Bacteriocin-Like Inhibitory Substances in Staphylococci of Different Origins and Species With Activity Against Relevant Pathogens

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Bacteriocins are antimicrobial peptides with relevance in the modulation of human and animal microbiota that have gained interest in biomedical and biotechnological applications. In this study, the production of bacteriocin-like inhibitory substances (BLIS) was tested among a collection of 890 staphylococci of different origins (humans, animals, food, and the environment) and species, both coagulase-positive (CoPS, 238 isolates of 3 species) and coagulase-negative staphylococci (CoNS, 652 isolates of 26 species). Of the 890 staphylococci, 60 (6.7%) showed antimicrobial activity by the spot-on-lawn method against at least one of the 25 indicator bacteria tested. BLIS-producer (BLIS+) isolates were detected in 8.8% of CoPS and 6.0% of CoNS. The staphylococcal species with the highest percentages of BLIS+ isolates were S. chromogenes (38.5%), S. pseudintermedius (26.7%), and S. warneri (23.1%). The production of BLIS was more frequently detected among isolates of pets, wild animals, and food. Moreover, 13 BLIS+ isolates showed wide antimicrobial activity spectrum, and 7 of these isolates (of species S. aureus, S. pseudintermedius, S. sciuri, and S. hominis) demonstrated antimicrobial activity against more than 70% of the indicator bacteria tested. The genetic characterization (by PCR and sequencing) of the 60 BLIS+ isolates revealed the detection of (a) 11 CoNS and CoPS isolates carrying putative lantibiotic-like genes; (b) 3 S. pseudintermedius isolates harboring the genes of BacSp222 bacteriocin; and (c) 2 S. chromogenes isolates that presented the gene of a putative cyclic bacteriocin (uberolysin-like), being the first report in this CoNS species. Antimicrobial susceptibility testing was performed in BLIS+ isolates and one-third of the CoNS isolates showed susceptibility to all antibiotics tested, which also lacked the virulence genes studied. These BLIS+ CoNS are good candidates for further characterization studies.

Keywords: bacteriocin, multidrug resistant (MDR) bacteria, BLIS, coagulase-negative staphylococci, coagulase-positive staphylococci
INTRODUCTION

Staphylococcus is a genus widely distributed in the environment and comprises important members of the major bacterial communities colonizing the skin and mucous membranes of humans and animals (Kranjec et al., 2020). This genus comprises a high diversity of species, traditionally separated into two major groups based on their ability to clot the plasma: coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS). CoNS is the broadest group, with more than 80% of species (Becker et al., 2015).

Although staphylococci often maintain a commensal or symbiotic relationship with their hosts, staphylococcal infections could develop in specific cases (de Freire Bastos et al., 2020). Some staphylococcal species are more related to human and animal infectious diseases, being Staphylococcus aureus one of the most important.

Individual bacterial strains living in highly competitive and polymicrobial environments have developed multiple types of interaction and self-defense mechanisms. The survival success can include different capabilities such as a higher specificity to a limited number of host attachment sites, a better ability to take up and metabolize micronutrients, or the production of antibacterial substances that inhibit competitors (Janek et al., 2016). The most widely distributed microbial defense mechanisms are bacteriocins, in most cases ribosomally synthesized peptides with antibacterial properties (França et al., 2021). From an ecological point of view, bacteriocins are synthesized to confer a selective advantage to the producer in terms of niche colonization ability since these molecules often display activity against closely related bacterial species (Heilbronner et al., 2021).

Staphylococci are no exception with respect to antimicrobial substances production. There is a wide variety of staphylococcal bacteriocins, highlighting the lantibiotic group (Class I), characterized by their post-translationally modifications, usually by enzymatic tailoring (Heilbronner et al., 2021). Moreover, other well-described antimicrobial peptides include those that remain unaltered (Class II bacteriocins), the heat-labile proteins differentiated between lytic and non-lytic bacteriocins (Class III) (Heng et al., 2007), and the cyclic molecules (Class IV) (de Freire Bastos et al., 2020; Newstead et al., 2020). Other antimicrobial peptides have recently been discovered in staphylococci isolates, such as those included in the sactipeptide group (de Freire Bastos et al., 2020) and the well-known fibepeptide lugdunin (a major non-ribosomal peptide, NRP) described by Zipperer et al. (2016). Moreover, bacteriocins previously described in other genera, such as the thiopetide Micrococcus P1, have been also detected in staphylococci (Carnio et al., 2000). In addition, there is another type of antimicrobial substance known as bacteriocin-like inhibitory substances (BLIS), which are not obtained in pure form or fully characterized, and they have also been reported in staphylococci (James and Tagg, 1991; Mak, 2018). Staphylococcus aureus is considered the most relevant CoPS in terms of bacteriocin production (generally, class II type bacteriocins), although CoNS bacteriocins (mostly lantibiotics) have been also reported in the literature (Bastos et al., 2009). Bacteriocin produced by staphylococci are active against pathogenic staphylococci, including methicillin-resistant S. aureus (Kranjec et al., 2020). These antimicrobial compounds could be of interest as food additives, therapeutic agents, or modulators of the microbiota (Soltani et al., 2021).

