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Exploiting the healthy microflora to develop new treatments for *Staphylococcus pseudintermedius* infection in canine atopic dermatitis

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Abbreviations:

ABC transporter: ATP-binding cassette transporter

AD: atopic dermatitis

agr: accessory gene regulator

AlbA: antilisterial bacteriocin subtilosin biosynthetic enzyme A

AlbB: antilisterial bacteriocin subtilosin immunity protein

AlbC: antilisterial bacteriocin subtilosin ABC-transporter subunit involved in immunity

AlbD: antilisterial bacteriocin subtilosin ABC-transporter subunit involved in immunity

AlbE: antilisterial bacteriocin subtilosin cyclase

AlbF: antilisterial bacteriocin subtilosin peptidase

AMP: antimicrobial peptide

AMR: antimicrobial resistant

antiSMASH: antibiotics and secondary metabolite analysis shell (genome mining tool)

AUC: area under curve

BAGEL: bacteriocin genome mining tool

BLAST: basic local alignment search tool

BLIS: bacteriocin-like inhibitory substance

CAD: canine atopic dermatitis

CFS: cell-free supernatant

CFU: colony forming unit

CoNS: coagulase-negative staphylococci

CoPS: coagulase-positive staphylococci

ECM: extracellular matrix

GRAS: generally regarded as safe

GWAS: genome-wide association study

HGT: horizontal gene transfer

LanA: generic designation for lanthipeptide precursor peptide

LanB: lanthipeptide dehydratase

LanC: lanthipeptide cyclase

LanD: lanthipeptide oxidative decarboxylase

LanE: lanthipeptide ABC-transporter subunit involved in immunity

LanF: lanthipeptide ABC-transporter subunit involved in immunity

LanG: lanthipeptide ABC-transporter subunit involved in immunity

LanH: lanthipeptide ABC-transporter subunit involved in immunity

LanI: lanthipeptide immunity protein

LanK: lanthipeptide regulatory histidine kinase

LanKC: lanthipeptide class III bi-functional dehydratase/cyclase

LanL: lanthipeptide class IV bi-functional dehydratase/cyclase

LanM: lanthipeptide class II bi-functional dehydratase/cyclase

LanP: lanthipeptide serine protease

LanR: lanthipeptide response regulator protein

LanT: lanthipeptide ABC-transporter subunit involved in immunity

MDR: multi-drug resistant

MGE: mobile genetic element

MLST: multilocus sequence type

MRS: methicillin-resistant *Staphylococcus*

MSCRAMMS: microbial surface components recognising adhesive matrix molecules

NSR: nisin-resistance protein

ORF: open reading frame

PBP: penicillin-binding protein

PBS: phosphate buffered saline

PFGE: pulsed-field gel electrophoresis

PSM: phenol-soluble modulin

sboA: generic designation for subtilosin precursor peptide

SCC*mec*: staphylococcal cassette chromosome *mec*

SNP: single nucleotide polymorphism

ST: sequence type

TSB: tryptone soy broth

TYGS: type strain genome server

WDA: well-diffusion assay

Genes:

aac(6”)-*aph*(2”): confers aminoglycoside resistance

aadD: confers aminoglycoside resistance

abrB: encodes subtilosin A transcription repression system

ant(4’)-*Ib*: confers aminoglycoside resistance

blaZ: confers resistance to some beta-lactams

ccrA, ccrB, ccrC: encode recombinases responsible for excision and integration of SCC*mec*

dfrC, dfrG: encode trimethoprim-susceptible dihydrofolate reductase (DHFR), conferring diaminopyrimidine resistance

fosB, fosD: confer fosfomycin resistance

fusB: confers fusidic acid resistance

glpT: confers fosfomycin resistance

gyrA/gyrB, grlA/grlB: encode DNA gyrase subunits; mutations mediate fluroquinolone resistance

mecA: encodes altered penicillin-binding protein, mediating beta-lactam resistance

mphC: confers macrolide resistance

msrA: encodes efflux pump mediating resistance to several classes of antibiotic including macrolide

mupA: confers mupirocin resistance

norA: confers fluoroquinolone resistance

resD, resE: encodes subtilosin A oxygen-limitation dependent signal transduction system

rpoB: encodes housekeeping gene RNA polymerase β

spa: encodes protein A

str: confers aminoglycoside resistance

tet(M), *tet*(K), *tet*(45): confer resistance to tetracyclines

tuf: encodes housekeeping gene elongation factor Tu

vgaALC: confers resistance to streptogramins

ydbI: encodes transport protein involved in subtilosin immunity

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Abstract

Staphylococcus pseudintermedius is a commensal microorganism and opportunistic pathogen, most frequently responsible for causing pyoderma and secondary skin infections in dogs with atopic dermatitis, one of the most common causes for antimicrobial prescriptions in small animal practice. The emergence of methicillin- and multidrug-resistant *S. pseudintermedius* strains has resulted in some infections being deemed untreatable by many veterinarians. The potential for zoonotic transmission also makes *S. pseudintermedius* a public health concern; thus, identifying novel antimicrobial treatments for *S. pseudintermedius* is a priority in human and animal health. Bacteriocins, ribosomally-synthesised peptides, provide an alternative to antibiotics, displaying potent, specific, antimicrobial activity against strains closely related to or sharing the same niche as the producing bacteria.

This study explored the skin microbiota of healthy dogs, investigating the prevalence of commensal staphylococci, and bacteriocin production by the species isolated.

Staphylococci were isolated from skin swabs of 121 healthy dogs; 166 isolates, belonging to 20 species, including two proposed novel species, were identified. Twelve methicillin-resistant isolates were identified, seven of which were multidrug-resistant, all belonging to coagulase-negative staphylococci species. Thirteen isolates belonging to eight species displayed inhibitory activity against *S. pseudintermedius* via deferred-antagonism assay. One *Staphylococcus devriesei* isolate, H14, inhibited ten clinically relevant *S. pseudintermedius* and two *Staphylococcus aureus* strains in cell-free supernatant assays, reducing growth by over 70%, including methicillin-resistant strains. Genome-mining identified two novel bacteriocin gene clusters from *S. devriesei* H14. Nine further novel bacteriocin gene clusters were identified, from *S. pseudintermedius, Staphylococcus warneri*, *Staphylococcus simulans*, and *Staphylococcus xylosus*. These bacteriocins show promise as novel therapies for *S. pseudintermedius* infections in canine atopic dermatitis.

Lay Summary

Staphylococcus pseudintermedius resides harmlessly on the skin of healthy dogs, but can cause infections in dogs with underlying conditions, such as atopic dermatitis, where secondary skin infections caused by *S. pseudintermedius* are the most frequent cause of antibiotic prescriptions in small animal practice. Many *S. pseudintermedius* strains display methicillin- or multidrug-resistance, resulting in extremely limited treatment options, increasing the need for alternative antimicrobial treatments. Bacteriocins are small peptides produced by bacteria that present a promising alternative to traditional antibiotics; they are active at very low concentrations, and act against strains closely related to or sharing the same niche as the producer strain.

The skin microbiota of healthy dogs were explored to investigate bacteriocin production by commensal staphylococci, and whether any bacteriocins identified were potential alternative treatments for *S. pseudintermedius* infections.

One hundred and sixty-six staphylococci isolates, belonging to 20 species, were isolated from 121 healthy dogs. Of these, thirteen isolates, from eight species, inhibited *S. pseudintermedius* growth via deferred-antagonism assay, a crude screen suggestive of production and secretion of bacteriocin-like substances. Only one isolate, belonging to the species *Staphylococcus devriesei,* inhibited *S. pseudintermedius* in cell-free supernatant assays, which allow further characterisation and quantification of bacteriocin-like substances produced. *S. devriesei* inhibited clinically relevant strains of *S. pseudintermedius* and *Staphylococcus aureus,* including multidrug-resistant strains. Bacteriocin-encoding genes were identified in the inhibitory *S. devriesei* strain. Further novel bacteriocin-encoding genes were identified in isolates belonging to *S. pseudintermedius, Staphylococcus warneri*, *Staphylococcus simulans*, and *Staphylococcus xylosus.* Further investigation will determine if the novel bacteriocins identified in this study might be suitable alternative therapies for *S. pseudintermedius* infection in canine atopic dermatitis.

“Exploiting the healthy microflora to develop new treatments for *Staphylococcus pseudintermedius* infection in canine atopic dermatitis”

1. Introduction

1.1 Staphylococcus pseudintermedius

Staphylococcus pseudintermedius is a Gram-positive, coagulase-positive bacteria (1,2), first recognised as a species in 2005 (3) following the re-classification of *Staphylococcus intermedius* into three species; *Staphylococcus delphini, S. intermedius,* and *S. pseudintermedius* (2). *S. pseudintermedius* resides on the cutaneous and mucosal membranes of healthy dogs (4–11), with the mouth, nose, groin and anus the most commonly colonised regions (4–10). Dogs are thought to become colonised by *S. pseudintermedius* shortly after birth, typically within the first 24 hours of life, via vertical transmission from the dam (12–17). It has been shown that *S. pseudintermedius* can be transmitted to puppies via the mother’s milk as well as through contact with the vagina during birth (15). There is evidence to support horizontal transmission between dogs within the same household (18), both direct and indirect, with the environment and humans acting as short-term reservoirs to facilitate the dispersal of *S. pseudintermedius* between healthy dogs (19–24). Reports suggest that 37-92% of healthy dogs are colonised by *S. pseudintermedius* (4,9,10,21,25–29); the variation in these figures is likely due to discrepancies in culturing methods (the use of selective enrichment versus directly plating samples), methods of identification (phenotypic or genotypic), as well as natural variation due to geographic region or dog breed (29). Dogs may be persistent (more than 75% of samples positive), intermittent (more than 25% but less than 75% of samples positive), or transient (less than 25% of samples positive) carriers of *S. pseudintermedius* (12); longitudinal studies have revealed that the majority of dogs appear to be persistently colonised (5,30). High levels of heterogeneity are seen among *S. pseudintermedius* isolates colonising healthy dogs, with most dogs colonised by at least two unrelated strains (29–34).

1.1.1 *S. pseudintermedius* in disease

Although a part of the normal cutaneous microbiota of dogs, *S. pseudintermedius* is an opportunistic pathogen (8,35), implicated in a range of infections, most commonly pyoderma, otitis externa, wound

and surgical site infections, and urinary tract infections (9,10,25,36–43). *S. pseudintermedius* skin infections are one of the most frequent causes of owners seeking veterinary attention for their pets (8), and a leading cause of antimicrobial prescriptions in small animal practice (44). These infections are typically associated with underlying conditions that impair host defences (45); atopic dermatitis is the main predisposing factor for *S. pseudintermedius* skin infections in dogs (35,46,47). Higher rates of *S. pseudintermedius* colonisation are seen in atopic dogs compared to their healthy counterparts, with carriage rates around 80% (25,26,34). Atopic dogs are also more likely to be colonised at more than one body site than healthy colonised dogs (34).

Carriage of *S. pseudintermedius* appears to be a risk factor for the development of infection; this is supported by both the higher carriage rate in atopic dogs, and the involvement of endogenous strains in infection (*i.e.* the same as those found carried on cutaneous and mucosal surfaces) (31–34). No particular strain(s) have been identified as being associated with infection (25,34,48–50); different bacterial strains belonging to the same species can display intraspecies diversity as a result of loss or exchange of genetic material, such as through horizontal gene transfer (HGT), insertion/deletion events (INDELs), and recombination (51). Previously, strain typing was carried out phenotypically, based on biochemical tests, serology, antimicrobial susceptibility testing, and colony morphology on different media (51); however, genetic methods allow more insight into the phylogenetic history and epidemiology of isolates, and of the diversity of the species overall (51). Whole genome sequences can be used to assess strains; however, this is costly and time consuming (51). Multilocus sequence typing (MLST), a widely used method for assessing phylogeny and epidemiology (52,53), assigns strain based on variation in six-seven conserved house-keeping genes (52,54). *spa*-typing is often used for rapid typing of nosocomial methicillin-resistant *Staphylococcus aureus* (55) and *S. pseudintermedius* (56–59); this method looks at the number of tandem repeats in the X region of protein A, encoded by *spa*, which is prone to spontaneous loss/gain of repeat motifs (59). *spa*-typing has higher discriminatory power than MLST, alongside lower costs, making it a popular technique in hospital laboratories (55). Pulsed-field gel electrophoresis (PFGE) involves digestion of the bacterial DNA with restriction enzymes, and separation of larger fragments (10kb-10Mb) using an alternating electric field (60); this is considered the current gold standard of strain typing (61), however it can be

time consuming and challenging to implement in some laboratories (62,63). Several of these techniques are used for strain-typing *S. pseudintermedius* isolates.

Several studies reported that a diverse population of *S. pseudintermedius* strains can be isolated from atopic lesions (34), suggesting that it is host factors that influence progression from carriage to infection (34). Whilst colonisation of the skin is typically harmless in healthy dogs, it is also the first stage towards infection (32–34,64–66); the skin is the first line of defence against pathogens, in atopic dermatitis the skin barrier is impaired, allowing *S. pseudintermedius* invasion and infection (45).

1.2 Pathogenesis of S. pseudintermedius infection in canine atopic dermatitis

Canine atopic dermatitis (CAD) is a predisposed inflammatory skin disease, characterised by pruritus, and typically associated with an immunoglobulin (Ig) E response against environmental allergens (67). CAD is not a single entity but rather a descriptive term for a clinical syndrome with several endotypes (68,69); clinical presentation and predisposing factors can vary, but the disease process typically involves skin barrier dysfunction, hypersensitivity to environmental allergens, and cutaneous inflammation (70–73). The clinical presentation, immunopathogenesis, and histological features of CAD strongly resemble those of its human counterpart (74), with the primary difference between human and canine AD being the causative agent of secondary infections; in humans, *Staphylococcus aureus* is the main pathogen involved, rather than *S. pseudintermedius*. CAD affects 10-15% of dogs; of these, 55-66% experience recurrent staphylococcal skin infections (75–78). Disease onset typically occurs in dogs under three years of age (75–81); there is no sex predilection, however certain breeds such as West Highland white terriers, Labrador retrievers, boxers, German shepherd dogs, and golden retrievers, are more frequently affected (75,76,78,80–82). The defining presentation of CAD is steroid-responsive pruritus, preceding other symptoms (75,78,79). Characteristic skin lesions are seen, both primary and secondary to self-trauma, including erythema, excoriations, furuncles, hyperpigmentation, lichenification, and self-induced alopecia (75–81). These affect certain areas of the body, most commonly the muzzle and periocular region, feet, axillae, inguinal and abdominal areas, perineum, and pinnae (75–80,83); however, the skin that appears macroscopically uninvolved has been shown to display subclinical inflammation, suggesting it is not just the visibly lesional skin affected (72,73,84). An IgE response against environmental allergens, such as pollen, house dust

mites, and mould spores, is characteristic of CAD, however serum IgE levels do not correlate to disease severity (85–87).

Canine atopic dermatitis, as with human AD, has a complex, multifactorial pathogenesis involving skin barrier dysfunction, immune dysregulation, and genetic abnormalities (68,70–73,84,88,89). Whilst it was previously hypothesised that genetic alterations to the immune response, particularly IgE production, were the main driver of AD ("inside-outside" theory), it is now widely suggested that impaired skin barrier function is the initial pathology (90,91). This results in increased penetrance of allergens, leading to an immunological cascade and cutaneous inflammation, further worsening the skin barrier function, creating a vicious cycle of impaired defence, immune hyperreactivity and inflammation ("outside-inside-outside" theory) (91,92). It is unclear whether cutaneous barrier dysfunction is the primary injury in CAD; in human AD, a loss-of-function mutation in the gene encoding filaggrin, a structural protein required for skin barrier function, is a major risk factor for developing the condition (93,94). Genome-wide association studies (GWAS) in dogs identified a filaggrin mutation implicated in disease in Labrador retrievers (95), but not in other breeds studied (85). However, decreased filaggrin expression and increased expression of enzymes involved in filaggrin metabolism have been reported in atopic dogs (68,85,96,97), suggesting impairments to filaggrin expression contribute to skin barrier dysfunction in CAD. Increased penetration of environmental and staphylococcal antigens is seen as a result of impaired skin barrier function (98). Mast cell degranulation in response to allergen-presentation increases epidermal barrier permeability, resulting in further allergen penetration and presentation (47,99). IgE production contributes to the initiation of an immunological cascade (100). Increased IgE levels against *S. pseudintermedius* antigens are seen in CAD-related pyoderma compared to non-atopic pyoderma, supporting the important role of IgE in the pathogenesis of CAD (101,102). The dendritic and mast cells that bind IgE are long-lived, meaning the immune system is primed for an allergic response against antigens for a prolonged period, hence the chronic and recurring nature of CAD (91). Following antigen presentation, a Th2-biased acute phase immune response is triggered, initiating the immune cascade seen in CAD (103–105). Keratinocytes are stimulated to upregulate production and release of pro-inflammatory mediators such as TSLP (thymic stromal lymphopoietin), TARC (thymus and activation regulated chemokine) and MIF (macrophage inhibition factor) (106,107); this leads to activation,

maturation and recruitment of macrophages, dendritic, mast and T cells (107–109). A pro-inflammatory cytokine cascade ensues, involving interleukin (IL)-25, IL-33, IL-4, IL-8, IL-31, and TNFα (110); IL-31 is also a pruritogenic cytokine (111). The consequent cutaneous inflammation further exacerbates barrier dysfunction; one mechanism of this is through the downregulation of filaggrin expression (112). Self-trauma and excessive grooming due to pruritus lead to further barrier damage, and dispersal of *S. pseudintermedius* from mucosal body sites that act as a reservoir (6). Increased proteolytic activity, altered lipid composition and aquaporin expression lead to epidermal structural changes, contributing to trans-epidermal water loss (TEWL), associated with impaired barrier function (113). Ceramide composition contributes to epidermal barrier dysfunction in human and canine AD (84,114–116); ceramides are found in the cellular lipid bilayer, and possess both structural and cell-signalling roles (117). Ceramide deficiency is associated with increased TEWL and barrier dysfunction in lesional skin of humans and dogs; this can be a primary impairment or secondary to inflammation (84,112,115,116). This cutaneous barrier impairment allows increased adherence and invasion of *S. pseudintermedius.*

S. pseudintermedius displays greater adherence to atopic epidermal cells, from both lesional and non-lesional skin, compared to cells from healthy skin (118–121). Some studies have suggested concentration-dependent adherence, or correlation to pruritus score or breed (118–120,122,123). One study demonstrated that *S. pseudintermedius* isolates obtained from lesional atopic skin displayed greater adherence to corneocytes than *S. pseudintermedius* isolates from healthy skin (118), however this was not corroborated by further studies (50), again suggesting it is host factors, rather than bacterial, influencing adherence of *S. pseudintermedius* to epidermal cells in atopic dogs. It has been suggested that changes to the cutaneous micro-environment following inflammation lead to more favourable conditions for adherence and proliferation of *S. pseudintermedius* (124). Adhesion involves interaction of bacterial ligands and host epidermal cells such as keratinocytes and corneocytes, and the extracellular matrix (ECM) (121). Staphylococci express an array of cell-wall anchored proteins that are involved in attachment to host tissues (41,46,125,126). MSCRAMMS (microbial surface components recognising adhesive matrix molecules), such as SpsD, and SpsL mediate adherence to the host ECM via the binding of ECM proteins fibrinogen, fibronectin, and cytokeratin (119,127,128), thus playing a fundamental role in colonisation of host tissue and the pathogenesis of skin infections.

In atopic dermatitis, increased expression of receptors, adhesins (such as endothelial intercellular adhesion molecule-1 and vascular cell adhesion molecule-1), and ECM proteins is seen in host epidermal tissue (127–134); fibronectin expression is upregulated, and distribution is altered, with fibronectin found on the stratum corneum of atopic but not healthy skin (127). This likely facilitates the increased adherence of *S. pseudintermedius* to atopic skin. Antimicrobial peptides (AMPs) are produced by host cells such as keratinocytes, and are involved in defence against bacterial invasion (135–139). Altered AMP production has been noted in atopic dogs (140), although the significance of this has yet to be elucidated. In human AD, decreased AMP expression is seen, particularly β-defensins and cathelicidins (135–137,141–144); this is not seen in other inflammatory skin disorders where bacterial infections are not a common complication (138,140,143). This suggests that downregulation of AMP expression plays an important role in staphylococcal invasion of host tissues in atopic skin infections.

S. pseudintermedius produces an array of virulence factors, many of which are similar to those possessed by *S. aureus,* that contribute to disease pathogenesis by promoting immune evasion, disruption of epithelial barriers, and enhancing inflammation (91,145–154) (Figure 1). Alongside MSCRAMMs and other cell-wall anchored components, *S. pseudintermedius* produces several secreted virulence factors, including superantigens, toxins, and enzymes (155–162). Approximately 25% of *S. pseudintermedius* isolates are thought to be able to produce superantigens (161); superantigens bypass normal immune response regulation, stimulating activation of a large numbers of T-cells, and consequently triggering a cytokine storm, enhancing the pro-inflammatory response and contributing to barrier dysfunction (161,162). Toxins produced by *S. pseudintermedius* include enterotoxins, cytotoxins, and exfoliative toxins (155,163,164). In a human study, *S. aureus* cytotoxins were only detected in lesional atopic skin, and not healthy skin, supporting the hypothesis that cytotoxins play a critical role in *S. aureus* skin infections in atopic individuals (165). This has not been investigated in *S. pseudintermedius,* but the similar virulence profile of the two species and pathogenesis of infection suggests cytotoxins could contribute to *S. pseudintermedius* infections in CAD (91,145,147–153). Staphylococcal exfoliative toxins have been found to trigger atopic lesions in humans; *S. pseudintermedius* produces three exfoliative toxins: SIET, EXI, and ExpB (166–169). Experimental intradermal injection of SIET in dogs induced erythema, exfoliation and crusting, similar

to the symptoms of pyoderma (166). Another study demonstrated that SIET intradermal injection induced no clinical or histopathological lesions in a neonatal mouse model (167), however intradermal injection of EXI caused exfoliative and erosive lesions and epidermal splitting (169). ExpB intradermal injection in dogs caused epidermal splitting and desmoglein-1 degradation (168). These results support the role of exfoliative toxins in *S. pseudintermedius* skin infections contributing to skin barrier dysfunction and development and severity of lesions in CAD. Production of several phenol-soluble modulins and exoenzymes, particularly proteases, contribute to the development of atopic infections (91,147–153,170,171). *S. pseudintermedius* isolates have been found to possess biofilm-forming ability (3,172–174), alongside *agr* system homologues (157,175), suggesting the ability to participate in quorum sensing; these characteristics contribute to the virulence of *S. pseudintermedius* and the pathogenesis of skin infections (176–179).

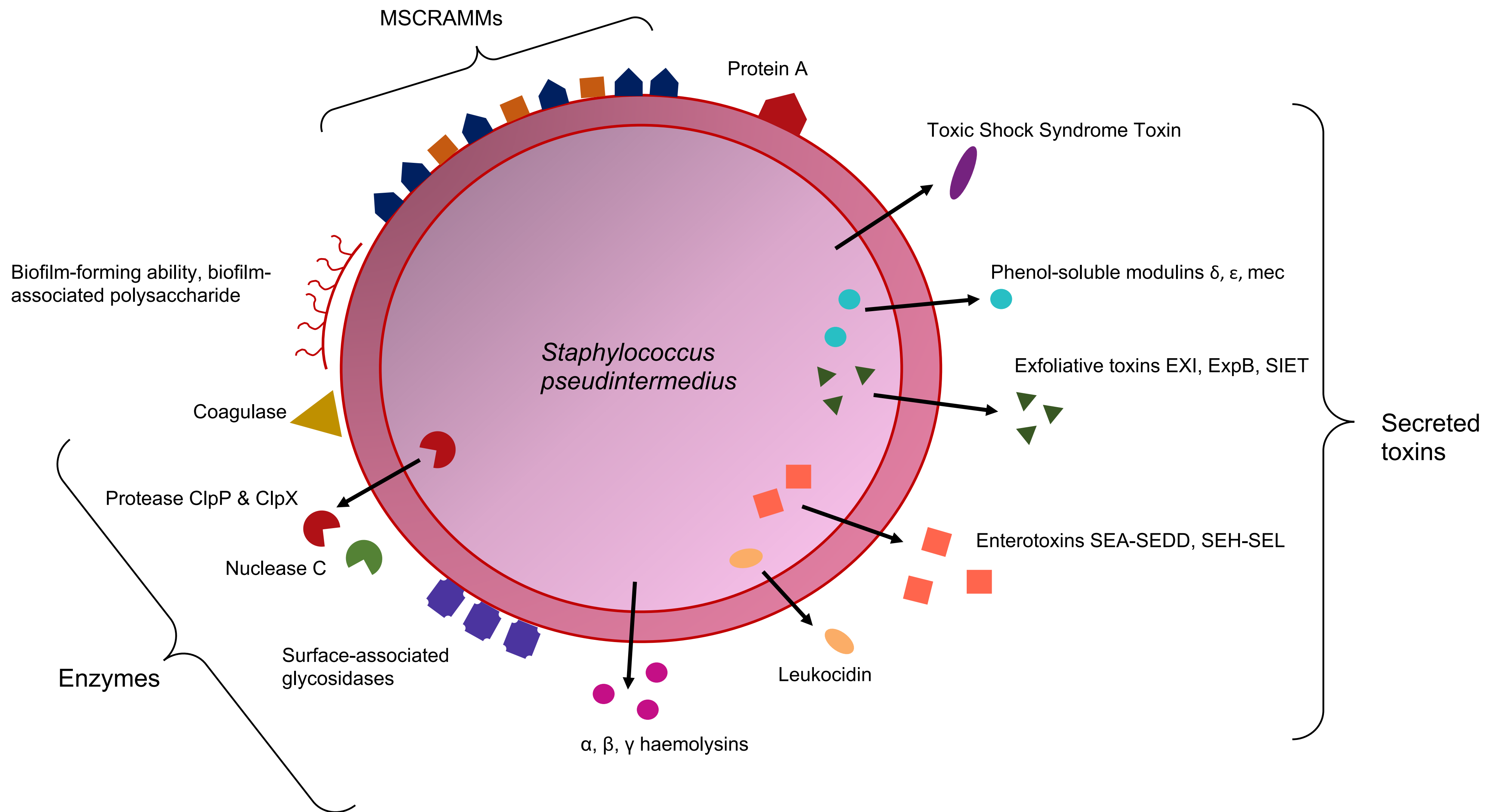

Figure 1. Virulence factors of *Staphylococcus pseudintermedius*

Although the underlying cause of skin barrier dysfunction in CAD is not entirely clear, it is central to the pathogenesis of the condition, contributing to the cycle of dysfunction and immune hyperreactivity and the consequent clinical signs. Treatment of CAD is focused on controlling the inflammatory and pruritogenic response in order to induce clinical remission (69). Rapidly acting, broadly targeting immunomodulatory agents are required; oral glucocorticoids, typically prednisone or prednisolone, are the drug of choice (69). The calcineurin-inhibitor ciclosporin may be prescribed to target acute exacerbations due to its rapid and broad effect, however glucocorticoids are often preferred (69). Whilst glucocorticoid therapy was previously rapidly tapered to avoid adverse effects, a "long and strong" approach is now favoured in order to achieve stable remission (69). Treatment of clinical exacerbations in CAD is a delicate balance; treatment must target and inhibit the pro-inflammatory immune response, however, this results in impaired defences, possibly increasing susceptibility to infections (180,181). Prolonged use of glucocorticoids can also result in damage to epidermal barriers and sebaceous gland atrophy, further increasing susceptibility to pathogenic invasion (182). However, it is essential to control the underlying inflammation and pruritus in order to restore normal skin barrier function. Once inflammation has subsided to mild levels, narrow-spectrum drugs are utilised; oclacitinib, a JAK inhibitor, or Lokivetmab, an anti-IL-31 monoclonal antibody are preferred choices (69). Both drugs inhibit cytokines involved in inflammation and pruritus, with fewer wide-spread immunomodulatory effects (69). Alongside pharmaceutical intervention, other complimentary therapies may be considered, including dietary supplementation with essential fatty acids (EFAs) (183) and nutrients such as choline, nicotinamide, histidine and inositol. These have been shown to have beneficial effects on epidermal barrier function (184), increasing the overall lipid content of the stratum corneum and improving the composition and ultrastructure (89). Emollient shampoos containing lipids, complex sugars, and antiseptics may also be recommended (98). It is important to note that these are not appropriate for use as monotherapies, but used in conjunction with immunomodulatory drugs have been shown to have modest improvements on severity of CAD (69). Identification and avoidance of triggers of clinical exacerbations is also important in the control of CAD; implementation of flea and house dust mite control regimes is recommended, alongside intradermal or allergen specific IgE testing if necessary to identify allergens (69). Antimicrobial therapy is required for the treatment of *S. pseudintermedius* infection, alongside control of atopic exacerbations. The International Committee on Allergic Diseases of Animals (ICADA) recommend

treatment with topical or systemic antimicrobials as per national or international guidelines (185,186). Most veterinarians opt for systemic therapy, with broad spectrum antimicrobials being the most frequently prescribed; amoxicillin-clavulanic acid, cefalexin, or clindamycin are often chosen for first-line treatment for bacterial skin infections (185,186). Antibiotic use, particularly broad-spectrum antimicrobials, is the main driver of selection for antimicrobial resistance (182,187), a growing issue in human and veterinary medicine (57,58,188,189); the rapid emergence of methicillin- and multi-drug resistant *S. pseudintermedius* has resulted in treatment of these infections being deemed a clinical challenge by veterinarians worldwide.

1.3 Antimicrobial resistance in S. pseudintermedius

Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) was first reported in the 1980's; since the first detected MRSP isolates in Europe in 2006 (57,58,188–190), the prevalence has rapidly increased, with MRSP now accounting for around 40% of all clinical *S. pseudintermedius* isolates (188,191–193). MRSP resides commensally on the skin and mucosa of 1.5-2% of healthy dogs (9,194), and 3.5-7% of dogs with CAD (9,195). Prior antibiotic treatment appears to be a risk factor for MRSP colonisation; one study showed that a large proportion of dogs treated for methicillin-susceptible *S. pseudintermedius* (MSSP) pyoderma were found to be MRSP carriers following treatment (191). MRSP now presents a major problem in veterinary medicine; following reports of MRSP strains resistant to all veterinary licensed antibiotics, infections caused by these organisms have been deemed untreatable by veterinarians (58,188,189,196).

Methicillin resistance is mediated by the *mecA* gene, located on staphylococcal chromosomal cassette *mec* (SCC*mec*) (39). This mobile genetic element contains a *ccr* recombinase gene complex, essential for integration into the genome and excision, alongside the *mec* gene complex (196,197). Other antimicrobial resistance genes may also be located on SCC*mec* (198–200). *mecA* encodes an alternative penicillin binding protein, PBP2a, conferring resistance to virtually all β-lactam antibiotics, including penicillins, lactam-lactamase inhibitor combinations, cephems, and carbapenems (201). SCC*mec* sequence can be used to "type" staphylococcal strains and establish their epidemiology (202). Various SCC*mec* types are found across MRSP lineages, demonstrating independent acquisition of these elements by different strains (58,197). SCC*mec* can be acquired via

horizontal gene transfer (HGT) (203); SCC*mec* elements closely resembling those found in *S. aureus* and coagulase-negative staphylococci (CoNS) including *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* have been detected in MRSP strains*,* highlighting the role of HGT from other staphylococci, particularly CoNS, in the emergence of MRSP (203). Novel SCC*mec* variants have been detected in two unrelated MRSP sequence types (STs), presenting evidence for the ongoing diversification of SCC*mec* elements via recombination (204). Multilocus sequence typing revealed considerable clonal diversity amongst MSSP lineages, with over 60 STs identified from a sample of 89 isolates (56,196,205,206), whilst MRSP lineages appear to display less diversity (205,206); two major endemic MRSP lineages are seen, with ST71 predominating in Northern Europe, and ST68 in North America (57,58,191,207–211).

Methicillin-resistance is often associated with a multi-drug resistant phenotype (39,57,58,188,197,207,212–215). The ability to readily accumulated antimicrobial resistance genes is a noted quality of staphylococci, including *S. pseudintermedius* (39,58)*.* Up to 30% of *S. pseudintermedius* isolates display multi-drug resistance (resistant to three or more classes of antimicrobials) (39,57,191,216); a study at a small animal dermatology referral clinic found 23% of *S. pseudintermedius* isolates to be resistant to five or more classes of antibiotics (188). It has been demonstrated that only a three-step process is required for MRSP isolates to accumulate a multi-drug resistant (MDR) genotype, this involves: acquisition of an SCC*mec* element, a Tn5405-like element, and single nucleotide polymorphisms (SNPs) in *gryA/grlA* (58,188,203,217)*.* Tn5405-like elements encode up to five antimicrobial resistance genes, conferring resistance to antibiotics including kanamycin, streptomycin, and streptothricin (203). SNPs in the *gyrA/gyrB* and *grlA/grlB* genes, encoding topoisomerase II and IV, respectively, confer resistance to fluoroquinolones (26,197). Fluoroquinolone resistance is associated with the global success of MDR MRSP lineages, with widespread fluoroquinolone use thought to promote the survival and spread of MRSP similar to that of nosocomial methicillin-resistant *S. aureus* lineages (58,205,218,219). MRSP lineages frequently display resistance to other antimicrobial classes, including macrolides, lincosamides, and tetracyclines (58,197). Two variants of the *tet* gene confer tetracycline resistance; *tet*(M) confers resistance to tetracycline, doxycycline and minocycline, whilst *tet*(K) confers resistance only to

tetracycline (181,212,220). As a result of MDR genotypes, therapeutic options for MRSP infections are extremely limited.

