach to shed light on the levels of delta-lysin or valinomycin production *in vivo*. We could show that the delta-lysins and valinomycin also pose some toxicity to the host, although the concentrations at which host cells (especially epithelial cells) show symptoms of damage are substantially higher than the concentrations at which *S. suis* killing is achieved (**Chapter 5**). Both valinomycin and delta-lysin were often described as molecules that primarily display cytotoxic or haemolytic effects on host cells [62, 63, 100-103], although the antimicrobial, antiviral and antitumor activities of valinomycin have also been reported [73]. Yet, it remains unclear what the exact biological and ecological role of these molecules might be, and in what manner they confer benefit to the producing *S. pasteuri* or *R. nasimurium* bacteria. **Chapter 6** aimed to expand the knowledge on valinomycin production by a commensal bacterium, such as R. nasimurium, and explored several possible effects this ionophore may have *in vivo*. Figure 2 shows a schematic overview of the potential roles of valinomycin production by *R. nasimurium* in the tonsil or small intestine. Broad-spectrum antimicrobial activity has been reported for valinomycin [103], and we observed growth inhibition of all tested strains of *S. suis* in a well-diffusion assay by valinomycin-containing extracts of *R. nasimurium*. However, most other bacteria that we tested were not susceptible, and similarly, a 100-fold difference in MIC was observed for pure valinomycin between closely related *Rothia* species (**Chapter 6**). Apparently, some bacteria are better able to resist the antimicrobial action of valinomycin, and this differential susceptibility may also be of importance *in vivo*. We also showed that valinomycin was able to reduce epithelial cell barrier function, induce apoptosis in epithelial cells, and induce inflammation and IL-1β production in macrophages, although epithelial cells were resistant to higher concentrations of valinomycin than immune cells. As shown in Figure 2, the exact biological function and effects of valinomycin production by *R. nasimurium in vivo* remains unclear, and is likely to depend on multiple environmental factors, such as K^+ concentration, bacterial density, nutrient availability, host defense molecules, etc.

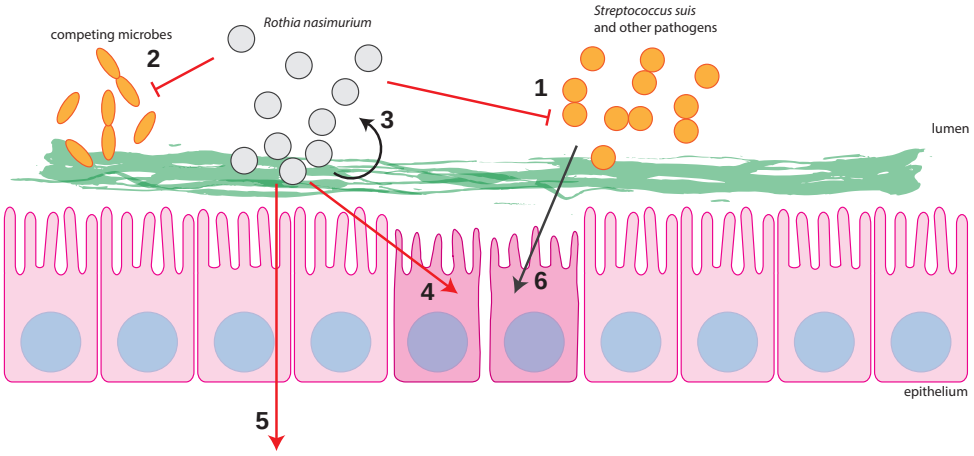

Figure 2. Simplified graphical representation of possible roles of valinomycin production by *Rothia nasimurium* on host mucosal surfaces. (1) valinomycin has an antimicrobial effect on *S. suis* and potentially on other pathogenic bacteria, (2) production of valinomycin could give *R. nasimurium* an advantage over competing microbes, (3) valinomycin may be involved in signalling within the *Rothia* biofilm community, possibly via induced potassium release, (4) cytotoxic effects on epithelial cells may cause inflammation and release nutrients for *R. nasimurium*, (5) the triggering of host inflammation responses may extend beyond the epithelial layer (6) *R. nasimurium* may promote infection by *S. suis* and other pathogens by damaging the epithelial cells or increasing barrier permeability.

