# The human skin microbiota is a rich source of bacteriocin producing staphylococci which kill human pathogens

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**Abstract**

The demand for novel antimicrobial therapies due to the threat posed by antimicrobial resistance has resulted in a growing interest in the protective role of our skin bacteria, and the importance of competition between bacteria on the skin. A survey of the cultivable bacteria on human skin was undertaken to identify the capacity of the skin microbiota to produce bacteriocins with activity against skin pathogens. Twenty-one bacteriocins produced by bacteria isolated from seven sites from each subject exhibited inhibition spectra ranging from broad to narrow range, inhibiting many Gram-positive bacteria, including opportunistic skin pathogens such as *Propionibacterium acnes* (recently renamed *Cutibacterium acnes*), *Staphylococcus epidermidis,* and methicillin-resistant *Staphylococcus aureus* (MRSA). Sequencing indicated that the antimicrobial-producing isolates were predominately species/strains of the *Staphylococcus* genus. Colony mass spectrometry revealed peptide masses which do not correspond to known bacteriocins. In an era where antibiotic resistance is of major concern, the inhibitory effect of novel bacteriocins from bacteria of skin origin demonstrates the antimicrobial potential that could be harnessed from within the human skin microbiota.

## Introduction

The human skin microbiome is home to hundreds of diverse bacterial species from the four phyla Actinobacteria, Proteobacteria, Firmicutes and Bacteriodetes, that act as part of the body's first line of defence against the external environment (Natsuga, 2014). The commensal microbiota of the skin contributes to host health and is thought to play a role in protecting the host against a wide range of infections. One such defence mechanism is the production of bacteriocins. Bacteriocins are ribsosomally synthesised, heat-stable antimicrobial peptides that are produced by bacteria which can exhibit both broad and narrow range inhibition spectra. Lantibiotics belong to class I bacteriocins which are heavily post-translationally modified - characterised by the presence of lanthionine/B-methyllanthionine residues (McAuliffe, Ross, & Hill, 2001). Bacteriocins may exert a diverse array of functions from colonising peptides which help the producer to become established in a niche, to killing peptides for eliminating competitors, or as signalling peptides through interactions with other bacteria and the immune system (Bastos, Ceotto, Coelho, & Nascimento, 2009; Dobson, Cotter, Ross, & Hill, 2012; Schauber & Gallo, 2008). The importance of these traits can be seen where imbalances in the ratio or composition of skin microbiota has been linked to skin diseases/infections such as psoriasis, atopic dermatitis, acne and impetigo (Grice, 2014; Otto, 2009; Yamasaki & Gallo, 2008; Zeeuwen, Kleerebezem, Timmerman, & Schalkwijk, 2013). It has been hypothesised that restoration of this natural microbial balance could potentially alleviate or prevent such skin infections and would reduce the use of antibiotics to treat such conditions (Sanford & Gallo, 2013).

*Staphylococcus* species are dominant bacterial colonisers of the skin and are divided into two main groups on the basis of coagulase activity. Coagulase triggers the coagulation of soluble fibrinogen forming insoluble fibrin, resulting in the formation of a clot. Thus, coagulase positive staphylococci, for example *Staphylococcus aureus* (*S. aureus*) which can reside on the skin surface as a commensal organism, are potentially pathogenic and are capable of producing haemolysins and a range of heat stable toxins. Conversely, the skin is also colonised by a diverse array of coagulase negative *Staphylococcus* species which form part of the normal commensal bacteria of the skin. The production of lantibiotics is abundant within commensal coagulase negative staphylococci, for example *Staphylococcus gallinarum, epidermidis* and *hominis* produce the lantibiotics gallidermin, epidermin and hominicin, respectively (Götz *et al*., 2014). Studies have characterised the human skin microbiome in

healthy and diseased states (Grice & Segre, 2011), and while little has been done in screening the human skin specifically for novel bacteriocins, there is increasing interest in the human skin microbiome as a source of competitive strains and novel antimicrobials. This has led to the discovery of a novel antibiotic, lugdunin, isolated from a nasal strain of *Staphylococcus lugdunensis* (Zipperer et al., 2016). Furthermore, the application of human skin commensals as sources of protection against *S. aureus* and skin neoplasia's (Nakatsuji et al., 2017, 2018), highlight the potential of human skin commensals as possible alternate solutions to antibiotics.

Here we describe one approach targeted specifically at the isolation of coagulase negative staphylococcal skin isolates and investigate their inhibitory activity against a range of skin pathogens associated with skin infections such as MRSA, atopic dermatitis and acne vulgaricus, as well as the causative agents of mastitis.

## Materials and Methods

### Isolation of antimicrobial producing skin isolates

Twenty healthy volunteers, 18-65 years of age, 13 females and 7 males, were recruited for this study which was approved by the Cork Clinical Research Ethics Committee under Protocol number APC071. Seven different areas of the body were swabbed, specifically the retro auricular crease (behind ear), the axillary vault (underarm), the inguinal crease (groin), the umbilicus (bellybutton), the gluteal crease (between lower back and buttocks), the alar crease (side of nose) and the toe web space (between toes). Each swab (Sarstedt swabs Amies, PS, Viscose) was dipped in sterile saline before swabbing the body area (Landers, Hoet, & Wittum, 2010) and swabs were stored for no more than 12 hours at 4°C before being homogenised in 2 mL of Maximum Recovery Diluent (MRD; Oxoid Ltd., Basingstoke, Hampshire, UK). Tenfold serial dilutions were performed in MRD and 100 µL aliquots were spread plated onto Mannitol Salt Agar (MSA; Oxoid Ltd.) and Brain Heart Infusion (BHI) agar (Merck, Darmstadt, Germany)**.** All plates were incubated aerobically at 37°C for 48h. After incubation, colonies were counted and colonies of different morphology and colour were streaked for purity and grown aerobically in 10 mL of BHI broth (Merck) at 37°C in a shaking incubator (311DS Labnet) overnight.

### Detection of antimicrobial activity

Spot bioassays for antimicrobial production were then carried out on the isolates by spotting 10 µL of the overnight culture onto BHI agar, the plates were incubated overnight at 37°C and then overlaid with MRS sloppy *Lactobacillus* MRS broth, (BD™ Difco™ Trafalgar Scientific Ltd, Leicester, United Kingdom, LE3 1UQ) containing 0.75% agar seeded with 0.25% of an overnight culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* LMG 6901 and grown anaerobically overnight (see Table 1 for optimal growth conditions of bacterial strains used in this study). The remaining colonies on BHI plates were also overlaid with *L. delbrueckii* ssp. *bulgaricus* LMG 6901. The MSA plates were overlaid with sloppy (0.75%) BHI agar seeded with an overnight inoculum of either 0.25% *Listeria innocua* DPC 3572 or 0.25% MRSA DPC 5645. Colonies from spot assays and plate overlays that exhibited zones of inhibition were then inoculated into BHI broth, grown overnight at 37°C and stocked in 20% glycerol at -80°C for further characterisation (Fig. 1).