MRSP is not only a concern in veterinary medicine; recently, the zoonotic potential of *S. pseudintermedius* has been highlighted. Several humans have been found to be colonised by *S. pseudintermedius,* both MR- and MSSP strains. This colonisation is typically transient, and associated with regular contact with dogs (19–21,24,221); owners of dogs with pyoderma have been found to be at higher risk of colonisation with MRSP compared to non-dog owners (221). Human carriage was not seen following clearance of pyoderma in the dogs. Studies have revealed the strains isolated from humans are usually closely phylogenetically related to, or identical to, strains found on dogs, suggesting dog-human transmission (20,24,42,221). MRSP carriage rates are particularly high amongst veterinary professionals, especially those working in dermatology referral centres (222). Nosocomial spread of MRSP strains within veterinary clinics has been reported (206,223,224), in some cases suspected to be facilitated by human carriage (24). Whilst carriage in humans is often harmless, there is concern regarding the potential for horizontal transfer of genetic resistance elements, which contributes to the emergence of MDR bacterial strains, thus posing a risk to public health (19,221). *S. pseudintermedius,* including MRSP, has also been implicated in human disease, most commonly skin and soft tissue infections, often associated with dog bite-wounds, but also implant-associated infections, pneumonia, and brain abscesses (10,42,225–227). In nearly all cases, contact with dogs was suspected to be the source of infection. It has been suggested that *S. pseudintermedius* involvement in human infections may be underestimated, with clinical isolates being mis-identified as *S. intermedius* or *S. aureus* by laboratories relatively frequently (2,39,228). The risk of human infection, alongside the dissemination of antimicrobial resistance determinants, makes MRSP a public health concern, and a threat to human and animal health. Thus, there is an urgent need for the development of alternative treatments for *S. pseudintermedius* infections.

1.4 Role of the microbiota in the pathogenesis of atopic skin infections

The microbiota is a complex microbial community residing on the surfaces of and within the host body (229,230), and is thought to play a significant role in both health and disease (231). The microbiota is an essential component of the skin barrier, which represents the first line of defence against

pathogens and allergens, with the bacteria residing on the surface contributing to the protective role (232). The skin microbiota protects from disease by helping to maintain skin barrier function, preventing colonisation by pathogens, and via immune modulatory functions (11,233–236). Commensal bacteria interact with the cutaneous immune system to maintain homeostasis (237–240); this is not only important in maintaining the ability to mount an appropriate immune response against pathogens (240,241), but also for the development of tolerance towards resident microbes (233,242–244). Failure to develop tolerance towards commensal organisms is associated with immune-mediated disorders, including atopic dermatitis (AD) (245,246). The early life microbiota in humans has been linked to protection from or predisposition towards developing AD (247–249); whilst previous studies focused on the gastrointestinal microbiota and its role in immune-education via the gut-skin axis (247,248), recent studies have revealed that there are significant differences in the skin microbiota composition of infants who go on to develop AD versus those who do not (241,249,250). In particular, a higher abundance of CoNS, including *Staphylococcus hominis* and *S. epidermidis* is associated with protection from the development of AD (249). No relationship between early life skin microbiota and development of AD has been revealed in dogs (251), but this is not an area that has been intensively studied, and so it is possible that the skin microbiota may be involved in predisposing dogs to AD, as is seen in humans.

Disruption to the normal microbiota results in a state known as dysbiosis, typically characterised by decreased microbial diversity and dominance of one species (252). Dysbiosis of the skin microbiota has been implicated in the pathogenesis of exacerbations of AD in humans and dogs (245,252–257). These specific, large-scale alterations to the skin microbiota during atopic exacerbations are thought to be associated with the establishment of staphylococcal skin infections (245,252–255,258). It has yet to be elucidated whether dysbiosis precedes immune and cutaneous barrier dysfunction, or if it is a consequence of this disruption (11,252,259). The inflammatory and hyperreactive immune response to allergens alters the cutaneous environment and could contribute to disruption of the microbiota; a study in dogs noted dysbiosis in experimentally allergen-challenged (lesional) atopic skin, but not in contralateral unchallenged (non-lesional) skin, suggesting that the cutaneous allergen response plays a key role in disruption to the microbiota (259). However, other studies have reported changes in the microbiota composition before the onset of atopic exacerbations (245), suggesting dysbiosis is key in

initiating atopic exacerbations, possibly due to downregulation of skin barrier maintenance, immune modulation and protection from infection by the microbiota (260). There is no clear “starting point” to the cycle of disruption to the epidermal barrier, cutaneous immune system, and skin microbiota; both endogenous and exogenous factors contribute to this complex disorder, however it is apparent that dysbiosis of the skin microbiota is central to exacerbations of atopic dermatitis and the establishment of staphylococcal infections in both humans and dogs (92,261).

The skin microbiota begins to develop shortly after birth; in dogs there is a high degree of variability in microbiota composition between individuals (11,262,263). This has been attributed to differences in environment (housed indoors or outdoors, rural or urban habitat) and hygiene, alongside changes in hormone levels related to age (251). Adult dogs displaying a more diverse population of bacteria in their microbiota (251), in healthy adult dogs, the composition of the skin microbiota is stable over time (263). The methods used for both collection and identification of bacteria from the skin can influence the results seen; studies vary in their techniques, with body location, sample type, and sample-area preparation not standardised, which may affect the species and relative abundances seen. Isolation methods yield different results depending on how samples are processed before plating and the choice of media, amongst other variable factors. As studies have moved from culture-based to genomic identification, using 16s rRNA sequencing, considerably greater diversity in the dog skin microbiota than previously thought has been revealed (11). The predominant phyla present within the cutaneous microbiota of dogs include Proteobacteria, Firmicutes, Actinobacter and Bacteroidetes (11,234,263–266); this resembles the phyla seen in humans (11), although greater diversity has been noted in dogs (267). Studies have reported the most abundant genera colonising the dog skin include *Acinetobacter* spp., *Pseudomonas* spp., *Streptococcus* spp., *Micrococcus* spp., *Clostridium* spp., and *Porphyromonas* spp. (11,21,234,251,264,265,268), with transient members of the microbiota including *Escherichia coli, Proteus mirabilis, Corynebacterium* spp. and *Bacillus* spp. (11). *Cutibacterium acnes* (previously *Propionibacterium*) has also been found to be abundant within the dog skin microbiota (251). *Staphylococcus* spp. are common colonisers of canine and human skin (11,230,234,264,265); as previously discussed, *S. pseudintermedius* is a readily isolated commensal coloniser of the skin microbiota of healthy dogs (4,10,11). CoNS species are also a major component of the dog and human skin microbiota (27,264,269,270); species such as *S. epidermidis,*

Staphylococcus, xylosus, Staphylococcus saprophyticus, and *S. hominis* can be found colonising healthy dogs (25,27,264).

During atopic exacerbations, atopic skin is characterised by a trend of decreased diversity in the microbiota (245,264,265,271,272); this is seen on both lesional and non-lesional skin (11,259,264,265), again demonstrating the involvement of macroscopically unaffected skin in atopic dermatitis. A decreased abundance of CoNS in atopic individuals compared to healthy controls has been noted in humans and dogs (253,265), whilst a decrease in Gram-negative commensals has been reported in humans only (273). A significant increase in the abundance of *S. pseudintermedius* is seen during exacerbations compared to the same sites on healthy dogs (9,259,261,264), with an inverse relationship between relative abundance of *S. pseudintermedius* and diversity on the skin (259,264), highlighting the involvement of the microbiota in the pathogenic overgrowth of *s. pseudintermedius* and consequent establishment of infection.

The microbiota exists in a delicate balance; disruption to the microbial community or their environment is a key mechanism in the establishment of *S. pseudintermedius* skin infections. The cutaneous inflammation associated with atopic exacerbations creates a microenvironment that favours the growth of coagulase-positive staphylococci (CoPS; *S. aureus* and *S. pseudintermedius)* (260). Reduction of the number of commensal organisms colonising the skin likely results from both the inflammation and the increased growth of *S. pseudintermedius* (253,274). The resulting deficiency in commensal organisms means that their protective role is downregulated, potentially contributing to the pathogenic proliferation of *S. pseudintermedius* and the consequent establishment of infection (234,260,264). Commensal bacteria protect from infection via several mechanisms; the most basic form of protection is due to competition for resources and adherence ligands on the skin surface (231,250,253,274–276). CoNS are the main species sharing an ecological niche with *S. pseudintermedius* and thus compete for the same limited nutrients (92), helping to control the growth of *S. pseudintermedius.* Commensal bacteria can impair pathogenic growth through the production of toxic metabolites (277,278); commensals such as *Dermacoccus* and *Deinococcus* spp. produce secondary metabolites with both anti-inflammatory and anti-microbial properties (279). A deficiency of both genera is seen in atopic skin in humans compared to healthy individuals, potentially contributing

to CoPS overgrowth in AD (256). *Cutibacterium acnes,* a resident of both healthy human and canine skin, possesses several lipases, which are utilised to digest sebum lipids into toxic fatty acids, which interfere with the growth of potentially pathogenic microorganisms, including CoPS (277,278). The commensal bacteria residing in the skin microbiota, particularly CoNS, have also been shown to disrupt the virulence of *S. aureus,* thus limiting its ability to establish infection. *S. epidermidis* secretes an array of proteases that can disrupt *S. aureus* biofilm formation and prevent surface colonisation (280–284). Other CoNS, including *S. hominis* and *Staphylococcus simulans,* have the ability to produce autoinducing peptides and phenol-soluble modulins (PSMs) that repress *S. aureus agr* signalling (276,285). Disruption of *agr*-mediated quorum sensing interferes with the ability of *S. aureus* to colonise host tissue and produce virulence factors such as PSMs that that contribute to barrier disruption and inflammation in AD (276,285,286). Certain CoNS-derived PSMs display the ability to selectively kill pathogens such as *S. aureus,* and can synergise with host AMPs to enhance antimicrobial activity (282). *S. pseudintermedius* shares many virulence factors with *S. aureus,* and so it is likely that these mechanisms may play a role in protection against *S. pseudintermedius* infection in healthy dogs. Thus, the reduction in commensal organisms, particularly CoNS, during dysbiosis contributes to the pathogenesis of *S. pseudintermedius* infections in atopic dermatitis.

The ability of commensal bacteria to not only prevent colonisation and growth of pathogens but also to inhibit processes central to virulence, such as quorum sensing and biofilm formation, has led to considerable interest in bacteriotherapy; targeted manipulation of the microbiota, utilising bacteria or bacterial components (287). Bacteriotherapy has shown great success in the treatment of multi-drug resistant *Clostridium difficile* infections in the gastrointestinal tract via transplantation of faecal material from a healthy donor to restore the microbiota (288). The central role of the skin microbiota in the pathogenesis of AD and *S. aureus*-mediated exacerbations has resulted in the exploration of manipulation of the skin microbiota as a novel therapeutic for AD in humans. Bacterial-derived lysates and enzymes, probiotics, and skin microbiota transplants have been proposed as possible treatment options (272,289–294). Following observations that certain Gram-negative organisms exert anti-inflammatory effects on human skin, Guineache *et al*. investigated the application of lysates from the Gram-negative bacterium *Vitreoscillia filiformis* to the skin, resulting in improvements to the clinical signs of AD compared to control treatments (295). Similarly, application of another Gram-negative

organism, *Roseomonas mucosa,* isolated from healthy human volunteers, led to reduced inflammation and erythema in an experimental mouse model of AD, whilst *R. mucosa* isolated from atopic volunteers did not lead to any improvement in clinical signs (272,273). Studies have explored the application of probiotic strains, such as *Lactobacillus salivarius* and *Lactococcus lactis,* to inhibit pathogenic growth, restore the microbiota, and decrease the severity of clinical signs in AD (292,293), however, these strains are not native to the skin and so are not adapted to adhere and proliferate in the cutaneous environment (292), limiting their therapeutic benefit in AD. Nakatsuji *et al.* (253) noted that a population of CoNS present in healthy human individuals was deficient in those with AD, and this deficiency was particularly evident on those colonised by *S. aureus;* those possessing a normal abundance of CoNS within the skin microbiota were not colonised by *S. aureus.* Application of these CoNS strains, belonging to the species *S. hominis* and *S. epidermidis,* in animal and human models greatly reduced *S. aureus* colonisation. The inhibitory strain *S. hominis* A9 was found to produce two antimicrobial compounds, *Sh*-lanticiotic-α and *Sh*-lanticiotic-β. Application of the purified substance or the producing strain inhibited *S. aureus in vitro* and *in vivo,* whilst application of a non-producing strain of *S. hominis* had no effect on *S. aureus,* demonstrating this protection against *S. aureus* colonisation is due to the production of *Sh*-lanticiotic-α and *Sh*-lanticiotic-β (253). These substances are bacteriocins; peptides secreted by many commensal bacteria to protect against pathogenic invasion and promote survival in competitive niches (296,297). The application of purified bacterial components is preferable to live bacterial transplantation as a therapeutic intervention, as this can be more precisely regulated, and removes any risk of horizontal gene transfer between strains, which is a mechanism for the dissemination of antimicrobial resistance elements or conversion to virulence of typically non-pathogenic strains (27,298–300). Bacteriocins have been suggested as alternative treatments for *S. aureus* infections, including those seen in atopic dermatitis (301); the similar virulence profile of *S. pseudintermedius* and the shared pathogenesis of canine and human AD means bacteriocins could present a suitable treatment for atopic *S. pseudintermedius* skin infections. The specific killing of pathogens such as *S. aureus* and *S. pseudintermedius*, including multi-drug resistance strains, whilst not disrupting commensal organisms and contributing to dysbiosis, allows for specific targeting of *S. pseudintermedius* and restoration of the microbiota (234,302). Thus, bacteriocins present a promising opportunity to exploit the microbiota for novel therapeutics against *S. pseudintermedius* infections in canine atopic dermatitis (235).

1.5 Bacterial-produced antimicrobial peptides

Bacteriocins are ribosomally synthesised antimicrobial peptides, secreted by bacteria to aid survival within an ecological niche (274,303–307). It is thought that up to 99% of bacteria strains produce bacteriocins (308), suggesting they are central to bacterial fitness. Bacteriocin-encoding genes can be located chromosomally, or more frequently, on mobile genetic elements such as plasmids (296,309); there is evidence to suggest horizontal transfer of bacteriocin genes within and between species (310). Bacteriocin resistance/immunity genes, encoding antagonistic receptors or efflux transporters, are expressed concomitantly with structural genes (304,311,312), deeming producing-strains resistant to their own bacteriocins. Bacteriocins are typically active against strains closely related to, or sharing a niche with, the producer strain (303,304), although some Gram-positive derived bacteriocins have been shown to demonstrate a broader spectrum of inhibitory activity, acting against a range of other Gram-positive organisms (313). The antimicrobial activity possessed by bacteriocins can be bacteriostatic or bactericidal. Bacteriocins produced by Gram-positive bacteria, such as staphylococci (referred to as “staphylococcins”), are categorised into four classes, each with several subclasses, based on size and structure (305,314) (Figure 2). Class Ia, lantibiotics, are the most abundant form of bacteriocins; ten have been identified from *Staphylococcus* spp. (310,315–320). These are post-translationally modified molecules, containing lanthionine or β-methyllanthionine residues, with a molecular weight below 5 kDa (304,313). Lantibiotics typically exert antimicrobial activity by inducing cell lysis, through either membrane potential-dependent permeabilisation or transmembrane pore formation (296,321,322), however some have been shown to inhibit peptidoglycan synthesis, thus inhibiting growth and division (323–326). Class III bacteriocins are large (>10 kDa), heat labile enzymes, with lytic (IIIa) or non-lytic (IIIb) activity (327,328). Lysostaphin, a class IIIa metalloprotease, produced by *S. simulans* biovar *staphylolyticus* ATCC1362, was one of the first staphylococcins to be discovered and studied (329). The inhibitory activity of lysostaphin is a result of hydrolysis of pentaglycine cross-links in the peptidoglycan cell wall of other bacteria (330,331). The mechanisms of action of other classes and subclasses of bacteriocins have yet to be elucidated.

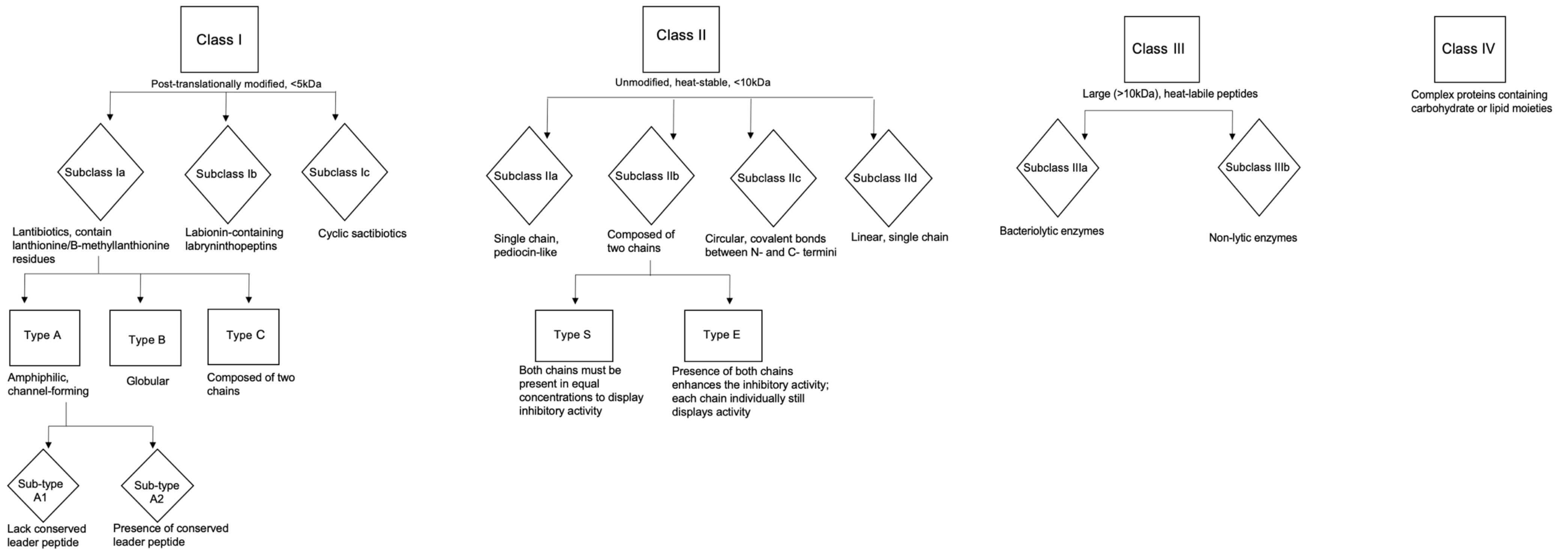

Figure 2. Classification of Gram positive-derived bacteriocins (Taken from Newstead *et al.* (301)).

Several bacteriocins derived from CoNS have demonstrated *in vitro* efficacy against a range of pathogens, including *S. aureus,* such as Pep5, epidermin, gallidermin, hominicin, hyicin 4244 and hyicin 3682, isolated from *S. epidermidis* 5, *S. epidermidis* Tü3298, *Staphylococcus gallinarum* F16/P57 Tü3298, *S. hominis* MBBL2–9, *Staphylococcus hyicus* 4244 and *S. hyicus* 3682, respectively (314–316,318,321,332–337). Pep5, epidermin, gallidermin and hominicin are effective against methicillin-resistant *S. aureus* strains, and the epidermin-like group of bacteriocins are also able to repress biofilm formation by *S. aureus,* a potentially valuable traits for an antimicrobial agent (318,321,338). Lysostaphin is effective against *S. aureus,* including MRSA, both *in vitro* and *in vivo,* showing eradication of *S. aureus* in rodent models of colonisation and infection, and in humans (331,339–349); application of lysostaphin to catheters was able to inhibit *S. aureus* biofilm formation and disrupt pre-formed biofilms (345,349). Epidermicin NI01 is also effective against *S. aureus* in greater wax moth and cotton rat models (350–352). Together the *in vivo* activity of lysostaphin and epidermicin NI01 suggests bacteriocins could potentially be used clinically. Capidermicin, a lantibiotic produced by *Staphylococcus capitis* CIT060, also displayed inhibitory activity against *S. pseudintermedius* (353); it is possible that other staphylococcins are active against *S. pseudintermedius* due to its resemblance to *S. aureus*, however most bacteriocins have not been tested for activity against *S. pseudintermedius.* Several bacteriocin-like inhibitory substances (BLIS) have been identified from staphylococci but not yet characterised (235,284,354–357), several of which are thought to represent novel bacteriocins, not matching any currently known bacteriocins in databases (235). In one study of 441 staphylococci, five species were shown to produce BLIS, however the use of genome mining tools revealed that 95 of the isolates possessed bacteriocin-related gene clusters, despite not displaying any antimicrobial activity (358). This suggests there are many staphylococcal-produced bacteriocins yet to be discovered or characterised, which may be of clinical use in human and veterinary medicine.

Table 1. Well-characterised bacteriocins isolated from *Staphylococcus* species, including those tested for *in vitro* inhibitory activity against coagulase-positive *Staphylococcus* species, with minimum inhibitory concentration (MIC) where provided.

Class	Subclass	Subtype	Bacteriocin	Producing strain	*In vitro* activity against (MIC)	References
I	Ia	A1	BacCh91	*S. aureus* CH9/DSM26258	*S. aureus* (4.0-6.0 µM), *S. pseudintermedius* (1.5 µM)	(359)
			Epicidin 280	*S. epidermidis* BN280		(317)
			Epidermin	*S S. epidermidis* Tü3298	*S. aureus*	(314,318)
			Epilancin 15X	*S. epidermidis* 15X154		(319)
			Epilancin K7	*S. epidermidis* K7		(320)
			Gallidermin	*S. gallinarum* F16/P57 Tü3298	*S. aureus* (1.25-8.0 µg/ml)	(321,333,334)
			Hominicin	*S. hominis* MBBL2-9	*S. aureus* (0.06-3.82 µg/ml)	(316,336)
			Hyicin 3682	*S. hyicus* 3682	*S. aureus*	(315,337)
			Nisin J	*S. capitis* APC2923	*S. aureus*	(310)
			Pep5	*S. epidermidis* 5	*S. aureus*	(309,314,318)
		A2	Nukacin ISK-1	*S. warneri* Nukadoko/*S. simulans* 3299		(360)
			Warnericin RB4	*S. warneri* RB4		(361)
	Ic		Hyicin 4244	*S. hyicus* 4244	*S. aureus*	(332,335)
II	IIb	S	Aureocin A70	*S. aureus* A70		(362)
		S	C55	*S. aureus* C55		(363)
	IId		Aureocin A53	*S. aureus* A53		(364)
			BacSp222	*S. pseudintermedius* 222	*S. aureus* (0.89-1.3 µM), *S. pseudintermedius* (0.16-2.56 µM)	(365)
			Capidermicin	*S. capitis* CIT060	*S. aureus* (3.1-10 µg/ml), *S. pseudintermedius* (10 µg/ml)	(353)
			Epidermicin NI01	*S.epidermidis* 224		(350)
III	IIIa		Endopeptidase ALE-1	*S. capitis* EPk1		(366)
			Lysostaphin	*S. simulans* biovar Staphylolyticus ATCC1362	*S. aureus* 0.002-100 µg/ml)	(329,348,367)
IV			Aureocyclin 4185	*S. aureus* 4185		(368)

Bacteriocins are used as antimicrobial agents in a variety of non-clinical settings; the bacteriocin nisin was granted "generally regarded as safe" status and is widely used in food preservation (369,370), and bacteriocin-enriched teat seals have been explored for the control of bovine mastitis (371,372), however fewer studies have investigated the use of bacteriocins as novel antimicrobials to treat bacterial infections in clinical settings. The absence of toxicity against eukaryotic cells and efficacy in *in vivo* models suggest bacteriocins could be suitable for clinical use in humans and other mammals (321,339,342–344,347,348,350). As peptides, bacteriocins are amenable to bioengineering to improve suitability as pharmaceutical agents, including improved potency, solubility, and stability (323,373). The narrow spectrum of activity displayed by bacteriocins minimises disruption to the microbiota and generates less selective pressure for the development of resistance (374), making them ideal candidates as antimicrobial treatments; the distinct mechanisms of action also means they are suitable for treatment of antibiotic resistant, including multi-drug resistant, infections (323,375). As abundant members of the skin microbiota of healthy dogs, CoNS protect from pathogenic invasion; bacteriocin production is likely a key mechanism by which they offer this protection, which is downregulated in conditions such as canine atopic dermatitis. Thus, it is possible this protection offered by the healthy microbiota can be exploited to develop alternative treatments for *S. pseudintermedius* infections in dogs. CoNS are prolific bacteriocin producers, and phylogenetic relatives of *S. pseudintermedius,* residing within the same niche, and so it is highly possible that bacteriocins produced by CoNS within the dog skin microbiota could be promising antimicrobial agents for the treatment of *S. pseudintermedius* infections. As such, this project aims to:

- Isolate and identify CoNS from the skin of healthy dogs.
- Investigate the antimicrobial resistance profiles of CoNS residing on the skin of healthy dogs.
- Identify isolates possessing antimicrobial activity against *S. pseudintermedius.*
- Identify and characterise the bacteriocins produced by CoNS strains that mediate the inhibition of *S. pseudintermedius* to assess their suitability as novel antimicrobials for *S. pseudintermedius* infection in canine atopic dermatitis.

2. Materials and methods

2.1 Sample collection

Skin swabs were collected from healthy dogs (*i.e.* not currently receiving treatment for any illness, had not received antibiotic treatment in the three months prior to sample collection, and without a history of skin conditions, including canine atopic dermatitis (CAD)), visiting The Hospital for Small Animals (Royal (Dick) School of Veterinary Studies, Edinburgh), and AniCura Animal Clinic (Stockholm, Sweden) for routine check-ups or procedures. A pooled swab was collected from the nares, axilla, groin, and perineum of each dog using charcoal transport swabs; swabs were rolled on each area for 20-30 seconds. Samples were stored at 4°C before being transported to the laboratory at ambient temperature. Swabs were inoculated into 2 ml Tryptone Soya Broth (TSB) (Oxoid, Basingstoke, UK) with 6.5% NaCl and incubated overnight at 37°C (static), before being supplemented with 20% glycerol (vol/vol); the addition of NaCl allows for crude selection of staphylococcal species which are relatively halotolerant, whilst glycerol prevents damage and lysis of bacterial cells when frozen. Inoculated broths were stored at -80°C until needed.

2.2 Bacterial isolation, identification and antimicrobial susceptibility testing

20 µl of inoculated broth was streaked onto mannitol salt agar (Oxoid, Basingstoke, UK) and incubated aerobically at 37 °C overnight. Colonies displaying distinct morphologies were picked and sub-cultured for single colonies onto Columbia horse blood agar (Oxoid, Basingstoke, UK), and incubated for 24 hours at 37°C, aerobically. Isolates were Gram stained, and any resembling staphylococci (Gram-positive clustered cocci) were identified using the VITEK®2 (using GP cards; BioMérieux, Basingstoke, UK). Samples for the VITEK®2 were prepared per the manufacturer's instructions; pure colonies were picked with sterile swabs and used to inoculate 3 ml of 0.45% saline, and vortexed to obtain a homogeneous solution. Saline was inoculated to a turbidity equivalent to 0.50-0.63 McFarland, measured using the Densichek Plus (BioMérieux, Basingstoke, UK). Antimicrobial susceptibility testing for 21 antimicrobials was carried out for all isolates using the VITEK®2 (using AST-GP80 cards).

Coagulase testing was carried out for any isolates identified as *S. schleiferi* to determine subspecies, per manufacturer's instructions (Biosera, Nuaille, France).

2.3 Screening for antimicrobial activity

2.3.1 Deferred antagonism assay

All CoNS isolates were screened for antimicrobial activity against *S. pseudintermedius.*
Spot-on lawn assays were carried out to identify inhibitory activity of CoNS isolates, as described in (376). Briefly; three colonies from each agar plate, containing a single pure CoNS isolate, were used to inoculate 10 ml of TSB. Inoculated broth was incubated for 18 hours at 37°C, shaken (200 rpm). 10 µl of inoculated broth was pipetted onto the surface of Muller-Hinton agar to form a “spot”; square, 12 cm diameter agar plates were used. Seven samples were spotted onto each agar plate, plus controls. *Lactococcus lactis* CECT4433 (nisin-producing) was used as the positive control; these were cultured in MRS broth for 18 hours statically at 30 °C. Non-inoculated TSB was used as the negative control. Following incubation, plates were overlaid with 50ml of soft (0.7%) Muller-Hinton agar seeded with 0.5 ml (~10^6 CFU) of one of the following indicator strains: methicillin-resistant *S. pseudintermedius* (MRSP1), or methicillin-susceptible *S. pseudintermedius* (MSSP1) (Table 2). Plates were incubated overnight at 37°C, and clear zones of inhibition were measured in millimetres using a ruler the following day.

CFU per ml was calculated for test CoNS and indicator *S. pseudintermedius* isolates during every assay using 10-fold serial dilutions in 0.85% saline to give dilutions of 10^{-1} to 10^{-6}. 20 µl of diluted culture was pipetted onto Columbia blood agar and incubated for 18 hours at 37°C, aerobically, before colonies were counted to calculate CFU. A dilution of 10^{-6} gave a countable number of colonies (30-300).

Table 2. Coagulase-positive *Staphylococcus* isolates used as indicator isolates in deferred antagonism and CFS microtiter assays. All strains are clinical isolates collected at The Hospital for Small Animals, R(D)SVS. Multilocus sequence type (ST) provided where known.

Study reference	Species	Methicillin susceptibility	Isolate	Source
MRSP1	*S. pseudintermedius*	resistant	6109/25111 (ST71)	canine pyoderma
MRSP2	*S. pseudintermedius*	resistant	6112/66182 (ST726)	canine pyoderma
MRSP3	*S. pseudintermedius*	resistant	6127/64107 (ST41)	canine pyoderma
MSSP1	*S. pseudintermedius*	susceptible	62130/54800 (ST1195)	canine pyoderma
MSSP2	*S. pseudintermedius*	susceptible	6125/66074 (ST1191)	canine pyoderma
MSSP3	*S. pseudintermedius*	susceptible	6134/64963 (ST1183)	canine pyoderma
MRSA	*S. aureus*	resistant	88744	canine wound
MSSA	*S. aureus*	susceptible	88300	canine wound
MRSS	*Staphylococcus schleiferi* subsp. *schleiferi*	resistant	p5309 (8117)	canine nasal swab
MSSS	*S. schleiferi* subsp. *schleiferi*	susceptible	78868/335221	canine pyoderma

Isolates that displayed inhibitory activity against either MRSP1 or MSSP1 via spot-on assay were screened for inhibitory activity against various indicator isolates using agar-based antagonism assays. Three assays were used; spot-on (as described above), well-diffusion (WDA) and cross-streak. For all assays, nisin$^+$ *L. lactis* and uninoculated TSB were used as positive and negative controls, respectively. Well-diffusion assays were carried out as follows; indicator strains were grown overnight in nutrient broth (Oxoid, Basingstoke, UK), at 37ºC (static), before being diluted in broth to ~10^5 CFU/ml, and swabbed across the surface of Muller-Hinton agar plates. Producer strains were grown in TSB overnight (37ºC, 200 rpm). Wells were punched in the agar plates using a sterile pipette tip; 10 µl of inoculated broth containing a producer strain was added to each well. Plates were incubated for

24 hours, before results were recorded. A cross-streak assay was carried out by growing producer strains overnight on Columbia horse blood agar (Oxoid, Basingstoke, UK), colonies were picked and diluted in PBS to a density equivalent to 0.5 McFarland turbidity standard. Indicator isolates were grown in TSB overnight (37ºC, 200 rpm) before being diluted in TSB to ~10^5 CFU/ml. 15 μl of inoculated PBS was streaked onto Muller-Hinton agar plates; three isolates were streaked onto each plate, with each streak approximately 5 mm thick. Plates were incubated for 24 hours at 37ºC, before the agar was turned over, and 20 μl of indicator isolate was spread. Plates were incubated for a further 24 hours, before results were recorded. Indicator strains used in the deferred antagonism assays shown in Table 3.