Combining different approaches to find novel bacterial antagonist against *S. suis*

Both approaches we undertook to identify microbial antagonists of *S. suis* have limitations and advantages. The outcome of culture-dependent identification of AMP producing antagonists of *S. suis* may be subject to several factors such as the selectivity of the culture conditions, number of screened colonies, and selection of target strains of *S. suis*. Furthermore, the genes that are responsible for the production of the antimicrobial compound may be regulated differently under laboratory growth conditions compared to *in vivo*. This might partly explain why many isolates that we initially selected, lost their ability to inhibit the growth of *S. suis* after continued culturing under laboratory conditions. One main advantage of the culture-dependent approach is the relative simplicity of isolating and purifying the antimicrobial compounds and linking this directly to the producing bacterial strain that can be characterized genetically. Culture-independent approaches, however, provide indirect information about bacterial antagonism and the 16S rRNA gene is in most cases unsuitable for the identification of bacteria to the species level. In addition, it offers evidence on the presence and abundance of bacterial taxa, but it does not provide any functional information.

The research described in this thesis demonstrates the suitability of culture-based approaches to identify natural products that can kill pathogenic bacteria. Together with the microbiota profiling results, a picture emerges where *S. suis* is part of a polymicrobial community, possibly occurring in biofilms, that may contain other pathogenic bacteria as

well as antagonistic species. It is likely that in newborn piglets, biofilm microbial composition depends on initial colonisation via the sow, the environment and the feed. Overcrowding and suboptimal ventilation may promote growth of pathogens, whereas more optimal initial growth conditions, including good animal welfare and hygiene practices, and healthy sows, may promote growth of bacteria that better suppress outgrowth of *S. suis* and other pathogens [104, 105]. It would thus be of interest to change focus of future research on suppressing *S. suis*, from the point of view of a single-pathogen problem to one of a polymicrobial nature, where *S. suis* is part of a complex of respiratory pathogens, bacteria, viruses and maybe fungi, that colonise the upper respiratory tract including the tonsillar areas. The bacterial part of the pathogen complex may be dispersed further into the upper GI tract via the piglet's feeding. Infection of piglets by *S. suis* via the tonsils might thus originate from bacterial (and viral) pathogens that entered via inhalation, and actual infection could be dependent on infection pressure by *S. suis* and other pathogens. Infection in the first weeks upon weaning may be stress-induced but may also be a result of a sudden shift from milk oligosaccharides to starch [43] in those pathogens that use CcpA to control carbohydrate metabolism. Moreover, it is likely that consumption of solid feed in addition sow's milk leads to a change in the microbiota composition. To illustrate this, LEfSE analysis showed that in a group of 21 animals that might have consumed solid food before weaning, based on an ambiguous visual inspection of the colour of their feces, three bacterial species (*Actinomyces denticolens*, *Fusobacterium nucleatum* and *Streptococcus ferus*) were enriched in the tonsillar areas compared to piglets that only appeared to have consumed sow's milk prior to weaning (**Chapter 4**). Polymicrobial infections of this nature are expected to be influenced by farm practices as well as pig contact with microbes colonising diverse biotic (sows and other piglets) and contaminating abiotic surfaces including the air circulation, walls and floor coverings.