Due to the growing interest in the identification and characterization of new antimicrobial agents, this study sought to determine the production of BLIS in a large collection of CoNS and CoPS isolates of different origins (human, animal, food, and the environment) and to characterize the BLIS-producer (BLIS⁺) isolates at the genomic level to determine the presence of bacteriocin encoding genes as well as antimicrobial resistance (AMR) and virulence genes.

MATERIALS AND METHODS

Bacterial Collection

A total of 890 Staphylococcus spp. isolates, both CoPS (n = 238, of three different species) and CoNS (n = 652, of 26 different species), recovered from diverse origins (humans, n = 27; food, n = 278; wild animals, n = 403; pets, n = 50; and the environment, n = 132), were included in this study. All isolates of humans and animals were obtained from nasal samples of healthy individuals (except 4 CoPS clinical isolates of humans) and were identified using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF, Bruker). Table 1 shows the species and origins of the isolates tested, and those showing antimicrobial activity. The isolates were obtained from previous studies performed by the OneHealth-UR research group of the University of La Rioja, and many of them were included in previously published papers (Gómez-Sanz et al., 2013; Gómez et al., 2017; Lozano et al., 2017; Mama et al., 2019; Ruiz-Ripa et al., 2019, 2020, 2021).

Screening of Antimicrobial Activity

The screening for the production of BLIS was performed with the complete staphylococci collection (n = 890 isolates) in agar diffusion assays by the spot-on-lawn method (Supplementary Figure 1). For that purpose, 25 indicator bacteria of different genera and species were used. Gram-positive and Gram-negative bacteria of 8 different genera (Staphylococcus, Enterococcus, Micrococcus, Streptococcus, Clostridiun, Listeria, Escherichia, and Pseudomonas) and 19 different species, including multidrug-resistant bacteria and relevant pathogens, were used as indicator bacteria (Table 2).

Agar Diffusion Assays (Spot-On-Lawn method)

All staphylococci to be tested for the production of BLIS and the 25 indicator isolates were grown on brain heart infusion (BHI) agar (Condalab, Spain) for 24 h at 37°C. Indicator isolates were resuspended in BHI broth at a concentration equivalent to 0.5 MacFarland turbidity. Then, 10 μl of each indicator bacteria were added to 5 ml of semisolid tryptic soy broth (SS-TSB) (BD, Difco, France) media supplemented with 0.3% of yeast extract and 0.7% of bacteriological agar (autoclaved and cooled until 45°C). After mixing, the SS-TSB media with the indicator bacteria were poured into a tryptic soy agar (TSA) plate (BD,
TABLE 1 | Species, origins, and bacteriocin-like inhibitory substances (BLIS) production capacity of the 890 CoPS and CoNS isolates included in this study.

| Type of Staphylococci | Species | Number of isolates of different origins tested/number of BLIS* isolates (%) |
|-----------------------|---------|-------------------------------------------------|
|                       |         | Total isolates | Human | Food | Wild animal | Pet | Environmental |
| CoPS                  | S. aureus | 147/5 (3.4%) | 11/0 | 72/2 (2.8%) | 57/2 (3.5%) | 0 | 7/1 (14.3%) |
|                       | S. pseudintermedius | 60/16 (26.7%) | 9/1 (11.1%) | 1/0 | 0 | 50/15 (30%) | 0 | 7/1 (14.3%) |
|                       | S. delphini | 31/0 | 0 | 19/0 | 12/0 | 0 | 0 | 0 |
| Total CoPS*           |         | 238/21 (8.8%) | 20/1 (5%) | 92/2 (2.2%) | 69/2 (2.9%) | 50/15 (30%) | 7/1 (14.3%) |
| CoNS                  | S. sciuri | 214/13 (3.1%) | 0 | 23/2 (8.7%) | 182/11 (6%) | 0 | 9/0 |
|                       | S. saprophyticus | 69/0 | 0 | 39/0 | 5/0 | 0 | 25/0 |
|                       | S. lentus | 48/0 | 0 | 16/0 | 28/0 | 0 | 4/0 |
|                       | S. xylosus | 48/3 (6.3%) | 0 | 7/0 | 24/3 (12.5%) | 0 | 17/0 |
|                       | S. epidermidis | 38/4 (10.5%) | 0 | 17/4 (23.5%) | 0 | 12/0 |
|                       | S. fleurettii | 29/0 | 0 | 14/0 | 0 | 2/0 |
|                       | S. chromogenes | 26/10 (38.5%) | 0 | 7/3 (42.8%) | 17/2 (11.6%) | 0 | 0 |
|                       | S. warneri | 26/6 (23.1%) | 1/0 | 24/6 (25%) | 0 | 0 | 1/0 |
|                       | S. vitulinis | 24/0 | 0 | 8/0 | 0 | 1/0 |
|                       | S. simulans | 22/1 (4.5%) | 0 | 16/0 | 3/1 (33.3%) | 0 | 3/0 |
|                       | S. arlettae | 21/0 | 0 | 0 | 0 | 21/0 |
|                       | S. cohnii | 16/0 | 0 | 2/0 | 2/0 | 0 | 12/0 |
|                       | S. equorum | 16/0 | 0 | 2/0 | 13/0 | 0 | 1/0 |
|                       | S. pasteuri | 9/0 | 0 | 8/0 | 0 | 0 | 1/0 |
|                       | S. hominis | 8/1 (12.5%) | 0 | 3/0 | 0 | 3/1 (33.3%) |
|                       | S. capitis | 6/0 | 0 | 1/0 | 1/0 | 0 | 4/0 |
|                       | S. hyicus | 6/1 (16.7%) | 0 | 2/0 | 17/2 (25%) | 0 | 0 |
|                       | S. succinus | 6/0 | 0 | 0 | 3/0 | 0 | 1/0 |
|                       | S. haemolyticus | 5/0 | 0 | 0 | 2/0 | 0 | 3/0 |
|                       | S. nepalensis | 5/0 | 0 | 0 | 0 | 5/0 |
|                       | S. kloosii | 3/0 | 0 | 0 | 3/0 | 0 | 0 |
|                       | S. schleiferi | 3/0 | 0 | 0 | 3/0 | 0 | 0 |
|                       | S. auricularis | 3/0 | 0 | 0 | 3/0 | 0 | 0 |
|                       | S. lugdunensis | 1/0 | 0 | 0 | 1/0 | 0 | 0 |
|                       | S. simulans | 1/0 | 0 | 0 | 0 | 0 |
| Total CoNS*           |         | 652/39 (6%) | 7/0 | 186/15 (8.1%) | 334/23 (6.9%) | 0 | 125/1 (0.8%) |