Table 3. Indicator species used in deferred-antagonism assays, including five Gram-negative organisms and eleven Gram-positive (*Streptococcus* species and CoNS)

Species	Strain
Aceintobacter baumannii	NCTC 12156
Escherichia coli	ATCC 25922
Klebsiella pneumoniae	NCTC 13438
Pseudomonas aureginosa	NCTC 10662
Streptococcus canis	NCTC 12191
Streptococcus uberis	CCUG 17930
Yersinia pseudotuberculosis	NCTC 10275
Staphylococcus chromogenes	NCTC 10530
Staphylococcus devriesei	DSM 25293
Staphylococcus epidermidis	NCTC 11047
Staphylococcus felis	DSM 7377
Staphylococcus haemolyticus	NCTC 11042
Staphylococcus hominis subsp. *hominis*	ATCC 27844
Staphylococcus saprophyticus subsp. *saprophyticus*	ATCC 15305
Staphylococcus simulans	NCTC 11046
Staphylococcus xylosus	ATCC 29971

2.3.2 Cell-free supernatant microtiter assays

Cell-free supernatant assays were used to further investigate inhibitory activity of isolates that demonstrated inhibitory activity in the deferred antagonism assay, along with species-matched non-inhibitory control counterparts. The use of cell-free supernatant distinguishes activity due to secreted antimicrobial compounds versus potential inhibition due to competition or toxic secondary metabolites,

whilst optical density readings obtained from the microplate reader allow for quantifiable bacterial growth and inhibition data. CoNS isolates were grown in TSB (37°C, 200rpm) for 18 hours. Crude cell-free supernatant (CFS) was obtained by centrifuging liquid cultures at 5,000rpm for 10 minutes, the supernatant was filtered using 0.22 µm syringe filters (Sartorius AG, Göttingen, Germany), transferred to a sterile tube, and the pellet disposed of. CFS was stored at -20°C until required.

Assays were carried out using flat-bottom 96-well plates; 100 µl of CFS was added to each well, containing 100 µl of indicator isolate (10^3 or 10^5 CFU) (Table 2). CFS derived from *L. lactis* CECT4433, and non-inoculated TSB were used as the positive and negative controls, respectively. Optical density was measured at 10-minute intervals during a 24-hour incubation period (37°C, 200rpm) using the CLARIOstar® microplate reader (BMG Labtech, Ortenberg, Germany). Raw and blank-corrected (using a TSB blank well) data was recorded. Isolates were tested in triplicate in each assay.

CFS assays were carried out using further indicator strains; including eight clinical CoPS isolates (Table 2) (all genome sequenced clinical isolates obtained from dogs visiting The Hospital for Small Animals, R(D)SVS), and 17 clinical *S. pseudintermedius* isolates (again collected from dogs visiting The Hospital for Small Animals, but currently without genome sequences available). Inhibitory activity against thirteen *S. pseudintermedius* and seven *S. aureus* isolates, obtained from the healthy dog skin swabs collected for this study, was also tested. All indicator strains were grown in TSB for 18 hours (37°C, 200rpm); 100 µl (10^5 CFU) was added to 100 µl CFS, and optical density measured as described above.

2.4 Characterisation of inhibitory activity

2.4.1 Effect of growth conditions on inhibitory phenotype

Growth conditions can influence gene expression; expression of bacteriocin-related genes has been shown to be upregulated in nutrient-limited conditions, particularly iron-limited, to enhance bacterial survival (235,275). Isolates that displayed inhibitory activity in the deferred antagonism assay were grown under nutrient-limited conditions to measure any effect on inhibitory phenotype. This was done via two methods: firstly, isolates were grown in TSB as described previously, but incubated for 48

hours rather than 18 hours to deplete nutrients within the broth. Secondly, isolates were grown in TSB with 2,2'-dipyridyl (Sigma-Aldrich, Gillingham, UK), an iron-chelating compound. Dipyridyl was prepared as a 300 µM solution in TSB and autoclaved to dissolve. Following growth in broth, CFS was obtained and used to screen for inhibitory activity against MRSP1 using microtiter assays, as previously described. Dipyridyl-free and dipyridyl-added positive and negative controls were used.

2.4.2 Semi-quantification of antimicrobial activity

Microtiter assays were repeated as described previously, using MRSP1 and MSSP1 (10^3 or 10^5 CFU) (Table 2) as the indicators, with CFS diluted two-fold in 0.85% saline to allow minimum inhibitory concentration of the CFS to be calculated. Isolates were tested in triplicate.

2.4.3 Self-immunity

A defining characteristic of bacteriocins is that the producer strain is immune to the inhibitory activity of the bacteriocin they have produced (303,304). Testing the spectrum of activity of inhibitory CFS against producing- and closely related strains reveals information about the nature of the inhibitory substances being produced. Microtiter assays were repeated as described previously, using the producing strains as the indicator strains. Isolates were tested in triplicate.

2.4.4 Enzymatic digestion

Cell-free supernatant was treated with various enzymes to characterise the active components in the CFS; loss of activity following protease or amylase treatment allows for presumptive identification of the active inhibitory substance in the CFS as a protein or carbohydrate, respectively (304). Trypsin, proteinase K, and amylase (Sigma-Aldrich, Gillingham, UK) were used for these assays. Enzymes were prepared as per manufacturer's guidelines. Briefly; trypsin was dissolved at 1 mg/ml in 1 mM hydrochloric acid, proteinase K at 1 mg/ml in water, and amylase at 0.5mg/ml in phosphate buffered saline. 10 µl of enzyme was added to 100 µl of CFS in 96 well plates and incubated at 37 °C for one hour; following incubation, 100 µl of MRSP1 (10^5 CFU) was added. Optical density readings were carried out for 24 hours using the CLARIOstar® microplate reader as described above. *L. lactis* and non-inoculated TSB controls were used as described previously; both enzyme-treated and non-treated controls were used to ensure enzyme treatment did not affect the growth of the indicator

strain. The assay was repeated using a two-hour incubation following addition of enzymes. Isolates were tested in triplicate in each assay.

2.5 Statistical analysis

Growth curve data collected using the CLARIOstar® microplate reader was analysed using GraphPad Prism8 (GraphPad Software, California, USA). Area under curve (AUC) analysis was carried out for all data. AUC as a percentage of the negative control AUC was calculated for all samples.

2.6 Genomic analysis

Isolates that showed inhibitory activity in the deferred antagonism assay were whole genome sequenced by MicrobesNG (Birmingham, UK) using Illumina HiSeq technology. Non-inhibitory isolates of the same species, isolated from the same dog where possible, were also selected for genome sequencing to allow for comparison and identification of inhibitory related genes. Isolates identified as demonstrating methicillin-resistance by the VITEK®2 were also sent for whole genome sequencing. Isolates were prepared for sequencing as described by MicrobesNG. Briefly; single colonies were mixed with 100 µl sterile saline, this was then streaked onto Columbia blood agar and grown aerobically overnight at 37°C. All culture was then scraped off the plates using a sterile loop and mixed into the bead tubes provided.

Whole-genome sequencing was carried out by MicrobesNG following DNA extraction as described: three beads were washed with extraction buffer containing lysostaphin and RNase A, and incubated at 37°C for 25 minutes. Proteinase K and RNase A were added and incubated for a further 5 minutes at 65°C. Genomic DNA was purified using an equal volume of SPRI beads and re-suspended in EB buffer. DNA was quantified in triplicates with the Quantit dsDNA HS assay in an Ependorff AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: two nanograms of DNA instead of one were used as input, and PCR elongation time was increased to 1 min from 30 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on

the Illumina HiSeq using a 250bp paired end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 (377). De novo assembly was performed on samples using SPAdes version 3.7 (378), and contigs were annotated using Prokka 1.11 (379) (taken from MicrobesNG, Birmingham, UK).

2.6.1 Species Identification

Species identification was performed using the whole genome sequence data. The RNA polymerase β (*rpoB)* and elongation factor Tu (*tuf)* genes were identified from isolates using Artemis (version 18.1.0) (380); the gene sequences were compared to those on GenBank using the BLASTn algorithm (National Centre for Biotechnology Information, Maryland, US) to identify the closest matches. These genes were chosen over 16S rRNA gene sequence data due to the lack of reliability when differentiating closely phylogenetically related species, particularly coagulase-negative staphylococci using the 16s rRNA gene sequence (381). Whole-genome based species identification was then confirmed using the Type (Strain) Genome Server (Leibinz-Institute DSMZ, Braunschweig, Germany; https://tygs.dsmz.de, (382)).

Isolates identified as potentially novel *Staphylococcus* species, based on whole genome and 16S rRNA analysis using the Type (Strain) Genome Server, were subject to further biochemical profiling in preparation for submission to culture collections. API® Staph testing (BioMérieux, Basingstoke, UK) was carried out by colleagues, per manufacturer's instructions.

2.6.2 Identification of antimicrobial resistance genes

Genome sequenced isolates were screened for antimicrobial resistance genes in their genome using the ResFinder 3.2 server (Center for Genomic Epidemiology, Lyngby, Denmark; https://cge.cbs.dtu.dk/services/ResFinder, (383)) (acquired antimicrobial resistance genes only; identification threshold 80%, minimum length 80%) and the Resistance Gene Identifier (RGI; Comprehensive Antibiotic Resistance Database; https://card.mcmaster.ca/analyze/rgi, (384)) (using the search criteria "perfect and strict hits only").

2.6.3 Genomic epidemiology of methicillin-resistant isolates

The multi-locus sequence type (MLST) (for *Staphylococcus epidermidis* isolates only as no MLST scheme is available for the other species in question) and staphylococcal cassette chromosome *mec* (SCC*mec*) type of isolates displaying genotypic methicillin-resistance was determined in order to investigate the source of isolates and SCC*mec* elements. SCC*mec* type was identified based on predicted genes and whole cassette homology, using the reference database, with an identification threshold of 90% and minimum length 60%. The MLST 2.0 (https://cge.cbs.dtu.dk/services/MLST,(385)) and SCC*mec*Finder 1.2 (International Working Group on The Classification of Staphylococcal Cassette Chromosome Elements, 2009; https://cge.cbs.dtu.dk/services/SCCmecFinder, (386)) databases were used (Center for Genomic Epidemiology, Lyngby, Denmark).

2.6.4 Identification of bacteriocin-related gene clusters

The genome mining web-based tools BAGEL4 (387) and antiSMASH 5.0 (388) were used to search for and identify bacteriocin-related gene clusters present in the genomes of sequenced isolates. In addition, a collection of previously genome sequenced clinical *S. pseudintermedius* isolates from The Hospital for Small Animals, R(D)SVS, were also screened for bacteriocin-related gene clusters. The antiSMASH search functions “KnownClusterBlast”, “SubClusterBlast” and “ActiveSiteFinder” were enabled, searched using “relaxed” strictness criteria. Regions highlighted as containing putative bacteriocin-related gene clusters were visualised using Artemis (380); the nucleotide and amino acid sequences of the coding sequences were compared to known genes from GenBank using the BLASTn and BLASTp algorithms (National Centre for Biotechnology Information, Maryland, US) in order to derive putative functions. Predicted mass and charge of hypothetical proteins were obtained from Artemis. Genome comparisons of inhibitory isolates harbouring bacteriocin-related gene clusters and non-inhibitory control isolates were carried out using the Artemis Comparison Tool (ACT version 18.1.0) (389). Where required, type-strain genomes were obtained from GenBank for comparison.

3. Results

3.1 Prevalence of staphylococci in the healthy canine skin microbiota

Multi-site swabs were collected from healthy dogs visiting veterinary clinics in Sweden (AniCura: AC) and Scotland (Small Animal Hospital, Royal (Dick) School of Veterinary Studies: RD). A total of 121 swabs were collected (one per dog; 71 from AC, 50 from RD); of these, staphylococci were isolated from 63 dogs (52.1%). Staphylococci were isolated twice as frequently from swabs collected from RD compared to those from AC: 38/50 (76.0%) and 25/71 swabs (35.2%), respectively. A total of 166 staphylococci isolates were obtained, belonging to 18 species (Table 4). In cases where the same species was isolated from the same swab multiple times, the antibiogram data for each individual isolate was used to determine if they were distinct isolates; where the antibiogram was the same, isolates of the same species, from the same dog, were classed as a single isolate.

Coagulase-positive staphylococci (CoPS; *S. pseudintermedius, S. aureus, S. schleiferi* subsp. *coagulans*) were isolated from 44 dogs (36.4%). *S. pseudintermedius* was isolated from 32 dogs (26.4%); of these, eight dogs harboured two or more *S. pseudintermedius* isolates, and 14 dogs harboured both *S. pseudintermedius* and *S. aureus.* Eight CoPS isolates could not be distinguished between *S. pseudintermedius* and *S. aureus* phenotypically. Isolates identified as *S. schleiferi* by the VITEK®2 system were further classified into subspecies based on coagulase testing: all were coagulase-positive, thus presumed to be *S. schleiferi* subspecies *coagulans.*

Coagulase-negative staphylococci (CoNS) were isolated from 41 dogs (33.9%: 12 from AC, 29 from RD), with 11 dogs harbouring more than one CoNS species, and both CoPS and CoNS species were isolated from 20 dogs. The most frequently isolated CoNS species were: *S. vitulinus* (AC), *S. epidermidis, S. warneri,* and *S. xylosus* (RD). Four isolates were indistinguishable between two CoNS species based on phenotype, and two isolates could not be distinguished between CoNS and species belonging to different genera (*Aerococcus* and *Gemella*, respectively) by the VITEK®2; these are classed as “low discrimination” (Table 4).

Table 4. Prevalence of staphylococci species isolated from swabs collected from 121 healthy dogs, collected from AniCura clinic (71 swabs) and The Small Animal Hospital, R(D)SVS (50 swabs).

	AniCura, Sweden		R(D)SVS, Scotland		Total	
***Staphylococcus* species**	**No. dogs species were isolated from**	**No. isolates**	**No. dogs species were isolated from**	**No. isolates**	**No. dogs species wereisolated from**	**No. isolates**
S. pseudintermedius	16	21	16	20	32	41
S. aureus	7	8	13	15	20	23
Low discrimination *S. pseudintermedius*/ *S. aureus*	5	5	3	3	8	8
S. schleiferi subsp. *coagulans*	-	-	2	2	2	2
S. vitulinus	7	11	3	3	10	14
S. epidermidis	1	1	13	19	14	20
S. warneri	1	1	8	12	9	13
S. saprophyticus	2	3	1	1	3	4
S. equorum	1	1	2	2	3	3
S. scuiri	1	1	-	-	1	1
S. lentus	1	1	-	-	1	1
S. auricularis	1	1	1	1	2	2
S. hominis	-	-	3	3	3	3
S. xylosus	-	-	7	18	7	18
S. haemolyticus	-	-	1	1	1	1
S. simulans	-	-	3	3	3	3
S. capitis	-	-	1	1	1	1
S. lugdunensis	-	-	1	1	1	1
S. chromogenes	-	-	1	1	1	1
Low discrimination CoNS	1	1	4	5	5	6

3.1.1 Antimicrobial resistance amongst staphylococci isolated from healthy dogs

Of the 166 staphylococci isolates, 97 displayed resistance to at least one antimicrobial (58.4%) (Table 5) based on phenotypic testing using the VITEK®2; 48 isolates, 35 CoNS and 13 CoPS, displayed resistance to two or more antimicrobials. Methicillin-resistance (MRS) was noted in 12 isolates, all CoNS, based on cefoxitin-resistance screening. Seven of the methicillin-resistant isolates were multi-drug resistant (MDR; resistant to drugs belonging to three or more antimicrobial classes). Six *S. xylosus* and two *S. pseudintermedius* isolates were resistant to antimicrobials from three or more classes, but were not methicillin-resistant. Resistance was most frequently seen against benzylpenicillin (45.2% of isolates), tetracycline (19.9%), clindamycin (7.2%) and erythromycin (9.0%); a relatively high proportion of *S. xylosus* isolates (13 of 18) displayed intermediate-resistance to erythromycin. Seven methicillin-resistant isolates (five *S. epidermidis*, one *S. vitulinus*, and one *S. xylosus*) were whole-genome sequenced for further analysis.

Table 5. Antimicrobial resistance of staphylococci isolates from healthy dogs visiting The Hospital for Small animals, R(D)SVS, and AniCura Stockholm, obtained from the VITEK®2 system. Table 5a shows CoPS isolates, 5b shows CoNS.

Table 5a

	No. isolates displaying antimicrobial resistance:				
		Species:	**CoPS**		
Class	**Antibiotic**	*S. pseudintermedius*	*S. aureus*	*S. schleiferi* subsp. *coagulans*	Low discrimination CoPS
β-lactams	Cefoxitin screen +				
	Benzylpenicillin	24	9		6
	Amoxacillin/clavulanic acid				
	Oxacillin	1			
	Cefalotin				
	Cefovecin				
	intermediate				
	Ceftiofur				
	intermediate				
Aminoglycosides	Gentamicin				
	intermediate	1			
	Kanamycin	1			
	Neomycin	1			
Macrolide	Erythromycin	4	1		
	intermediate		1		
(Fluoro)quinolones	Enrofloxacin				
	intermediate		1		
	Marbrofloxacin		1		
	intermediate				
	Pradofloxacin				
	intermediate		1		
Lincosamines	Inducible clindamycin				
	Clindamycin	3			
	intermediate				
Tetracyclines	Tetracycline	8	3		1
	Doxycycline	7			
	intermediate		1		
Other	Nitrofurantoin	1			
	intermediate				
	Chloramphenicol	1			
	Trimethoprim/sulfamethoxazole				
Pan-susceptible		15	12	2	2

Table 5b

	No. isolates displaying antimicrobial resistance:								
		Species:							
Class	**Antibiotic**	*S. vitulinus*	*S. epidermidis*	*S. warneri*	*S. saprophyticus*	*S. equorum*	*S. scuiri*	*S. hominis*	*S. lentus*
β-lactams	Cefoxitin screen +	2	9						
	Benzylpenicillin	3	15	2	4		1	1	
	Amoxacillin/clavulanic acid	2	9						
	Oxacillin	2	9						
	Cefalotin								
	Cefovecin	2	6		4				
	intermediate								
	Ceftiofur	1	2		1				
	intermediate	1	6		2	1			
Aminoglycosides	Gentamicin								
	intermediate		1						
	Kanamycin		1						
	Neomycin								
Macrolide	Erythromycin		7	1	2				
	intermediate			2		1			1
(Fluoro)quinolones	Enrofloxacin								
	intermediate		1						
	Marbrofloxacin		1						
	intermediate								
	Pradofloxacin								
	intermediate		1						
Lincosamines	Inducible clindamycin		1			1			
	Clindamycin	1	2	3					1
	intermediate					1			
Tetracyclines	Tetracycline	3	8	3	1				
	Doxycycline		3						
	intermediate								
Other	Nitrofurantoin								
	intermediate								
	Chloramphenicol								
	Trimethoprim/sulfamethoxazole		1						
Pan-susceptible		9	4	6		2		2	

5b continued

	No. isolates displaying antimicrobial resistance:	Species:							
Class	**Antibiotic**	*S. auricularis*	*S. xylosus*	*S. haemolyticus*	*S. simulans*	*S. lugdunensis*	*S. capitis*	*S. chromogenes*	Low discrimination CoNS
β-lactams	Cefoxitin screen +		1						
	Benzylpenicillin		9		1				
	Amoxacillin/clavulanic acid								
	Oxacillin		1						
	Cefalotin								
	Cefovecin								
	intermediate		1						
	Ceftiofur								
	intermediate								
Aminoglycosides	Gentamicin								
	intermediate								
	Kanamycin								
	Neomycin								
Macrolide	Erythromycin								
	intermediate		13						
(Fluoro)quinolones	Enrofloxacin								
	intermediate								
	Marbrofloxacin								
	intermediate								
	Pradofloxacin								
	intermediate								
Lincosamines	Inducible clindamycin		1						
	Clindamycin		2						
	intermediate								
Tetracyclines	Tetracycline		5						1
	Doxycycline								
	intermediate								
Other	Nitrofurantoin		4						
	intermediate		1						1
	Chloramphenicol								
	Trimethoprim/sulfamethoxazole								
Pan-susceptible		2	2	1	2	1	1	1	5

3.2 Antimicrobial activity of staphylococci isolates

3.2.1 Deferred antagonism assays

All 144 phenotypically-identified CoNS were screened for inhibitory activity against *S. pseudintermedius* via an agar-based deferred antagonism assay, using the spot on method; this allows for preliminary identification of inhibitory activity due to production of excretory substances. Two indicator strains of *S. pseudintermedius* were used: methicillin-resistant *S. pseudintermedius* 6109/25111 (MRSP1), and methicillin-sensitive *S. pseudintermedius* 62310/54800 (MSSP1). Indicator isolates were diluted to ~10^6 CFU per plate. Thirteen test isolates, obtained from nine dogs, displayed inhibitory activity against at least one of the indicator strains (Table 6). In this initial screen, any clear zone of inhibition resulted in classification of the isolate as inhibitory (Figure 3). The thirteen inhibitory isolates, and five species-matched non-inhibitory isolates from this study were whole-genome sequenced for further analysis.

Table 6. Canine staphylococcal isolates displaying inhibitory activity against *S. pseudintermedius* via deferred antagonism assay. Isolates identified as *S. schleiferi* using the VITEK®2 were later identified to subspecies level via coagulase testing.

Isolate	Species	Inhibition of:
S13/1	*S. vitulinus*	MRSP1
S13/5	*S. vitulinus*	MRSP1
S25/3	*S. vitulinus*	MRSP1
H6/1	*S. haemolyticus*	MRSP1
H9/1	*S. warneri*	MSSP1
H14/1	*S. warneri*	MRSP1, MSSP1
H14/2	*S. warneri*	MRSP1
H14/3	*S. warneri*	MRSP1, MSSP1
H16/1A	Low discrimination (*S. hominis/S. auricularis*)	MSSP1
H16/2	*S. simulans*	MRSP1, MSSP1
H24/1A	*S. schleiferi* (ssp. *coagulans*)	MSSP1
H32/4	*S. epidermidis*	MSSP1
H34/3	*S. epidermidis*	MSSP1

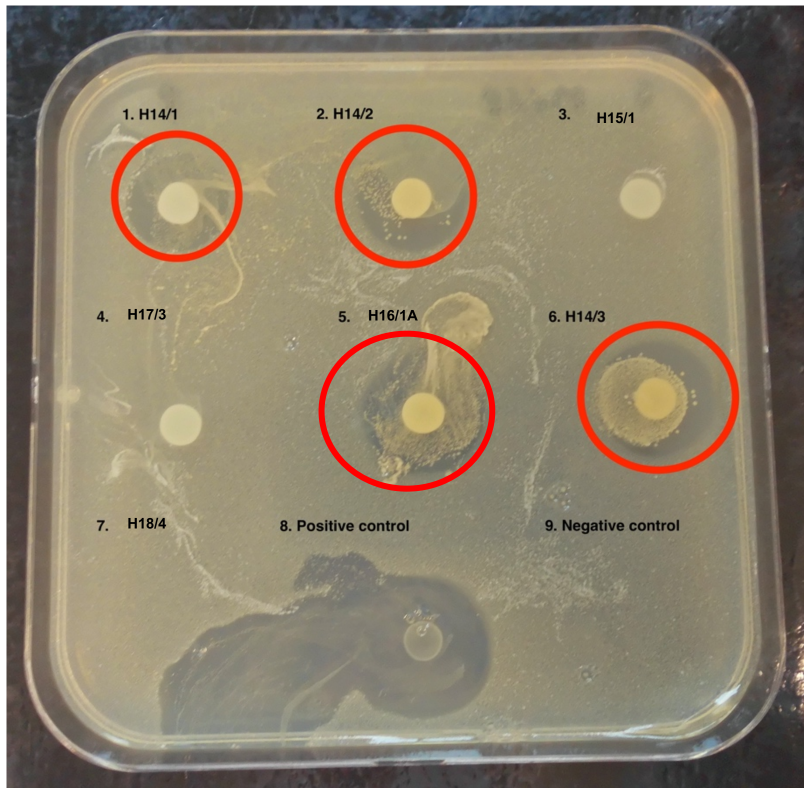

Figure 3. Agar plate displaying spot-on deferred antagonism assay, using methicillin-resistant *S. pseudintermedius* (MRSP1) as the indicator strain. Zones of inhibition circled in red. *L. lactis* CECT4433 was used as the positive control, and tryptone soy broth as the negative control.

The thirteen isolates that demonstrated inhibitory activity against MRSP1 or MSSP1 in the spot-on assay were also screened for activity against a range of different indicator species (Table 3) via well-diffusion and spot-on assays. No isolates demonstrated inhibitory activity against any of the five Gram-negative (*K. pneumoniae, E. coli, A. baumannii, P. aureginosa, Y*. pseudotuberculosis) or two Gram-positive species (*Str. canis* and *Str. uberis*) tested in either assay.

3.2.2 Cell-free supernatant growth assays

The 13 isolates that displayed inhibitory activity against *S. pseudintermedius* in the deferred antagonism assay (Table 6) were further investigated for antimicrobial substance production via cell-free supernatant (CFS) assays, alongside five isolates that did not display inhibitory activity during the initial deferred antagonism assays but belonged to the same species as the inhibitory isolates. The

use of CFS-based assays is an independent confirmation of inhibitory activity, and demonstrates that inhibitory activity seen is due to production of a secreted substance. Of the 18 isolates tested, only CFS from the isolates H14/1, H14/2 and H14/3 demonstrated a notable reduction in growth of MRSP1 or MSSP1, (Table 7, Figure 4); as defined by a reduction in growth of more than 50%, based on the percentage reduction of the area under the curve (AUC) of growth curves compared to that of the negative-control treated indicator growth.

Table 7. Mean area under curve (AUC) of 24-hours growth curves treated with cell-free supernatant from 18 staphylococci isolates, plus positive control (nisin+ *L. lactis)* and negative control (TSB). Technical replicates were recorded, *i.e.* three wells were used per isolate in a single assay. Percentage reduction of AUC based on the negative control treated growth curve AUC is shown; reductions of >50% are shown in bold. MRSP1 (10^5 CFU) and MSSP1 (10^3 CFU) were used as indicator strains.

CFS derived from Isolate	MRSP1		MSSP1	
	AUC	**% reduction in AUC**	**AUC**	**% reduction in AUC**
Negative control (TSB)	37.98	-	31.09	-
Positive control (*L. lactis*)	12.33	**67.5**	7.993	**74.29**
H6/1	34.48	9.30	24.11	22.45
H6/2	35.47	6.30	29.64	4.66
H9/1	34.83	8.30	30.24	2.77
H14/1	14.52	**61.80**	7.564	**75.67**
H14/2	12.79	**66.30**	7.769	**75.01**
H14/3	13.99	**63.20**	7.543	**75.74**
H16/1A	37.36	1.60	31.27	-0.58
H16/2	36.85	3.00	29.89	3.86
H16/4A	37.03	2.50	30.84	0.80
H24/1A	35.11	7.60	30.13	3.09
H25/1A	36.03	5.10	30.49	1.93
H32/4	37.3	1.80	32.09	-3.22
H33/1	35.41	6.80	30.82	0.87
H34/3	31.38	17.40	26.13	15.95
S13/1	34.34	9.60	28.93	6.95
S13/2	33.64	11.40	29.91	3.80
S13/5	34.59	8.90	29.69	4.50
S25/3	33.2	12.60	30.5	1.90

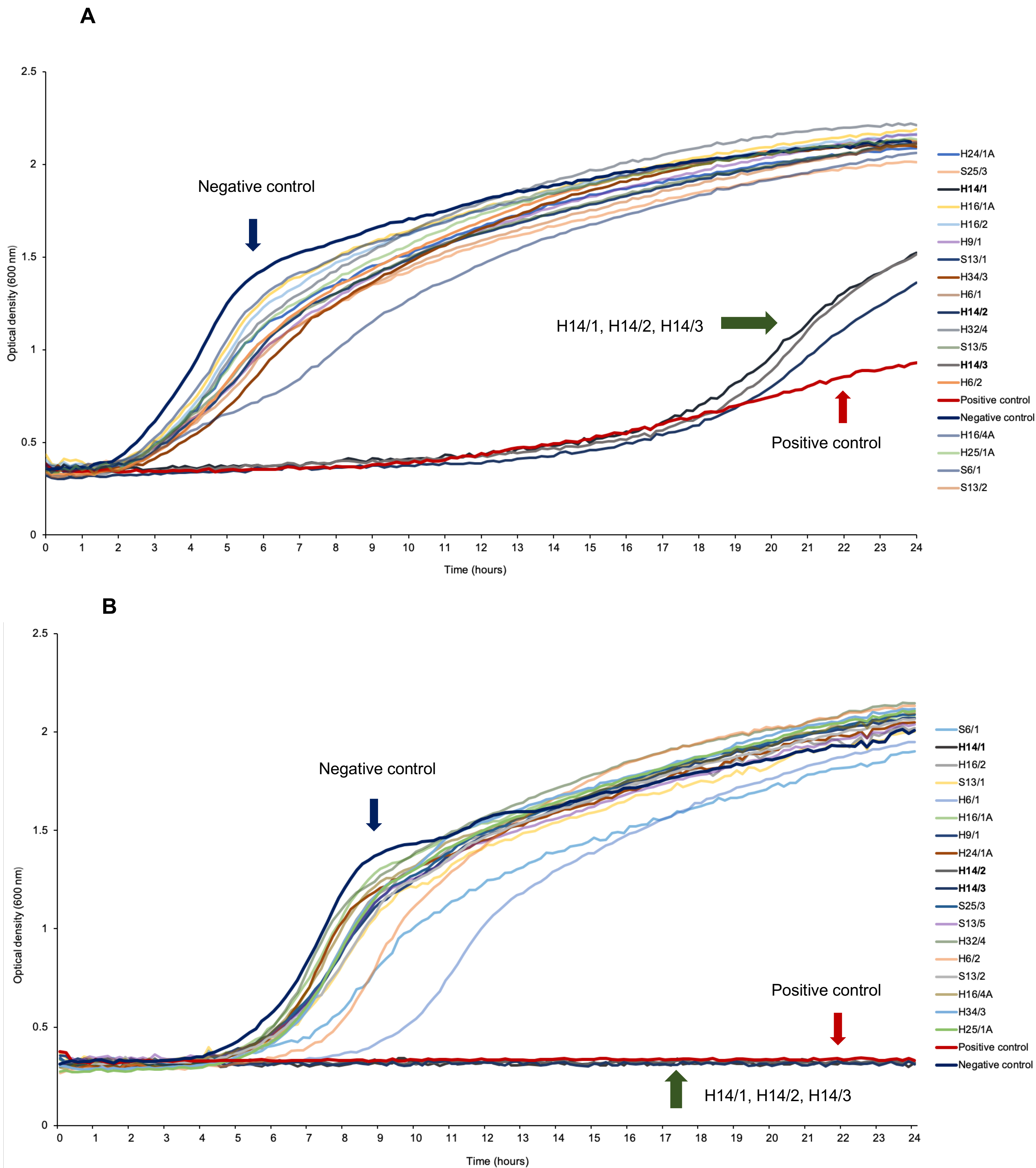

Figure 4. 24-hour growth curves showing the optical density of A) methicillin-resistant *S. pseudintermedius* (MRSP1, 10^5 CFU) and B) methicillin-susceptible *S. pseudintermedius* (MSSP1, 10^3 CFU), treated with one of 18 staphylococcal cell-free supernatants, TSB negative control, or nisin-producing *L. lactis* positive control. OD readings were taken at 10-minute intervals. 96-well plates were incubated at 37°C, shaken at 200 rpm, for the duration of the experiment. Test isolates displaying inhibition labelled in bold.

The 13 initial inhibitory isolates were also screened for inhibitory activity against eight clinical isolates obtained from veterinary cases (Table 2), using the CFS assay as described previously. CFS from the isolates H14/1, H14/2 and H14/3 inhibited the four tested *S. pseudintermedius* and both *S. aureus* indicator strains (which altogether included three methicillin-resistant strains), but neither of the two *S. schleiferi* subsp. *schleiferi* strains (Table 8). The CFS from the other 10 isolates did not displaying inhibitory activity against any of the tested indicators using the criteria of >50% reduction in AUC (Table 8).

Table 8. Mean area under curve (AUC) of 24-hours growth curves of eight clinically-relevant staphylococci treated with cell-free supernatant from 13 staphylococci isolates, plus positive control (nisin+ *L. lactis)* and negative control (TSB), recorded in triplicate from a single assay. Percentage reduction of AUC based on the negative control treated growth curve AUC is shown; reductions of >50% are shown in bold. 10^5 CFU per well was used for all indicator strains.