### Characterisation of antimicrobial activity of skin isolates

#### Well diffusion assays (WDA)

Cell free supernatants (CFS) were prepared from 10 mL overnight cultures of putative antimicrobial producing strains (isolated from BHI and MSA plates) by centrifugation (in Sorvall Legend RT) at 4000xg for 20 minutes following overnight growth in BHI broth in a shaking incubator at 37°C (Ryan, Rea, Hill, & Ross, 1996). To eliminate inhibition due to acid, the CFS were neutralised to pH 6.9-7.2 using 1M NaOH. Wells were bored in MRS agar plates, previously seeded with 0.25 % inoculum of an overnight culture of *L. delbrueckii* ssp. *bulgaricus* LMG 6901, and 50 µL of each neutralised supernatant was added to the wells. The plates were incubated anaerobically at 37°C overnight and observed for zones of inhibition around the well (Ryan et al. 1996). Strains showing zones of inhibition were selected for further study.

#### 16S rRNA sequencing

Genomic DNA was extracted from the antimicrobial producing strains (grown overnight in 10ml BHI broth at 37°C) using Sigma Aldrich DNA purification kit as described by the manufacturer (Sigma-Aldrich Ireland Limited, Vale Road, Arklow, Co. Wicklow, Ireland). For 16S rRNA sequencing, universal primers Uni F 5'-AGAGTTTGATCCTGGCTCAGG-3' and Uni R 5'-ACGGCAACCTTGTTACGAGT-3' were used for PCR reactions (run conditions: initial denaturation: 94°C x 5 minutes, cycling conditions: 30 cycles of 94°C x 40 seconds, 55°C x 30 seconds, 72°C x 1 minute, final extension: 72°C x 10 minutes) to initially identify the antimicrobial producing strains. To confirm species, degenerate primers targeting the *dnaJ* gene (Shah et al., 2007) forward primer: 5'-GCCAAAAGAGACTATTATGA-3' and reverse primer: 5'-ATTGYTTACCYGTTTGTGTACC-3') were used for the PCR reactions following conditions described by Shah et al., (2007). Quantification of the extracted DNA and PCR products was conducted using Qubit 2.0 fluorometer (Thermo Fisher Scientific, 168 Third Avenue Waltham,

MA USA 02451). Sequencing was conducted by Genewiz (Hope End, Takeley, Essex, CM22 6TA, United Kingdom) and analyses of the sequencing data were performed utilising Lasergene 8 software (DNAStar Inc., Madison, WI). Basic local alignment search tool (BLAST) on the National Centre for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov) was used to compare the sequencing data of the isolates to existing genomic data.

**Heat stability and protease sensitivity**

To establish the heat stability of bacteriocins produced, CFS were incubated for 10 minutes at a range of temperatures: 37°C, 60°C, 70°C, 80°C, 90°C, 100°C and for 15 minutes at 121°C before performing a WDA in 1.5% MRS agar seeded with 0.25% *L. delbrueckii* ssp. *bulgaricus* LMG 6901 as previously described, see supplementary information figure S2 A.

To investigate whether the antimicrobials produced were proteinaceous in nature, the CFS of all antimicrobial producing isolates were incubated with Proteinase K (Sigma) to a final concentration of 20 mg/mL at 37°C for 1 hour and tested for antimicrobial activity by WDA using *L. delbrueckii* ssp. *bulgaricus* LMG 6901 as the target organism as described above. The corresponding CFS, mixed with an equivalent volume of water, was used as a control, see supplementary information figure S2 B and C.

**Cross Immunity**

To determine the relatedness of the bacteriocins produced by the strains, cross immunity assays were performed as follows: CFS of all producing strains were tested against each other by conducting WDA assays as previously described using each producing strain as a producer and also as a target strain. Each producing strain was inoculated individually (at 0.25%) into BHI agar which was allowed to solidify and 50 µL of the pH neutralised CFS of each strain was added to wells of all plates.

**Spectrum of Inhibition**

A spectrum of inhibition was completed on pH adjusted CFS of all antimicrobial producing isolates using 28 indicator strains by WDA as described previously. The target strains, media and incubation temperatures are outlined in Table 1. All indicator strains were diluted to the same optical density, ($OD_{600\ nm}$ 0.8), using Biochrom Libra S2 Collimeter (Biochrom Ltd., Cambourne Business Park, Cambridge, United Kingdom, CB23 6DW) before seeding the agar. Indicator strains employed to detect antimicrobial activity and their optimal growth conditions are listed in Table 1. Zone size was measured as follows: area of zone $\pi r^2$ - area of well $\pi r^2$ in millimetres.

**Pulsed Field Gel Electrophoresis**

All isolates identified as *Staphylococcus* species were finger printed using Pulsed Field Gel Electrophoresis (PFGE) as described by Bannerman et al. with minor modifications (Bannerman et al., 1995). Cultures were inoculated at 0.02% and grown in a shaking incubator overnight in 5 mL BHI broth at 37$^{o}$C. The overnight culture (0.7 mL) was centrifuged for 2 minutes at 4500xg in an Eppendorf Centrifuge. The supernatant was discarded, and the cell pellets were washed in 1 mL of sterile TE buffer (0.1 M Tris Cl, 0.15 M NaCl, 0.1M EDTA pH7.5) and recentrifuged. Washed cells were resuspended in 0.3 mL of sterile EC buffer (6 mM Tris-HCl, 1 M NaCl, 0.1 M EDTA, 1% Sarkosyl). A solution of lysostaphin (1 mg/mL) was prepared in 20 mM sodium acetate (pH4.5) and 2 µL was added to the cell suspension and then vortexed. A 2% Sea Plaque agarose (Bio-Rad Laboratories Ltd., Watford, Hertfordshire United Kingdom, WD17 1ET) solution was prepared in EC buffer and 300 µL added to 300 µL lysostaphin-cell suspension mixed gently to avoid the formation of bubbles and then quickly pipetted into PFGE plug molds. The agar plugs were allowed to solidify at room temperature for 10 minutes. When solidified, the plugs were incubated in 3 mL EC buffer overnight at 37°C. After overnight incubation, the EC buffer was removed and replaced with 3 mL TE buffer (10 mM Tris Cl, 1 mM EDTA) and incubated for 1 hour at 55°C without shaking. Prior to electrophoresis, the plugs were cut into small slices 2x5 mm and transferred into Eppendorf tubes containing 125 µL of 20U SmaI restriction enzyme (New England Biolabs, 75-77 Knowl Piece, Wilbury Way, Hitchin, Herts, United Kingdom, SG4 0TY) in buffer. Plugs were incubated shaking overnight (300 rpm) at 25°C. Following digestion, trimmed slices of plug were loaded onto the PFGE comb, fixed with 1% PFGE grade agarose (Bio-Rad Laboratories) in 0.5X Tris-borate EDTA (TBE) buffer. Lambda PFGE marker (New England Biolabs) was loaded onto the gel as described above and used as reference ladder. The gel was run in 0.5X TBE buffer using a CHEF-DR II®PFGE apparatus using the following gel running parameters: initial pulse: 5 seconds, final pulse 40 seconds, voltage 200 V or 6 V/cm for 20 hours at 12°C-14°C. The gel was stained for 2 hours with Ethidium bromide (0.5 µg/mL), destained for 1 hour in distilled water and then photographed using GelDoc-It Imager (Ultra-Violet Products Ltd., Cambridge, UK).