Total (CoPS + CoNS)* 890/60 (6.7%) 27/1 (3.7%) 278/17 (6.1%) 403/25 (6.2%) 50/15 (30%) 132/2 (1.5%)

*Statistically significant differences with \( p \leq 0.05 \), depending on the origin of the isolates.

Difco, France), which was also supplemented with 0.3% of yeast extract. Staphylococci to be tested for the production of BLIS were spotted on prepared plates with each of the indicator isolates and were incubated in aerobic conditions for 24 h at 37°C. Anaerobic conditions were used when the indicator isolate was Clostridium perfringens, and Columbia agar with 5% sheep blood (bioMérieux, France) was used instead of TSA when indicator isolate was Streptococcus suis. Inhibition halos were checked and measured in millimeters (mm).

DNA Isolation
A colony of fresh culture bacteria was resuspended in 45 µl of sterile Milli-Q water and 5 µl of lysozyme (1 mg/ml). After incubation (10 min, 37°C), 45 µl of Milli-Q water, 150 µl of Tris-HCl (0.1 M, pH = 8), and 5 µl of proteinase K (2 mg/ml) were added. Finally, the total volume was incubated (10 min, 60°C), boiled (5 min, 100°C), and centrifuged (3 min, 12,000 rpm). The supernatant was used for PCR assays.

Detection of Bacteriocin Encoding Genes in BLIS+ Isolates
The presence of 23 bacteriocin structural genes was tested by PCR and sequencing in the 60 BLIS+ isolates (aurA, aucA, epaA, sacA/sacB, gdmA, bacSp222, nsj, hyiA, hycS, bacCH91, bsaA2, lugD, aclA, ale-1, lss, nukA, nkqA, eciA, pepA, elxA, elkA, ecda, orf4) and also the precursor gene for the NRP lugdunin (lugD). Moreover, three bacteriocin gene families were considered based on the nucleotide sequences of some bacteriocin structural genes with high similarities. These families were BS (BsaA2 and BacCH91), GEST (Gallidermin, Epidermin, and Staphylococcin T), and NUK (Nukacin KQU-131, Nukacin 3299, and Nukacin ISK1). Primers were designed for the detection of some bacteriocins and the bacteriocin families. The primer design was made with the online Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, United States) (Untergasser et al., 2012). To optimize primer creation, bacteriocin gene and peptide sequences were analyzed.
using Geneious and MegaX (refer to Supplementary Table 1 for primer sequences and PCR conditions).

Characterization of Antimicrobial Resistance, Virulence, and Molecular Typing of BLIS+ Isolates

The antimicrobial susceptibility testing, the detection of antibiotic resistance and virulence genes, and/or the molecular typing (spa, MLST, and/or agr) of 43 of the 60 BLIS+ isolates included in this study were performed in previous studies (Supplementary Table 2). The initial characterization of these isolates was used and partially completed in this study. The remaining 17 BLIS+ isolates were completely characterized in this study based on the criteria indicated in the following sections.

Antimicrobial Resistance Phenotype/Genotype

The susceptibility to 13 antimicrobial agents was evaluated in the BLIS+ isolates, including the following antibiotics: penicillin, cefoxitin/oxacillin, erythromycin, clindamycin, gentamicin, tobramycin, spectomycin, tetracycline, ciprofloxacin, chloramphenicol, linezolid, trimethoprim–sulfamethoxazole, and fusidic acid. The disk diffusion results for all antimicrobial agents were interpreted using the European Committee on Antimicrobial Susceptibility Testing criteria (EUCAST, 2021).