	MRSA 88744		MSSA 88300		MRSS 8117		MSSS 78868/335221	
CFS derived from isolate	**AUC**	**% reduction in AUC**	**AUC**	**% reduction in AUC**	**AUC**	**% reduction in AUC**	**AUC**	**% reduction in AUC**
Negative control (TSB)	34.69	-	36.24	-	35.11	-	33.21	-
Positive control (*L. lactis)*	8.43	**75.71**	9.59	**73.53**	8.84	**74.81**	8.70	**73.79**
H14/1	8.39	**75.81**	8.19	**77.41**	25.29	27.97	26.49	20.23
H14/2	8.44	**75.66**	8.26	**77.2**	26.99	23.13	20.41	38.54
H14/3	7.67	**77.90**	7.75	**78.61**	29.87	14.92	26.77	19.39
S13/1	24.97	28.02	26.17	27.79	27.36	22.07	24.36	26.65
S13/5	30.51	12.05	31.15	14.05	29.14	17.00	24.47	26.32
S25/3	25.07	27.73	31.54	12.97	26.84	23.55	26.82	19.24
H6/1	28.41	18.10	30.17	16.75	29.09	17.15	27.00	18.70
H9/1	26.55	23.46	30.87	14.82	24.87	29.17	27.58	16.95
H16/1A	33.36	3.83	31.10	14.18	32.39	7.75	30.69	7.60
H16/2	32.60	6.02	31.31	13.60	30.71	12.53	25.31	23.79
H24/1A	30.27	12.74	32.20	11.15	30.26	13.81	29.34	11.65
H32/4	33.70	2.85	34.14	5.79	32.26	8.12	30.81	7.23
H34/3	32.74	5.62	35.59	1.79	28.69	18.29	25.5.0	23.22

Table 8. continued

Supernatant derived from Isolate	MRSP2 6112/66182 AUC	% reduction in AUC	MSSP2 6125/66074 AUC	% reduction in AUC	MRSP3 6127/64107 AUC	% reduction in AUC	MSSP3 6134/64963 AUC	% reduction in AUC
Negative control (TSB)	30.07		28.99		30.85		30.95	
Positive control (*L. lactis*)	8.40	**72.07**	8.55	**70.52**	8.28	**73.17**	8.41	**72.82**
H14/1	8.03	**73.28**	7.85	**72.94**	7.872	**74.48**	7.91	**74.44**
H14/2	7.54	**74.92**	7.46	**74.29**	7.443	**75.87**	7.476	**75.84**
H14/3	7.73	**74.31**	7.88	**72.84**	8.001	**74.06**	7.713	**75.08**
S13/1	24.08	19.91	24.36	15.99	28.96	6.11	24.81	19.83
S13/5	21.36	28.95	21.45	26.02	28.53	7.51	25.81	16.59
S25/3	24.74	17.71	23.66	18.4	28.71	6.93	24.48	20.89
H6/1	26.53	11.76	26.71	7.88	29.44	4.56	28.14	9.06
H9/1	26.32	12.46	27.02	6.81	28.36	8.06	26.87	13.17
H16/1A	28.89	3.91	26.18	9.71	28.82	6.57	29.02	6.22
H16/2	27.82	7.47	27.88	3.85	30.33	1.67	29.21	5.61
H24/1A	27.62	8.13	26.62	8.20	28.56	7.41	28.64	7.45
H32/4	28.18	6.27	30.44	-4.98	30.97	-0.41	31.49	-1.76
H34/3	22.22	26.09	22.36	22.88	28.88	6.37	23.26	24.83

3.3 Characterisation of inhibitory activity

3.3.1 Effect of growth conditions on antimicrobial activity

A lack of inhibitory activity in the CFS assay was observed for ten of the isolates that inhibited at least one indicator strain of *S. pseudintermedius* via agar-based assays, with only CFS from H14/1, H14/2 and H14/3 displaying inhibitory activity. As AMP-related gene expression is influenced by environmental factors, particularly nutrient availability (235,275), isolates were grown in two nutrient-depleted mediums in order to mimic a natural environment and investigate any effect this had on inhibitory activity in CFS assays. Isolates were grown in TSB for 48 hours, rather than 18 hours, and in TSB supplemented with dipyridyl, an iron-chelating agent. 48-hour incubation had no effect on inhibitory activity, with only the isolates H14/1, H14/2 and H14/3 displaying inhibitory activity against MRSP1 (AUC reduction of 59.38% compared to negative control). The carryover of dipyridyl from the growth media used for the inhibitory isolates into the harvested CFS inhibited the growth of *S. pseudintermedius* growth (73.9% decrease in growth based on AUC compared to TSB without dipyridyl), meaning no conclusions can be drawn from the CFS with dipyridyl data as inhibitory effect of dipyridyl cannot be distinguished from that of the CFS. Further assays were carried out using only H14/1, H14/2 and H14/3, as they are the only isolates that demonstrate activity via both deferred antagonism and CFS assays.

3.3.2 Spectrum of activity of inhibitory isolates

CFS derived from H14/1, H14/2 and H14/3 was tested for inhibitory activity against 17 further clinical *S. pseudintermedius* isolates, demonstrating activity (>50% reduction in AUC) against 13 of the isolates (Table 9). Twenty-one CoPS, isolated from the healthy dogs used in the
is study, were also tested (7 *S. aureus,* 14 *S. pseudintermedius*). No inhibition of these *S. aureus* was seen, whilst three of the 14 *S. pseudintermedius* isolates carried by healthy dogs were inhibited, with reductions in AUC of 65.9%, 75.6%, and 72.1% compared to the negative control.

The isolates H14/1, H14/2 and H14/3 were screened for activity against nine CoNS indicator species (Table 3) via deferred antagonism assay using the cross-streak method, allowing for rapid testing of inhibition against a larger range of indicators. No inhibition against any of the species tested was seen in this assay.

Table 9. Mean area under curve (AUC) of 24-hour growth curves of clinical *S. pseudintermedius* isolates treated with 100 µl of cell-free supernatant derived from H14/1, H14/2 and H14/3 (shown as a mean of the three), plus positive control (nisin+ *L. lactis)* and negative control (TSB). Percentage reduction of AUC based on the negative control treated growth curve AUC is shown; reductions of >50% are shown in bold. 10^5 CFU per well were used for all indicator strains.

Indicator	AUC	% reduction in AUC	AUC positive control	AUC negative control
MRSP 6106	7.68	**75.13**	8.08	30.87
MRSP 6107	11.57	**61.9**	8.22	30.37
MRSP 6108	21.34	35.18	7.77	32.93
MRSP 6109	7.87	**73.34**	8.37	29.50
MRSP 6110	16.83	44.81	7.79	30.49
MRSP 6111	7.60	**74.33**	8.46	29.62
MRSP 6112	13.69	**56.58**	8.57	31.52
MSSP 6125	7.573	**74.82**	7.79	30.07
MSSP 6126	7.67	**76.48**	8.68	32.62
MRSP 6127	7.67	**73.35**	8.31	28.77
MSSP 6128	14.96	**53.9**	8.36	32.46
MSSP 6129	8.72	**71.77**	8.12	30.90
MSSP 6130	13.74	**53.99**	8.06	29.86
MSSP 6131	22.79	28.96	7.98	32.08
MSSP 6132	20.56	37.70	7.89	33.00
MSSP 6133	12.84	**57.77**	7.95	30.41
MSSP 6134	7.89	**75.55**	8.46	32.26

3.3.3 Semi-quantification of cell-free supernatant activity

CFS assays were repeated as before, using two-fold dilutions of CFS to obtain a crude value for minimum volume required to display inhibitory activity. 100 µl of CFS from the isolates H14/1, H14/2 and H14/3 resulted in notable inhibition (>50% reduction in AUC) of both MRSP1 and MSSP1, with >70% reduction in growth of MRSP1 (Table 10). 50 µl of H14/2 and H14/3 was also able to reduce the growth of MRSP1 by >50%, however this was not seen in MSSP1 or from treatment with H14/1.

Table 10. Area under curve (AUC) of 24-hour growth curves of MRSP1 (10^3 CFU) and MSSP1 (10^5 CFU), treated with CFS from H14/1, H14/2, H14/3, nisin+ *L. lactis* CFS (positive control) or TSB (negative control). CFS was diluted two-fold in PBS to give volumes of 6.25 µl to 100 µl, to determine crude minimum inhibitory volumes. Percentage reduction in AUC compared to negative control indicators was calculated; reductions of AUC of >50% shown in bold.

Supernatant derived from isolate	Volume/µl	MRSP1 AUC	MRSP1 % reduction in AUC	MSSP1 AUC	MSSP1 % reduction in AUC
Negative control (TSB)	100	29.93	-	34.88	-
Positive control (*L .lactis)*	100	8.396	**71.95**	8.57	**75.43**
H14/1	100	7.76	**74.08**	10.00	**71.33**
	50	15.20	49.21	20.66	40.77
	25	24.36	18.61	30.29	13.16
	12.5	26.36	11.93	34.23	1.86
	6.25	28.12	6.05	33.65	3.53
H14/2	100	8.392	**71.96**	10.85	**68.89**
	50	11.59	**61.28**	19.96	42.78
	25	24.70	17.48	32.84	5.85
	12.5	25.67	14.23	33.97	2.61
	6.25	27.63	7.68	34.33	1.57
H14/3	100	7.458	**75.08**	14.82	**57.51**
	50	12.15	**59.41**	20.26	41.92
	25	25.11	16.10	32.28	7.45
	12.5	26.40	11.79	34.39	1.41
	6.25	28.45	4.94	33.52	3.90

3.3.4 Self-immunity

CFS from each of the isolates H14/1, H14/2 and H14/3 were tested against themselves and each other to establish if they demonstrated self-immunity, a characteristic of bacteriocin-producing isolates. The CFS did not show inhibitory activity (reduction of >50% of AUC) against one another, although a decrease of approximately 20% of the AUC was seen (inhibition by positive control shown by a reduction of 73.11% of the AUC). The lack of notable inhibition against on another is suggestive of bacteriocin production, and that it is the same AMP being produced by the three isolates.

3.3.5 Enzymatic digestion

Loss of inhibitory activity due to enzymatic digestion offers insight into the structure of the substance responsible for the inhibition, based on the target of the enzyme that deactivates it. CFS from H14/1, H14/2 and H14/3 was treated with two proteolytic enzymes (proteinase K, trypsin), and amylase, which digests carbohydrates, in order to investigate the nature of the antimicrobial substance present in the CFS. Untreated CFS demonstrated a reduction of growth of the indicator (MRSP1) of over 70%, in line with previous assays. Treatment with each of the three enzymes individually resulted in reduced antimicrobial activity, with treated CFS only reducing growth by: 31.32% (proteinase K treated), 35.25% (trypsin treated), and 33.09% (amylase treated), suggesting partial digestion of proteinaceous and carbohydrate moieties resulting in partial loss of activity. Enzyme treated negative controls were included for each of the enzymes; no reduction in indicator growth was seen, showing that enzyme treatment does not inhibit bacterial growth and confound results. However, none of the three enzyme treatments resulted in a loss of activity of the positive control (nisin-producing *L. lactis* CFS), which would be expected following proteinase K or trypsin treatment, due to the peptide nature of nisin, the active component of the positive control CFS. As a result, the enzyme-treatment assay was repeated as before, but also including positive and negative controls treated with 50 µl of each enzyme. Treatment with 50 µl did not have an effect on the negative control (*i.e.* 50 µl of enzyme does not inhibit bacterial growth) or positive control, where loss of activity would be expected. Effect on CoNS CFS was variable in the repeated assay; proteinase K treatment caused some loss of activity, but less than was seen in the first experiment (CFS caused 44.73% reduction in bacterial growth compared to 31.2% in the previous experiment, and ~70% when untreated). Amylase-treated CFS showed a very slight reduction in activity (67.77% decrease in bacterial growth, vs ~70% untreated CFS), and trypsin-treated CFS showed no loss of activity, with bacterial inhibition the same as untreated CFS. Prolonged storage of enzyme preparations between experiments may have altered their stability and activity. The 10 µl enzyme-treated negative controls in the repeat assay all showed inhibition of bacterial growth (>70% decrease in AUC), suggesting a confounding variable or error occurred, meaning no conclusions can be drawn from the enzymatic digestion.

3.4 Genomic analysis of staphylococci isolates

3.4.1 Genotypic species identification

Isolates displaying inhibitory or methicillin-resistant phenotypes, and non-inhibitory species matches, were sent for whole genome sequencing by MicrobesNG (Birmingham, UK). A total of 27 isolates were sequenced; seven methicillin-resistant isolates, thirteen inhibitory isolates, and seven species-matched non-inhibitory isolates. Of these isolates, 24 belonging to seven *Staphylococcus* species, and three low-discrimination CoNS isolates (based on phenotypic identification by the VITEK®2 system). Isolates were subject to identification based on *rpoB* and *tuf* gene nucleotide sequences. The phenotypic identification was confirmed based on genotype for 18 isolates; for six isolates, the genotypic identification did not match, and for two isolates no genotypic match was found (Table 11).

All 27 genome sequenced isolates were uploaded to the Type (Strain) Genome Server (Leibinz-Institute DSMZ, Braunschweig, Germany) for whole genome based taxonomic analysis. For 24 isolates, the species identification provided corresponded to the identification obtained from the *rpoB* and *tuf* sequences. The three isolates denoted * in Table 11 were classified as potentially novel *Staphylococcus* species based on phylogeny, with no significant species matches obtained.

Table 11. Genotypic species identification of staphylococci isolates for which genotypic and phenotypic identification did not correspond. The top match for each gene only are shown. Percentage identity cut offs for a significant match are 93.6% for *rpoB* and 97% for *tuf* (381). Isolates denoted with * were identified as potentially novel species.

Sample ID	Phenotypically identified species	Genotypically identified species	Based on gene	Query Coverage (%)	Percentage identity (%)	Accession Number
H6/1	*Staphylococcus haemolyticus*	*Staphylococcus pseudintermedius*	*rpoB*	23	100	MF679124.1
		Staphylococcus pseudintermedius	*tuf*	67	100	MF679012.1
H6/2	*Staphylococcus haemolyticus*	*Staphylococcus pseudintermedius*	*rpoB*	23	100	MF679124.1
		Staphylococcus pseudintermedius	*tuf*	67	100	MF679012.1
H8/1*	*Staphylococcus warneri*	*Staphylococcus devriesei*	*rpoB*	23	96.78	MF679100.1
		Staphylococcus devriesei	*tuf*	67	99.38	MF678988.1
H14/1	*Staphylococcus warneri*	*Staphylococcus devriesei*	*rpoB*	23	100	MF679100.1
		Staphylococcus devriesei	*tuf*	67	99.38	MF678988.1
H14/2	*Staphylococcus warneri*	*Staphylococcus devriesei*	*rpoB*	23	100	MF679100.1
		Staphylococcus devriesei	*tuf*	67	99.38	MF678988.1
H14/3	*Staphylococcus warneri*	*Staphylococcus devriesei*	*rpoB*	23	100	MF679100.1
		Staphylococcus devriesei	*tuf*	67	99.38	MF678988.1
H16/1A*	low discrimination *Staphylococcus hominis* -	no significant matches	*rpoB*	-	-	-
	Staphylococcus auricularis	no significant matches	*tuf*	-	-	-
H16/4A*	low discrimination *Staphylococcus hominis* -	no significant matches	*rpoB*	-	-	-
	Staphylococcus auricularis	no significant matches	*tuf*	-	-	-

3.4.2 Identification of novel *Staphylococcus* species

Whole genome- and 16S rRNA- analysis involving determination of closely related strain type, pairwise comparison of genome sequences, phylogenetic inference, and type-based species and subspecies clustering using TYGS revealed three isolates belonging to two novel *Staphylococcus* species. The novel species represented by the isolate H8/1 is most closely related to *Staphylococcus devreisei,* whilst the isolates H16/1A and H16/4A belong to a novel species most closely related to *Staphylococcus felis* (Figure 5); these species have been proposed as '*Staphylococcus caledonicus'* and '*Staphylococcus canis',* respectively. A DNA-DNA hybridisation value (dd_4) of <70%, against the most closely related species, is considered the threshold for classification as a novel species (390); the dd4 value of H16/1A and H16/4A against *S. felis* is 22.0%, and for H8/1 against *S. devriesei* is 50.6%. API® testing was carried out for further characterisation and classification (Table 12). Genome sequence data has been submitted to NCBI GenBank, and type strains deposited into two culture collections (Culture Collection University of Gothernburg, Sweden, and National Collection of Type Cultures, UK), in preparation for publication.

Table 12. API® STAPH test results for two proposed novel species; H16/1A 'S. *canis'* and H8/1 'S. *caledonicus'*, and the most closely phylogenetically related species, *S. felis* and *S. devriesei*, respectively. Results are from four independent replicates. Tests were carried out per manufacturer's instructions (BioMérieux, Basingstoke, UK).

Test	**H16/1A** ***('S. canis')***	**DSM 7377** **(*S. felis*)**	**H8/1** ***('S. caledonicus')***	**DSM 25293** **(*S. devriesei*)**
Acid production from:				
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	+	+	-	-
D-Maltose	+	-	+	+
D-Lactose	+	+	-	+
D-Trehalose	+	+	+	+
D-Mannitol	+	+	+	+
Xylitol (Acidification)	-	-	-	-
D-Melibiose	-	-	-	-
D-Raffinose	-	-	-	-
D-Xylose	-	-	-	-
D-Saccharose (sucrose)	-	-	+	v (+ in $^{3}/_{4}$)
Methyl-α Dglucopyranoside	-	-	-	-
N-Acetylglucosamine	v (+ in $^{2}/_{4}$)	v (+ in $^{2}/_{4}$)	-	-
Reduction of nitrates to nitrites	+	+	+	v (+ in $^{2}/_{4}$)
Alkaline phosphatase	+	+	v (+ in $^{2}/_{4}$)	-
Voges-Proskauer	v (+ in $^{2}/_{4}$)	+	v (+ in $^{3}/_{4}$)	+
Arginine dihydrolase	-	+	+	+
Urease	+	+	v (+ in $^{1}/_{4}$)	+

v = variable

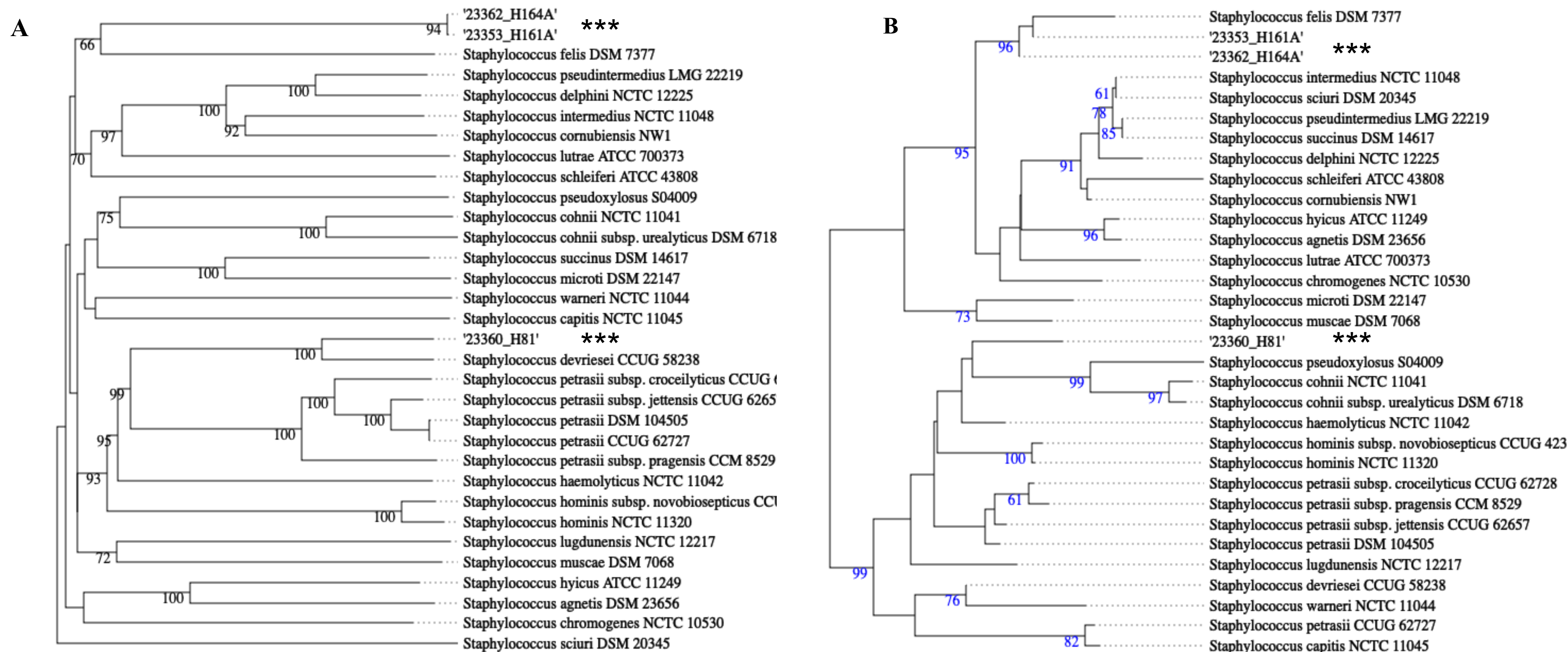

Figure 5. Phylogenetic tree showing the position of two novel species (represented by H8/1, and H16/1A H16/4A, respectively). Tree inferred with FastME 2.1.6.1 from Genome BLAST Distance Phylogeny (GBDP) distances calculated from genome sequences (5a) and 16s rDNA gene sequences (5b). The branch lengths are scaled in terms of GBDP distance formula d5. The numbers above branches are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 72.1% (5a) and 61.6% (5b) The tree was rooted at the midpoint. (Produced by Type Strain Genome Server, DSMZ, Leibniz Institute, Germany).

3.4.3 Identification of antimicrobial resistance genes

The 27 whole genome sequenced isolates were investigated for presence of antimicrobial-resistance associated genes; 23 of the isolates harboured at least one antimicrobial resistance gene (Table 13). For some of the genes present, the corresponding phenotype was unknown as the antimicrobials in question were not tested when screening for resistance using the VITEK®2 system, these included: fosfomycin (14 isolates harbouring fosfomycin resistance gene(s)), aminocoumarins (9 isolates harbouring resistance genes), streptogramins (3 isolates harbouring resistance genes), fusidic acid (5 isolates harbouring resistance genes) and mupirocin (2 isolates harbouring resistance genes). Six isolates harboured *dfr*C, conferring resistance to diaminopyrimidines such as trimethoprim. Trimethoprim was only tested in combination with sulfamethoxazole (TMP/SMX); one isolate displayed phenotypic trimethoprim-sulfamethoxazole resistance, this isolate harboured the gene variant *dfr*G, as well as *dfr*C, with the former most likely conferring the TMP-SMX resistance. Six isolates harboured *nor*A, conferring fluoroquinolone resistance, but did not demonstrate phenotypic resistance to any of the three fluorquinolones tested (marbrofloxacin, enrofloxacin, pradofloxacin). Three isolates harboured lincosamide resistance genes, and one harboured an aminoglycoside resistance gene, with no corresponding phenotypic resistance seen. Two *S. vitulinus* isolates (isolated from the same dog) were both shown to harbour *mec*A2, however only one of the isolates was identified as phenotypically methicillin-resistant. One Isolate displayed phenotypic tetracycline resistance, with no corresponding gene detected. One *S. xylosus* isolated was phenotypically identified as methicillin-resistance, also displaying resistance tetracycline, and intermediate resistance to erythromycin and nitrofurantoin, however no genotypic resistance to these classes were detected.

Table 13. Antimicrobial resistance genotypes and phenotypes of 27 sequenced staphylococci isolates from healthy dogs, obtained from ResFinder 3.2 server (Center for Genomic Epidemiology, Lyngby, Denmark) and RGI (Comprehensive Antibiotic Resistance Database, Alcock *et al*., 2020). Where two percentages are shown, the first is obtained from ResFinder and the second from RGI. Antimicrobial resistance phenotypes were obtained from the VITEK2 system. Phenotypes shown in brackets signal intermediate-resistance. For isolates denoted by a *, the resistance gene detected was also noted to confer resistance to macrolide, lincosamide, tetracycline, oxazolidinone, phenicol, pleuromutilin drugs, based on identification from RGI.

Isolate	Species	Resistance Gene	Resistance Conferred	% Identity	% length		Resistance phenotype
H6/1	*S. pseudintermedius*	*blaZ*	beta lactam	99.88 / 94.31	100		sensitive
H6/2	*S. pseudintermedius*	*blaZ*	beta lactam	98.91 / 96.8	100		sensitive
H8/1	*novel sp.*	-					tetracycline
H9/1	*S. warneri*	*gyrB*	aminocouramin	92.38	100.16	RGI only	sensitive
S13/1	*S. vitulinus*	*fosD*	fosfomycin	88.33 / 84.17	100		sensitive
S13/2	*S. vitulinus*	*fosD*	fosfomycin	88.33 / 84.17	100		sensitive
S13/5	*S. vitulinus*	*fosD*	fosfomycin	88.33 / 84.17	100		sensitive
H14/1	*S. devriesei*	*vgaALC*	streptogramin B*	100	100		sensitive
H14/2	*S. devriesei*	*vgaALC*	streptogramin B*	100	100		clindamycin, tetracycline
H14/3	*S. devriesei*	*vgaALC*	streptogramin B*	100	100		clindamycin (erythromycin)
H15/1	*S. epidermidis*	*blaZ*	beta lactam	99.62	92.4	ResFinder only	**methicillin-resistant,**
		mecA	beta lactam	100 / 99.7	100		erythromycin, doxycycline
		fosB	fosfomycin	99.53	100	ResFinder only	tetracycline
		fusB	fusidic acid	100	100		
		mphC	macrolide	100 / 98.66	100		
		tetK	tetracycline	99.93	100	ResFinder only	
		*tet*45	tetracycline	61.14	100.22	RGI only	
		msrA	macrolide, lincosamide, streptogramin B, tetracycline, oxazolidinone, phenicol, pleuromutilin	99.05 / 98.57	100		
		dfrC	diaminopyramidine	100	100	ResFinder only	
		norA	fluoroquinolone	100	100	ResFinder only	
		gyrB	aminocouramin	93.31	100	ResFinder only	
H16/1A	novel sp.	-					sensitive
H16/4A	novel sp.	-					sensitive

Table 13. continued

Isolate	Species	Resistance Gene	Resistance Conferred	% Identity	% length		Resistance phenotype
H16/1B	*S. simulans*	*blaZ*	beta lactam	99.88 / 94.66	100		benzylpenicillin
		str	aminoglycoside	99.88	100	RGI only	
		glpT	fosfomycin	87.08	99.78	ResFinder only	
H16/2	*S. simulans*	*blaZ*	beta lactam	99.88 / 94.66	100		benzylpenicillin
		glpT	fosfomycin	87.08	99.78	ResFinder only	
H21/1	*S. epidermidis*	*aac*(6')-*aph*(2'')	aminoglycoside	99.93 / 100	100		**methicillin-resistant,**
		aadD	aminoglycoside	100	100	ResFinder only	kanamycin, marbrofloxacin,
		str	aminoglycoside	99.88	100	ResFinder only	tetracycline (gentamycin,
		tetK	tetracycline	100	100		enrofloxacin, pradofloxacin)
		fusB	fusidic acid	100	100 / 98.59		
		fosB	fosfomycin	99.77	100	ResFinder only	
		blaZ	beta lactam	99.88 / 95.02	100		
		mecA	beta lactam	100 / 99.70	100		
		dfrC	diaminopyramidine	100	100	RGI only	
		mupA	mupirocin	99.61	100	RGI only	
		gyrB	aminocouramin	93.31	100	RGI only	
		ant(4')-*Ib*	aminoglycoside	99.61	101.19	RGI only	
		norA	fluoroquinolone	100	100	RGI only	
H23/1A	*S. epidermidis*	*blaZ*	beta lactam	100 / 95.02	100		**methicillin-resistant,** tetraycline
		mecA	beta lactam	100 / 99.70	100		
		fosB	fosfomycin	96.27	100	ResFinder only	
		fusB	fusidic acid	100	100		
		tetK	tetracycline	100	100		
		dfrC	diaminopyramidine	98.76		RGI only	
		gyrB	aminocouramin	93.16	100	RGI only	
		norA	fluoroquinolone	99.48	111.37	RGI only	

Table 13. continued

Isolate	Species	Resistance Gene	Resistance Conferred	% Identity	% length		Resistance phenotype
H24/1A	*S. schleiferi* subsp. *coagulans*	-					sensitive
H24/2	*S. schleiferi* subsp. *coagulans*	-					sensitive
H25/1A	*S. epidermidis*	*blaZ*	beta lactam	100 / 95.02	100		**methicillin-resistant,** erythromycin, tetracycline, doxycycline, trimethoprim-sulfamethoxazole
		mecA	beta lactam	100 / 99.70	100		
		fosB	fosfomycin	100	100	ResFinder only	
		fusB	fusidic acid	100	100		
		mphC	macrolide	100 / 98.66	100		
		msrA	macrolide, lincosamide, streptogramin B	99.05 / 98.57	100		
		*tet*K	tetracycline	99.93	100	ResFinder only	
		*tet*45	tetracycline	61.14	100.22	RGI only	
		dfrG	trimethoprim	99.8 / 99.39	100		
		dfrC	diaminopyramidine	100	100	RGI only	
		norA	fluoroquinolone	100	100	RGI only	
		gyrB	aminocouramin	93.31	100	RGI only	
S25/3	*S. vitulinus*	*mecA*2	beta lactam	99.75	100	ResFinder only	sensitive
S25/3M	*S. vitulinus*	*mecA*2	beta lactam	99.75	100	ResFinder only	**methicillin-resistant**, tetracycline
H32/4	*S. epidermidis*	*blaZ*	beta lactam	99.88 / 95.02	100		benzylpenicillin, tetracycline
		fosB	fosfomycin	99.77	100	ResFinder only	
		gyrB	aminocouramin	93.31	100	RGI only	
		norA	fluoroquinolone	100	100	RGI only	
		dfrC	diaminopyramidine	100	100	RGI only	

Table 13. continued

Isolate	Species	Resistance Gene	Resistance Conferred	% Identity	% length		Resistance phenotype
H33/1	*S. epidermidis*	*blaZ*	beta lactam	99.88 / 95.02	100		**methicillin-resistant**
		mecA	beta lactam	100 / 99.55	100		
		fosB	fosfomycin	99.77	100	ResFinder only	
		dfrC	diaminopyramidine	100	100	RGI only	
		norA	fluoroquinolone	100	100	RGI only	
		mupA	mupirocin	99.61	100	RGI only	
		gyrB	aminocouramin	93.31	100	RGI only	
H34/1	*S. epidermidis*	*blaZ*	beta lactam	99.76 / 94.31	100		benzylpenicillin
		fosB	fosfomycin	99.53	100	ResFinder only	
		dfrC	diaminopyramidine	100	100	RGI only	
		norA	fluoroquinolone	100	107.24	RGI only	
		gyrB	aminocouramin	93.31	100	RGI only	
H34/3	*S. epidermidis*	*blaZ*	beta lactam	100 / 94.31	100		benzylpenicillin
		fosB	fosfomycin	100	100	ResFinder only	
		fusB	fusidic acid	100	100		
		dfrC	diaminopyramidine	100	100	RGI only	
		norA	fluoroquinolone	99.48	100	RGI only	
		gyrB	aminocouramin	93.31	100	RGI only	
H39/4B	*S. xylosus*	*glpT*	fosfomycin	86.38	99.34	RGI only	**methicillin-resistant,** tetracycline (erythromycin, nitrofurantoin)

3.4.4 Genomic epidemiology of methicillin-resistant isolates

Following whole genome sequencing, the genomic epidemiology of the seven methicillin-resistant isolates (five *S. epidermidis* (MRSE), one *S. vitulinus* (MRSV), and one *S. xylosus* (MRSX)) was investigated. Multilocus sequencing typing (MLST) was carried out for MRSE isolates using the MLST 2.0 database from the Center For Genomic Epidemiology (Lyngby, Denmark) (Table 14). All isolates were investigated for presence and typing of SCC*mec* elements (Table 13). The MRSV isolate did not contain a SCC*mec* element but the *mec*A gene was present. Neither *mecA*/*mecC* nor a SCC*mec* element were detected from the MRSX isolate H39/4B.

Table 14. Multilocus sequence type and SCC*mec* type of methicillin-resistant *S. epidermidis* isolates, obtained from SCC*mec*Finder (Center for Genomic Epidemiology). The identification threshold for SCC*mec* identification was 90%, with a minimum length of 60%, using the reference database. SCC*mec* type was based on predicted genes and whole cassette homology.