**Activity Units**

For the 13 different antimicrobial producing strains identified from PFGE, bacteriocin activity units (AU) were calculated as described by Nilsen et al. 2002 based on the WDA. Briefly, 50 µL of CFS was diluted 2 fold and dilutions were dispensed into wells of plates seeded with indicators

listed in Table 4. AU were calculated by multiplying the reciprocal of the lowest dilution of the CFS, which produced zones of inhibition by 20 in order to obtain AU/mL, see Table 4. This assay was performed in duplicate.

**MALDI TOF Mass Spectrometry**

Colonies from the 13 antimicrobial producing skin isolates were mixed with 50 µL propan-2-ol 0.1% TFA, vortexed three times and centrifuged at 16000xg for 30 seconds. MALDI TOF mass spectrometry was performed on the CFS using an Axima TOF$^2$ MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). An aliquot (0.5 µL) of matrix solution (α-cyano 4-hydroxy cinnamic acid, 10 mg ml$^{-1}$ in acetonitrile-0.1% (v/v) trifluoroacetic acid) was deposited onto the target and left for 10 seconds before being removed. The residual solution was allowed to air-dry and 0.5 µL of sample solution was deposited onto the pre-coated sample spot; 0.5 µL of matrix solution was added to the deposited sample and allowed to air-dry. The sample was then analysed in positive-ion linear mode and peptide masses compared to the bacteriocin database, 'Bactibase' to identify putative bacteriocins.

**Results and Discussion**

The human skin microbiome is home to trillions of bacteria but is predominately colonised by members of the genus *Staphylococcus* (Otto, 2010). The skin is the defensive barrier that separates our internal organs from the external world. It acts as a first line of defence against pathogens, and the layers of skin help prevent harmful substances from accessing the body (Sanford & Gallo, 2013; Zeeuwen, Kleerebezem, Timmerman, & Schalkwijk, 2013). Significant therapeutic potential has been identified for the staphylococcal genus, including the production of class 1a lantibiotics such as epidermin, nukacin, gallidermin and aureocin (Netz et al., 2002; Sashihara et al., 2000; Schnell et al., 1989, 1992), as well as a novel antibiotic lugdenin (Zipperer et al., 2016). Consequently, a culture-based approach was adopted in this study to isolate antimicrobial-producing bacteria with a particular focus on those that exhibit inhibitory activity against bacteria involved in the pathogenesis of skin infections. These include MRSA, *Staphylococcus epidermidis*, *Cutibacterium acnes* and a number of mastitic pathogens, such as *Streptococcus uberis* (*St. uberis*), *St. bovis, St. agalactiae, St. dysgalactiae* and *S. aureus*.

**Detection and isolation of antimicrobial producing skin isolates**

Twenty volunteers of mixed age, gender and race were recruited to provide swabs from seven body locations. The body sites were chosen based on the varied bacterial compositions of different skin areas (Grice & Segre, 2011). Superficial swabs were employed in this study because the swabbing method was less invasive, and was reported to yield a largely similar microbiota profile to that associated with skin scraping of epidermis or punch biopsy of the full thickness of epidermis and dermis when next generation sequencing methods were used (Grice et al., 2008). Over 90,000 colonies were initially screened for antimicrobial activity; these were isolated from 7 sites from 20 individuals (140 sites) using the agar overlay technique on the original isolation plates. Samples were serially diluted and spread plated onto the surface of BHI and MSA agar plates and all plates which contained visible growth were counted as potential antimicrobial producers, and overlaid with *L. delbrueckii* ssp. *bulgaricus* or MRSA / *L. innocua* respectively. At this stage of the study the genetic relationship between the colonies was not determined. Spot bioassays on selected individual colonies and original plate overlays identified 101 possible antimicrobial producing isolates (see supplementary information for more details). To confirm that an antimicrobial substance was secreted into the growth medium, neutralised CFS was tested against *L. delbrueckii* ssp. *bulgaricus* LMG 6901 by WDA and this reduced the number of possible bacteriocin producers to 25, giving an observed frequency of 0.03% from the 90,000 colonies screened in this study. Due to small zone sizes four of the antimicrobial producers were discarded. *L. delbrueckii* ssp. *bulgaricus* LMG 6901 was selected as the primary indicator for all assays, due to its insensitivity to acid thus reducing the frequency of false positives (Casey et al., 2004). While it is now accepted that many bacteria in natural ecosystems have the genetic potential to produce bacteriocin-like inhibitors, their detection in vitro is largely dependent on the screening methods undertaken. The low frequency observed in this study may be contributed to the fact that only *L.* delbrueckii ssp. *bulgaricus* was used as the primary indicator bacteria.

**Identification of antimicrobial producing skin isolates**

16SrRNA sequencing of the 25 antimicrobial producing isolates revealed four were *Bacillus* species - *Bacillus licheniformis* (2), *Bacillus endophyticus* and *Bacillus safensis* - but the remainder were coagulase negative *Staphylococcus* species. However, further analysis by amplification of the *DnaJ* gene was required to further differentiate between *Staphylococcus* species. Five *Staphylococcus* species were identified, including *S. capitis* (9), *S. hominis* (4), *S. epidermidis* (1), *S. simulans* (1), and *S. warneri* (6) (Table 2). *S. capitis*, a frequent coloniser of human skin, was the most prevalent species isolated (43%) followed by *S. warneri* (29%), which has been reported to be a less frequent skin coloniser (Otto, 2010) (Otto, 2011). Twelve strains (~50%) of antimicrobial producing staphylococci were isolated from the toe web-space body area. The four antimicrobials omitted from study as they were considered poor producers were *Bacillus* species.