Applications with the endogenous microbiota to reduce the risk for *S. suis* infection in piglets

As mentioned previously, a stable and diverse microbiota may confer health benefits to the host and provide colonisation resistance against pathogenic bacteria [55, 56]. The research described in **Chapter 4-6** emphasises that certain members of the endogenous microbiota may display direct or indirect antagonism against *S. suis*. Conversely, co-occurring complexes of opportunistic or pathogenic bacteria may promote the outgrowth of *S. suis*. Neonatal piglets become colonised by *S. suis* as part of their developing natural microbiota at farrowing via vertical transmission from the sow [27, 28]. *S. suis* mostly causes morbidity and mortality in piglets post-weaning, a period associated with a high level of stress, dietary change to starch-rich feed [27, 34, 35]. During early life the porcine microbiota composition is flexible and influenced by environmental factors, and weaning has been shown to have an important impact on microbiota composition [44]. We hypothesise that modulation of the microbiota during the early life of a piglet may be used to reduce the risk of *S. suis* associated invasive post-weaning disease. Currently, similar approaches with probiotic supplements are

being used in pigs to improve animal performance and overall health, although for most applications the underlying mechanism of action that confers the benefits is unknown [106-109].

As the tonsils and small intestine have been shown to be important niches colonised by *S. suis*, and potentially also provide a point of entry for systemic disease, alteration of the tonsillar and small intestinal microbiota is anticipated to have an impact on *S. suis* abundance. One or multiple bacterial isolates that showed direct inhibition of *S. suis* may be administered to piglets through addition to the feed or drinking water, or by oral or intranasal application of a bacterial suspension in the period between farrowing and weaning. Alternatively, administration of a more complex microbial consortium, composed of the most abundant key bacterial taxa found in healthy pigs, could be considered. Given that these endogenous bacterial strains were isolated from piglets, there is a high probability that they may successfully colonise, or at least remain present in sufficient abundance until after the post-weaning period in which piglets are most vulnerable to infections by *S. suis*.

The feasibility of such a strategy to reduce *S. suis* load, and consequently risk for invasive disease, could be experimentally shown by using caesarean-derived piglets in a specific-pathogen free (SPF) clean environment. In that way, the microbiota composition of the neonatal piglets is less likely to vary due to the colonisation by sow-associated microbes. 16S- or whole-genome shotgun sequencing could be used to observe colonisation, changes to the microbiota, and determine the effect on *S. suis* abundance. Obviously, this approach may also be suitable for other porcine opportunistic pathogens.

Over the last decade, the fundamental scientific and applied importance of small molecule metabolites and peptides in microbe-host and microbe-microbe interactions within the microbiota have become clear [110, 111]. In addition to roles in bacterial communication [112], these molecules also modulate host health and disease by (in)directly influencing host physiology including the development of the immune system in new-borns. Discovering these microbiota-derived biologically active small molecules and the genes that encode them are essential requirements for understanding the microbiota-host interplay and are needed for developing bacterial communities with therapeutic potential to prevent or cure infections with bacterial pathogens. Future work in host-microbe interactions should therefore include research aimed at elucidating the exact function(s) of bacteriocins or secondary metabolites, for instance using isogenic mutants, to assay their expression and activity *in vivo*. Such work requires effective animal models, preferably using the same host organism from which the original metabolite- or peptide-producing isolates were sampled.

Taking an unbiased ecosystem approach, where microbial communities are considered to function as complex networks, makes it possible to appreciate the microbial ecology where a microbe, such as *S. suis,* is just one small component of the entire microbiome. Integration of multiple unbiased whole-genome-sampling technologies: (meta-)genomics, metabolomics, proteomics, and transcriptomics of microbiota and host help to draw a chart of the whole microbiome. Such “charts” visualise hidden parts of the microbiome, they may for instance

show predicted networks of co-occurring opportunistic or pathogenic bacteria, but may also suggest mutualistic interactions between commensals, e.g. multiple *Lactobacillus* species including known probiotics, that may have (indirect) beneficial effects on the host. Combining unbiased -omics platforms with culture-dependent screens therefore appear to have true potential to contribute to finding alternatives for conventional antibiotics within the endogenous microbiota.