Sample ID	Multilocus Sequence Type	SCC*mec* Type
H15/1	ST48	IVg(2B)
H21/1	ST5	Iva(2B)
H23/1A	ST230	IV(2B&5)/V(5C2&5)
H25/1A	ST54	Ivg(2B)
H33/1	ST89	V(5C2&5)

3.5 Identification of bacteriocin-related biosynthetic gene clusters

3.5.1 Genome mining

All whole-genome sequenced isolates (thirteen inhibitory, seven species-matched (based on phenotypic identification) non-inhibitory, and seven methicillin-resistant) were uploaded to two genome mining tools, BAGEL4 (387) and antiSMASH (388), to identify putative bacteriocin-related gene clusters. Bacteriocin-related gene clusters were identified by genome mining sites based on the presence of core biosynthetic or structural gene homologs. Putative bacteriocin gene clusters were identified in six of the inhibitory isolates, belonging to four species (Table 15); *S. devriesei* H14/1 H14/2 and H14/3 were the only strains to demonstrate inhibitory activity against *S. pseudintermedius* via cell-free supernatant assay. A non-inhibitory isolate, *S. xylosus* H39/4B, was also found to harbour a putative bacteriocin-related gene cluster, and the non-inhibitory species matched isolate for *S.*

pseudintermedius H6/1 (H6/2) possessed the same bacteriocin-related gene clusters despite the lack of inhibitory activity observed in the deferred-antagonism assay. Nine putative bacteriocin-related gene clusters were identified; of these, five were predicted to encode lanthipeptides (class Ia), one Sactipeptide (Ic), and three class II bacteriocins.

Table 15. Bacteriocin-related gene clusters identified using genome mining tools BAGEL4 and antiSMASH, from whole genome sequenced isolates

Isolate	Species	Class	Cluster size (bp)	Identified with
H6/1 H6/2	*S. pseudintermedius*	Ia	22,456	antiSMASH
		Ia	12,912	antiSMASH
		Ic	21,334	BAGEL4, antiSMASH
H9/1	*S. warneri*	II	20,063	antiSMASH
		Ia	22,159	BAGEL4
H14/1 H14/2 H14/3	*S. devriesei*	Ia	17,407	BAGEL4
H16/2	*S. simulans*	Ia	23,855	BAGEL4, antiSMASH
		II	20,246	BAGEL4
H39/4B	*S. xylosus*	II	49,234	BAGEL4, antiSMASH

Three putative bacteriocin gene clusters were identified in the two *S. pseudintermedius* H6 isolates; two of the bacteriocins were predicted to be type III lanthipeptides, each containing one lanthionine bridge, with predicted molecular weights of 1451 Da and 1297 Da, respectively (Table 16). The first of the putative lanthipeptides was encoded in a cluster containing three transport related genes, whilst the second lanthipeptide cluster contains one major facilitator transport gene, amongst other biosynthetic genes. A subtilosin-like sactipeptide cluster was also found, with the cluster containing antilisterial subtilosin biosynthesis genes *alb*E and *alb*D homologs, and an ABC-family transporter. The core subtilosin-like peptide was an amino acid sequence match for a subtilosin-A family bacteriocin derived from *S. pseudintermedius* (100% identity, 100% query coverage, accession WP_115830367.1).

S. warneri H9/1 harboured two bacteriocin gene clusters; the first encodes a warnericin-like peptide, and ABC-family transporter, alongside other biosynthetic genes. The second encodes a putative class II bacteriocin, comprised of two structural peptides (Table 16). This cluster also contains ATP-binding

and ABC-family proteins, for transport and immunity, a LanC-like synthetase, and a serine peptidase. A putative lactococcin 972-like bacteriocin cluster was identified in *S. xylosus* H39/4B; this bacteriocin was a BLASTp match for a lactococcin-972 family bacteriocin derived from *S. xylosus* (100% identity, 100% query coverage, accession WP_042361972.1). A lactococcin-972-like cluster was also identified from *S. simulans* H16/2; the *S. simulans* and *S. xylosus* lactococcin-like bacteriocins showed peptide sequence homology of 51/93 amino acids. The second bacteriocin gene cluster identified in *S. simulans* H16/2 contains the lanthipeptide biosynthetic gene homologs *lan*B and *lan*C, and a *lan*T ABC-transporter/ATP-binding permease homolog, however no structural lanthipeptide gene was identified. Amino acid sequences of putative structural genes are shown in Table 16, putative gene clusters are shown in Figure 6.

Table 16. Peptide sequences of putative bacteriocin structural genes identified from staphylococci isolates via genome mining. No sequence is available for H16/2 lanthipeptide as no structural gene was detected; two sequenced provided for H9/1 class IIb bacteriocin as these bacteriocins are dimers comprised of two peptide chains.

Isolate	Class	Type	Amino acid sequence	Predicted mass (Da)
S. pseudintermedius H6/1, H6/2	Lanthipeptide(1)		MKAILELAKLPKEELVSNVR	1451
	Lanthipeptide(2)		MKGELKMNRVLKLQKLNAEFETKNAK	1297
	Sactipeptide	Subtilosin-like	MEKGIMVSNKGCSTCSIGAACLIDGPLPDFEVMGIT GIFGLTS	4365
S. warneri H9/1	Lanthipeptide	Warnericin-like	MQFITDLIKKAVDFFKGLFGNK.	2561
	II	b	MFKNENLETVIPSFEDLSFEEMRALQGA	698
			MMITMFENENLETVIPSFEDLSFEEMKALQGDGEVQ AETTPACGVA	4048
S. devriesei H14/1, H14/2, H14/3	Lanthipeptide	Hominicin-like	MKDREILVEQLRNSNFGSENFNHPSGESELNMELV SNENEVNAEITPATPFTPSITEITASVIAT	7164
	II	d, BacSp222-like	MAGLLRFLLSQGRALYNWAKSNAGKVWEWLKSGA TYEQIKEWIEQALGWR	5855
S. simulans H16/2	II	Lactococcin 972-like	MGTTTIANAYEWAESGKWSHGIGSTYVWSYYTHNSY GHDSTAIGKYRSDSGYTTAGKQARASAKKAWWGNQ AYYRVY	10560
S. xylosus H39/4B	II	Lactococcin 972-like	MKKTIISILFTGIVVFGSGTVAKAVTIYAEGGLWNY GVGSSYVWSYYNHNSKAHGSTAIGKYASYSGKTRAG VQARASAPKAYWGNQTYYKVY	10091

0 bp 11,500 bp 23,000 bp

A

B

C

D

E

F

G

H

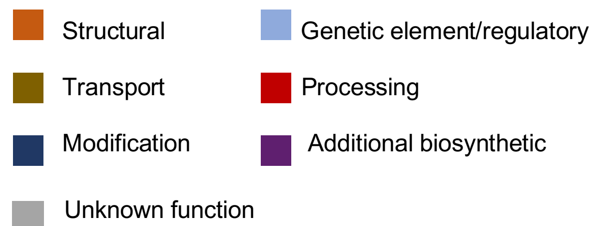

Figure 6. Biosynthetic gene clusters of eight putative bacteriocins: A) Lanthipeptide 1 B) Lanthipeptide (2) and C) Sactipeptide, from *S. pseudintermedius* H6. D) Lactococcin-like, from *S. xylosus* H39/4B, E) Lanthipeptide, and F) Class IId bacteriocin, from *S. warneri* H9/1. G) Lactococcin-like, and H) lanthipeptide, from *S. simulans* H16 (see Table 15).

3.5.2 *S. devriesei* bacteriocin gene clusters

Hominicin-like gene cluster

Two putative bacteriocin gene clusters were identified in the isolates *S. devriesei* H14/1, H14/2 and H14/3. A hominicin-like bacteriocin was identified with the genome mining tool BAGEL4, but not by antiSMASH. Sequences of the hominicin-like cluster from H14/1, H14/2 and H14/3 were downloaded from BAGEL4 and run through a BLASTn multi-sequence alignment. Hominicin-like cluster sequences displayed 100% identity and query coverage to one another, thus demonstrating the same cluster present in all three isolates; as such, *S. devriesei* H14/1 was selected as the representative sample. The encoded structural peptide is comprised of 65 amino acids, with a predicted molecular mass of 7164 Da. The peptide is predicted to belong to bacteriocin class I, lanthipeptides, which are encoded as pre-peptides, before undergoing cleavage and post-translational modification. The cleaved leader peptide, located at the N-terminus, is characterised by high net charge, whilst the active core peptide (located at the C-terminus) is characterised by the presence of unevenly distributed cysteine (C), serine (S), and threonine (T) residues, the latter of which are dehydrated during post-translational modification to form dehydroalanine and dehydrobutyrine, respectively. The pro-peptide shows amino acid sequence similarity to hominicin, a lantibiotic produced by *Staphylococcus hominis* MBBL2-9 (Figure 7). No amino acid sequence similarity is seen to the other staphylococcal-produced lantibiotics, gallidermin, epidermin, epicidin and Pep5.

Putative *S. devriesei* H14/1 bacteriocin: MKDREILVEQLRNSNFGSENFNHPSGESELNMELVSNENEVNAEITPATPFTPSITEITASVIAT

Hominicin: ITPATPFTPAITEITAAVIAX

Figure 7. Amino acid sequence of uncharacterised *S. devriesei* H14 bacteriocin 1, with sequence similarity to hominicin shown in yellow. Amino acid residues in the pro-peptide leader sequence are shown in pink (positive charge) and blue (negative charge). The glycine motif shown in green is predicted to precede the pro-peptide cleavage site.

Lanthipeptide subtypes are determined based on the class of modifying system present, the most common being LanBC or LanM. The Lan enzyme present in the hominicin-like gene cluster was given contradicting annotations (Table 17); BAGEL4 identified the enzyme as a LanC-type, whilst via BLAST the enzyme was identified as LanM-type. No LanB-like enzyme was identified by BAGEL4 or through manually searching the genome region for conserved motifs characteristic of LanB enzymes

(391). Open reading frame (ORF) 11 encodes a serine protease, typically functioning in the cleavage of the pre-peptide to form the active pro-peptide. No immunity or transport genes were identified; one set of genes can function in dual roles, with immunity conferred by efflux of the active peptide. Bacteriocin-related proteases with dual functions have previously been reported (313), with proteolytic N-terminals and membrane/ATP-binding C-terminals functioning in protein export; it is possible a multi-functioning protein explains the lack of immunity and transport genes identified in the hominicin-like gene cluster. The role of other processing genes present, such as flavoproteins, methyl- and amino-transferases, and oxidoreductases, is unclear in the context of bacteriocin production, but their presence has been noted in bacteriocin gene clusters identified from other species, such as *Frankia* spp., *Geobacillus* spp., and *Streptomyces* spp. (391), suggesting a conserved role. The Hominicin-like gene cluster from *S. devriesei* H14/1 is shown in Figure 8.

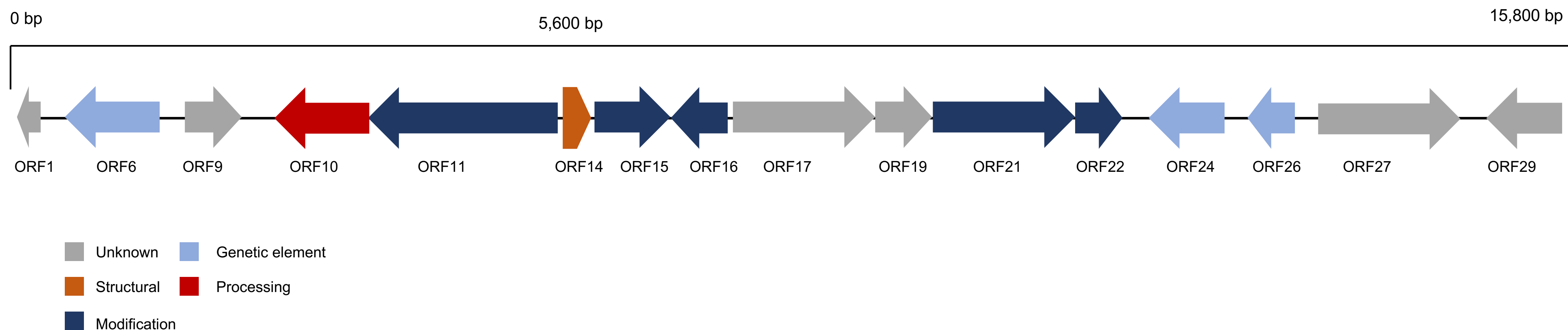

Figure 8. Schematic of the biosynthetic gene cluster of hominicin-like bacteriocin identified from *S. devriesei* H14/1. Predicted gene functions can be seen in Table 16.

Table 17. Biosynthetic gene cluster of hominicin-like bacteriocin identified in *S. devriesei* H14/1. Predicted functions and sequence matches obtained from BAGEL4 annotation (where available; based on UniProt reference clusters) BLASTp search results; structural peptide match derived from BAGEL4 database (no accession available). Multiple predicted functions are listed for some genes due to the discrepancies between matches.

ORF	Gene	Gene size (bp)	No. amino acids	Predicted molecular mass (Da)	Predicted function	Sequence match	% Identity	% Query coverage	Accession/UniRef
1 (c)	02311	153	50	5412	Hypothetical protein	*S. epidermidis*	98	100	WP_173020229.1
6 (c)	02312	936	311	36897	Replication initiator protein A	*S. hominis*	93.57	100	WP_145444616.1
					Hypothetical protein	*Lactobacillus helveticus*	27.71	-	P17212
9	02313	774	257	30068	DUF536 domain-containing protein	*Staphylococcus* (multiple species)	86.38	98	WP_049399516.1
10 (c)	02314	930	309	35202	S8 family serine peptidase	*S. pseudintermedius*	52.92	99	WP_157980674.1
					Major intercellular serine protease	*Bacillus subtilis*	38.74	-	P11018
11 (c)	02315	1551	516	61350	DUF4135 domain-containing protein	*Staphylococcus felis*	43.03	95	WP_116050704.1
					Type 2 lanthipeptide synthetase LanM	*Staphylococcus chromogenes*	32.47	69	WP_133170691.1
					Lantibiotic modifying enzyme (LanC)	*Bacillus cereus*	28.16	-	B9J4P0
14 (c)	02316	197	65	7164	Hypothetical protein	*S. felis*	32.2	90	WP_115902469.1
					Hominicin		90	-	-
15	*ubi*G	828	275	32465	Methyltransferase domain-containing protein	*Staphylococcus delphini*	43.64	98	WP_096666177.1
					Putative methyltransferase	*Geobacillus* sp.	28.8	-	C5D4V7
16 (c)	02318	723	240	27294	SDR family oxidoreductase	*Staphylococcus saprophyticus*	55.81	53	WP_119651505.1
					Uncharacterised oxidoreductase	*Thermatoga maritima*	26.6	-	Q9WYG0
17	02319	1194	397	46770	Hypothetical protein	*S. warneri*	37.24	47	WP_142396233.1
19	02320	732	243	28473	Hypothetical protein	*Bacillus ginsengihumi*	42.73	93	WP_163174598.1
21	*arg*D	1230	409	46947	Aminotransferase class II-fold pyridoxal phosphate-dependent enzyme	*S. pseudintermedius*	52.37	99	WP_110148682.1
					Acetylornithine aminotransferase	*S. epidermidis*	28.35	-	Q8CSG1

Table 17. continued

ORF	Gene	Gene size (bp)	No. amino acids	Predicted Molecular mass (Da)	Predicted function	Sequence match	% Identity	% Query coverage	Accession
22	02322	696	231	26982	Flavodoxin family protein	*S. aureus*	46.62	64	NGG19649.1
					Iron-sulfur flavoprotein	*Archaeoglobus fulgidus*	31.21	-	O28836
24 (c)	*yidC*_3	843	280	32757	Membrane protein insertase YidC	*Staphylococcus microti*	75.12	100	WP_044361027.1
					Membrane protein insertase YidC	*S. epidermidis*	57.08	-	Q5HMD1
26 (c)	*bin*3	618	205	23973	Putative transposon Tn552 DNA-invertase bin3	*S. aureus*	88.27	-	P20384
					Recombinase family protein	*Staphylococcus* sp.	92.35	95	WP_070597466.1
27	02325	1209	402	47788	Hypothetical protein	*S. hominis* 19A	99.59	100	CP031277.1
29 (c)	02326	822	273	32838	Hypothetical protein	*Staphylococcus* sp.	98.06	100	WP_070860669.1

BacSp222-like gene cluster

A structural bacteriocin gene in the *S. devreisei* isolates H14/1, H14/2 and H14/3 was identified via Artemis inspection, located ~10,900 bp downstream of the hominicin-like gene cluster; the gene was not identified by the genome mining tools BAGEL4 and antiSMASH. The cluster sequence was identical in all three isolates, thus H14/1 was selected as the representative sample. The structural gene encoded a 50 amino acid peptide, with a predicted mass of 5855 Da. The bacteriocin peptide shows similarity to BacSp222, a class IId bacteriocin produced by *S. pseudintermedius* 222, with five amino acid substitutions (Figure 9). No other known bacteriocins display homology to BacSp222 beyond arbitrary similarities (365). ORFs on either side of the structural gene were investigated to derive function and possible role in BacSp222-like bacteriocin production (Table 18).

Putative *S. devriesei* H14/1 bacteriocin 2: `MAGLLRFLLSQGRALYNWAKSNAGKVWEWLKSGATYEQIKEWIEQALGWR`

BacSp222: `MAGL-RFLLSKGRALYNWAKSHVGKVWEWLKSGATYEQIKEWIENALGWR`

Figure 9. Amino acid sequence of uncharacterised *S. devriesei* H14/1 bacteriocin 2, showing similarity to BacSp222. Amino acid differences between the two peptides shown in yellow.

The ORFs investigated did not show any similarity to bacteriocin biosynthetic genes via BLASTn and BLASTp, however seven of the ORFs, including the BacSp222-like structural gene, showed nucleotide sequence resemblance to *S. pseudintermedius* 222 plasmid p222, which encodes the BacSp222 gene cluster (365). The BacSp222 cluster contains genes encoding YdbS-like and YdbT-like immunity proteins; none of the ORFs localised to the BacSp222-like structural gene from *S. devriesei* H14 show any similarity to *ydb*I or *ydb*I (365,392). An ABC transporter (resembling the ABC transporter DrrA, based on annotation from MicrobesNG using Prokka 1.11, obtained from Artemis) was identified in the *S. devriesei* H14/1, H14/2 and H14/3 isolates (four ORFs downstream of the BacSp222-like structural gene); an ABC transporter is located in the same position in the BacSp222 cluster (Figure 10), supporting the similarity between the putative cluster from the three *S. devriesei* H14 isolates and the original BacSp222 cluster.

Neither the hominicin-like or BacSp222-like structural or related genes were identified in the *S. devriesei* type strain (GenBank accession NZ_UHCZ01000002.1, visualised using ACT).

Figure 10. Schematic of A) Putative biosynthetic gene cluster of Bacsp222-like bacteriocin identified from *S. devriesei* H14/1. Predicted gene functions can be seen in Table 17. B) BacSp222 biosynthetic cluster (365).

Table 18. Putative biosynthetic gene cluster of BacSp222-like bacteriocin identified in *S. devriesei* H14/1, based on ORFs surrounding the BacSp222 structural gene. Gene functions and sequence matches assigned based on Artemis annotation (where no accession provided), and BLASTn and BLASTp searches.

ORF	Gene	Gene size (bp)	No. amino acids	Predicted size (Da)	Predicted function	Sequence match	% Identity	% Query coverage	Accession
-4	Soj	795	264	30186	Sporulation initiation inhibitor J	-	-	-	-
					Unknown	*S. simulans* bv. Staphylolyticus NRRL B2629 plasmid pACK1	99.5	100	GU228571.2
					YhjQ protein	*S. epidermidis* IS-250	100	100	EID37185.1
-3	02335	201	66	7590	Unknown Hypothetical protein	*S. warneri* WB224 plasmid pWB224 *Terrabacteria* group	99.5 100	100 100	CP053472.1 WP_000896836.1
-2 (c)	02336	1419	472	56469	Unknown	*S. pseudintermedius* 222 plasmid p222	47.71	96	CP011490.1
					PH-domain containing protein	*S. pseudintermedius*	56.99	38	WP_143951482.1
-1 (c)	02337	483	160	19049	Unknown	*S. pseudintermedius* 222 plasmid p222	47.27	96	CP011490.1
					PH-domain containing protein	*Bacillus* sp.	100	97	WP_003234318.1
1	*BacSp*222	153	50	5855	**Bacteriocin structural protein**	*S. pseudintermedius* 222 plasmid p222	92	100	CP011490.1
					Class II bacteriocin	*Staphylococcus* sp.	92	100	WP_143212018.1
2 (c)	02339	282	93	11281	Unknown	*S. pseudintermedius* 222 plasmid p222	60.42	51	CP011490.1
					Hypothetical protein	*S. pseudintermedius*	79.73	79	WP_140858999.1
3 (c)	02340	294	97	11409	Unknown	*S. pseudintermedius* 222 plasmid p222	67.01	100	CP011490.1
					DUF2089 family protein	*S. pseudintermedius*	79.12	93	WP_182281184.1
4	02341	690	229	26157	Unknown	*S. pseudintermedius* 222 plasmid p222	66.81	100	CP011490.1
					Hypothetical protein	*S. pseudintermedius*	84.72	100	WP_110165577.1
5	02342	906	301	33725	Daunorubicin/doxorubicin resistance ATP-binding protein	-	-	-	-
					Unknown	*S. pseudintermedius* 222 plasmid p222	75.9	99	CP011490.1
					ABC transporter ATP-binding protein	*S. pseudintermedius*	77.31	79	WP_110165576.1

3.6 Inhibitory activity of MRS isolates

Seven whole-genome sequenced methicillin-resistant CoNS isolates were screened for bacteriocin-related genes; one isolate, *S. xylosus* H39/4B was found to harbour a lactococcin-like bacteriocin gene cluster (Table 15). This isolate was screened for inhibitory activity against a range of clinical indicators (Table 3), and MRSP1 and MSSP1 using the well-diffusion and spot-on deferred-antagonism assays. *S. xylosus* H39/4B was shown to inhibit both MRSP1 and MSSP1 via well-diffusion assay, despite not displaying inhibitory activity against these *S. pseudintermedius* strains in the initial spot-on assay previously or in this independent repeat. This highlights issues regarding discrepancies between results depending on which deferred antagonism assay used (well-diffusion, spot-on, cross-streak), despite all working in the same way (*i.e.* demonstrating inhibition due to production of a secreted substance).

3.7 S. pseudintermedius bacteriocin gene clusters among sequenced isolates

Following the identification of an inhibitory, bacteriocin encoding isolate as *S. pseudintermedius* (initially identified as *S. haemolyticus*), BAGEL4 and antiSMASH were used to screen 58 previously sequenced clinical *S. pseudintermedius* isolates for bacteriocin-related gene clusters. Three distinct clusters were identified; a lactococcin-972 family encoding cluster was identified in 32 of the isolates (Figure 11). This *S. pseudintermedius* lactococcin-like bacteriocin showed no homology to the lactococcin-like family bacteriocins identified from *S. simulans* H16/2 nor *S. xylosus* H39/4B (Table 16).

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MKKKFLSTLTAGLLVVFIGATSLVSAESVHAEGGIWNHGVGSKYVWSYYSHNGKYHTSTAIGKYRSDSGATKPGEEAQASAEKRWWWRNEAYYSVL
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Figure 11. Lactococcin-972 family bacteriocin (WP_037541931.1). Identified in 32 clinical *S. pseudintermedius* isolates.

A subtilosin A-like bacteriocin cluster was identified in 22 of the clinical *S. pseudintermedius* isolates; this bacteriocin is identical to the subtilosin-like bacteriocin identified in *S. pseudintermedius* H6 (Table 16). A hominicin-like bacteriocin was identified in three of the isolates; the core peptide showed no homology to either of the two lanthipeptides identified in *S. pseudintermedius* H6/1 and H6/2 (Table 16). The bacteriocin shows 50/65 amino acid sequence homology to the hominicin-like

bacteriocin derived from *S. devriesei* H14 (Figure 12), and 16/21 amino acid sequence homology to hominicin.

A) LKENEILINQLRNSSFTDADFKHPSGESNLNMELVSDANEVNAEITPATPFTPSVVEITASVIAT

B) MKDREILVEQLRNSNFGSENFNHPSGESELNMELVSNENEVNAEITPATPFTPSITEITASVIAT

C) ITPATPFTPAITEITAAVIAX

Figure 12. A) amino acid sequence of hominicin-like bacteriocin derived from *S. pseudintermedius*. Similarity to B) hominicin-like bacteriocin derived from *S. devriesei* H14/1 shown in yellow. C) amino acid sequence of *S. hominis* hominicin. Amino acids shared between all three hominicin(-like) bacteriocins shown in green, similarity between B) and C) shown in blue.

3.7.1 Inhibitory activity of genome-sequenced *S. pseudintermedius*

Following genome mining of clinical *S. pseudintermedius* strains to identify any harbouring bacteriocin-related genes, a selection of isolates encoding bacteriocins were screened for inhibitory activity against MRSP1 and MSSP1 via well-diffusion and spot-on assays (Table 19). Three bacteriocin-harbouring *S. pseudintermedius* isolates were found to inhibit MRSP1 or MSSP1 via at least one assay. *S. pseudintermedius* 9654 (subtilosin-like$^+$), 9655 (hominicin-like$^+$), and 10914 (lactococcin-like$^+$, hominicin-like$^+$) inhibited both MRSP1 and MSSP1 via well-diffusion assay; however, in the spot-on assay, only *S. pseudintermedius* 9654 was seen to inhibit MRSP1 and MSSP1, demonstrating the discrepancies between results depending on screening assay used. These results still suggest that all three putative bacteriocins identified in *S. pseudintermedius* isolates potentially have the ability to inhibit the growth of other *S. pseudintermedius* strains.

Table 19. Selection of bacteriocin-encoding *S. pseudintermedius* isolates tested for antimicrobial activity against two *S. pseudintermedius* indicators via deferred antagonism assays. Isolates harbouring each of three identified bacteriocins individually and in combination were chosen to identify which bacteriocin(s) mediate antimicrobial activity.

Strain	Bacteriocin gene cluster present	Inhibition of:	
		Well-diffusion	Spot-on
6106 /62623	None	-	-
6107 /62517	Lactococcin-like	-	-
6109 /25111	Lactococcin-like, subtilosin-like	-	-
6120 /22069	Lactococcin-like, subtilosin-like	-	-
6129 /59214	Lactococcin-like	-	-
7014 /64052	Lactococcin-like	-	-
9648 /74573	Subtilosin-like	-	-
9650 /74839	Hominicin-like	-	-
9651 /74825	Lactococcin-like	-	-
9653 /75449	Lactococcin-like, subtilosin-like	-	-
9654 /75633	**Subtilosin-like**	MRSP1, MSSP1	MRSP1, MSSP1
9655 /75698	**Hominicin-like**	MRSP1, MSSP1	-
10755 /76609	Subtilosin-like	-	-
10914 /77256	**Lactococcin-like, hominicin-like**	MRSP1, MSSP1	MSSP1

4. Discussion

4.1 Staphylococci in the healthy dog skin microbiota

The healthy microbiota plays a central role in protecting from disease (231); dysbiosis of the skin microbiota is associated with exacerbations and subsequent skin infections in both human and canine atopic dermatitis (245,254). This is likely a result of decreased diversity and diminished populations of resident bacteria that mediate the protective role via skin barrier maintenance, immune modulation, and prevention of pathogenic invasion (11,233–235). The role of the microbiota in protecting from disease has resulted in an increased interest in bacteriotherapy; the use of live bacteria or bacterial-derived components as novel treatments for bacterial infections or dysbiosis-mediated conditions (287). Coagulase-negative staphylococci (CoNS) have become an area of interest in bacteriotherapy for treatment of human and canine atopic exacerbations, following the recently revealed role of CoNS in protection from development of AD in human infants (249), and the early success of CoNS-derived topical therapy for *S. aureus* infections in AD (253). CoNS are a component of the dog skin microbiota (27,264,269,270), sharing a niche with *S. pseudintermedius,* and are known to produce an array of antimicrobial peptides (AMPs) that can inhibit the growth of coagulase-positive staphylococci (CoPS) (301), thus making them a promising area of study when looking for novel therapies for *S. pseudintermedius* in CAD.

In this study, the staphylococci populations present on the skin of 121 healthy dogs were explored. Staphylococcal species were isolated from 52.1% of the dogs sampled; this is in line with previous literature that reported isolation of staphylococci from 55-65% of dogs (270,393,394), whilst other studies had isolation rates of >90% (22,27). The difference in isolation frequency of staphylococci is likely a result of differences in sample collection and culturing methods (270). The isolation rate was twice as high in swabs collected from The Small Animal Hospital, R(D)SVS (“RD”), as compared to those collected from AniCura, Stockholm (“AC”). It is possible this was due to the transportation of samples from AC at ambient temperature, and the slightly modified processing methods between the two locations (swabs from AC inoculated into TSB with 6.5% NaCl and glycerol upon arrival and stored at -80 C, versus directly streaking onto Mannitol salt agar and frozen on beads for the samples from RD). *S. pseudintermedius* was isolated from 26.4% of samples; this is lower than the isolation rates of 37-92% cited in previous studies (4,9,10,21,25–27,29,395), however this is likely a result of

the use of culture-based isolation, and the focus being on isolating a diverse range of colonies in order to study CoNS species, rather than the sole identification *S. pseudintermedius*. 25% of dogs found to have *S. pseudintermedius* present in this study harboured two or more *S. pseudintermedius strains* (based on antibiogram data), in line with previous studies showing heterogenous populations of two or more strains colonising healthy dogs (29–34). The use of molecular-based sequence typing would offer more insight into the strain diversity of *S. pseudintermedius* present on the skin of healthy dogs. *S. aureus* was isolated from 16.5% of dogs in this study; this is higher than previous reports of *S. aureus* carriage in 4.3-12% of healthy dogs (9,31,396,397).

CoNS were isolated from 33.9% of dogs, with a total of 15 species isolated. *S. vitulinus, S. epidermidis, S. warneri* and *S. hominis* were the most frequently isolated species (Table 4); several of these have previously been reported as frequent canine skin colonisers (25,27,264,269). Initial species identification was carried out using the VITEK®2 system, which uses phenotypic-based analysis. The VITEK®2 system was unable to discriminate between *S. aureus* and *S. pseudintermedius* for eight isolates, and between two or more CoNS species, or CoNS and a species belonging to a different genus, for six isolates, highlighting some limitations of the VITEK®2 system. Following whole genome sequencing of 27 isolates, genotypic identification was carried out using whole genome-based analysis with the Type (Strain) Genome Server (Leibinz-Institute DSMZ, Braunschweig, Germany), and using *rpoB* and *tuf* gene sequences; these genes are considered more reliable for identifying closely phylogenetically related staphylococcal species (381), with *tuf* sequence based identification considered the gold standard single gene approach for identifying CoNS species (398,399). Phenotypically, the 27 genome sequenced isolates were identified as belonging to seven species, and three low discrimination CoNS isolates. Five of these isolates had been incorrectly identified by the VITEK®2; three isolates initially identified as *S. warneri* were shown to belong to *S. devriesei,* upon genotypic identification (Table 11). Two isolates phenotypically identified as *S. haemolyticus* were revealed to be *S. pseudintermedius* following genotypic identification; whilst it is challenging to distinguish between CoNS species phenotypically, and so results must be accepted tentatively, the inability to distinguish between *S. pseudintermedius* and CoNS raises concerns about misidentification of clinical *S. pseudintermedius* isolates in microbiology laboratories using the VITEK®2 system.

Three isolates were identified as belonging to two novel species (Table 11, Figure 5); two low discrimination *S. hominis/S. auricularis* belong to the proposed novel species "*Staphylococcus felis",* most closely related to *Staphylococcus canis,* whilst an isolate identified as *S. warneri* was identified to belong to another proposed novel species "*Staphylococcus caledonicus",* phylogenetically closest to *S. devriesei.* The identification of two novel species reveals yet more diversity within the *Staphylococcus* genus.

The skin microbiota plays an important role in maintaining health and preventing disease; it is essential to gain insight into the diversity and abundance of species residing in the microbiota in order to understand how they mediate protection, and how they can be exploited in order to prevent and treat disease.