**Characterisation of antimicrobial producing skin isolates**

A characteristic of bacteriocins is that they are relatively heat stable and are susceptible to degradation by proteolytic enzymes (Jack, Tagg, & Ray, 1995). Antimicrobial activity in the CFS from all strains was eliminated following proteinase K treatment indicating that the antimicrobials were proteinaceous in nature (see supplementary information fig. S2). In addition, all antimicrobials were stable up to 100 °C for 10 minutes and showed only a 50% reduction in activity after autoclaving at 121 °C for 15 minutes (see supplementary information fig. S2).

One of the features of bacteriocin production is that the genome of producers must harbour immunity gene(s) which protect producers from the antimicrobial effects of the bacteriocin they produce (Cotter, Hill, & Ross, 2005; Yang, Lin, Sung, & Fang, 2014). Cross immunity assays were performed to determine if the producing strains were sensitive to the antimicrobial activity of the other producers (Fig. 2). To determine the genetic relatedness of the 21 staphylococci isolated, the strains were fingerprinted using PFGE and similarities were categorised based on the procedure described by Tenover et al., (1995). Strains of the same species are closely related, in some cases only differing from each other by a single DNA band for example *S. hominis* strains APC 2925, APC 2924 and *S. capitis* strains APC 2934 and APC 2918 (Fig. 3). However, the PFGE analysis revealed 13 genetically different antimicrobial producing staphylococci - three *S. hominis*, four *S. warneri*, one *S. epidermidis*, one *S. simulans* and four *S. capitis*.

Combining data from cross immunity assays and PFGE analysis revealed that antimicrobials produced by skin isolates of the same species and of the same or similar pulsotype were immune to each other; suggesting they are the same strain and are most likely producing the same bacteriocin. For example, two *S. hominis* strains APC 2925 and APC 2939, isolated from different body areas (toe web-space and gluteal crease) of the same subject, yielded the same pulsotype (lanes 1 and 3 on PFGE gel), had the same immunity pattern as another *S. hominis* strain, APC 2924, from a different subject whose pulsotype differed by just one SmaI digestion DNA band (lane 2 on PFGE gel). This was also the case for two *S. capitis* strains from two different subjects (APC 2934 and APC 2918, lanes 13 and 14 of PFGE gel). This shows that some different strains which are closely related can produce the same bacteriocin.

PFGE profiles demonstrated that the same pulsotype can be shared across a number of individuals, with the same *S. capitis* pulsotype isolated from four different subjects (Fig. 3, lanes 15- 19) and three subjects sharing the same *S. warneri* pulsotype; APC 2942, APC 2937 and APC 2940 (Fig. 3, lanes 5, 7 and 9). Interestingly, we also showed that the same subject can carry more than one strain of the same species; *S. warneri* APC 2947 and APC 2940 were isolated from subject seven. These strains were isolated from different locations on the body and exhibited different pulsotypes – (Fig. 3, lanes 8 and 9). *S. warneri* APC 2947 had a very different pulsotype to other *S. warneri* strains isolated in this study and while it was immune to the bacteriocin produced by others of the same species, it had a different immunity pattern in that it was inhibited by *S. capitis* skin isolates unlike other *S. warneri* (Fig. 2; Fig 3, lane 8).

Bacteriocins such as thuricin and nisin have either a narrow or broad spectrum of activity, respectively (Rea et al., 2010; Shin et al., 2016), and both of these characteristics may be of benefit to the producing strain depending on the niche inhabited by the bacterium. In a crowded environment like the skin, bacteriocin production might help producing strains establish themselves in a particular skin environment. For example, the production of narrow spectrum bacteriocins may confer a competitive advantage to strains, allowing them to inhibit closely related species that would be competing for similar nutrients on the skin (Dobson, Cotter, Ross, & Hill, 2012). Table 3 shows the spectrum of inhibition of the bacteriocins produced against a panel of bacteria including skin pathogens such as *Cutibacterium acnes, S. epidermidis, Corynebacterium xerosis* and MRSA. The majority of *S. warneri* exhibited the broadest spectrum of activity, inhibiting nine of the indicator strains; while the inhibitory activity of three strains (APC 2930, APC 3477 and APC 2926) was shown to exhibit a narrow inhibition spectrum - inhibiting only the primary indicator *L. delbrueckii* ssp. *bulgaricus*.

While the majority of our commensal or resident skin microbiota are beneficial to skin health, occasionally the microbes that inhabit our skin can develop into opportunistic pathogens, for example *S. lugdunensis* and *S. capitis* (Böcher, Tønning, Skov, & Prag, 2009; Cameron et al., 2015). Even the most abundant species of *Staphylococcus* on human skin, *S. epidermidis,* can become pathogenic if the conditions are suitable (Cogen, Nizet, & Gallo, 2008). Disruptions to the microbial composition of the skin are responsible for skin conditions such as psoriasis, atopic dermatitis, impetigo and acne, and sometimes these imbalances can also prevent healing of chronic wounds (Grice, 2014). Two *S. capitis* strains (APC 2934 and APC 2918) isolated in this study exhibited inhibitory activity of 160 AU/mL against *S. epidermidis* (DPC 5990) (Tables 3 and 4).

*S. aureus* in particular is known for developing antibiotic resistance (Chambers & Deleo, 2010). MRSA was first identified in 1960 and since then has become one of the most prevalent antibiotic resistant pathogens worldwide and is characterised by swollen red lesions containing pus following infection of skin wounds, it also causes many other diseases, including pneumatic diseases that can result in mortality in the elderly. A study investigating incidence of MRSA in *S. aureus* isolates in Prince of Songkhla Hospital, Thailand found that 60.9% of the *S. aureus* isolates

were methicillin resistant (Indrawattana et al., 2013) while a study on skin and soft tissue infections carried out in medical centres in Europe revealed that the most prevalent pathogen was MRSA (22.5%) (Johnson, 2011). MRSA is first treated with glycopeptide antibiotics such as vancomycin, (Santajit & Indrawattana, 2016). However the development of novel antimicrobial therapies for methicillin resistant and vancomycin resistant *S. aureus* are included in the second highest priority group in a list of priority pathogens published by the WHO in a recent report describing antimicrobial resistance as a 'global emergency' (http://www.who.int/antimicrobial-resistance/en/). Alternative interventions to alleviate the problems posed by MRSA and other antimicrobial resistant pathogens are urgently required. *S. capitis* strains APC 2934 and APC 2918 with very similar pulsotypes (Fig 3, lanes 13 and 14) were isolated in this study from two different subjects, from different body areas inguinal crease and alar crease, and were immune to each other's antimicrobial activity (Fig 2). These strains exhibited inhibitory activity, 80 AU/mL and 40 AU/mL respectively, against a strain of the skin pathogen MRSA (DPC 5645) (Table 3 and 4).