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APPENDICES

Summary

The increasing prevalence of antibiotic resistance in pathogenic bacteria and the potential future implications for human and animal morbidity and mortality, health-care costs and economic losses pose an urgent worldwide problem. As a result, exploration of alternative strategies to combat antibiotic resistant bacteria have intensified over the last decades. The use of naturally occurring antimicrobial peptides (AMPs) has received considerable scientific and commercial interest due to their antimicrobial activity and plethora of possible therapeutic applications. The work described in this thesis focused on the study of AMPs and other bioactive molecules produced by bacteria as potential alternatives to prevent or treat infections with pathogenic bacteria.

In the first part of the thesis, the antimicrobial potency of eukaryotic AMPs and the responses they elicit in bacteria were studied. **Chapter 2** described the potential for a selection of frog skin AMPs and analogues for development into novel topical therapeutics to treat skin infections caused by antibiotic resistant bacteria. We showed that several natural AMPs and analogues displayed promising growth-inhibiting activity against multi-drug resistant pathogenic bacteria whereas these AMPs or analogues did not inhibit growth of probiotic or commensal strains. In **Chapter 3** the generic and adaptive resistance mechanisms in *Streptococcus suis* upon exposure to sub-lethal concentrations of a model cationic AMP, the human cathelicidin LL-37, were studied. We found significantly altered expression of genes associated with two-component signalling, membrane transport and carbohydrate uptake, as well as upregulation of genes involved in adhesion, pilus formation, and resistance against bacteriocins or small peptides.

The second part of this thesis aimed to increase knowledge about the role of the porcine microbiota in *S. suis* abundance, with the purpose to identify commensal bacteria that displayed strong and selective antagonism against this pathogen, possibly *via* the production of AMPs. In **Chapter 4** we identified bacterial taxa that show strong positive or negative correlations with the abundance of *S. suis* and other porcine pathogens. The results indicated that colonisation by *S. suis* was correlated with a group of other opportunist pathogens, including species belonging to the genera *Actinobacillus*, *Actinomyces*, *Pasteurella* and *Streptococcus* which are are known to cause respiratory disease. The results also revealed that the microbiota from the tonsil or small intestine contain bacteria that might display (in) direct antagonism against *S. suis*. Based on this result, we screened the tonsillar and small intestinal microbiota for isolates that could specifically inhibit the growth of *S. suis*. **Chapter 5** described the isolation and identification of a *Staphylococcus pasteuri* isolate producing two α-helical peptides that possess narrow-spectrum antimicrobial activity against multiple serotypes of *S. suis*. In **Chapter 6** we characterized a *Rothia nasimurium* strain from the palatine tonsil that produces the non-ribosomally synthesized ionophore antibiotic valinomycin, and investigated the effects of this cyclic peptide on other bacteria and host cells. **Chapter 7** summarizes and discusses the key results of the research described in this thesis and describes possible directions for future research.

This thesis increased the understanding of the role of host- and microbiota-derived biologically active small molecules in microbe-microbe and microbe-host interplay. Such knowledge may contribute to the development of novel therapeutic solutions to treat antibiotic resistant bacteria, such as beneficial microbial communities (i.e. next-generation probiotics) or biotechnological applications of natural or modified AMPs.

Samenvatting

De toename van antibioticaresistentie van ziekteverwekkende bacteriën vormt een urgent wereldwijd probleem vanwege de bedreiging van de volksgezondheid, de mogelijke toekomstige implicaties voor de humane en veterinaire geneeskunde en de bijkomende economische schade. Gedurende de afgelopen decennia is daarom het onderzoek naar alternatieve strategieën voor de aanpak van resistente bacteriën geïntensiveerd. Veel wetenschappelijke en commerciële aandacht is er voor het mogelijke therapeutische gebruik van antimicrobiële peptiden (AMPs) die van nature voorkomen. Dit proefschrift behandelt het onderzoek van AMPs en andere door bacteriën geproduceerde bioactieve moleculen als mogelijke alternatieven voor het voorkomen of bestrijden van infecties met pathogene bacteriën.