4.1.1 Antimicrobial resistance amongst staphylococci

Antimicrobial resistance is a growing issue in both human and veterinary medicine, with the emergence and dissemination of methicillin- and multi-drug resistant strains resulting in infections that are challenging and costly to treat (58). Methicillin-resistance is mediated by the presence of *mec*A, encoding an alternative penicillin-bind protein (PBP2a), which confers resistance to nearly all β-lactam antibiotics (201). *mec*A is located on the mobile genetic element (MGE) staphylococcal cassette chromosome SCC*mec*, which is comprised of the *mec* and *ccr* complexes. These contain *mecA* and regulatory genes *mec*I and *mec*R1, and either *ccr*A and *ccr*B, or *ccr*C, respectively (198,400,401). The *ccr* complex encodes recombinases responsible for integration into the genome, facilitating the mobility of SCC*mec* (402). The rest of the cassette is comprised of variable components, such as additional antibiotic or metal resistance genes, and "J" regions of unknown function (198,199). The presence of SCC*mec* is frequently associated with a multi-drug resistant phenotype (197,207,212,214,403), likely due to the ability of staphylococci to acquire resistance-related genetic elements (58,203). SCC*mec* is distributed among staphylococci via horizontal gene transfer (HGT), and can undergo recombination to form novel types (203,204,400,404,405). SCC*mec* typing, based on the *mec* and *ccr* types present, allows insight into the epidemiology of methicillin-resistant organisms, and an understanding of how SCC*mec* is spread between bacteria (406,407). To date,

there are eleven SCCmec types identified from methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE) (408,409), however SCC*mec* types identified from other MR-CoNS are less well characterised, and show considerable variation not adequately described in the current typing scheme, which is based on MRSA (409). Whilst MRSA and MRSP are prominent pathogens, implicated in a range of infections in humans and animals, it is important not to overlook MR-CoNS. SCC*mec* typing and further investigation of *mec* variations revealed that the *mec* gene likely originated in the coagulase-negative organism *Staphylococcus fleurettii,* and that CoNS played a major role in the emergence and distribution of SCC*mec*-harbouring methicillin-resistant organisms, including MRSA and MRSP (400,404,405,410). MR-CoNS pose a public health concern, not only due to the opportunistic nature of many of these species, resulting in infections often in immunocompromised patients or in healthcare settings (298,411,412), but also due to their role as reservoirs of SCC*mec* and other antimicrobial resistance determinates. Many CoNS isolated from healthy dogs are strains associated with human carriage (27); the shared population of CoNS between humans and dogs highlights the risk to both species, due to the risk of transmission of both potentially pathogenic bacteria strains and of antimicrobial resistance elements between bacteria populations, contributing to the virulence of the organisms (28,194,270,413). It is thus important to monitor antimicrobial resistance in bacteria, including both clinical and carriage isolates, in order to understand the spread of resistance and manage the risk to human and animal health.

58.4% of staphylococci isolated in this study displayed resistance to at least one antibiotic tested (Table 5); a previous study by Wedley *et al.* (270) reported a similar percentage (54.4%) of staphylococci isolates from healthy dogs displaying resistance, however β-lactams were excluded from their value and so the prevalence of resistance was likely higher than seen in this study. Another study reported 100% of staphylococci isolates demonstrated resistance to one or more antimicrobials (393); the staphylococci isolated in that study were obtained from dogs with otitis externa, an often recurrent condition, requiring treatment with antibiotics. Previous treatment with antibiotics is associated with higher carriage of antimicrobial resistant strains of bacteria (191), possibly explaining the high frequency antibiotic resistance seen. Staphylococci in this study most commonly displayed resistance to: benzylpenicillin (45.2% of isolates), tetracycline (19.9%), erythromycin (9.0%), and clindamycin (7.2%); high prevalence of resistance to these antibiotics among staphylococci isolated

from dogs has been reported previously (27,269,270,393), with 16-35% of isolates displaying tetracycline resistance (27,269,270). Tetracyclines, macrolides, and lincosamides are the least frequently prescribed antibiotics in companion animals in Europe, according to the European Medicines Agency (European Surveillance of Veterinary Antimicrobial Consumption, 2018) census, suggesting the widespread resistance to these antimicrobials among staphylococci isolated from dogs is due to transfer of mobile genetic elements between strains. Resistance to these classes of antimicrobials is mediated via three mechanisms: i) modification of antibiotic target site, ii) efflux of antibiotic, iii) enzymatic degradation of antibiotic (414–417); more than one of these mechanisms may facilitate resistance in any one strain.

Molecular basis of antimicrobial resistance

Antibiotic resistance related genes were identified in 23 of 27 whole genome sequenced isolates (Table 13). Tetracycline resistance genes were detected in three of six phenotypically-resistance isolates; *tet*(K) was present in all three genomes, encoding a major facilitator family efflux pump, mediating resistance to tetracycline only (unlike the variant *tet*(M) which mediates resistance to tetracycline, doxycycline, and minocycline) (181,220). One isolate was found to also harbour the variant *tet*(45), first identified in *Bhargavaea cecembensis* found in chicken litter (418). Presence of *tet*(45) was associated with decreased susceptibility to doxycycline, alongside resistance to tetracycline (419). No *tet* variant or other resistance-related genes were identified in the remaining four tetracycline resistant isolates. Macrolides and lincosamides share a similar mode of action, binding ribosomal subunits to inhibit protein synthesis, despite being chemically distinct (420). As a result, several genes facilitate resistance against both classes (416,417). Two ABC-family efflux transporters, encoded by *vgaALC* and *msrA* (421,422), were identified in isolates resistant to clindamycin (lincosamide) and erythromycin (macrolide), respectively. Both of these gene variants were first identified in CoNS species, with *vg*aALC first identified in *S. haemolyticus,* and *msrA* in *S. epidermidis*; both genes have now been identified in a range of species, including CoPS (420,421). These transporters both also facilitate streptogramin resistance (416,417,420,422), however isolates were not tested for phenotypic streptogramin resistance in this study. A second macrolide resistance-related gene was identified in phenotypically resistant isolates; *mphC*, encoding a phosphotransferase, which inactivates macrolide antibiotics by phosphorylating the substrate

recognition site, preventing binding of the ribosome (416,422,423). Many of the genes described here are found on mobile genetic elements, such as plasmids and transposons (414,420), facilitating the dissemination of resistance-related genes through staphylococcal populations, although the genomic context of these genes was not examined in detail in this study.

Genes encoding resistance to fosfomycin, aminocoumarins, fusidic acid, and mupirocin were identified in 14, 9, 5 and 2 of the sequenced isolates, respectively. Three genes conferring fosfomycin resistance were identified; *fosB* was most frequently identified (8 of 14 fosfomycin-resistance gene harbouring isolates), alongside the variant *fosD*. These plasmid-encoded metallo-enzymes inactivate fosfomycin (424–426). The third gene encodes a mutant variant of glycerol-phosphate-permease; this protein acts as a transporter, responsible for uptake of fosfomycin into the bacterial cell, where it inhibits the first step of peptidoglycan synthesis, inhibiting bacterial growth. The mutant protein, encoded by *glpT* does not function, and so a loss of uptake is seen, mediating resistance to the drug (426,427); this mutation was first seen in *Escherichia coli,* but can now be found in a range of bacteria species (426). The phenotypic susceptibility to fosfomycin is unknown for the isolates, as it is not included in the companion animal screening antimicrobial panel in the VITEK®2 system. Six isolates harboured trimethoprim-associated resistance genes, however only one of these isolates displayed phenotypic trimethoprim-sulfamethoxazole (TMP/SMX) resistance. All six isolates harboured *dfr*C; this encodes a trimethoprim-susceptible dihydrofolate reductase (DHFR), an evolutionary precursor of the trimethoprim resistance gene variant *dfrS1* (428), explaining why carriage of this gene was not associated with phenotypic TMP/SMX resistance. The single isolate that did display phenotypic resistance also harboured *dfr*G, a plasmid-borne DHFR variant (429,430), which confers high-level resistance to trimethoprim (431). Six isolates harboured *nor*A, which mediates fluoroquinolone resistance, but did not display phenotypic resistance to the fluroquinolones tested; this was also noted in isolates harbouring lincosamide and aminoglycoside-related resistance genes, demonstrating that carriage of resistance-related genes does not always confer resistance to the target drugs (415,432). This can be due to lack of promotors, preventing expression of genes, or partial deletions or missense SNPs affecting transcription but still allowing detection of the (partial) gene by molecular methods (270,433), highlighting the importance of using both phenotypic- and molecular screening methods when investigating antimicrobial resistance amongst bacteria populations.

Methicillin-resistance among commensal staphylococci

Up to 4.5% of healthy dogs are reported to be colonised by MRSP (28,39,189,213,407,434); however, no MRSP was detected from dogs in this study. Several previous studies have also reported that no carriage of MRSP was detected (27,194,394,413,435), suggesting methicillin-resistance is not common among *S. pseudintermedius* isolated from healthy dogs, despite the growing prevalence of methicillin-resistance in clinical *S. pseudintermedius* isolates (9). Phenotypic methicillin-resistance (based on cefoxitin screen) was detected in 12 of 92 CoNS isolates obtained from dogs in this study, with eight dogs (6.6%) harbouring methicillin-resistant CoNS strains. Methicillin-resistance has been reported in 5.5-58% of CoNS isolates from dogs (27,269,270), with Schmidt *et al.* (27) citing 58% of CoNS isolates displayed phenotypic methicillin resistance, whilst only 42% harboured *mecA*, again showing the importance of using molecular and phenotypic antimicrobial resistance screening in order to obtain an accurate picture of resistance amongst isolates. The CoNS species demonstrating methicillin-resistance in this study were: *S. epidermidis* (MRSE; 9 isolates), *S. vitulinus* (MRSV; 2 isolates), and *S. xylosus* (MRSX; 1 isolate). Five of the nine MRSE isolates were multi-drug resistant (resistant to antibiotics belonging to three or more classes). Of the MR-CoNS, five MRSE, one MRSV and one MRSX were whole genome sequenced and subjected to further molecular characterisation. Multi-locus sequence typing (MLST) was carried out on the five MRSE isolates; all five isolates harbour known SCC*mec* types, and belong to known STs (Table 14), with the assigned STs associated with human infection or carriage. ST5 and ST54 are both single locus variants of ST2 (408,436–439), the most frequently identified ST in MRSE (412,440), suggested to be the founding ST of many prominent nosocomial MRSE strains (436,441). Both ST5 and ST54 are associated with carriage in healthy people, and implicated in infections in humans, including catheter-related bloodstream infections (CRBSIs) (415,440,442,443), one of the most common nosocomial infections (412). The remaining STs identified, ST48, ST89, and ST230, are most frequently associated with carriage in healthy individuals (437,444). Previous studies have showed that around 20% of healthy humans and dogs are colonised by MRSE (27,445); although a lower percentage of colonised dogs was seen in this study, it may be due to sample location (skin), whilst other studies exploring MRSE carriage investigate nasal colonisation (413). The prevalence of MRSE amongst both clinical and commensal isolates is a concern due to the central role of MR-CoNS as reservoirs of antimicrobial

resistance-related genetic elements, particularly MRSE, which is implicated in the emergence and continuous diversification of MRSA strains (405,412,437).

No SCC*mec* element was detected in the MRSV isolate, however a *mecA* variant, *mecA*2, was identified in both MRSV and a phenotypically susceptible *S. vitulinus* isolate; *mec* variants isolated from *S. vitulinus* and other closely related species have previously been found in the absence of an SCC*mec* element (446). *mecA*2 shows >90% nucleotide homology to *mec*A, and is thought to be an evolutionary precursor of *mecA* (447). Carriage of *mec*A2 does not typically confer methicillin resistance (448–451), with methicillin-resistance in *mec*A2-harbouring isolates often attributed to a secondary mechanism, such as hyper-production of β-lactamases, alternative structural alterations to non-binding domains of PBPs, or through mutations in promotors resulting in increased protein expression (198,452–455); it is possible one of these alternative mechanisms is responsible for facilitating resistance in the mecA2-harbouring MRSV in this study. No *mecA* (or variant) or SCC*mec* were detected in MRSX, the isolate also displayed tetracycline resistance, and intermediate erythromycin and nitrofurantoin resistance, with no resistance genes identified. As with MRSV, this possibly suggests a secondary mechanism of resistance, or the presence of novel, non-typeable resistance elements. The prevalence of antimicrobial resistance in staphylococci isolated from healthy dogs, including the presence of non-typeable elements is evidence of the continuous diversification and distribution of antimicrobial resistance-related genetic elements between bacterial populations, presenting a growing issue in human and veterinary medicine.

4.2 Bacteriocins as novel antimicrobial therapies

The commensal bacteria residing on the skin help to prevent pathogenic growth and invasion; this is largely via the production of a range of antimicrobial peptides (AMPs), enzymes, and toxins (235,353,456). As a result, the microbiota has become a useful resource in the search for novel antimicrobial substances that can be used therapeutically in the age of antibiotic resistance (235,275). One of the largest and most diverse groups of AMPs are bacteriocins; ribosomally synthesised peptides, typically displaying antimicrobial activity, although some may also demonstrate additional functions (296,307,365,457). Bacteriocins are seemingly ubiquitous, produced by up to 99% of

bacteria (308,458), with the majority yet to be discovered (458). Production of bacteriocins is reported to be more frequent amongst bacteria residing on the skin, compared to those in the gastrointestinal (GI) tract (459); this is likely due to their central role in improving bacterial survival and fitness in harsh and competitive environments (274,459), particularly in nutrient-deprived conditions, such as the skin, whilst the GI tract is more nutrient-rich, thus lessening the need for energy-costly mechanisms to aid fitness.

The bacteriocin nisin, isolated from *L. lactis,* was granted “generally regarded as safe” (GRAS) status and approved for use as a food bio-preservative, due to its antimicrobial activity against a range of Gram-positive pathogens (306,369,370,460). Focus has widely been on the use of bacteriocins in food safety, however some bacteriocin-based therapies have been implemented in veterinary medicine, including nisin and lacticin topical formulations to prevent bovine mastitis (371,372,461). There is now considerable interest in the use of bacteriocins as clinical antimicrobials in human and veterinary medicine, due to; the abundance of bacteriocins, their potency (typically effective at nanomolar concentrations) (305,323), and amenability to bioengineering, due to their ribsomally synthesised peptide nature (323,373,462), making them ideal candidates as novel antimicrobials (456,463–466). Bacteriocins are often effective against multi-drug resistant bacteria, and against biofilms, with some displaying greater efficacy than traditional antibiotics (348,349,467,468); these are highly desirable traits. Early reports suggest low or no cytotoxicity against eukaryotic cells (321,350,469,470), however this is an area lacking in research, which requires further exploration before bacteriocins can be used in clinical medicine. Bacteriocins typically possess a narrow spectrum of activity, acting on closely phylogenetically related species, and those sharing an ecological niche (303,304); when looking at clinical antimicrobials, this is an advantage, as it minimises disruption to the microbiota and limits the development and spread of resistance amongst bacteria (456,466). The heterogeneous nature of bacteriocins is also an asset, as the diverse range of structures, mechanisms of action, and target organisms increases their clinical value, and helps to minimise the development of resistance by allowing the use of multiple bacteriocins with distinct cellular targets and mechanisms (466).

Coagulase-negative staphylococci have become an area of particular interest when investigating bacteriocins as they are prolific bacteriocin producers, with over 20 known bacteriocins and several more currently uncharacterised; many CoNS-derived bacteriocins demonstrate antimicrobial activity against clinically relevant pathogens, including *S. aureus* and *S. pseudintermedius* (reviewed in (301)). A deficiency in bacteriocin-producing commensal bacteria, including CoNS species, is thought to be implicated in the pathogenic overgrowth of CoPS in atopic dermatitis (253,265); a study by Nakatsuji *et al.* (253) demonstrated that application of producing strains or the purified bacteriocins derived from them was able to inhibit *S. aureus* growth and help restore the normal microbiota in atopic patients. This is the basis for our project, in which we hypothesised that bacteriocins derived from CoNS residing on the skin of healthy dogs may present promising novel therapies for *S. pseudintermedius* infection in canine atopic dermatitis. The frequency of bacteriocin production within this genus, the efficacy of other CoNS-derived bacteriocins against CoPS, and the shared ecological niche of skin-dwelling CoNS and *S. pseudintermedius,* increasing the likelihood of bacteriocins targeting *S. pseudintermedius* whilst minimising disruption to other commensal communities on the skin, suggests that CoNS-derived bacteriocins are a promising area of investigation (235,353,355,456).

4.2.1 Investigating bacteriocin production by commensal CoNS

A popular method for screening bacteria for production of bacteriocins and other antimicrobial substance is via agar-based (deferred) antagonism assays, such as spot-on, well-diffusion, and cross-streak assays, or modified variations of these methods (284,314,353,376,471), where visible zones of inhibition demonstrates the secretion of antimicrobial substances. Whilst these techniques do have limitations, such as the inability to discriminate between bacteriocins and other antimicrobial substances (296), and limited ability to obtain quantitative data, they are useful assets for preliminary and crude screening of antimicrobial activity, allowing a large number of isolates to be investigated in a simple and non-time consuming manner. In this study, all isolates identified as coagulase-negative staphylococci by the VITEK®2 were screened using a spot-on assay, with two clinically relevant *S. pseudintermedius* strains used as indicators, one methicillin-resistant and one methicillin-susceptible. Thirteen isolates displayed inhibitory activity against at least one of the strains (Table 6), identifiable by a clear zone of inhibition around the “spot” of producer strain growth. The percentage of isolates

identified as inhibitory (9%) was notably lower than that of other studies screening CoNS via agar-based assays, which reported 86.5-94% of isolates demonstrating inhibitory activity (284,353). The higher frequency of inhibition in these studies is likely due to the larger selection of indicator strains used; a study that used only a single indicator strain found only 0.11% of 90,000 screened CoNS displayed inhibition (235). Although the use of a broader selection of indicators is more likely to result in identification of inhibitory activity, we were only interested in those that show inhibition against *S. pseudintermedius* strains of clinical relevance, and so use of more indicator species would not have been beneficial when trying to identify inhibitory isolates active against *S. pseudintermedius*. Following whole genome sequencing, it was revealed two of the inhibitory isolates were coagulase-positive species, with one identified as *S. pseudintermedius*, and one as *S. schleiferi* subsp. *coagulans,* and one inhibitory isolate belonged to a novel *Staphylococcus* species.

The thirteen inhibitory isolates, and non-inhibitory species-matched controls, were investigated further using cell-free supernatant (CFS) assays, allowing for collection quantitative data, and further characterisation of active antimicrobial substances present in the CFS. Only three isolates, *S. devriesei* H14/1-3, displayed any antimicrobial activity via CFS assay (Table 7, Figure 4). It is not clear why the ten other isolates that displayed inhibitory activity via deferred-antagonism did not in the CFS assay, although this has been noted in previous studies (358,472). Jang *et al.* (472) shared a similar observation, in which a strain of *S. epidermidis* was inhibitory in live agar-based assays, but if heat-killed or using CFS no inhibition was noted. They were able to extract and purify an active compound from the bacteria; they hypothesised that the inhibitory bacteriocin is expressed but not exported, thus only present in the cytoplasm (472). However, both deferred-antagonism and CFS assays demonstrate activity due to secreted substances, so this is not likely to be the reason for the lack of inhibition in CFS assays in this case. Producer strains were grown in identical conditions for use in the spot-on assay and for harvesting the CFS, and so a difference in environment or conditions, which often regulate expression of AMPs, again is unlikely to be the cause (235,275,353,473). Isolates were grown in nutrient-limited conditions before harvesting CFS to see if this induced antimicrobial activity; this was achieved via 48-hour incubation before harvesting CFS, and addition of dipyridyl, an iron chelating agent, to growth media. A longer incubation period, resulting in depletion of nutrients, did not induce antimicrobial substance production in these isolates.

Iron-limited conditions have been shown to induce AMP production in other CoNS (275), however residual dipyridyl present in the CFS inhibited the growth of the indicator strains, producing inconclusive results. Zipperer *et al.* (275) also observed that production of the novel antimicrobial lugdunin was only seen on solid media; this is a possible cause for the lack of antimicrobial substance production for our isolates, as CFS was harvested directly from broth culture. The same two indicator *S. pseudintermedius* strains were used for both assays; isolates were tested against a range of other clinically relevant CoPS strains (Table 2) via CFS assay, but again, no inhibition was noted (Table 8). It is most likely that for the majority of the inhibitory strains, the active antimicrobial substance is only produced when the competitor/target strain is present, as production is energy costly and so not constitutive (274).

CFS derived from *S. devriesei* H14/1-3 demonstrated notable inhibition against *S. pseudintermedius,* decreasing growth by up to 75% based on area under curve (AUC) analysis of growth curve data (Figure 4). A volume of 50 or 100 µl of CFS was needed to observe inhibition (Table 10). The same antimicrobial activity was seen against 16 of 21 clinical *S. pseudintermedius* strains, isolated from canine pyoderma cases, including methicillin-resistant strains (Table 9). *S. devriesei* H14/1-3 CFS also inhibited methicillin-resistant and methicillin-susceptible *S. aureus* test strains, but not *S. schleiferi* subsp. *coagulans* (Table 8). CFS was then tested for inhibitory activity against commensal CoPS, isolated from the skin of healthy dogs in this study; *S. devriesei* H14/1-3 displayed inhibitory activity against 0/7 *S. aureus* isolates tested and 3/14 *S. pseudintermedius*. Whilst it appears *S. devriesei* H14/1-3 is less active against carriage strains, desirable for an antimicrobial as results in less disruption to the microbiota, it is typically the same strains of *S. pseudintermedius* found on the skin that result in opportunistic infection in atopic dogs (34). Some CoNS-produced AMPs are able to selectively disrupt virulence, interfering with pathogenic growth (276,282,283,285). A better understanding of the active substance present in the CFS and its mechanism of action will help to further the understanding of why it appears more active against certain strains, when epidemiologically many are closely related or the same.

Crude characterisation of the active substance in the CFS can be carried out with basic assays; a defining feature of bacteriocins is that the producing-strain is immune to its own bacteriocin (303,304).

CFS from *S. devriesei* H14/1-3 had no inhibitory activity against itself, suggestive of a bacteriocin. Enzyme treatment can offer insight into biochemical properties of the active substance; a loss of antimicrobial activity following treatment with proteases, amylase, or lipase is suggestive of peptide, carbohydrate, and lipid moieties, respectively (304). Bacteriocins are peptides, very rarely containing carbohydrate structures, and never reported to contain lipid elements. Loss of activity due to digestion by proteases is often used to classify AMPs as “bacteriocin-like inhibitory substances” (296). Two proteases, proteinase K and trypsin, and amylase, were used to treat *S. devriesei* H14/1-3 CFS, however inconsistent results mean that no conclusions about the nature of the substance can be drawn based on the data. Extraction and purification of the active substance, in order to reveal molecular mass and structure, and to confirm antimicrobial activity of the purified substance, are the preferable next steps to identify and characterise the antimicrobial substance produced by *S. devrisei* H14/1-3.

Live bacteria agar-based and cell-free supernatant assays are valuable tools when searching for antimicrobial substances, however these approaches have limitations. Antimicrobial substance production is often regulated by growth conditions (235,275,353,473), and so whilst antimicrobial activity may not be observed in one set of conditions, it may be induced when tested at different temperatures, pH, nutrient availability, or growth conditions (235,353). Production of some bacteriocins is regulated by an oxygen-limitation signal transduction system, meaning production is only seen under anaerobic conditions (474). As typically narrow spectrum antimicrobials, a range of indicator strains is often required to identify bacteriocin production; an absence of antimicrobial activity in these assays does not mean that the isolates tested do not possess the ability to produce antimicrobial substances, but that expression is not induced under the set of test conditions used (235,275,353,473). Due to this, it is often advantageous to combine wet-lab screening for antimicrobial activity with bioinformatic analysis for the identification of bacteriocin and antimicrobial peptide related genes. A bioinformatics-based approached also allows more insight into the nature of antimicrobial peptides, revealing properties such as molecular mass, structure, and thus class, in the absence of proteomic analysis.

4.3 *In silico* analysis of bacteriocin-related gene clusters

Bacteriocins are encoded in biosynthetic gene clusters containing other genes essential for their production, such as those encoding core synthetic enzymes, proteins involved in transport and immunity, and additional accessory modification enzymes. These clusters often also contain regulatory genes. Bacteriocin core synthetic enzymes typically contain highly conserved motifs; the presence of these motifs is utilised in web-based genome mining tools, such as BAGEL4 and antiSMASH, which identify putative bacteriocin clusters based on conserved motifs (387,473,475). The introduction of genome mining tools has helped to advance the search for bacteriocins, as they overcome the test condition-dependent nature of experimental agar-based screening (353,466,473,476); however, a purely genomic approach still faces limitations. The presence of bacteriocin-related gene clusters in the genome of bacteria does not always mean the encoded protein is produced or active (235,353,477); transcription regulation systems may be dependent on conditions, or the genes encoded may be pseudogenes, or contain nonsense point mutations (466). Genome mining is also based on current knowledge of bacteriocins, using known conserved motifs as driver sequences to identify putative novel bacteriocin encoding genes (391,473,476), meaning bacteriocins from less well studied classes, unrelated novel sequences, or short sequences may be overlooked (391,477). It also cannot be determined whether the encoded bacteriocins are of any clinical usefulness based on peptide sequences and genes present, as activity and target strains are unknown (391,463,476–478); thus, again, a combination of *in vitro* screening for antimicrobial activity, and *in silico* analysis for the presence of AMP-related genes is the most effective and efficient way to screen for AMP production and identify potentially novel AMPs, this approach has aided the discovery of many novel bacteriocins (353,391,463,466,473,477).

BAGEL4 and antiSMASH were used in this study to search the genome sequences of the thirteen initially inhibitory isolates, plus additional non-inhibitory isolates. Bacteriocin-related gene clusters were identified in six of the inhibitory isolates (Table 15), and two non-inhibitory isolates (*S. xylosus* H39/4B and *S. pseudintermedius* H6/2). A total of nine bacteriocin-related gene clusters were identified via genome mining, with an additional putative cluster found in *S. devriesei* H14/1, H14/2 and H14/3 by manually searching genome annotations using Artemis. No clusters were identified in the remaining seven isolates that initially displayed inhibition in deferred antagonism assay; this may

be due to clusters being overlooked by genome mining tools, or inhibition due to production of other non-bacteriocin antimicrobial substances. Four of the bacteriocin-encoding species identified harboured more than one bacteriocin-related gene cluster; it is not uncommon for bacteria to encode multiple bacteriocins (353,358,391). Lanthipeptides (class Ia) were the most frequently identified class of bacteriocin in these isolates, followed by class II bacteriocins, with one sactipeptide (class Ic) identified. As one of the isolates initially displaying inhibitory activity, and then found to harbour multiple bacteriocin-related gene clusters, was revealed to be *S. pseudintermedius* (rather than a CoNS species) following genotypic identification, it was decided to also screen the genomes of 58 clinical *S. pseudintermedius* isolates, collected from patients visiting The Hospital for Small Animals (R(D)SVS). Three bacteriocin-related gene clusters were identified; a class II bacteriocin, identified in 32 isolates, a sactipeptide, identified in 22 isolates, and a lanthipeptide, identified in 3 isolates, with the majority containing more than one of the clusters. All clusters identified were investigated further to reveal more about the nature of the bacteriocins encoded.

4.3.1 Lanthipeptide gene clusters

Lanthipeptides are the most abundant type of bacteriocin (479–481), with lanthipeptide-encoding gene clusters identified across an array of bacterial phyla, particularly Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, and Cyanobacteria (391,476,482,483). The name lanthipeptide is derived from “lanthionine containing peptides”, as this class of bacteriocin is characterised by the presence of lanthionine and the heavy posttranslational modification the peptides undergo (484); lanthipeptides that display antimicrobial activity are often referred to as lantibiotics (484,485). CoNS species are considered a rich source of lantibiotics (235,253,296,338,368), with several well-characterised lantibiotics isolated from these species (Table 1), particularly *S. epidermidis,* which produces five known lantibiotics. Bacteriocin production, including lanthipeptides, is a strain-specific trait, rather than species-specific, and so many distinct bacteriocins can be isolated from one species. Lanthipeptides can serve in a range of biological functions, including antifungal and antiviral, morphogenetic, antinociceptive and antiallodynic (486–489), but are primarily of interest due to their antibacterial nature. Lanthipeptides exert antimicrobial activity by several different mechanisms; most frequently, they cause membrane-potential dependent permeabilisation, or transmembrane pore formation, resulting in cell lysis (296,321,322). Epidermin, gallidermin, and other related lanthipeptides

are able to inhibit peptidoglycan synthesis through interactions with lipid II, a highly conserved peptidoglycan precursor (323–325). This sub-group also possess the ability to inhibit biofilm formation, by disrupting teichoic acid biosynthesis (321,327). The lantibiotic pep5 induces autolysis of the target cell by binding lipoteichoic acids, resulting in activity of cell wall-targeting lytic enzymes (296). Interaction with conserved targets allows lanthipeptides to demonstrate activity against a broader spectrum of target organisms compared to other bacteriocins, often acting against many Gram-positive organisms (313,473). Several staphylococcal-derived lanthipeptides demonstrate *in vitro* efficacy against *S. aureus* and other Gram-positive organisms; their ability to target biofilm formation, and act against antibiotic resistant strains, due to their distinct cellular targets, make lanthipeptides one of the most promising classes of bacteriocin for use as clinical antimicrobials.

Lanthipeptides are encoded as a precursor peptide (often designated "LanA"); the encoded peptide is larger than the final peptide, containing a leader peptide at the N-terminus, often around 25 residues in length (463), that is cleaved to form the final biologically active peptide (303,463,484,490,491). The pro-peptide, located at the C-terminal, is rich in serine (Ser), threonine (Thr) and cysteine (Cys) residues; these are key sites for posttranslational modification and recognition by biosynthetic enzymes (491,492). Serine and threonine residues are dehydrated to form didehydroalanine (Dha) and didehydrobutyrine (Dhb), respectively (483,484). Intramolecular Cys thiols are added to the dehydrated amino acids, forming thioether cross links, producing the characteristic (methyl)lanthionines for which lanthipeptides are named (493). Lanthipeptides can be further divided into subclasses based on the enzymes that carry out the posttranslational modifications (484,493). Type I lanthipeptides are modified by the LanBC system (485); the LanB dehydratase catalyses the dehydration of Ser and Thr, whilst the LanC cyclase facilitates the addition of Cys thiols and the subsequent cyclisation of the peptide (484,494,495). Phylogenetic analysis of LanB and LanC encoding genes revealed distinct cluster patterns amongst bacterial phyla, demonstrating distinct evolutionary origins of the two enzymes (482). It is the presence of a LanB enzyme that defines type I lanthipeptides, as LanC enzymes have been identified in other subclasses (463). Dehydration and cyclisation are carried out by a single, dual-function enzyme, LanM, in type II lanthipeptides (496–499). LanM contained an N-terminus dehydratase domain, and a C-terminus cyclase domain; this is homologous to LanC (496–499), whilst the dehydratase domain displays no homology to LanB (500).

LanM-type lanthipeptides are more frequently identified in bacteria belonging to Proteobacteria and Cyanobacteria (501,502). Type III and IV lanthipeptides are modified by single, tri-functional enzymes, termed LanKC and LanL, respectively (496,500,503). These lanthipeptides and their modification enzymes are less well characterised than other subclasses. Both LanKC and LanL contain N-terminal phosphoSer/phospoThr lyase domains, central Ser/Thr kinase domains, and C-terminal cyclisation domains (496,500,503). The N-terminus and central domains of LanKC and LanL are shared, whilst they differ in C-terminal domains; the LanKC C-terminus domain is unique among lanthipeptide modification enzymes, due to the lack on conserved Cys-Cys-His/Cys zinc-binding motif (483,488,496). Lanthipeptides can be broadly categorised based on similarities in the unmodified precursor peptide sequence (391); type I lanthipeptide subgroups include nisin-like, epidermin-like, pep5-like, streptin-like, and planosporocin-like (322). Similarities in pre-peptide sequences often do not reflect the similarity or relatedness of the biosynthetic enzymes, however (391,463,482), and so grouping this way should not be used to try and derive evolutionary origin or phylogenetic history of lanthipeptide synthetic gene clusters.

Other genes present in lanthipeptide biosynthetic clusters include those encoding LanP, a serine protease responsible for cleavage of the leader peptide following posttranslational modification (499,504), some clusters may instead contain an S8-class subtilin-like protease to function in this role (505). Many contain a two-component regulatory system, consisting of a histidine kinase (LanK) and a transcription regulator, LanR (463,484,506). Genes encoding proteins involved in immunity and transport are present; often these may be the same proteins sharing a function (500), as immunity is often facilitated by active transport of the peptide out of the cell, by a LanEFG ATP-binding cassette (ABC) transporter (507). Cleavage of the leader peptide to form the active lanthipeptide is often coupled with export, as an innate defence mechanism (463). Immunity may be mediated by the independent LanI system, encoding an extracellular lipoprotein, that binds and sequesters the active peptide (463,507). In some clusters, neither LanI nor LanEFG are present (463,500), suggesting other immunity and/or transport mechanisms yet to be discovered. Type IV (LanL) gene clusters contain LanT and LanH, the ATP-binding and membrane permease subunits of an ABC transport, but no other immunity genes, nor any regulatory or protease elements (500). This demonstrates that not every element is required in a gene cluster to produce a functioning bacteriocin, and that lanthipeptide

gene clusters may vary significantly in the enzymes and other proteins encoded. Additional 'accessory' genes may be present in lanthipeptide gene clusters, tailoring the peptide typically following cleavage of the leader peptide (391,463). The epidermin synthetic gene cluster contains the tailoring protein LanD, a flavoprotein functioning in the oxidative decarboxylation of Cys residues localised at the C-terminus (508). The subgroup formed of epicidin 280 and epilancins K7 and 15X are characterised by a lactyl group at the N-terminal of the cleaved, active propeptides (463,509). Other accessory biosynthetic enzymes include oxidoreductases, o-methyltransferases, aminoglycoside phosphotransferases, phosphate acetyltransferases, and carboxymethyltransferases (391,509); the exact role of many of these enzymes in lanthipeptide synthesis is unclear, but the genes encoding these enzymes have been frequently identified in lanthipeptide gene clusters in *Frankia, Geobacillus, and Streptomyces* sp. (391). Lanthipeptide biosynthetic gene clusters can often be located on plasmids and other mobile genetic elements (MGEs), and many gene clusters contain multiple transposases (391); this may explain the mobility and diversity amongst bacteriocin-related gene clusters, many of which are thought to have been shared between species via horizontal gene transfer (HGT) (296,309,310,357), and possibly undergoing recombination or independent acquisition of individual modification enzymes, resulting in the varied composition of these gene clusters and their non-linear phylogenetic history.