Many adolescents and adults will experience the effects of acne vulgaricus at some point in their lives (Webster, 2002). Patients suffering from mild acne are often prescribed topical antibiotics or antiseptics at the beginning of treatment, such as clindamycin, erythromycin, benzoyl peroxide, topical retinoid or a combination of these products. However if these are ineffective, or if the acne is classified as moderate to severe, then patients are often prescribed systemic treatments including oral antibiotics including tetracyclines, erythromycin, trimethoprim–sulfamethoxazole hormone therapy and retinoid-isotreretinoin, often for long periods (Raithi, 2011). According to '*TIME Health'*, acne patients stay on antibiotics for four times the recommended time period. Eight skin isolates in this study displayed inhibitory activity of 40 AU/ mL against *Cutibacterium acnes*, which is a leading cause of acne vulgaricus (see Table 3 and 4). Seven of these isolates were *S. capitis*, however, there were three distinct pulsotypes. *S. hominis* APC 3365 was also active against *C. acnes.*

*Corynebacterium xerosis* is a Gram positive aerobic bacterium, and a commensal of the human skin. *C. xerosis* can cause bacteremia, skin infections, endocarditis and pneumonia in immune-compromised patients (Pessanha, Farb, Lwin, Lloyd, & Virmani, 2003). Three *S. hominis* and five *S. warneri* demonstrated inhibitory activity of 40 AU/mL and 80 AU/mL against *C. xerosis* (Table 3 and 4), and against other *Corynebacterium* species (results not shown). PFGE analysis revealed five distinct pulsotypes (two *S. hominis* and three *S. warneri* strains (Fig. 3)). In addition to highlighting the therapeutic potential of coagulase negative staphylococci on human skin by inhibiting skin pathogens, we also observed the ability of skin commensals to inhibit other pathogens such as *Listeria monocytogenes* (Table 3, APC 2924, APC 2925, APC 2939- 2 distinct strains from PFGE, Fig. 3) and *Streptococcus* species involved in bovine mastitis, such as *St. uberis, St. agalactiae* and *St. dysgalactiae* (Table 3). While traditionally *S. aureus* was the most common cause of mastitis in dairy cows, more recently *St. uberis* has been shown to be increasingly associated with infection (Kromker, et al., 2014). In this study, three *Staphylococcus* species, *S. hominis, S. warneri* and *S. capitis* inhibited a range of *Streptococcus* species (*St. uberis, St. bovis, St. agalactiae* and *St. dysgalactiae*) which have been implicated in bovine mastitis (Table 3) with AU ranging from 20 to 320 AU/mL (Table 4). *St. uberis* is of particular interest as it has been increasingly shown to be one of the causative agents of bovine mastitis in the UK. This shows that the antimicrobial producing human skin isolates may also have applications in veterinary medicine for the treatment of mastitis given their ability to inhibit both *Staphylococcus* and *Streptococcus* species, and could aid in the search for non-antibiotic solutions to mastitis treatment to reduce antibiotics in the food chain. Mastitis in humans is also a major health issue (Michie, Lockie, & Lynn, 2003). *S. epidermidis* and streptococci are among the most prevalent causative agents in this infection, found in 87.6% and 68.6% of mastitic human breast milk samples, respectively (Marín, Arroyo, Espinosa-Martos, Fernández, & Rodríguez, 2017). Bacteriocin producers from human skin, identified in this study, exhibited inhibitory activity against staphylococcal and streptococcal species thus highlighting their potential as live bio-therapeutics for the treatment of human mastitis.

Colony mass spectrometry can be used to identify antimicrobial peptides if they can be matched to known peptide masses in the Bactibase database. However, in this case the peptide masses detected did not match any known bacteriocins, suggesting these isolates are potentially producing novel bacteriocins. The Maldi-TOF MS profiles of the 13 antimicrobial producing staphylococci are shown in Supplementary figure S3.

**Conclusion**

There is a need to identify new antimicrobial strains with the potential to outcompete pathogens in the skin environment. The skin is a protective defence shielding us from the external environment (Yamasaki & Gallo, 2008). This study has highlighted that the skin microbiota is home to many bacteriocin producing strains which may indicate that the skin microbiota is an important tool in our fight against antimicrobial resistance. Indeed, this screening has resulted in the isolation of a set of 13 novel bacteriocin producing human skin isolates with potential to restore imbalances of skin microbiota as well as inhibit skin pathogens such as MRSA and *Cutibacterium acnes*. More importantly, these strains may prove useful as probiotics for topical skin applications to provide colonization resistance by replacement of skin pathogens and particularly MRSA. Further characterisation studies will be carried out on these bacteriocin- producing skin isolates.

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**Conflict of interest**

The authors declare no competing interests.

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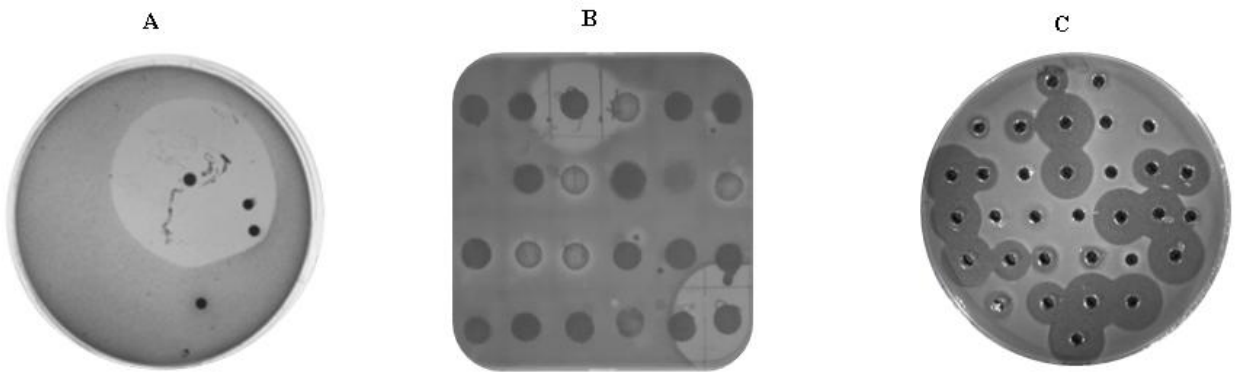