The presence of the following resistance genes was tested by single PCR according to the corresponding phenotypes of AMR: blaZ, mecA, mecC, erm(A), erm(B), erm(C), erm(T), mcr(A), mph(C), icsu(A), icsu(B), vga(A), sal(A), aac6’-Ie-aph(3’)-Ia, ant(4’)-Ia, str, ant(6), tet(L), tet(M), tet(K), fex(A), fex(B), cat(C194), cat(C221), cat(C223), fusB, fusC, fusD, dfrA, dfrD, dfrG, and dfrK (Ruiz-Ripa et al., 2020).

Multidrug resistance (MDR) was considered when staphylococci presented resistance to at least three different families of antibiotics. In this sense, clindamycin resistance due to the intrinsic presence of the sal(A) gene in S. sciuri was not considered for MDR classification, unless an additionally acquired resistance gene was found (Ruiz-Ripa et al., 2020).

Virulence Factors

The presence of relevant virulence genes, such as the leukocidin genes lukSF-PV, lukM, lukED, and lukPQ, the toxic shock syndrome toxin 1 (tst), and the exfoliative toxins A, B, and D (eta, etb, and etd), respectively, was studied by single PCR and confirmed by amplicon sequencing in all CoNS and S. aureus isolates. S. pseudintermedius isolates were screened for the presence of the leukocidin gene lukSF-I, the exfoliative genes siet, expA, and expB, and the enterotoxin genes si-ent and sec-canine by PCR (Gómez-Sanz et al., 2013).

Positive controls from the collection of the University of La Rioja were included in all PCR assays.

Molecular Typing

Spa-typing was carried out by PCR and amplicon sequencing in all S. aureus isolates. The spa sequences were analyzed using Ridom Staph-Type software version 1.5.21 (Ridom GmbH, Münster, Germany). Multilocus sequence typing (MLST) was performed on S. aureus and S. pseudintermedius isolates, as well as on a selected S. epidermidis isolate (Bannoehr et al., 2007; Ruiz-Ripa et al., 2019). All isolates carrying the mecA gene were subjected to SCCmec typing by multiplex PCRs, and agr-typing was characterized following standard methodology in all S. aureus and S. pseudintermedius isolates (Zhang et al., 2005; Perreten et al., 2010).

Statistical Analysis

The Pearson’s chi-square test was used to explore significant differences in the antimicrobial activity of the isolates tested. Comparisons between BLIS production and origins (human, food, wild animal, pet, and environment) were carried out in the total collection of 890 staphylococci, as well as in the CoPS and CoNS isolates, considered in a separate way. Moreover, the relationship between the rate of BLIS production and the staphylococcal species was studied. Analyses were carried out using SPSS statistical software version 26.0 (IBM®, SPSS Inc., Chicago, IL, United States) and significance was set at p ≤ 0.05.

RESULTS

Bacteriocin-Like Production in Isolates of Different Species and Origins

Bacteriocin-like production assays performed using the spot on the lawn method revealed that 60 out of 890 isolates tested (6.7%)
showed antimicrobial activity (BLIS+) against at least one of the 25 indicator isolates (Table 1). Differences were observed in the percentage of BLIS+ isolates between CoPS (n = 21 of 238 isolates, 8.8%) and CoNS (n = 39 of 652 isolates, 6%), and among staphylococcal species (number of BLIS+ isolates/total number of tested isolates): S. aureus (5/147), S. pseudintermedius (16/60), S. sciuri (13/214), S. hyicus (1/6), S. hominis (1/8), S. chromogenes (10/26), S. epidermidis (4/38), S. warneri (6/26), S. xylosus (3/48), and S. simulans (1/22). No BLIS+ isolates were detected among the remaining staphylococcal species. The comparison of the rates of BLIS+ isolates by staphylococcal species showed statistically significant differences, and the higher rates were detected for the following species: S. chromogenes (38.5%), S. pseudintermedius (26.7%), and S. warneri (23.1%). When the origin of the isolates and the rate of BLIS+ isolates were considered, statistically significant differences were observed for the total collection of staphylococci, as well as for the collections of CoPS and CoNS (p ≤ 0.05). The highest frequency of BLIS+ isolates was found in isolates obtained from pets (n = 15, 30%), followed by those of wild animals (n = 25, 6.2%) and of food samples (n = 17, 6.11%) (Table 1 and Figure 1).

Antimicrobial Activity of BLIS+ Isolates

The antimicrobial profile of the 60 BLIS+ isolates against the 25 indicator bacteria is summarized in Table 3. Antimicrobial activity was detected against all the species of Gram-positive indicator bacteria tested; nevertheless, none of the BLIS+ isolates showed activity against the Gram-negative indicator bacteria used in this study (Escherichia coli and Pseudomonas aeruginosa). The indicator bacteria with the highest inhibition rates were as follows: S. delphini (inhibited by 62% of BLIS+ isolates), S. pseudintermedius (55%), M. luteus (38%), and C. perfringens (35%).