Lanthipeptide gene clusters identified

Five lanthipeptide biosynthetic gene clusters were identified from the 13 isolates that displayed inhibitory activity via deferred-antagonism assay. Two lanthipeptide clusters were identified in *S. pseudintermedius* H6/1, these were also present in the non-inhibitory isolate *S. pseudintermedius* H6/2. As no inhibition was observed from *S. pseudintermedius* H6/1 via CFS assays, it is likely there are transcription regulation systems controlling the expression of these lanthipeptides, explaining the inconsistent inhibitory activity between agar and CFS assays. The core pre-peptide sequence of both lanthipeptides showed no homology to any known lanthipeptides; the predicted final molecular masses were 1451 and 1297 Da, based on predicted modifications via antiSMASH. antiSMASH predicted both lanthipeptides to be type III (LanKC). A LanC-type enzyme was identified in one of the lanthipeptides; the cyclase domain of LanKC does not display homology to other LanC's, and so it is an inconsistency for this lanthipeptide to be classed as both type III but with LanC present. No LanB

was detected, suggesting this cluster is more likely to be type II (LanM) or type IV (LanL), as the cyclase domains of these multi-functional modification enzymes are homologous to LanC, explaining why they were recognised as LanC by genome mining tools, which identify conserved motifs and domains. No Lan enzymes were identified in the second lanthipeptide cluster, making it hard to gain any further insight into the nature of this lanthipeptide. A warnericin-like lanthipeptide cluster was encoded by *S. warneri* H9/1; an ABC-family transporter was identified, but no other modification enzymes. This lanthipeptide is possibly a natural variant of warnericin produced by *S. warneri* RB4 (361), but without further comparison of the gene clusters it is not possible to draw conclusions. A lanthipeptide gene cluster was identified in *S. simulans* H16/2, containing LanB, LanC, and LanT (ABC-family transporter), but no pre-peptide (LanA) was identified. This is not uncommon, due to the short open reading frames encoding the pre-peptides (391,510), and the variation that can be found between LanAs in different bacteria. However, as no conserved motifs were identified, it suggests that any pre-peptide gene present encodes a novel lanthipeptide with no similarity to other known lanthipeptides. ORFs localised both upstream and downstream of the *lan* genes were searched using BLAST to try and identify *lan*A, however none resembled any other lanthipeptide structural genes. The independent acquisition of various *lan* elements means it is possible that no *lan*A is present despite the presence of other biosynthetic genes; another bacteriocin cluster is encoded by *S. simulans* H16/2, so it is possible that the inhibitory activity seen via deferred antagonism was due to the second encoded bacteriocin.

A lanthipeptide identified as similar to hominicin was identified in three clinical *S. pseudintermedius* isolates; this lanthipeptide shared 16/21 amino acids with the hominicin partial peptide sequence, but no other similarities were noted across the gene cluster. No homology in pre-peptide sequence or biosynthetic enzymes were observed between this hominicin-like lanthipeptide and the two lanthipeptides identified in *S. pseudintermedius* H6. Clinical *S. pseudintermedius* isolates found to harbour bacteriocin-related gene clusters were screened for inhibitory activity against MRSP1 and MSSP1 via deferred-antagonism assays (both well-diffusion and spot-on); three isolates displayed inhibitory activity, two of which harboured the hominicin-like gene cluster. However, one isolate harboured a second gene cluster, and the third isolate did not encode the hominicin-like lanthipeptide but did harbour a third distinct bacteriocin gene cluster. Thus, it is not clear if the hominicin-like

lanthipeptide mediates the antimicrobial activity, and further investigation of the bacteriocin-harbouring *S. pseudintermedius* strains is needed.

A hominicin-like lanthipeptide was identified from *S. devriesei* H14/1-3; this lanthipeptide is of particular interest as *S. devriesei* H14/1-3 isolates displayed potent antimicrobial activity against multiple clinically relevant *S. pseudintermedius* strains, including MRSP, via deferred-antagonism and CFS assays. This activity is possibly due to the expression of this lanthipeptide. A pre-peptide of 65 amino acids was encoded; the pre-peptide possesses a predicted mass of 7164 Da, however this prediction is based on mass before posttranslational modification and cleavage of the leader sequence, and so is not likely to be accurate. Lanthipeptides typically have a molecular mass below 5000 Da. The pre-peptide contained characteristic Ser, Thr, and Cys residues at the C-terminus, and a leader peptide containing highly charged amino acids (positively charged; lysine - K, arginine - R, negatively charged: aspartic acid - D, glutamic acid – E). A conserved glycine motif was present, localised towards the centre of the peptide, thought to be a recognition site for LanP, essential for cleavage of the leader peptide. The pre-peptide shared 18/21 amino acids with hominicin, produced by *S. hominis* MBBL2-9 (Figure 7). Only a short, partial peptide sequence is available for hominicin, making it hard to estimate similarity, as this is typically based on the full ~60 amino acid pre-peptide sequence. No pre-peptide sequence similarity was observed between this hominicin-like lanthipeptide and other staphylococcal-derived lanthipeptides, including galidermin, epidermin, epicidin 280, and pep5. This hominicin-like lanthipeptide does display 50/65 amino acid similarity to the hominicin-like lanthipeptide identified among genome-sequenced *S. pseudintermedius* in this study (Figure 12). As mentioned previously, pre-peptide sequence similarity is not always the most insightful way to compare relatedness of lanthipeptides, and so the rest of the hominicin-like gene cluster identified in *S. devriesei* H14/1-3 was investigated further. A Lan modification enzyme was encoded, however this received contradicting annotation from different comparison methods (BAGEL4 annotation vs BLAST search vs BLASTp) (Table 17); BAGEL4 identified the enzyme as LanC, whilst BLAST searches resulted in a significant match to LanM. Due to the absence of LanB, and the homology of LanC and the cyclase domain of LanM enzymes, it is likely this is a LanM (type II) lanthipeptide, and the identification as LanC was a false recognition of the shared conserved motifs in cyclase domains. An S8-family serine peptidase was identified, functioning in the cleavage of the leader peptide following

modification, to form the final bioactive peptide. No immunity or transport related genes were identified, however the identified cluster contained five genes encoding proteins of unknown function, so it is possible that transport and/or immunity proteins are encoded, but were not recognised due to lack of conserved motifs or a novel nature. Several tailoring enzymes were identified, including a methyltransferase, oxidoreductase, aminotransferase, and flavoprotein, all previously reported to be present in lanthipeptide clusters, despite unclear functions. Putative genes that may be involved in regulation ("replication initiator") and mobility ("transposon invertase/recombinase") were identified. No similarities were noted between this gene cluster and that encoding the homincin-like lanthipeptide from *S. pseudintermedius.* The general lack of homology between proteins encoded in this biosynthetic cluster and other lanthipeptide gene clusters suggest the lanthipeptide encoded by *S. devriesei* H14/1-3 is a novel lanthipeptide; purification of the lanthipeptide to characterise and confirm bioactivity are the next steps required to investigate this as a possible novel antimicrobial for the treatment of *S. pseudintermedius* infections in veterinary medicine.

4.3.2 Sactipeptide gene clusters

Sactipeptides are extensively post-translationally modified peptides, characterised by the presence of cross-links between the thiol group of Cys residues and the α-carbon of acceptor amino acids (511). Their classification has been debated, but currently they are considered as Class Ic bacteriocins (305,332,484). Nearly all known sactipeptides have been identified from *Bacillus* species (512–515), however recently the sactipeptide hyicin 4244 was isolated from the coagulase-negative *Staphylococcus hyicus* 4244 (332). Subtilosin A, first isolated from *B. subtilis,* is considered the prototype sactipeptide (516). A subtilosin A-like sactipeptide was identified by genome mining in *S. pseudintermedius,* and from 22 of the clinical *S. pseudintermedius* genomes screened; the encoded pre-peptide and the biosynthetic clusters present were identical in both *S. pseudintermedius* H6 and clinical *S. pseudintermedius.*

Subtilosin A, hyicin 4244, and the subtilosin A-like sactipeptide were all encoded as 43 amino acid pre-peptides (typically denoted "sboA") (332,517,518); like lanthipeptides, these undergo proteolytic cleavage of the leader peptide (located at the N-terminus) to form the final active peptide (519). The enzyme AlbA catalyses the formation of thioether links (516,520). Processing enzymes AlbF and AlbE

are responsible for cleavage of the leader peptide, and macrocyclisation via the formation of covalent bonds between-terminus asparagines and C-terminus glycines (517,518). The final, bioactive subtilosin A peptide is comprised of 35 amino acid residues, with a molecular mass of 3.4 kDa (515,520). AlbBCD are immunity-related proteins; AlbC and AlbD form the two components of an ABC-transporter, whilst the function of AlbB is unclear, possibly acting as an extracellular receptor for sequestering the active peptide, as is seen in lanthipeptides (517,518). An oxygen-limitation dependent signal transduction system, encoded by *resD* and *resE* induces the expression of the *sbo-alb* operon (474); a second, independent transcription repression system, encoded by *abrB,* is also present in the subtilosin A biosynthetic gene cluster (474).

The subtilosin A-like pre-peptide identified here in *S. pseudintermedius* shares 29/43 amino acid residues with subtilosin A, and 32/43 with hyicin 4244. Genes encoding AlbA, AlbC, AlbD and AlbE homologs were detected in the subtilosin A-like cluster from *S. pseudintermedius*, alongside tailoring enzymes, a transcription regulator, and several genes encoding hypothetical proteins. The genes encoding the hyicin 4244 cluster show 42-70% identity to those encoding subtilosin A (332). Despite the similarities, the sequence differences in the biosynthetic clusters suggest the subtilosin A-like sactipeptide harboured by *S. pseudintermedius* is distinct from hyicin 4244 and subtilosin A. However, this subtilosin A-like sactipeptide from *S. pseudintermedius* has already been annotated in sequenced *S. pseudintermedius* genomes, but has not yet been characterised or published (GenBank accession accession WP_115830367.1).

4.3.3 Class II bacteriocin gene clusters

Class II bacteriocins are not as well characterised as those belonging to class I. They are unmodified peptides (303,364,521), divided into four subclasses: IIa, containing a conserved YGNGVXC domain; IIb, composed of two peptide chains; IIc, leaderless peptides; and IId, single chain, linear, non-pedocin like peptides (511). There are two known class IIb bacteriocins produced by *Staphylococcus* species; C55 and aureocin A70, both identified from *S. aureus* (362,364,521). Other class II bacteriocins produced by staphylococci belong to class IId, all members of the aureocin A53-like subgroup, comprising of founding member aureocin A53, capidermicin, and epidermicin NI01, derived

from *S. aureus*, *S. capitis*, and *S. epidermidis*, respectively (350,353). The *Lactococcus*-produced bacteriocins lacticin Q and Z also belong to the aureocin A53-subgroup (353).

A bacteriocin gene cluster identified in *S. warneri* H9/1 was presumed to encode a class IIb bacteriocin, due to the presence of two structural bacteriocin genes. However, genes encoding a LanM modification enzyme and a serine protease were also present; these are characteristic of lanthipeptide clusters, and as class II bacteriocins are unmodified, no modification enzymes are expected to be present. Further investigation into the gene cluster and the proteins encoded is needed to reveal whether the identification of two structural peptides was incorrect, or if this possibly represents a novel subclass of lanthipeptide.

Lactococcin 972-like bacteriocins were identified in *S. xylosus* H39/4B, *S. simulans* H16/2, and 32 of the clinical *S. pseudintermedius* genomes. The three lactococcin 972-like bacteriocins and the clusters they are encoded in showed no similarity, suggesting three distinct bacteriocins. Although discovered in the 1990's, lactococcin 972 is poorly characterised; a heat-sensitive, pH-stable peptide, lactococcin 972 belongs to class IId (522), displaying antimicrobial activity towards other *Lactococcus* species via lipid II binding-mediated cell wall synthesis inhibition (522,523). The lactococcin 972 gene cluster also contains two genes predicted to encode an ABC transporter (524); the rest of the gene cluster has not been studied further. The lactococcin 972-like bacteriocin from *S. xylosus* H39/4B has previously been identified (accession WP_042361972.1), however it is possible that the two lactococcin 972-like bacteriocins from *S. pseudintermedius* and *S. simulans* H16/2 represent novel bacteriocins.

BacSp222 is a class IId bacteriocin, first identified from *S. pseudintermedius* 222 (365). BacSp222 displays limited similarity to the aureocin A53 subgroup bacteriocins, being of a similar molecular mass, and sharing formylated N-terminal methionines, abundant tryptophan residues, and lack of cysteine residues (365). However, it is not a member of this subgroup, and is considered fairly unusual, in part due to the highly cationic nature of the peptide and low lysine content (365). BacSp222 is also multifunctioning, displaying characteristics of a virulence factor as well as an antimicrobial peptide (365). BacSp222 displayed antimicrobial activity against pathogens including *S.*

aureus and *S. pseudintermedius* (365). Another unusual trait was the ability to inhibit the producing strain, although a much higher concentration (2.1 μM) was needed compared to that needed to inhibit a non-producing *S. pseudintermedius* strain (0.16 μM) (365). At concentrations higher than those needed for antimicrobial activity, BacSp222 demonstrates cytotoxicity against eukaryotic cells, whilst at sub-inhibitory concentrates, an immune modulatory role is observed (365). Both cytotoxicity and antimicrobial activity of this peptide are thought to be mediated by massive membrane disruption (rather than pore-formation based), resulting in cell lysis (365). BacSp222 is encoded on a plasmid, the gene cluster also encoded YdbS- and YdbT-like immunity proteins (365), similar to those found in the capidermicin gene cluster (353), and an ABC transporter displaying some resemblance to that found in epidermicin NI01 (350,365). A BacSp222-like peptide was identified in *S. devriesei* H14/1-3; this was not detected by either of the genome mining tools, however the encoded peptide shared 45 of 50 amino acids with BacSp222 (Figure 9). The encoded bacteriocin has a predicted mass of 5855 Da, whilst BacSp222 has a mass of 5922 Da (365). ORFs surrounding the BacSp222-like structural gene were investigated to assess their functions (Table 18); no genes displaying homology to the immunity genes *ydbS* or *ydbT* were identified. An ABC transporter was detected downstream of the structural gene, in the same position as the ABC transporter present in the BacSp222 cluster. No other predicted functions were noted based on BLASTp searches, however all the genes were a nucleotide sequence match for *S. pseudintermedius* 222 plasmid 222, which encodes the BacSp222 cluster. The BacSp222 cluster is reported to be organised unlike any other bacteriocin gene clusters identified in *Staphylococcus* or *Lactococcus* species (365), possibly explaining why this cluster was not identified by genome mining tools. The databases utilised by these tools often lack data for many recently discovered staphylococcal bacteriocins, and primarily contain information about lanthipeptides, with data for other classes lacking. This is a limitation of genome mining, as many bacteriocin gene clusters may be overlooked due to the lack of data for comparison and identification.

Multiple bacteriocin gene clusters were identified in both CoNS and *S. pseudintermedius* isolates; this is not unexpected, due to the abundant array of bacteriocins, belonging to all four classes, produced by staphylococci. Lanthipeptides appear to be the most frequently encoded bacteriocins; most of the bacteriocin gene clusters identified in this study encoded lanthipeptides. Although bacteriocins produced by *S. pseudintermedius* have been shown to inhibit other *S. pseudintermedius* strains (365),

the general lack of inhibitory activity observed by the bacteriocin-harbouring *S. pseudintermedius* in this study suggests that this is not the most promising avenue when looking for bacteriocins with antimicrobial activity against *S. pseudintermedius* infections. The bacteriocin-harbouring CoNS did not demonstrate inhibition against other non-staphylococci in the deferred antagonism assay, although again this result is expected as bacteriocins typically only act on closely phylogenetically related species. *S. devriesei* H14/1-3 isolates did not inhibit any of the CoNS indicator strains tested via deferred antagonism; this is promising, as it suggests the antimicrobial substance responsible for the observed inhibitory activity is relatively narrow spectrum, and will result in minimal disruption to the microbiota, an important trait in any antimicrobial but of particular importance when looking for therapies for use in atopic dermatitis, where dysbiosis contributes to pathogenesis of the condition (245,257). The lack of activity via CFS of many of the bacteriocin-harbouring isolates makes purification of antimicrobial substances a challenge, as the growth conditions must first be optimised to induce expression, if any, of the bacteriocin encoded. It is possible that it is a different antimicrobial substance, rather than the encoded bacteriocins, responsible for any inhibitory activity observed; purification of the bacteriocins is required to confirm antimicrobial activity before any further investigation can take place.

As *S. devriesei* H14/1-3 demonstrated potent antimicrobial activity against multiple clinically relevant *S. pseudintermedius* strains, whilst exerting minimal effect on commensal microbiota-dwelling species, it seems the most promising area for further investigation. Two bacteriocins were encoded by this strain; both must be purified and tested for antimicrobial activity, to confirm which bacteriocin(s), if either, is the active substance of interest. The purified bacteriocin can then be taken forward to further test suitability as a novel antimicrobial therapy for *S. pseudintermedius* infection in canine atopic dermatitis.

4.4 Future directions and development of antimicrobial therapies

The critical next step in this study is the purification of the active antimicrobial substance produced by the isolates *S. devriesei* H14/1-3; this will allow further insight into the nature of the substance, such as size and structure, and independent confirmation of antimicrobial activity. The information gained about the physical properties can be combined with the known genetic information to ascertain if the

active substance is the hominicin-like or BacSp222-like encoded bacteriocins, or an uncharacterised substance, not identified by genome mining.

Identifying antimicrobial substances is only the first step in developing novel antimicrobial therapies. Testing substances for cytotoxicity against eukaryotic cells is important to ensure substances are not likely to have harmful or adverse effects if used clinically; typically, this is first carried out *in vitro* using various eukaryotic cell lines, including dermal and renal derived cell lines (350,354,470). The majority of bacteriocins that have been screened in this manner displayed no cytotoxicity (350–352,354,469,470,525), however there have been reports of bacteriocins possessing cytotoxic effects (526), including BacSp222 (365); this is a concern if the active substance produced by *S. devriesei* H14/1-3 is a BacSp222-like peptide, as this is a characteristic that may be shared. The interaction between BacSp222 and immune cells is also of concern when developing antimicrobials for use in atopic dermatitis-related infections, as peptides with any immunogenic effects will contribute to the immunological cascade and inflammatory cycle that increases susceptibility to *S. pseudintermedius* infection in atopic dogs. Thus, both screening for cytotoxicity and immunogenic effects is paramount in the search for novel antimicrobials for treatment of these infections.

If an absence of cytotoxicity is observed *in vitro,* then subsequent stages in the development of therapeutics is the use of *in vivo* models; this is important not only to further ensure no toxicity or adverse effects are observed, but also to monitor efficacy in an environment mimicking that which will be the target, in this case, the dog skin (527). Activity of compounds can be altered under physiological conditions, limiting the use of certain substances, or requiring alterations to the substance or the delivery method to improve efficacy *in situ.* The pH instability and protease susceptibility of most bacteriocins may result in decreased efficacy under physiological conditions; the susceptibility of bacteriocins to digestive enzymes limits them to topical use only (465,528), however this is the preferred delivery route for an antimicrobial treatment for *S. pseudintermedius* skin infections. The susceptibility to digestive enzymes also improves their safety, particularly in veterinary use, as this limits the likelihood of adverse effects or undesired disruption of the gastrointestinal microbiota if accidentally consumed (528). The stability of bacteriocins can be improved by bioengineering, however, recent developments in nanotechnology-based delivery systems for drugs

and peptides, such as liposome capsules, can improve stability and enhance activity whilst reducing cytotoxicity and adverse effects, eliminating the need to bioengineer the peptide itself (529–531). Once the safety and efficacy of a novel substance has been ensured, a lengthy and complex process involving many stages, including double-blind, placebo-controlled clinical trials, the final obstacle in the development of bacteriocin-based antimicrobial therapies is the large-scale production and purification of the desired bacteriocin. This can be a challenge, although the peptide nature of bacteriocins allows for heterologous expression, typically in *E. coli,* or bioengineering to improve production yield (532–534).

Although novel antimicrobial drugs, including bacteriocin-based therapies, are promising tools in the age of antibiotic resistance, these should be approached with caution, to avoid the widespread development of resistance as is seen in traditional antibiotics. Nisin has been used in food preservation for several decades, with minimal reports of resistance developing (500), however resistance to bacteriocins is not unheard of; two mechanisms of nisin resistance have been noted, in both non-producing *L. lactis* strains, and in the veterinary pathogen *Streptococcus agalactiae* (535,536). The first involved alterations to the target cell envelope, mediating non-specific resistance (456,536), including nisin resistance. A specific nisin-resistance protein (NSR) has been identified, which cleaves nisin at the C-terminus, deactivating the peptide, sometimes coupled with an ABC transporter, facilitating efflux (323,324,536,537). Resistance to the class IIa bacteriocin pedocin has been seen via loss of the pedocin receptor (324,538). These mechanisms fall into the broad categories of antibiotic resistance (modification of target, inactivation of drug, efflux of drug), and there are concerns that non-innate mechanisms of resistance (e.g. encoding enzymes that inactivate) may be disseminated via HGT (324,539). However, despite this, reports of bacteriocin resistance are infrequent, and it is suspected that mutations that mediate resistance are more costly to bacterial fitness than antibiotic resistance related variations, hence the lack of widespread resistance (324).

Bacteriocins and other antimicrobial substances, many of which are derived from commensal bacteria, present the essential next generation of antimicrobial therapies for human and veterinary use, helping to overcome the issue of multi-drug resistant bacterial infections. If prudence is applied to the use of these substances, they will be a valuable asset in medicine over the coming decades.

5. Conclusion

The skin is host to a diverse population of bacteria, that play a central role in maintaining the health of their host; dysbiosis of this community is implicated in a range of diseases. Many of these commensal bacteria protect from invasion and proliferation of pathogenic species by the production of antimicrobial peptides, such as the abundant class of ribosomally-synthesised peptides, bacteriocins. There is a particular interest in coagulase-negative staphylococci (CoNS) as frequent producers of bacteriocins that inhibit coagulase-positive staphylococci (CoPS), with potential as novel antimicrobial treatments for infections caused by CoPS species.

This study aimed to explore the population of coagulase-negative staphylococci residing on the skin of healthy dogs, to identify those with the ability to inhibit the growth of the frequent canine pathogen *S. pseudintermedius.* A diverse community comprised of 18 staphylococci species was identified from 121 dogs; of these, 9% demonstrated the ability to inhibit clinical *S. pseudintermedius* strains via agar-based deferred antagonism assay. The low frequency of inhibition noted likely reflects the limited selection of growth conditions strains were tested under, which can induce or repress expression of bacteriocins. Isolates *S. devriesei* H14/1, H14/2 and H14/3 were found to secrete an antimicrobial substance that possesses the potent ability to inhibit multiple clinical *S. pseudintermedius* strains, with minimal effect on other commensal species. Genome mining revealed *S. devriesei* H14/1-3 encoded two putative novel bacteriocins; it is likely that one of these is responsible for the antimicrobial activity observed, and may be able to be developed into a novel antimicrobial therapy for the treatment of *S. pseudintermedius* infections in canine atopic dermatitis.

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Appendix A.

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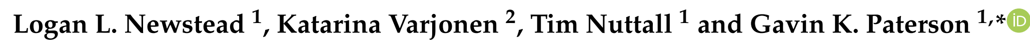
Review

Staphylococcal-Produced Bacteriocins and Antimicrobial Peptides: Their Potential as Alternative Treatments for *Staphylococcus aureus* Infections



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Abstract: *Staphylococcus aureus* is an important pathogen of both humans and animals, implicated in a wide range of infections. The emergence of antibiotic resistance has resulted in *S. aureus* strains that are resistant to almost all available antibiotics, making treatment a clinical challenge. Development of novel antimicrobial approaches is now a priority worldwide. Bacteria produce a range of antimicrobial peptides; the most diverse of these being bacteriocins. Bacteriocins are ribosomally synthesised peptides, displaying potent antimicrobial activity usually against bacteria phylogenetically related to the producer strain. Several bacteriocins have been isolated from commensal coagulase-negative staphylococci, many of which display inhibitory activity against *S. aureus in vitro* and *in vivo*. The ability of these bacteriocins to target biofilm formation and their novel mechanisms of action with efficacy against antibiotic-resistant bacteria make them strong candidates as novel therapeutic antimicrobials. The use of genome-mining tools will help to advance identification and classification of bacteriocins. This review discusses the staphylococcal-derived antimicrobial peptides displaying promise as novel treatments for *S. aureus* infections.

Keywords: bacteriocins; antimicrobial peptides; *Staphylococcus*; *Staphylococcus aureus*; MRSA

1. Introduction

Staphylococcus aureus is a frequent opportunistic pathogen of humans and animals that is capable of causing a variety of infections including skin and soft tissue infections, mastitis, urinary tract infections (UTIs), osteomyelitis, meningitis, food poisoning, biofilm-associated infections or septicaemia [1–3]. These can range from trivial and self-limiting to severe and life-threatening. *S. aureus* is a leading cause of nosocomial infections, implicated in 30% of infectious endocarditis cases [4,5], and the second most common cause of hospital-acquired pneumonia [6,7]. However, *S. aureus* is also a commensal organism, with 20–30% of humans persistently colonised nasally by the bacteria [8–10]. There is an epidemiological link between nasal carriage of *S. aureus* and subsequent infection with the carriage strain, especially among hospitalised individuals [9,11]. Risk factors for *S. aureus* infection include prolonged hospitalisation (especially intensive care), surgery, orthopedic and nursing implants, compromised immunity, skin barrier defects, and inflammatory diseases such as atopic dermatitis. The pathogenicity of *S. aureus* is attributed to an array of virulence factors, which include toxins such as enterotoxins, exfoliative toxins, and Panton-Valentine leucocidin (PVL) [12,13]. *S. aureus* can cause disease in healthy individuals as a result of expression of these virulence factors [1,13]. *S. aureus* also has the ability to form biofilms both on medically implanted devices and on tissue [14]; these characteristics allow

S. aureus to invade tissue and disseminate, causing systemic disease. The emergence of antimicrobial resistance makes the treatment of *S. aureus* infections a clinical challenge, with many strains displaying methicillin-resistance (MRSA) or multidrug resistance (MDR) [15]. Methicillin-resistance is typically mediated by *mecA*, or less frequently by *mecC*, located on the staphylococcal chromosomal cassette *mec* (SCC*mec*), and is associated with resistance to virtually all β-lactam antibiotics [15–17]. Multidrug resistance is typically defined as acquired resistance to three or more classes of antibiotic, with some *S. aureus* strains possessing resistance to all available antibiotics [18]. Topical mupirocin application is often used to eradicate nasal MRSA colonisation pre-operatively to prevent infections, however there are reports of increasing mupirocin resistance [19]. As such, finding alternative treatments for MRSA infections is a public health priority worldwide [20].

S. aureus, including MRSA, can be isolated from healthy and diseased animals, from companion animals to livestock [21–24]. *S. aureus* infection has serious welfare implications; some of the most severe infections can be seen in food animals, such as poultry, where the bacteria can cause comb necrosis, chondronecrosis and septicaemia [25,26], and in dairy cattle, where it is one of the causative agents of mastitis [27,28]. *S. aureus* causes chronic, sub-clinical intramammary infection in cattle, resulting in increased somatic cell count in the milk, and as such, decreased milk quality, in addition to decreased milk yield, increased veterinary and labour costs, and loss due to culling [27–29]. As a result, bovine mastitis is one of the most economically important diseases in animals. Colonisation and infection in animals also poses a threat to human health, so called livestock-associated MRSA (LA-MRSA), due to the risk of zoonotic transmission, via the food chain or through direct contact [30]. This represents the third recognised epidemiological form of human MRSA along with hospital-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA). Holistic approaches such as improved biosecurity on farms, vaccine development, and selective breeding for animals resistant to pathogens, have yet to succeed in the control of *S. aureus* infections in animals [31–35], increasing the urgent need for the development of novel antimicrobials.

Bacteriocins as Novel Antimicrobials

In recent years, the importance of the natural microbiota in health and disease has been highlighted [20,36–40]. In particular, the normal diverse healthy-state microbiota may help regulate inflammation and help prevent colonisation and invasion by potentially pathogenic organisms [41]. One of the ways by which commensal bacteria regulate colonisation by invasive pathogens is via bacteriocin production [20]. Bacteriocins are ribosomally synthesised peptides that display antimicrobial activity against bacteria closely related to the producer strain, but to which the producer strain itself is resistant [42,43]. Bacteriocin resistance genes are typically present concomitantly with bacteriocin structural genes. The mechanisms of resistance include antagonistic bacteriocin receptors or specialised ATP-binding cassette efflux transporters [43–45]. As the target strains and producer strains typically share an ecological niche, these specific resistance mechanisms contribute to producer strain survival [43,46–48]. Bacteriocin production is an important trait for bacterial fitness, allowing competition against other microorganisms within a niche [49]. However, bacteriocin activity is more complex, with some shown to act as signaling peptides in both quorum sensing systems or interaction with the host immune system [48,50,51]. Some bacteriocins are multifunctional, such as BacSp222 produced by *Staphylococcus pseudintermedius* 222, which features bacteriocin activity, cytotoxicity towards eukaryotic cells and immunomodulating properties [52]. The seeming ubiquity of bacteriocins, despite the energetic costs of production, supports the theory that they are important to bacterial success beyond their role as antimicrobial peptides, and up to 99% of bacteria are thought to produce at least one bacteriocin [53]. Bacteriocins have become an important target in the search for novel antimicrobials as a result of their abundance and activity against a range of pathogens.

Bacteriocins possess several advantages over traditional antibiotics as a treatment for bacterial infections. Firstly, they typically possess a very narrow spectrum of activity, resulting in less disruption to the microbiota, which can increase susceptibility to pathogenic invasion and has been

associated with several inflammatory or metabolic diseases [54]. Narrow spectrum antimicrobials also generate less selective pressure for the development of resistance in non-target organisms [55]. The mechanism of action of bacteriocins is distinct from most antibiotics, meaning they are effective against antibiotic-resistant strains of bacteria [56]. Many bacteriocins are also able to target quiescent cells as well as those actively dividing [57,58]. As ribosomally synthesised peptides, they are amenable targets for bioengineering, and can be modified relatively easily to improve characteristics such as potency, solubility, and stability [56,59]. They also show antimicrobial activity at very low concentrations compared to antibiotics (typically nanomolar concentrations) [46]. As peptides, they are susceptible to digestive enzymes; this improves their safety and minimizes disruption to the gastrointestinal microbiota but might limit them to parenteral or topical administration [54]. Several bacteriocins, such as nisin, have been approved for use as food bio-preservatives and granted generally regarded as safe (GRAS) status [60,61]. However, despite their use in the food industry, it is only recently that attention has been turned to potential use of bacteriocins as alternative antimicrobial therapies. Many *Staphylococcus* species have been shown to produce bacteriocins (Table 1), although bacteriocin production is a strain-specific, not a species-specific, trait [62]. Coagulase-negative *Staphylococcus* spp. (CoNS) are commonly found in the commensal skin microbiota [63]. As bacteriocins typically display antimicrobial activity against strains closely phylogenetically related or within the same niche as the producer, staphylococcal bacteriocins (referred to as staphylococcins) could be promising candidates for the treatment of *S. aureus* infections [64,65]. This review will explore fully and partially characterised staphylococcins, and their therapeutic potential as novel alternatives to traditional antimicrobials in the treatment of *S. aureus* infections.

2. Staphylococcins

A large number of bacteriocins have been isolated from *Staphylococcus* species. *S. aureus* is a prolific bacteriocin producer, with approximately 10 bacteriocins and bacteriocin-like inhibitory substances (BLIS) identified [46,66,67]. Six well-characterised bacteriocins have been isolated from *Staphylococcus epidermidis* [46,64,68–70]. Many other CoNS produce bacteriocins, and several have been shown to exert inhibitory activity against *S. aureus*, making them promising candidates for further research. Gram-positive bacterial derived bacteriocins tend to be highly cationic heat stable molecules [71,72]. Staphylococins are most commonly encoded on plasmids or other mobile genetic elements, although they can be chromosomally encoded [51,73].