**Figure 1:** A: Overlay of an original plate containing dilution of a toe swab with *L. delbrueckii* ssp. *bulgaricus* DPC 6901. B: Spot assay of 10 µL of inoculum from colonies of varying morphology isolated from swab dilution plates, overlaid with *L. delbrueckii* ssp. *bulgaricus* DPC 6901. C: Well diffusion assay of antimicrobial producing skin isolates in agar seeded with *L. delbrueckii* ssp. *bulgaricus* DPC 6901 to confirm positive result in spot assay was due to antimicrobial substance secreted into CFS.

| APC number ↓→ | 2925 | 2924 | 2939 | 3365 | 2942 | 2922 | 2937 | 2940 | 2947 | 2930 | 3477 | 2926 | 2934 | 2918 | 2923 | 2941 | 3480 | 2946 | 2932 | 2927 | 3481 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2925 | | | | | | | | | | | | | | | | | | | | | |
| 2924 | | | | | | | | | | | | | | | | | | | | | |
| 2939 | | | | | | | | | | | | | | | | | | | | | |
| 3365 | | | | | | | | | | | | | | | | | | | | | |
| 2942 | | | | | | | | | | | | | | | | | | | | | |
| 2922 | | | | | | | | | | | | | | | | | | | | | |
| 2937 | | | | | | | | | | | | | | | | | | | | | |
| 2940 | | | | | | | | | | | | | | | | | | | | | |
| 2947 | | | | | | | | | | | | | | | | | | | | | |
| 2930 | | | | | | | | | | | | | | | | | | | | | |
| 3477 | | | | | | | | | | | | | | | | | | | | | |
| 2926 | | | | | | | | | | | | | | | | | | | | | |
| 2934 | | | | | | | | | | | * | | | | | | | | | | |
| 2918 | | | | | | | | | | | * | | | | | | | | | | |
| 2923 | | | | | | | | | | | | | | | | | | | | | |
| 2941 | | | | | | | | | | | | | | | | | | | | | |
| 3480 | | | | | | | | | | | | | | | | | | | | | |
| 2946 | | | | | | | | | | | | | | | | | | | | | |
| 2932 | | | | | | | | | | | | | | | | | | | | | |
| 2927 | | | | | | | | | | | | | | | | | | | | | |
| 3481 | | | | | | | | | | | | | | | | | | | | | |

**Figure 2:** Cross immunity assays using CFS from all the antimicrobial producing strains tested against themselves and each other with the list of antimicrobial producing isolates down the left hand side and the indicator strains across the top of the Table. The diagonal green line ■ reveals that the antimicrobial producer is immune to its own antimicrobial as expected. The red box ■ represents inhibition of the indicator strain indicating that these strains might be different or sensitive to each other. ■(orange) = small/ medium hazy zones. ■(yellow) = very small zones but sensitive. White boxes represent no inhibition revealing target strains not inhibited by the producing strain, indicating they are immune (related) to the bacteriocins produced by other producers. *=initially sensitive but bactostatic activity observed.

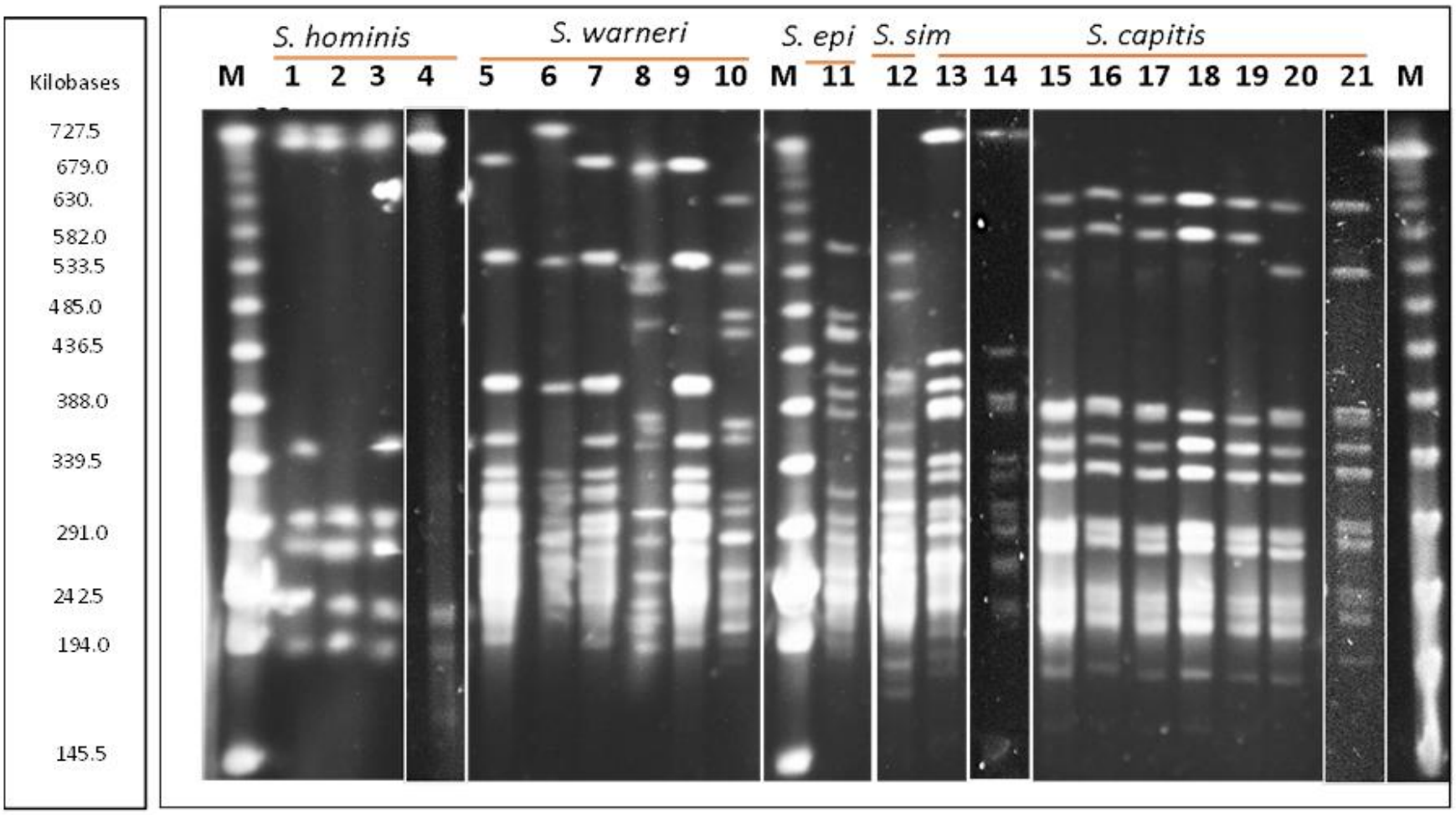


**Figure 3:** PFGE macro-digestion patterns of staphylococcal skin isolates following genomic digestion with Sma1. Lanes 1-4= Staphylococcus hominis: APC 2925, 2924, 2939 and 3365; lanes 5-10= Staphylococcus warneri: APC 2942, 2922, 2937, 2947, 2940 and 2930; lane 11= Staphylococcus epidermidis: APC 3477; lane 12= Staphylococcus simulans APC 2926; lanes 13-21= Staphylococcus capitis APC 2934, 2918, 2923, 2941, 3480, 2946, 2932, 2927 and 3481.