Moreover, our results reflected intense bioactivities both in CoPS and CoNS, detecting strains of some species (S. pseudintermedius, S. aureus, S. hominis, and S. sciuri) with antimicrobial activity against more than 70% of the 25 indicator bacteria tested. In addition, high diversity was found in the number of indicator isolates inhibited by the BLIS+ isolates.

**FIGURE 1** Distribution of the 60 BLIS+ isolates per origin, indicating the species and the number of isolates in which antimicrobial activity was detected.
| Indicator bacteria (No isolates) | No of BLIS[^+^] isolates[^b^] | |
|---------------------------------|-------------------------------|--|
| S. pseudintermedii (16)         | 10 2 2 2 0 0 2 1 2 1 1 | 18/30 |
| S. aureus (5)                   | 10 2 2 2 0 0 0 0 0 0 | 8/13 |
| S. sciuri (13)                  | 10 1 0 0 1 1 1 0 0 1 | 13/22 |
| S. chromogenes (10)             | 10 2 0 1 0 0 0 0 0 0 | 33/55 |
| S. warneri (6)                  | 9 0 0 0 0 0 0 0 0 0 | 13/22 |
| S. epidermidis (10)             | 9 2 2 6 0 0 0 0 0 0 | 37/62 |
| S. sciuri (12)                  | 9 2 1 6 0 0 1 0 0 0 | 9/15 |
| S. delphini (13)                | 9 0 0 0 1 1 1 0 0 0 | 9/15 |
| S. haemolyticus (6)             | 8 0 0 0 0 0 0 0 0 0 | 10/17 |
| S. lugdunensis (11)             | 8 1 0 0 0 0 1 0 0 0 | 15/25 |
| S. sciuri (6)                   | 7 2 2 2 0 0 0 0 0 0 | 11/18 |
| E. casseliflavus (2)            | 7 1 2 0 0 0 0 0 0 0 | 11/18 |
| E. faecium vanA (2)             | 7 1 2 0 0 0 0 0 0 0 | 11/18 |
| E. gallinarum (2)               | 7 1 2 0 0 0 0 0 0 0 | 11/18 |
| E. hirae vanA (2)               | 6 2 2 2 0 0 0 0 0 0 | 14/23 |
| L. monocytogenes (2)            | 6 1 2 2 0 0 0 0 0 0 | 10/17 |
| M. luteus (2)                   | 6 0 0 0 0 0 0 0 0 0 | 23/38 |
| S. suis (4)                     | 5 0 0 0 0 0 0 0 0 0 | 19/32 |
| C. perfringens (1)              | 4 0 0 0 0 0 0 0 0 0 | 21/35 |
| E. coli/P. aeruginosa (4)       | 4 0 0 0 0 0 0 0 0 0 | 0 |

[^a^]MRSA, methicillin resistant S. aureus; MSSA, methicillin-susceptible S. aureus; MRSP, methicillin-resistant S. pseudintermedius; MSSP, methicillin-susceptible S. pseudintermedius; vanA, acquired mechanism of vancomycin resistance.

[^b^]The number of staphylococcal isolates that showed antimicrobial activity against each indicator bacteria is indicated.

[^c^]The number of the total staphylococcal isolates that showed antimicrobial activity against each indicator bacteria and percentage respect to the 60 BLIS[^+^] isolates is represented.
of each staphylococcal species. For example, one BLIS+ S. aureus isolate was able to prevent the growth of up to 21 indicators, while two BLIS+ S. aureus isolates only inhibited the growth of two indicator isolates. In the remaining staphylococcal species, the following ranges of the number of indicator isolates inhibited were detected among BLIS+ isolates: S. pseudintermedius (1–18), S. sciuri (1–22), S. chromogenes (1–10), S. epidermidis (1–7), and S. warneri (1–5). In the case of the species S. xylosus, the three BLIS+ isolates only showed antimicrobial activity against M. luteus.

For an overview of the antimicrobial activity of the 60 BLIS+ isolates, three levels of activity were established based on the percentage of indicator bacteria inhibited by each BLIS+ staphylococci: high activity (H-Act, activity against > 70% of the 25 indicator bacteria tested), medium activity (M-Act, 20–70%), and low activity (L-Act, < 20%) (Figure 2). In all staphylococcal species with BLIS+ isolates, there were H-Act or M-Act BLIS+ isolates, except for S. xylosus. Moreover, BLIS+ isolates with H-Act were identified for the species S. aureus (20% of BLIS+ isolates), S. pseudintermedius (19% of BLIS+ isolates), S. sciuri (15% of BLIS+ isolates), and S. hominis (the unique BLIS+ isolate of this species).

A total of 13 isolates among the total collection of 60 BLIS+ isolates showed high or medium antimicrobial activities, and their antimicrobial profiles against relevant indicator bacteria are presented in Table 4. Some of these 13 BLIS+ isolates produced high inhibition halos (larger than 10 mm in diameter) against methicillin-resistant S. pseudintermedius (MRSP), methicillin-resistant S. aureus (MRSA), vancomycin-resistant enterococci (VRE), L. monocytogenes, C. perfringens, or S. suis, among others.