Gram-positive and Gram-negative bacteriocins have distinct classification systems; there are four classes of Gram-positive bacteriocins, each containing several sub-classes (Figure 1) [46,74]. The majority of staphylococcins belong to class Ia, also known as lantibiotics. These are small (<5 kDa), post-translationally modified peptides, containing lanthionine or β-methyllanthionine residues [43,71], and possess relatively broad spectrum activity for bacteriocins, typically demonstrating antimicrobial activity against a range of Gram-positive organisms [71]. Lantibiotics are the most extensively studied class of bacteriocins, and as a result, their mechanism of action is relatively well understood. The majority of lantibiotics cause bacterial cell lysis and death via membrane potential-dependent permeabilisation or transmembrane pore formation [51,75,76]. The lantibiotic epidermin and its natural variant gallidermin can also inhibit peptidoglycan biosynthesis by binding membrane-bound lipid II, a peptidoglycan precursor [56,77–79]. These bacteriocins bind distinct sites from those targeted by the antibiotic vancomycin, allowing them to maintain efficacy against vancomycin-resistant bacterial strains [80]. The epidermin group of bacteriocins also have the potential to inhibit biofilm formation due to their ability to disrupt teichoic acid biosynthesis [75,81]. Pep5, a bacteriocin produced by *S. epidermidis*, binds negatively charged lipoteichoic acids, initiating autolysis of the target cell due to release and activation of cell wall hydrolysing enzymes [51]. This demonstrates that bacteriocins can inhibit target strains through several mechanisms, both bacteriostatic and bactericidal.

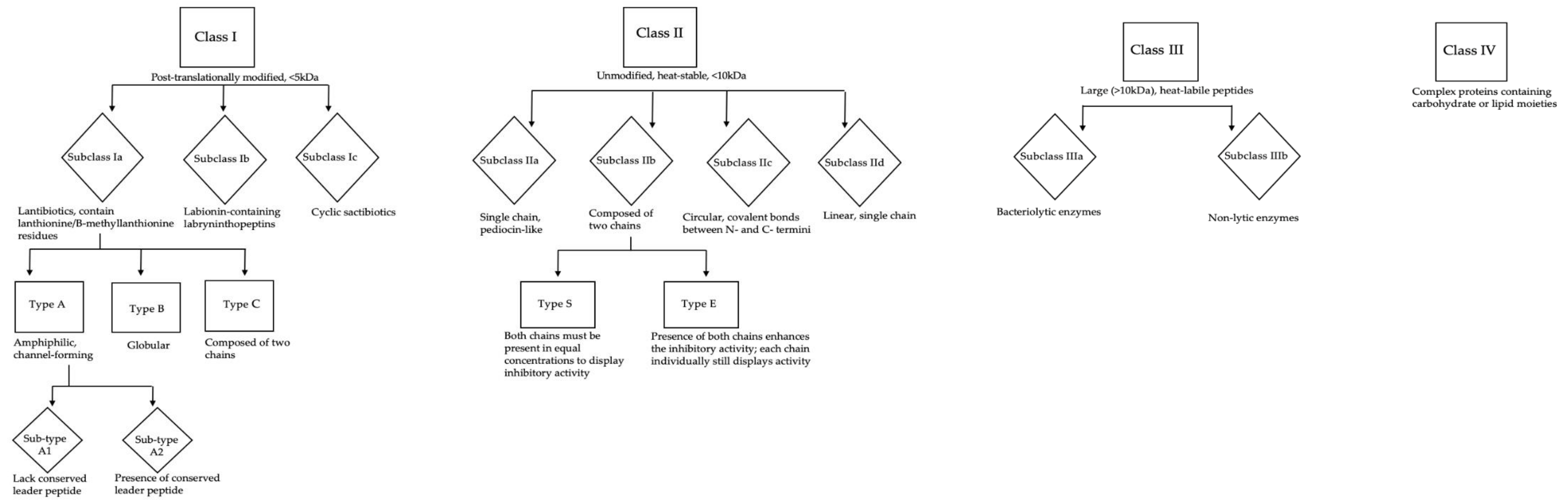

Figure 1. Classification of Gram positive-derived bacteriocins.

Subclass Ic, the sactibiotics, are extensively post-translationally modified bacteriocins characterised by the presence of cross-links between the thiol group of cysteine residues and the α-carbon of acceptor amino acids [42,82]. Only a handful of sactibiotics have been characterised, nearly all from *Bacillus* spp. However the first staphylococcal-derived sactibiotic, hyicin 4244, was recently discovered by Freitas De Souza Duarte et al. [83]. Many of the first described sactibiotics were circular, leading to the initial classification of sactibiotics as class IV [84]. However, the existence of linear sactibiotics has resulted in some discrepancy regarding the position of sactibiotics within bacteriocin nomenclature; it has been suggested they represent a novel class, class V, but currently they are tentatively considered a subgroup of class I [84,85]. As more sactibiotics are isolated and characterised, a robust classification of these substances may be elucidated.

The remaining classes of bacteriocin are not as well characterised as the class I peptides. Class II, compromised of four subclasses, contains fewer members than seen in class I. Most belong to class IId; linear, single chain peptides, typically unmodified and of molecular mass below 10 kDa [66]. Class IIb contains bacteriocins composed of two chains. These bacteriocins can be further defined as type E, where both components show inhibitory activity alone but the presence of both enhances this activity. However, the two class IIb staphylococcins (C55 and aureocin A70 both produced by *S. aureus*) are type S (synergy) meaning that both chains must be present in equimolar proportions for bacteriocin activity [46,72].

Class III bacteriocins are large (>10 kDa), heat-labile proteins in two sub-classes; IIIa, the bacteriolytic enzymes, and IIIb, non-lytic enzymes [81,86]. The *in vitro* and *in vivo* efficacy of lysostaphin, a class IIIa metalloprotease, against a range of pathogens has been studied since the 1960s [87]. The catalytic domain of lysostaphin has three distinct functions (a glycylglycine endopeptidase, an endo-B-N-acetyl glucoamidase, and an N-acetyl-muramyl-L-alanine amidase) allowing it to hydrolyse peptidoglycan components, particularly pentaglycine cross-links [88,89]. These are not typically seen in CoNS, making lysostaphin specific for actively growing and quiescent *S. aureus* [88–90].

Class IV is the final class of staphylococcins. These bacteriocins are poorly characterised and complex proteins, containing carbohydrate or lipid moieties [46,91]. Currently there is only one known staphylococcin in this group; aureocyclicin 4185, isolated from *S. aureus* 4185. This is a cyclic bacteriocin, thought to be cationic, with high hydrophobic residue content. There is little known about its mechanism of action or spectrum of activity [91].

Whilst several staphylococcins have been well characterised (Table 1), there are also many other that are only partially characterised with undefined structures, genetics and activities. Few have been tested for antimicrobial activity against pathogens, although some produced by CoNS have shown promising anti-*S. aureus* activity *in vitro* and *in vivo*.

Table 1. Well-characterised bacteriocins isolated from *Staphylococcus* species.

Class	Subclass	Subtype	Bacteriocin	Producing Strain	Inhibits *S. aureus:* Strain (MIC)	*in vivo* Model	References
I	Ia	A1	BacCh91 *	*S. aureus* CH9/DSM26258	*in vitro*: ATCC25923, Newman, M-122, RN4220 (4.0–6.0 µM)		[92]
			Epicidin 280 *	*S. epidermidis* BN280			[70]
			Epidermin *	*S. epidermidis* Tü3298	*in vitro*		[74,93]
			Epilancin 15X *	*S. epidermidis* 15X154			[94]
			Epilancin K7 *	*S. epidermidis* K7			[69]
			Gallidermin *	*S. gallinarum* F16/P57 Tü3298	*in vitro*: ATCC29213, CCUG35601 (1.25–8.0 µg/mL)		[75,95,96]
			Hominicin	*S. hominis* MBBL2–9	*in vitro*: ATCC25923, ATCC11435, CCAR M3501 (0.06–3.82 µg/mL)		[97,98]
			Hyicin 3682	*S. hyicus* 3682	*in vitro*		[99,100]
			Nisin J	*S. capitis APC2923*	*in vitro*		[20]
			Pep5	*S.epidermidis 5*	*in vitro*		[73,74,93]
		A2	Nukacin ISK-1 **	*S. warneri* Nukadoko/*S. simulans* 3299			[101]
			Warnericin RB4	*S. warneri* RB4			[102]
	Ic		Hyicin 4244	*S. hyicus* 4244	*in vitro*		[83,103]
II	IIb	S	Aureocin A70	*S. aureus* A70			[104]
		S	C55 *	*S. aureus* C55			[67]
	IId		Aureocin A53 *	*S. aureus* A53			[66,74]
			BacSp222 **	*S.pseudintermedius*	*in vitro:* DSM26258, MRSA USA300, KB/8568, ATCC25923 (0.89–1.30 µM)		[52]
II	IId		Capidermicin	*S. capitis* CIT060	*in vitro:* NCDO1499, DPC5297, Newman, RF122 (3.1–10 µg/mL)		[105]
			Epidermicin NI01 *	*S. epidermidis* 224	*in vitro:* 1195, MRSA s37, MRSA s41, MRSA s71 (1.0–2.0 µg/mL)	greater wax moth, cotton rat	[64,106,107]
III	IIIa		Endopeptidase ALE-1 †	*S. capitis* EPk1			[108]
			Lysostaphin **	*S. simulans* biovar Staphylolyticus ATCC1362	*in vitro* (0.002–100 µg/mL)	rat, mouse, cotton rat, rabbit, human	[87,88,90,109–119]
IV			Aureocyclicin 4185	*S. aureus* 4185			[91]

Chemical structure available from: * https://www.bactibase.hammamilab.org; ** https://www.ncbi.nlm.nih.gov/Structure; † https://www.rcsb.org; all other chemical structures available from references stylised in bold.

2.1. Studies Showing in vitro Inhibitory Activity against Staphylococcus aureus

Multiple techniques have been used to screen bacterial isolates for bacteriocin production *in vitro*. These include spot-on lawn assays where test producer strains are pipetted in small volumes onto the surface of agar plates, which are overlaid with soft agar containing the indicator (target) strain. Well-diffusion assays can be carried out using whole bacteria or, more commonly, cell-free supernatants [74,120]. The limitation of these assays is that they cannot discriminate between inhibitory activity due to bacteriocins or other antimicrobial substances, such as phenol-soluble modulins or organic acids [51]. The use of whole live bacteria also limits the quantitative data that can be obtained, as these assays cannot provide a minimum inhibitory or bactericidal concentration (MIC/MBC). Some studies utilise inhibition zone or density measurements to calculate arbitary units (AU) of inhibition, however these measurements are hard to standardise and are of less value than MICs. Partially purified protein and peptide antimicrobial substances are often tested for stability and activity under different conditions, such as pH, temperature and following proteolytic digestion by enzymes such as proteinase K or trypsin. Proteolysis-associated loss of activity confirms their protein or peptide structure [43]. Based on these results it is then reasonable to presume the antimicrobial substance is a bacteriocin-like inhibitory substance (BLIS), however, molecular and genomic analysis should be carried out to confirm the molecule is a bacteriocin and further characterise and classify it.

The lantibiotics Pep5 and epidermin, both produced by *Staphylococcus epidermidis*, were shown to inhibit 14 and 13 of 16 test strains of *S. aureus*, respectively, including the endemic Brazilian MRSA clone A/22C. Pep5 also inhibited a mupirocin-resistant strain [74]. Further studies showed that Pep5 inhibited 63% and epidermin 87% of 165 *S. aureus* isolates from bovine mastitis cases in South America [85]. Hyicin 3682 from *S. hyicus*, a member of the epidermin-like group, inhibited 15 of 16 *S. aureus* test strains [99,100]. Hominicin from *S. hominis* displayed potent activity against multiple strains including *S. aureus* ATCC 25923, MRSA ATCC 11435, and vancomycin-intermediate *S. aureus* CCARM 3501 [97,98], at MICs of 0.06μg/mL, 0.96 μg/mL, and 3.82 μg/mL, respectively [98]. BacCh91, produced by *S. aureus* CH91, inhibited four test strains of *S. aureus* (ATCC25293, Newman, M-122 and RN4220), with an MIC of 4.0–6.0 μM [92]. Gallidermin, isolated from poultry-associated *Staphylococcus gallinarum*, has been shown to be bactericidal against both MRSA and methicillin-sensitive *S. aureus* (MSSA) [96,121]. Gallidermin demonstrated both an MIC and MBC of 1.25 μg/mL against MSSA, and 1.56 μg/mL against MRSA [96]. Gallidermin was also able to inhibit biofilm formation of *S. aureus* SA113 at 0.16× the MIC [75]. Biofilm inhibition by gallidermin is due to repression of biofilm related genes *atl* and *ica*, encoding autolysin and polysaccharide intercellular adhesin (PIA), respectively. These gene products are involved in attachment to surfaces and cell aggregation, important steps in biofilm formation. However, gallidermin was not as effective against pre-formed biofilms, requiring 8× MIC to display inhibitory activity with 0.1–1.0% 'persister' cells still remaining [75]. The activity against planktonic cells and biofilm formation at low concentrations combined with the absence of cytotoxicity against fibroblasts or peripheral blood mononuclear cells, suggests gallidermin is a promising candidate as a therapeutic antimicrobial agent.

Recently, a natural variant of the lantibiotic nisin (nisin J) was isolated from *Staphylococcus capitis* APC2923 [122]. Nisin is a well-characterised bacteriocin first isolated from *Lactococcus lactis*; there are now at least ten known natural nisin variants produced by various *Lactococcus*, *Streptococcus* and *Blautia* spp. [123–128]. Nisin J appears resemble streptococcal nisin variants more closely than lactococcal variants [122]. Nisin J inhibited staphylococcal isolates, including *S. aureus*, with greater efficacy than nisin A or Z [122]. Like other nisin variants, the nisin J-encoding gene cluster resides on a plasmid; this has led to the suggestion that the gene cluster has been acquired via horizontal gene transfer, possibly explaining why nisin variants are isolated from several species [122].

A bacteriocin produced by *Staphylococcus hyicus* 4244 (hyicin 4244) was shown to have inhibitory activity against other staphylococcal species [83]. This bacteriocin inhibited ten clinical *S. aureus* isolates from humans and cattle, and demonstrated efficacy against MRSA and MDR strains. Hyicin 4244 also

showed potential as an *S. aureus* biofilm-inhibiting agent. Genome analysis and further characterisation showed it belonged to class Ic, the sactibiotics [103], the first staphylococcin in this subclass.

BacSp222 is a class II staphylococcin produced by *S. pseudintermedius* [52]. This tryptophan residue rich bacteriocin showed no resemblance in peptide sequence to other bacteriocins beyond limited similarities to class II bacteriocins such as epidermicin NI01 and lacticin Q and Z. BacSp222 inhibited four *S. aureus* test strains, including MRSA and *S. aureus* CH91, the producer strain of bacteriocin BacCH91, with an MIC of 0.89–1.30 μM. BacSp222 possesses some unusual characteristics for a bacteriocin; it is resistant to protease digestion and is active against the producer strain, although the MIC required (2.1 μM) was much higher than the MIC against a non-producer *S. pseudintermedius* strain (0.16 μM). Capidermicin and epidermicin NI01 are also class II bacteriocins, both belonging to the aureocin A53-subgroup [64,105]. Capidermicin inhibited all four test strains of *S. aureus* (NCDO1499, DPC5297, Newman, and RF122) with an MIC of 3.1–10 μg/mL, as well as *S. pseudintermedius* (MIC 10 μg/mL) [105]. Epidermicin NI01 inhibited MRSA *in vitro* and was not toxic to erythrocytes or dermal fibroblasts, even at a concentration of 100× the MIC [64], making epidermicin NI01 a promising candidate treatment for *S. aureus* and *S. pseudintermedius* infections. The latter being a prominent pathogen in companion dogs, particularly in pyoderma [129], with antimicrobial resistance, including methicillin-resistance and MDR isolates presenting a challenge to treatment [130–132]. As with *S. aureus*, bacteriocins and related products may have a valuable role in new approaches to tackle this pathogen.

Many studies have demonstrated the efficacy of lysostaphin against *S. aureus*. Zygmunt et al. [114] showed lysostaphin inhibited 16 MRSA isolates with 4–8× the potency of synthetic β-lactams. Lysostaphin inhibited 111 clinical MRSA isolates in a study by Huber and Huber [116], with a subsequent study by von Eiff et al. [90] showing inhibition of 429 MRSA and MSSA strains, isolated from both commensal nasal swabs and cases of bacteraemia. Lysostaphin was also shown to kill biofilm-associated *S. aureus* cells and disrupt the biofilm extracellular matrix [115]. Catheters coated with lysostaphin showed complete clearance of *S. aureus* compared to control catheters, where an average of 493 CFU were recovered. The inhibitory activity of lysostaphin was maintained on the catheters for at least four days post-coating, suggesting lysostaphin is able to bind to plastic surfaces and retain anti-staphylococcal activity for several days [111]. Due to this, lysostaphin has potential use as a preventative and treatment for biofilm-associated infections. The promising inhibitory activity of lysostaphin against *S. aureus* led to investigations of its efficacy *in vivo*.

2.2. Models of in vivo Bacteriocin Therapy for Staphylococcus aureus Infection

Animals are often used as models of human disease to determine the safety and efficacy of treatments under physiological conditions [133]. Lysostaphin has been widely tested in a range of *in vivo* systems. A single intravenous injection of lysostaphin decreased *S. aureus* bacterial load and increased survival rates in rodent models of infection, including mastitis, peritonitis and sepsis [117–119]. In mouse models of renal disease, a single intravenous dose of lysostaphin (from 1.56 mg/kg to 50 mg/kg) significantly reduced viable *S. aureus* bacterial counts from renal lesions by 95% compared to an untreated control [112], whilst another study showed a 39–78% reduction in *S. aureus* bacterial burden and a 55–65% decrease in mortality, dependent on the dose [113]. Rabbit models were used to test the efficacy of lysostaphin against *S. aureus* associated aortic valve endocarditis [88,109]. Both studies demonstrated a reduction in *S. aureus* counts following administration of lysostaphin, with a single dose showing a 3.7–6.63 $\log_{10}$ and 7.27–8.5 $\log_{10}$ CFU/g decrease in bacterial counts compared to antibiotic-treated and untreated controls, respectively [88,109]. In one case, this result was seen three days post-treatment [109] whilst the second study noted that by 30 h post-treatment there was no difference in *S. aureus* counts between treated and control animals [88]. It is possible that the choice of vehicle and route of administration affects the duration of lysostaphin activity *in vivo*; this was further highlighted in a cotton rat model of *S. aureus* colonisation of the nares, where 0.5% lysostaphin in a petroleum-based vehicle eradicated MRSA and MSSA in 93% of subjects whilst lysostaphin in a PBS solution resulted in eradication in only 33% [57]. There has been a single trial of lysostaphin to

eradicate nasal colonisation in humans comparing three treatment groups with an intranasal spray of 0.5% lysostaphin in saline 3 × daily for five days, intranasal neomycin/polymyxin B/bacitracin spray 3 × daily for five days, or no treatment [110]. 40% of the lysostaphin-treated group were cleared of *S. aureus* colonisation, compared to 6% of the antibiotic-treated and 3% of the untreated group. The effect of lysostaphin appeared to be transient, however, with re-colonisation seen by Day 11. It is possible that if delivered in a different vehicle, a longer-lived effect may be seen and this is a promising direction for further studies.

A relatively new model has been introduced for first-line *in vivo* testing, using *Galleria mellonella* (greater wax moth) larvae. These are an alternative to mammalian models as their immune system shows a high degree of structural and functional similarities to mammals [134]. These models are more accessible, inexpensive and ethical than using experimental mammals [106]. This model was used to test the efficacy and safety of epidermicin NI01 for *S. aureus* infection, which was non-toxic to the larvae and increased survival compared to untreated controls [106]. However, no quantified data for *S. aureus* bacterial burden before and after treatment was provided, which would be helpful in understanding its efficacy as an antimicrobial agent. Epidermicin NI01 was also tested in a cotton rat model of *S. aureus* nasal colonisation [107]; the nares of cotton rats structurally resemble those of humans, making it a useful model [135]. Subjects were treated with a single dose of 0.8% epidermicin NI01, twice daily treatment for three days with 0.04% epidermicin NI01, 0.2% epidermicin NI01, 2% mupirocin, or a vehicle control. A single dose of 0.8% epidermicin NI01 was most effective, resulting in a significant reduction in nasal MRSA burden and eradication in three of five test subjects [107]. Epidermicin NI01 is therefore a potential novel therapeutic for *S. aureus* nasal colonisation.

3. Other Antimicrobial Substances with Anti-*Staphylococcus aureus* Activity

3.1. Bacteriocin-Like Inhibitory Substances

A partially purified antimicrobial substance has been derived from *Staphylococcus pasteuri* RSP-1 [65]. Cell-free supernatant (CFS) from *S. pasteuri* RSP-1 was found to inhibit 11 out of 14 *S. aureus* test strains [65]. Live-dead assays suggest this substance is bactericidal, causing membrane damage and subsequent cell death in target cells. Antimicrobial activity of the CFS was lost following proteolytic digestion, whilst nuclease, amylase and lipase had no effect, confirming the substance is proteinaceous. It was heat stable up to 121 °C and at a range of pH, although a gradual loss of activity was seen with increasing pH. These characteristics are suggestive of a bacteriocin. The substance, named pasteuricin, has a molecular weight of 5 kDa [65], suggesting it belongs to bacteriocin class I or II, but further characterisation is needed.

Staphylococcus capitis TE8 isolated from the skin microbiota of humans showed antimicrobial activity against a range of Gram-positive organisms, including *S. aureus*, but had no effect on Gram-negative organisms [136]. Partially purified CFS extract also demonstrated this activity, which was lost with proteinase K digestion, suggesting the inhibitory effect was mediated by production of a BLIS. Genomic analysis revealed *S. capitis* TE8 possesses multiple antimicrobial peptide (AMP) gene clusters, including those encoding an epidermicin-like peptide, a gallidermin-like peptide, and several phenol-soluble modulins [136]. The epidermicin-like peptide seen may be capidermicin, an epidermicin variant recently isolated from *S. capitis* CIT060 [105]. However, it is possible that the BLIS and gene-clusters possessed by *S. capitis* TE8 are novel bacteriocins.

Nakatsuji et al. [62] explored the abundance of AMP production in the skin microbiota of humans with atopic dermatitis (AD) and healthy controls; they found that AMPs were common in the microbial communities of healthy subjects, but not those with AD. The application of AMP-producing *S. hominis* or *S. epidermidis* to the skin of AD subjects significantly decreased *S. aureus* burden on the skin compared to untreated and vehicle-treated controls, supporting the protective role of AMP-producing CoNS within the skin microbiota. Further investigation of commensal CoNS isolates revealed a strain of *S. hominis* (A9) with potent antimicrobial activity against *S. aureus*. Application of *S. hominis* A9 to

sanitised pig skin coated with 1×10^5 CFU/cm^2 *S. aureus* or to mice colonised with *S. aureus* significantly decreased *S. aureus* counts, with application twice daily for one week eliminating *S. aureus* colonisation in the mouse model. In contrast, application of killed *S. hominis* A9 or a non-inhibitory control strain of *S. hominis* had no effect. Genomic and biochemical analysis revealed *S. hominis* A9 produces two AMPs, predicted to be lantibiotics based their on structure and amino acid composition. These AMPs, named *Sh*-lantibiotic-α and *Sh*-lantibiotic-β, were encoded within a gene cluster containing *lanM*, *lanC*, and *lanT* homologs. These genes were not detected in non-inhibitory *S. hominis* strains. Purified *Sh*-lantibiotic-α and *Sh*-lantibiotic-β inhibited *S. aureus* on sanitised pig skin at a concentration of 0.5 nM, whilst concentrations up to 10 nM had no effect on *S. hominis* A9, the producer strain. *Sh*-lantibiotic-α and *Sh*-lantibiotic-β were able to suppress clinical *S. aureus* isolates, including MRSA USA300, but had no effect on commensal species isolated from the skin such as *Propionibacterium acnes*, *S. epidermidis*, and *Corynebacterium minutissimim*. This potent anti-*S. aureus* activity with limited disruption to microbiota make *Sh*-lantibiotic-α and *Sh*-lantibiotic-β promising candidates for further development as novel therapeutics for *S. aureus* infection in AD and other skin conditions.

3.2. Inhibitory Staphylococcal Strains

Several strains of CoNS inhibit *S. aureus* in agar-based antagonism assays. Although the antimicrobial substances responsible have not been isolated, most are presumed to be BLIS. *Staphylococcus succinus* AAS2 CFS potently inhibited *S. aureus* in well-diffusion assays [137]. Another study [138] found that 28 of 243 *Staphylococcus* isolates produced antimicrobial substances; all were susceptible to proteolytic digestion and thus classified as BLIS. BLIS-producing isolates included *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. pseudintermedius*, *S. aureus*, and *S. agnetis*. All the BLIS-producing isolates harboured *nukA* or *bsaA2* genes, suggesting these BLIS are related to nukacin ISK-1 or Bsa (a member of the epidermin-like lantibiotics). Purification, classification, and further testing of the inhibitory activity against *S. aureus* is needed to determine their potential as anti-*S. aureus* agents. 77 of 89 *Staphylococcus* isolates from nasal swabs of 37 human volunteers were shown to have inhibitory activity [139]. These isolates belonged to six species: *S. epidermidis*, *S. aureus*, *S. hominis*, *S. lugdunensis*, *S. warneri*, and *S. capitis*. Only two of the total 77 strains, however, showed inhibitory activity against *S. aureus*. 96% of the *S. epidermidis* strains produced BLIS, but these were not further investigated to determine if they were novel or one of the already isolated bacteriocins from this species. A recent study demonstrated AMP production by 21 CoNS strains, belonging to five species; *S. capitis*, *S. hominis*, *S. simulans* and *S. warneri* [20]. Of these, four *S. warneri* strains and one *S. hominis* strain were able to inhibit *S. aureus* [20]. Two strains belonging to *S. capitis*, APC2934 and APC2918, were able to inhibit both *S. aureus* and MRSA test strains [20]. These strains did not possess the structural genes encoding nisin J and colony mass spectrometry did not match the peptides produced by these *S. capitis* strains to any listed on BACTIBASE [20], suggesting these are potentially novel bacteriocins.

Carson et al. [140] investigated 441 non-aureus staphylococci (NAS) isolates; 40 of the isolates showed inhibitory activity against a bovine mastitis *S. aureus* strain; of these, 23 also inhibited MRSA. These strains belonged to *S. capitis*, *S. chromogenes*, *S. epidermidis*, *S. pasteuri*, *S. simulans* and *S. xylosus*. Only five of these species inhibited *S. aureus* in well-diffusion assays using chloroform-extracted cell-free supernatant; all five supernatants were inactivated by proteinase K, suggesting the active components are BLIS secreted by the bacteria. The genomes of the 441 NAS were studied for the presence of bacteriocin biosynthetic gene clusters. 105 clusters were identified from 95 NAS isolates, belonging to 16 species (Table 2), but there was no obvious clustering based on phylogeny or bacteriocin class. Ten of the NAS genomes encoded two clusters, belonging to different classes, suggesting these isolates have the potential to produce two bacteriocins [140]. This data shows that the 95 isolates possessing bacteriocin gene clusters have the potential to produce bacteriocins. However, only 40 of the isolates displayed inhibitory activity *in vitro*. The discrepancy between presence of bacteriocin genes and production of bacteriocins is likely due to the influence of growth conditions on bacteriocin production; the availability of nutrients, presence of stressors, temperature, and choice of media can all

affect bacteriocin production [20]. This highlights the importance of screening methods when trying to identify bacteriocin-producing bacteria, suggesting that there may be many more strains capable of producing bacteriocins that have not yet been discovered. Genome-mining tools, such as antiSMASH and BAGEL [141,142], are able to identify bacteriocin gene clusters in bacterial genomes, highlighting those harbouring the potential to produce bacteriocins. These techniques will be invaluable in the search for novel bacteriocins especially as the availability of sequenced genomes increases.

Table 2. Strains of *Staphylococcus* found to harbour bacteriocin gene-clusters from 441 non-aureus *Staphylococcus* isolates analysed, the number of isolates possessing bacteriocin production genes that displayed inhibitory activity, and the number of isolates displaying in vitro inhibitory activity against *S. aureus* strains isolated from bovine mastitis cases. Each cluster encodes one bacteriocin [140].

	Class I			**Class II**
	Lantibiotics	**Sactibiotics**	**Lasso Peptides**	
Number of Bacteriocin Clusters Identified	29	3	4	69
Number of Isolates that the Clusters are Present in	29	3	2	68
The Species that the Clusters are Present in	*S. capitis* *S. chromogenes* *S. cohnii* *S. epidermidis* *S. equorum* *S. gallinarum* *S. sciuri* *S. simulans* *S. succinus* *S. vitulinus*	*S. capitis*	*S. fleurettii* *S. sciuri*	*S. equorum* *S. gallinarum* *S. haemolyticus* *S. hyicus* *S. saprophyticus* *S. sciuri* *S. simulans* *S. succinus* *S. warneri* *S. xylosus*
Number of Isolates Showing Inhibitory Activity *in vitro*	15	2	1	9

3.3. Staphylococcal-Produced Antimicrobial Substances

Staphylococcus species produce a range of other secretory-AMPs (sAMPs) alongside bacteriocins, and several of these non-bacteriocin AMPs show promise as therapeutic agents for *S. aureus* infections. Esp is a serine protease produced by some *S. epidermidis* strains [143]. It was noted that presence of certain *S. epidermidis* strains within the nasal cavity appeared to influence *S. aureus* nasal colonisation. The CFS of these strains inhibited *S. aureus in vitro*, leading to the purification and identification of Esp. Application of purified Esp or Esp-producing *S. epidermidis* to the nasal cavities of *S. aureus* carriers eliminated *S. aureus* colonisation. Esp is effective against *S. aureus* biofilms, cleaving autolysin-derived murein hydrolases [144] and preventing the release of DNA, one of the structural components of *S. aureus* biofilm extracellular matrices [145,146]. Esp also targets *S. aureus* surface proteins, disrupting host-pathogen interactions [147], allowing Esp to be active against biofilm-forming and planktonic *S. aureus* cells. This suggests Esp could be a very promising antimicrobial agent.

Lugdunin is a novel antimicrobial, isolated from *S. lugdunensis* IVK28 [148]. It is only produced under iron-limiting conditions on solid agar, again highlighting the importance of growth conditions of producer strains when isolating antimicrobial substances. Lugdunin was encoded by all the *S. lugdunensis* strains analysed, suggesting production is species specific rather than strain specific [148]. Lugdunin is a complex, non-ribosomally synthesised peptide, containing a tryptophan moiety, with no resemblance to any known antimicrobial substances [148]. Lugdunin became the founding member of a new class of antibiotics, the thiazolidine-containing peptide antibiotics. It is suggested that it exerts its antimicrobial activity by depleting bacterial energy resources [148]. Lugdunin demonstrated potent inhibitory activity against a range of Gram-positive organisms, including MRSA and glycopeptide-intermediate *S. aureus* [148]. This antibiotic displayed no toxicity towards human

neutrophils or erythrocytes, and retained activity in human serum. Lugdunin was also able to reduce or eradicate *S. aureus* in a mouse model [148]. Together these examples demonstrate the range of antimicrobial substances produced by commensal staphylococci, and their potential as novel treatments for *S. aureus* infection. It is highly likely more remain to be discovered.

4. Conclusions and Future Directions

The commensal bacteria residing in the microbiota play a vital role in protecting the host from invasion of pathogenic organisms. This protective activity is often mediated by bacteriocins, which are ribosomally synthesised peptides produced by bacteria that possess antimicrobial activity. Bacteriocins may be a valuable tool in the future fight against antimicrobial-resistant pathogens due to their novel mechanisms of action, narrow spectrum of activity, and ability to be bioengineered to improve specific qualities desirable in biopharmaceutical agents. Many bacteriocins produced by staphylococci display potent activity against S. aureus *in vitro*; however, the lack of cytotoxicity testing of many bacteriocins is a limitation when assessing their therapeutic usefulness. Although many bacteriocins demonstrate cytotoxicity against eukaryotic cells, often in a dose-dependent manner, this does not entirely eliminate their potential as candidates for treatment of *S. aureus* infections including those caused by methicillin-resistant strains in humans and animals. Thorough evaluation of potential cytotoxic effects and pharmacodynamics of the substance, weighed against its efficacy, is required to determine suitability as an anti-*S. aureus* agent. Genome-mining techniques will facilitate the search for bacteriocin-producing bacterial strains, overcoming some of the limitations of agar assay-based methods, and helping eliminate some discrepancy in the classification of these substances.

Elucidation of the mechanisms of action of bacteriocins, especially those belonging to classes II-IV, alongside further testing of their efficacy under physiological conditions is required to determine their suitability for therapeutic use. It is important to note that although resistance among target strains to these peptides has yet to be witnessed under laboratory conditions, resistance mechanisms are widespread in producer strains. Therefore, prudence must be exercised if and when bacteriocins and related products are utilised clinically to avoid the spread of resistance and loss of efficacy.

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