Het eerste gedeelte van het proefschrift is gewijd aan de antimicrobiële activiteit van eukaryote AMPs en de reacties van bacteriën op deze peptiden. **Hoofdstuk 2** beschrijft een selectie van AMPs afkomstig van kikkerhuid als een beginpunt voor verdere ontwikkeling van deze peptiden tot mogelijk therapeutische toepassing bij de behandeling van huidinfecties met resistente bacteriën. We laten zien dat een aantal van nature voorkomende AMPs en analoge peptiden veelbelovende antimicrobiële activiteit vertonen tegen verscheidene resistente pathogenen, maar niet tegen commensale of probiotische bacteriën. In **Hoofdstuk 3** onderzochten we de reactie en algemene en adaptieve resistentie mechanismes van *Streptococcus suis* na blootstelling aan subletale concentraties van humaan cathelicidine LL-37. We vonden significant veranderde expressie van genen die betrokken zijn bij twee-component-systemen, membraan transport en opname van koolhydraten, alsmede een verhoogde expressie van genen die te maken hebben met adhesie, pilus formatie en resistentie tegen bacteriocines of kleine peptiden.

Het tweede gedeelte van dit proefschrift heeft als doel om de kennis omtrent de rol van de microbiota bij de aanwezigheid en talrijkheid van *S. suis* in varkens te vergroten, met het oog op identificatie van sterke en specifieke antagonisten tegen deze pathogeen (mogelijkerwijs door middel van AMP productie). In **Hoofdstuk 4** identificeren we bacteriële taxa die sterke positieve of negatieve correlatie laten zien met de talrijkheid van *S. suis* en andere pathogene bacteriën. Kolonisatie van biggen met *S. suis* correleert met de aanwezigheid van een groep opportunistische bacteriën behorende tot de geslachten *Actinobacillus*, *Actinomyces*, *Pasteurella* en *Streptococcus* die vaak betrokken zijn bij respiratoire ziektes in varkens. De resultaten laten ook zien dat (in)directe antagonistische bacteriën van *S. suis* een onderdeel van de microbiota in de keelholte en dunne darm kunnen uitmaken. Hierop voortboordurend hebben we de microbiota van de keelholte en dunne darm getest op aanwezigheid van bacteriën die de groei van *S. suis* konden tegengaan. **Hoofdstuk 5** beschrijft de isolatie en identificatie van een *Staphylococcus pasteuri* stam die twee α-helische peptiden met smalspectrum activiteit tegen *S. suis* uitscheidt. In **Hoofdstuk 6** bespreken we een *Rothia nasimurium* stam afkomstig uit de keelholte die de niet-ribosomaal gesynthetiseerde ionofoor valinomycine produceert en hebben we de activiteit van dit cyclische peptide op

humane en bacteriële cellen onderzocht. **Hoofdstuk 7** vat de belangrijkste bevindingen van dit proefschrift samen met aanbevelingen voor mogelijk toekomstig onderzoek.
Dit promotieonderzoek draagt bij aan kennis over de rol van kleine peptiden met biologische activiteit afkomstig van de gastheer of de microbiota in de interacties tussen gastheer en microben en microben onderling. Zulke kennis zou bij kunnen dragen aan de ontwikkeling van nieuwe, alternatieve oplossingen om antibioticum-resistente bacteriën te bestrijden, zoals heilzame microbiële gemeenschappen (volgende-generatie probiotica) of biotechnologische toepassingen van natuurlijke of aangepaste AMPs.