**Bacteriocin Encoding Genes Detected in BLIS+ Isolates**

The presence of 23 bacteriocin-encoding genes was analyzed by PCR and sequencing in the collection of 60 BLIS+ isolates. At least one bacteriocin gene was detected in 16 of these isolates. The characteristics of these isolates are shown in Table 5. The bacteriocin encoding genes detected were as follows: (1) lantibiotic-like (n = 11 isolates, five CoNS and six CoPS); (2) uberolysin-like (two CoNS isolates); and (3) the BacSp222 (three CoPS isolates). Ten of these staphylococci were susceptible to all antibiotics tested.

**Antimicrobial Resistance, Virulence, and Molecular Typing of the 60 BLIS+ Staphylococcal Isolates**

The AMR phenotype/genotype and the virulence gene content results of the 60 BLIS+ isolates are shown in Supplementary Table 2 and summarized in Table 6. A great diversity of phenotypes and genotypes of antibiotic resistance was detected in the whole collection of 60 BLIS+ isolates. Among the CoNS, 30.7% showed susceptibility to all antibiotics tested, while 12.8% were MDR. Notably, clindamycin and fusidic acid were the most common AMR phenotypes observed in CoNS. The sal(A) gene (solely found among the S. sciuri isolates) and the ini(A) gene were detected among clindamycin-resistant isolates.

In contrast, only one CoPS isolate was susceptible to all the antimicrobials tested, whereas 33.3% of them were MDR and showed mainly resistance to the following antibiotics/resistance genes: penicillin/blaZ, erythromycin-clindamycin/erm(B), and tetracycline/tet(M). Virulence factors were only detected among CoPS isolates, mainly in the species S. pseudintermedius, where the detection of the leukocidin and the siet and se-int enteroroxin genes was frequent.

**DISCUSSION**

Antimicrobial resistance is a relevant health problem worldwide that needs the development of new strategies. The human–animal–environment interface is currently being considered under the OneHealth approach to better understand the ecology and dissemination of AMR and to control the silent pandemic, as is considered by some authors (Swift et al., 2019; Penna et al., 2021). In this context, the use of probiotics or new antimicrobial compounds, such as bacteriocins, is an interesting alternative to the use of conventional antibiotics. These peptides might become important biological weapons, especially against emerging drug-resistant bacteria, and they might be used in different areas, such as the food industry, medicine, veterinary, and agriculture (de Freire Bastos et al., 2020).

The *Staphylococcus* genus has been widely described in the literature as a bacteriocin producer. Several studies have described staphylococccins in strains from food products, such as milk (Hycin 3682, Aureocin A70, and Aureocin A53) (Netz et al., 2001; Netz et al., 2002; Carlin Fagundes et al., 2017), fermented food (Micrococcin P1 and Nukacin ISK-1) (Cundiffle and Thompson, 1981; Sashihara et al., 2000), fish (Nukacin KQU-131) (Wilaipun et al., 2008), livestock (BacCH91 and Gallidermin) (Kellner et al., 1988; Wladyka et al., 2013), and also in strains from pets (BacSp222) (Wladyka et al., 2015). However, very few studies have reported bacteriocins produced...
TABLE 4 | Antimicrobial profile of the 13 BLIS\(^+\) isolates with high**/medium* antimicrobial activities\(^a\) against relevant indicator bacteria\(^b\).

| Indicator bacteria (No isolates)\(^b\) | No of indicator bacteria inhibited |
|--------------------------------------|----------------------------------|
|                                      | S. pseudintermedius | S. aureus | S. sciuri | S. hominis | S. chromogenes | S. warneri | S. epidermidis | S. hyicus | S. simulans |
|                                      | C8189** | C8478** | C8479** | C5802** | X3011** | X3041** | C5835** | C9838* | C9581* | X2969* | X3009* | C9585* | C9832* |
| Human                               | 3       | 3       | 3       | 3       | 3       | 3       | 3       | 2       | 2       | 0       | 2       | 0       | 3       |
| Pet                                  | 3       | 3       | 3       | 3       | 3       | 3       | 3       | 3       | 2       | 0       | 2       | 0       | 3       |
| Environmental                       | 6       | 6       | 6       | 6       | 9       | 9       | 9       | 3       | 2       | 0       | 2       | 0       | 0       |
| Food                                | 6       | 6       | 6       | 6       | 6       | 6       | 6       | 5       | 2       | 0       | 0       | 0       | 0       |
| Wild animal                         | 3       | 3       | 3       | 3       | 3       | 3       | 3       | 2       | 0       | 0       | 0       | 0       | 0       |
| Total Staphylococcus spp. (10)      | 6       | 6       | 6       | 6       | 9       | 9       | 9       | 3       | 3       | 0       | 2       | 2       | 10      |
| VR-Enterococcus (3)                 | 3       | 3       | 3       | 3       | 3       | 3       | 3       | 2       | 0       | 0       | 0       | 2       | 0       |
| VS-Enterococcus (3)                 | 3       | 3       | 3       | 3       | 3       | 3       | 3       | 2       | 0       | 0       | 0       | 0       | 2       |
| Total Enterococcus spp. (6)         | 6       | 6       | 6       | 6       | 6       | 6       | 6       | 5       | 2       | 0       | 0       | 0       | 0       |
| L. monocytogenes (1)                | 1       | 1       | 1       | 1       | 1       | 1       | 1       | 1       | 0       | 0       | 0       | 0       | 0       |
| M. luteus (1)                       | 1       | 1       | 1       | 0       | 1       | 1       | 1       | 1       | 1       | 1       | 1       | 1       | 0       |
| S. suis (4)                         | 3       | 3       | 3       | 4       | 4       | 4       | 4       | 0       | 4       | 0       | 0       | 0       | 0       |
| C. perfringens (1)                  | 1       | 1       | 1       | 1       | 1       | 1       | 1       | 0       | 1       | 1       | 0       | 1       | 0       |
| E. coli (1)/P. aeruginosa (1)       | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       |
| Total number/% of indicator bacteria inhibited | 18/72% | 18/72% | 18/72% | 21/84% | 22/88% | 22/88% | 18/72% | 10/40% | 8/32% | 5/20% | 7/28% | 9/36% | 10/40% |