Lanes 4, 15 and 22 were taken from other PFGE gels with the same- lambda marker (M) and inputted into this gel. epi = epidermidis ; sim = simulans.

Artefact in lane 3 below first band is not a SmaI DNA band

**Table 1:** Growth conditions of the indicator strains used in this study.

| Species | Strain | Growth conditions | | |
|---|---|---|---|---|
| | | **Temp (°C)** | **Atmosphere** | **Growth media** |
| *Bifidobacterium longum* subsp. *infantis* | ATCC 15697 | 37 | Anaerobic* | mMRS |
| *Bifidobacterium longum* subsp. *longum* | DSM 20097 | 37 | Anaerobic* | mMRS |
| *Corynebacterium xerosis* | DPC 5629 | 37 | Aerobic | BHI |
| *Corynebacterium variablis* | NCIMB 702097 | 30 | Aerobic | BHI |
| *Cutibacterium acnes* | LMG 16711 | 37 | Anaerobic* | mRCM & RCA |
| *Enterococcus faecalis* | ATCC 19433 | 37 | Anaerobic* | MRS |
| *Fusobacterium varium* | DSM 19868 | 37 | Anaerobic* | BHI |
| *Lactobacillus delbrueckii* subsp. *bulgaricus* | LMG 6901 | 37 | Anaerobic* | MRS |
| *Lactobacillus fermentum* | APC 2582 | 37 | Anaerobic* | MRS |
| *Lactobacillus rhamnosus* | APC 3483 | 37 | Anaerobic* | MRS |
| *Listeria innocua* | DPC 3572 | 37 | Aerobic | BHI |
| *Listeria monocytogenes* | DPC 5788 | 37 | Aerobic | BHI |
| *Listeria monocytogenes* | DPC 6893 | 37 | Aerobic | BHI |
| *Listeria monocytogenes* | NCTC 5348 | 37 | Aerobic | BHI |
| *Pseudomonas aeruginosa* | NCIMB 8295 | 37 | Aerobic | BHI |
| *Pseudomonas aeruginosa* | APC 2064 | 37 | Aerobic | BHI |
| *Pseudomonas fluorescens* | DPC 6056 | 30 | Aerobic | BHI |
| *Pseudomonas putita* | ATCC 17522 | 37 | Aerobic | BHI |
| MRSA | DPC 5645 | 37 | Aerobic | BHI |
| *Staphylococcus aureus* | ATCC 25923 | 37 | Aerobic | BHI |
| *Staphylococcus aureus* | DPC 7016 | 37 | Aerobic | BHI |
| *Staphylococcus capitis* | APC 2923 | 37 | Aerobic | BHI |
| *Staphylococcus epidermidis* | DPC 5990 | 37 | Aerobic | BHI |
| *Staphylococcus simulans* | APC 3482 | 37 | Aerobic | BHI |
| *Streptococcus agalactiae* | ATCC 13813 | 37 | Aerobic | BHI |
| *Streptococcus bovis* | DPC 6491 | 37 | Aerobic | GM17 |
| *Streptococcus dysgalactiae* | APC 3484 | 37 | Aerobic | BHI |
| *Streptococcus uberis* | DPC 5344 | 37 | Aerobic | BHI |

mMRS= modified MRS, mRCM= modified Reinforced Clostridium Media (made following ATCC Medium:2107 Modified Reinforced Clostridial Agar/ broth (pre-reduced) protocol), RCA= Reinforced Clostridium Agar.

*=Anaerobic conditions, where appropriate, were achieved through the use of anaerobic jars and Anaerocult ® A gas packs (Merck, Darmstadt, Germany).

ATCC= American Type Culture Collection, APC= APC Microbiome Ireland culture collection, DPC= Teagasc culture collection, WSLC= Weihenstephan Listeria Collection, LMG= Laboratorium voor Microbiologie, Universteit Gent, Belgium

**Table 2:** Identification of antimicrobial producing strains isolated from skin.

| **Strain Identity** | **Strain number** | **Subject no.** | **Body area** | **PFGE lane no.** |
|---|---|---|---|---|
| *Staphylococcus hominis* | APC 2925 | SM019 | toe web-space | 1$ |
| *Staphylococcus hominis* | APC 2924 | SM012 | toe web-space | 2 |
| *Staphylococcus hominis* | APC 2939 | SM019 | gluteal crease | 3$ |
| *Staphylococcus hominis* | APC 3365 | SM020 | gluteal crease | 4 |
| *Staphylococcus warneri* | APC 2942 | SM009 | axillary vault | 5* |
| *Staphylococcus warneri* | APC 2922 | SM001 | toe web-space | 6 |
| *Staphylococcus warneri* | APC 2937 | SM006 | gluteal crease | 7* |
| *Staphylococcus warneri* | APC 2947 | SM007 | toe web-space | 8 |
| *Staphylococcus warneri* | APC 2940 | SM007 | gluteal crease | 9* |
| *Staphylococcus warneri* | APC 2930 | SM015 | toe web-space | 10 |
| *Staphylococcus epidermidis* | APC 3477 | SM015 | toe web-space | 11 |
| *Staphylococcus simulans* | APC 2926 | SM002 | toe web-space | 12 |

| | | | | |
|---|---|---|---|---|
| *Staphylococcus capitis* | APC 2934 | SM003 | inguinal crease | 13 |
| *Staphylococcus capitis* | APC 2918 | SM001 | alar crease | 14 |
| *Staphylococcus capitis* | APC 2923 | SM007 | toe web-space | 15[¤] |
| *Staphylococcus capitis* | APC 2941 | SM010 | umbilicus | 16[¤] |
| *Staphylococcus capitis* | APC 3480 | SM009 | umbilicus | 17[¤] |
| *Staphylococcus capitis* | APC 2946 | SM008 | toe web-space | 18[¤] |
| *Staphylococcus capitis* | APC 2932 | SM008 | toe web-space | 19[¤] |
| *Staphylococcus capitis* | APC 2927 | SM015 | toe web-space | 20[π] |
| *Staphylococcus capitis* | APC 3481 | SM015 | toe web-space | 21[π] |

[$, *, ¤, π] These symbols are used to reflect identical PFGE patterns from Fig. 3.