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Curriculum Vitae

Rogier Aäron Gaiser was born on August the 27th in Delft, The Netherlands. After the completion of high school at St. Stanislascollege in Delft in 2002, he started his BSc Biology studies at Wageningen University, followed by a MSc Biology at the same university with a specialisation in Immunology, Microbiology and Molecular Biology. For his MSc thesis and internship, he spent one year in the laboratory of Jeroen Saeij at Massachusetts Institute of Technology in the United States of America. His MSc thesis, supervised by Prof. Huub Savelkoul from the Cell Biology and Immunology group, was about identifying and testing genes and associated proteins of the protozoan parasite *Toxoplasma gondii* that are involved in host cell signalling modulation. Following his graduation, Rogier worked for several months as a research assistant at the University Medical Centre (UMC) Utrecht in the group of Prof. Debbie van Baarle on Eppstein-Barr virus (EBV) and cytomegalovirus (CMV) reactivation and T-cell reconstitution in allogeneic stem-cell transplantation patients. In 2010, he started as an early stage researcher in the Marie Curie Initial Training Network "STARS" (Scientific Training in Antimicrobial Research) at the Centre for Biological Research (CIB) of the Spanish National Research Council (CSIC) in Madrid, Spain. The topic of his research project was "Eukaryotic antimicrobial peptides as an alternative to conventional antibiotics" and he was supervised by Dr. Paloma López Garcia and Dr. Luis Rivas. In 2013, Rogier joined the FP7-funded Marie Curie ITN "TRAIN-ASAP" as early stage research fellow and PhD candidate in the laboratory of Host-Microbe Interactomics at Wageningen University, under the supervision of Prof. Jerry Wells and Dr. Peter van Baarlen. The research undertaken during his PhD project is described in this thesis entitled "Antimicrobial peptides and the interplay between microbes and host: "Towards preventing porcine infections with *Streptococcus suis*".

Overview of Completed Training Activities

Conferences and meetings

Fundacion Lilly 18th Symposium: The Microbiome, San Lorenzo el Escorial, Spain, 2010
2nd Workshop on Prebiotics, Probiotics and Health, Madrid, 2010 (poster)
10th Symposium on Lactic Acid Bacteria, Egmond aan Zee, The Netherlands, 2011 (poster)
STARS international Antimicrobial Drugs Conference, Madrid, Spain, 2013 (poster)
ICOHAR International Conference on One Health Antimicrobial Resistance, Copenhagen, Denmark, 2015 (oral)
ALW Platform Moleculaire Genetica, Lunteren, The Netherlands, 2013
WIAS science day, Wageningen, The Netherlands, 2013
WIAS science day, Wageningen, The Netherlands, 2016 (oral)
STARS 1st network meeting, Valencia, Spain, 2010 (oral)
STARS 2nd network meeting, Amsterdam, The Netherlands, 2011 (oral)
STARS 3rd network meeting, Siena, Italy, 2011 (oral)
STARS mid-term review meeting, Amsterdam, The Netherlands, 2011 (oral)
STARS 4th network meeting, Riga, Latvia, 2012 (oral)
STARS 5th network meeting, Madrid, Spain, 2013 (oral)
TRAIN-ASAP 2nd scientific meeting, Olot, Spain, 2013 (oral)
TRAIN-ASAP mid-term review meeting, Paris, France, 2013 (oral)
TRAIN-ASAP 3rd scientific meeting, Ulcinj, Montenegro, 2014 (oral)
TRAIN-ASAP 4th scientific meeting, Arona, Italy, 2015 (oral)

Courses

Ethics and Philosophy in Life Sciences, WIAS course, Dieren, The Netherlands 2012
Utrecht University Summerschool: Infection meets Immunity, Utrecht, The Netherlands, 2012
Analysis of microarray and RNA Seq expression data, Erasmus MC, Rotterdam, The Netherlands, 2014
Statistics for the Life Sciences, WIAS course, Wageningen, The Netherlands, 2015
STARS training event: Personal Skills training, F. Little, Valencia, Spain, 2010
STARS training event: Writing and Presenting a Scientific Paper, M. Grossman, Riga, Latvia, 2012
Project and Time Management, Wageningen, The Netherlands, 2012
Information Literacy including EndNote Introduction, Wageningen, The Netherlands, 2012
Reviewing a Scientific Paper, WGS course, Wageningen, The Netherlands, 2014
TRAIN-ASAP Pharmaceutical industry workshop, Olot, Spain, 2013
TRAIN-ASAP media training event, Warwick, UK, 2014
TRAIN-ASAP SME training event, KtedoGen, Arona, Italy, 2015
External training periods (UCC, Ireland: 2 months; KU, Denmark: 2,5 months)

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