\(^a\)High activity** (>70%); medium activity* (20–70%) of the number of indicator bacteria inhibited.

\(^b\)MR, methicillin-resistant; MS, methicillin-susceptible; VR, vancomycin-resistant; VS, vancomycin-susceptible.
by staphylococci from wild animals, except for some studies on animals from the marine environment (Prichula et al., 2021). Regarding the available information about the environment, the soil is the most widely studied natural source of antimicrobial peptides, and Bacillus is the most representative genus of soil bacteriocin producers (Zimina et al., 2020).

In humans, bacteriocin production has been detected in clinical and commensal Staphylococcus isolated from the skin (Capidermicin, Nisin), and Staphylococcus C55 (Dajani and Wannamaker, 1969; Lynch et al., 2019; O’Sullivan et al., 2020) and nose (Nukacin IVK45 and Lugdunin) (Janek et al., 2016; Wannamaker, 1969; Lynch et al., 2019; O’Sullivan et al., 2020) (Capidermicin, NisinJ, and Staphylococcin C55) (Dajani and Wannamaker, 1969; Lynch et al., 2019; O’Sullivan et al., 2020) (Capidermicin, NisinJ, and Staphylococcin C55) (Dajani and Wannamaker, 1969; Lynch et al., 2019; O’Sullivan et al., 2020). In this study, production of BLIS was found in 6.7% of isolates obtained with respect to the number of total isolates tested in each origin); these BLIS

| Species                  | Strain | Origin | Antimicrobial levela | Bacteriocins detected | Antimicrobial resistance phenotype-[genotype]b | Virulence content | spa-MLST-CC/age |
|--------------------------|--------|--------|----------------------|-----------------------|-----------------------------------------------|-------------------|-----------------|
| S. pseudintermedius      | C4502  | Pet    | M-Act                | Lantibiotic-like      | SXT-[tet]\(a\)                                | se-int, sea1, sed1-sel, \(\ futuristic\) | ST160/III       |
|                          | C8189  | Human  | H-Act                | Bacsp222              | ERY-CLL-[erm(B)]                              | \(\ futuristic\)  | ST241/III       |
|                          | C8478  | Pet    | H-Act                | Bacsp222              | ERY-CLL-[erm(B)]                              | \(\ futuristic\)  | ST241/III       |
|                          | C8479  | Pet    | H-Act                | Bacsp222              | ERY-CLL-[erm(B)]                              | \(\ futuristic\)  | ST241/III       |
| S. aureus                | C5802  | Environment | H-Act              | Lantibiotic-like       | PEN-[blaZ]                                    | \(\ futuristic\)  | t843-ST130-CC130/III |
|                          | C6770  | Wild animal | L-Act              | Lantibiotic-like       | Susceptible                                   | –                 | t11255/CC5/II   |
|                          | C8609  | Wild animal | L-Act              | Lantibiotic-like       | Susceptible                                   | –                 | t11225-CC425/II |
|                          | X3410  | Food   | L-Act                | Lantibiotic-like       | Susceptible                                   | –                 | 110234/I        |
| S. chromogenes           | C8838  | Wild animal | M-Act              | Uberolysin-like        | Susceptible                                   | –                 | NSc             |
|                          | C8958  | Wild animal | M-Act              | Uberolysin-like        | Susceptible                                   | –                 | NSc             |
|                          | C9977  | Wild animal | M-Act              | Lantibiotic-like       | Susceptible                                   | –                 | NSc             |
|                          | C9976  | Wild animal | M-Act              | Lantibiotic-like       | Susceptible                                   | –                 | NSc             |
| S. epidermidis           | C8009  | Food   | M-Act                | Lantibiotic-like       | PEN-[blaZ], mph(C)                            | –                 | ST1025          |
| S. xylosus               | C9255  | Wild animal | L-Act              | Lantibiotic-like       | PEN-[blaZ]                                    | –                 | NSc             |
|                          | C9588  | Wild animal | M-Act              | Lantibiotic-like       | Susceptible                                   | –                 | NSc             |
|                          | C9926  | Wild animal | M-Act              | Lantibiotic-like       | Susceptible                                   | –                 | NSc             |

Some authors consider bacteriocin production to be a common characteristic of bacteria since most of them can produce these antimicrobial peptides (Cotter et al., 2005). Bacteriocin production has been reported in the literature for CoPS and CoNS, and S. aureus and S. epidermidis have been highlighted as highly prevalent producer species or at least with the better-characterized bacteriocins (Bastos et al., 2009).