**Table 3:** Spectrum of inhibition of bacteriocin producing skin isolates against indicator strains subjected to well diffusion assays. +<50 $mm^2$; ++50-150$mm^2$; +++150-249$mm^2$; ++++ 250-349$mm^2$; 350-449$mm^2$; ++++++>450 (Calculated as area of zone $\pi r^2$ - area of well $\pi r^2$ in millimetres).

*S. hominis* | *S. epidermidis* (*S. warneri*, *S. simulans*, *S. capitis*)

| | | Producer strains | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2925 | 2924 | 2939 | 3365 | 2942 | 2922 | 2937 | 2947 | 2940 | 2930 | 3477 | 2926 | 2934 | 2918 | 2923 | 2941 | 3480 | 2946 | 2932 | 2927 | 3481 |
| *Corynebacteria xerosis* | DPC 5629 | ++ | ++ | ++ | - | +++ | +++ | +++ | +++ | +++ | - | - | - | - | - | - | - | - | - | - | - | - |
| *Cutibacterium acnes* | LMG 16711 | - | - | - | ++ | - | - | - | - | - | - | - | - | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | - |
| *Lactobacillus bulgaricus* | LMG 6901 | ++ | ++ | ++ | ++++ | +++++ | +++++ | +++++ | +++++ | +++++ | +++ | +++++ | + | ++ | ++ | +++++ | +++++ | +++++ | +++++ | +++++ | +++++ | +++++ |
| *Lactobacillus fermentum* | APC 2582 | ++ | ++ | ++ | + | +++ | ++ | ++ | ++ | ++ | - | - | - | ++ | ++ | + | + | + | + | + | + | + |
| *Listeria monocytogenes* | DPC 6893 | ++ | ++ | ++ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| MRSA | DPC 5645 | - | - | - | - | - | - | - | - | - | - | - | - | ++ | ++ | - | - | - | - | - | - | - |
| *Staphylococcus aureus* | ATCC 25923 | - | - | - | - | - | - | - | - | - | - | - | - | ++ | ++ | - | - | - | - | - | - | - |
| *Staphylococcus aureus* | DPC 7016 | - | - | - | ++ | ++ | ++ | ++ | ++ | ++ | - | - | - | ++ | ++ | - | - | - | - | - | - | - |
| *Staphylococcus epidermidis* | DPC 5990 | - | - | - | - | - | - | - | - | - | - | - | - | +++++ | +++++ | - | - | - | - | - | - | - |
| *Staphylococcus simulans* | APC 3482 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| *Streptococcus agalactiae* | APC 1055 | ++ | ++ | ++ | - | +++ | +++ | +++ | +++ | +++ | - | - | - | - | - | + | + | + | + | + | - | - |
| *Streptococcus agalactiae* | ATCC 13813 | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | - | - | - | - | - | - | - | - | - | - | - | - |
| *Streptococcus bovis* | DPC 6491 | ++ | ++ | ++ | + | ++ | ++ | ++ | ++ | ++ | - | - | - | - | - | +++ | +++ | +++ | +++ | +++ | - | - |
| *Streptococcus dysgalactiae* | APC 3484 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | - | - | - | - | - | - | - | - | - | - | - |
| *Streptococcus uberis* | DPC 5344 | - | - | - | - | +++ | +++ | +++ | +++ | ++ | - | - | - | - | - | +++ | +++ | +++ | +++ | +++ | ++ | ++ |

Other strains tested against but were not inhibited can be seen on Table 1.

**Table 4:** Activity Units per mL from well diffusion assays of the 13 different bacteriocin producing skin isolates against indicator strain. (*S. hominis* APC 2925, 2924, 3365; *S. warneri* APC 2922, 2937, 2947, 2930; *S. epidermidis* APC 3477; *S. simulans* APC 2926; *S. capitis* APC 2934, 2918, 2923, 2927).

| **Indicator strains** | | Producer strain | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **2925** | **2924** | **3365** | **2922** | **2937** | **2947** | **2930** | **3477** | **2926** | **2934** | **2918** | **2923** | **2927** |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| *Corynebacterium xerosis* | DPC 5629 | 40 | 40 | - | 80 | 80 | 80 | - | - | - | - | - | - | - |
| *Cutibacterium acnes* | LMG 16711 | - | - | 40 | - | - | - | - | - | - | 40 | 40 | 40 | - |
| *Lactobacillus bulgaricus* | LMG 6901 | 80 | 80 | 320 | 640 | 640 | 640 | 160 | 320 | 40 | 80 | 80 | 1280 | 640 |
| *Lactobacillus fermentum* | APC 2582 | 40 | 40 | 20 | 80 | 80 | 80 | - | - | - | 20 | 20 | 40 | 20 |
| *Listeria monocytogenes* | DPC 6893 | 40 | 40 | - | - | - | - | - | - | - | - | - | - | - |
| MRSA | DPC 5645 | - | - | - | - | - | - | - | - | - | 80 | 40 | - | - |
| *Staphylococcus aureus* | ATCC 25923 | - | - | - | - | - | - | - | - | - | 40 | 40 | - | - |
| *Staphylococcus aureus* | DPC 7016 | - | - | - | 80 | 20 | 40 | - | - | - | 40 | 40 | - | - |
| *Staphylococcus epidermidis* | DPC 5990 | - | - | - | - | - | - | - | - | - | 160 | 160 | - | - |
| *Staphylococcus simulans* | APC 3482 | - | - | - | - | - | - | - | - | - | - | - | 40 | 40 |
| *Streptococcus agalactiae* | APC 1055 | 20 | 20 | 20 | 320 | 80 | 80 | - | - | - | - | - | 20 | - |
| *Streptococcus agalactiae* | ATCC 13813 | 20 | 20 | - | 80 | 80 | 80 | - | - | - | - | - | - | - |
| *Streptococcus bovis* | DPC 6491 | 40 | 40 | 20 | 40 | 40 | 80 | - | - | - | - | - | 320 | - |
| *Streptococcus dysgalactiae* | APC 3484 | 40 | 40 | - | 80 | 40 | 40 | - | - | - | - | - | - | - |
| *Streptococcus uberis* | DPC 5344 | - | - | - | 80 | 40 | 40 | - | - | - | - | - | 40 | 20 |