Statistically significant differences were detected in this study between the origin of the isolates and the production of BLIS. Considering the total collection of Staphylococcus tested, BLIS production was more frequently detected among isolates of pets (30%), food (6.1%), and wild animals (6.2%) (percentages obtained with respect to the number of total isolates tested in each origin); these BLIS+ isolates represented 25%, 28.3%, and 41.7% of the 60 BLIS+ isolates, respectively. The high rate of BLIS production detected among isolates of pets might be explained by the fact that all the isolates analyzed from this originally belonged to the species S. pseudintermedius, one of the higher BLIS+ species detected in our study, as previously indicated. In humans, only one of the 27 tested isolates (3.7%) showed BLIS production, and this isolate was S. pseudintermedius, which interestingly was obtained from the clinical sample of a human cohabitating with pets. Higher frequencies of bacteriocin production have been previously reported in nasal staphylococci of human origin (80%) when bacteria of the nasal ecosystem were used as indicator bacteria (Janek et al., 2016). A wide diversity of variables could explain the differences observed in bacteriocin production rates, but the different origins and species of the staphylococci tested for and the different indicator bacteria employed might be involved.

Most of the known bacteriocins have a narrow spectrum of activity, often active against closely related bacteria (Cotter et al., 2013); nevertheless, in relation to staphylococci, a wide variety of antimicrobial profiles against pathogens have been described depending on the peptide (Streptococcus, Enterococcus, Enterococcus...).
S. capitis

Freire Bastos et al., 2020). In our study, 16 of the 60 BLIS
more gene clusters responsible for bacteriocin production (de
of interest for further in-depth characterization.

L. monocytogenes

presented antimicrobial activity against
Corynebacterium

CIP , ciprofloxacin; CHL, chloramphenicol; FUS, fusidic acid; SXT, trimethoprim–sulfamethoxazole.

S. delphini

Janek et al., 2016).

to be maintained in complex ecosystems in different hosts
might indicate that this genus needs bacteriocins as a strategy
Neisseria

is an important pathogen in poultry production
is an emerging
S. suis

or

B. perfringens,

which

S. pseudintermedius

13 0 ERY

10 8 CIP

4 0 PEN

S. epidermidis

1 0 TET

S. hyicus

1 1 Susceptible

S. xylosus

3 0 PEN-I-

S. hominis

1 1 Susceptible

S. hyicus

1 1 Susceptible

S. simulans

1 0 TET-I-

Total CoPS

21 1 PEN-12.0X1.0-ERY-CL5.0-

17-STR-0T5.0-CHL-3.0-

SXT-FA-01

Total CoNS

39 12 PEN-8.0X6.0-ERY-I-

13-TO5.0-0T6.0-

CIP-0ST7.0-FA-11

Virulence gene content

Table 6

Antimicrobial resistance phenotype/genotype and virulence gene content in the 60 BLIS+ isolates recovered in this study.

| Species                  | Number of isolates | Number of isolates susceptible to all antibiotics tested | Antimicrobial resistance phenotype a,b | Antimicrobial resistance genotype b | Virulence gene content b |
|--------------------------|--------------------|--------------------------------------------------------|---------------------------------------|------------------------------------|--------------------------|
| S. pseudintermedius      | 16                 | 1                                                      | PEN10, OX1.0-ERY7-CL5.0-STR-0T5.0-CHL3.0-0ST5.0-FA-01 | blaZ10, mecA1, ermB1, aacD4, tetM6, catGC221.0, aacC2-0, ina7, ant(B)-la1 | lukSF-I-F16, sec15, seA12, sec12, sec11, expB1 |
| S. aureus                | 5                  | 3                                                      | PEN2-ERY-CL5.1 | blaZ2, mrr(A)1, emc(C), emc(T)1, nua(A)1 | lukSF-I, lukED1, etD21 |
| S. sciuri                | 13                 | 0                                                      | ERY-CL5.13-FA-0T5.0-CHL3.0 | emcB1, mrr(A)1, saI(A)13, nua(A)1 | -- |
| S. chromogenes           | 10                 | 8                                                      | CIP-0T5.0 | tatM1, tatK1, tetL1 | -- |
| S. warneri               | 6                  | 2                                                      | PEN2-ERY-CL5.1-0T5.0 | tetZ2, tatK2, emcB2 | -- |
| S. epidermidis           | 4                  | 0                                                      | PEN2-FOX2-ERY3.0-0ST7.1-0T5-FA-01 | mecA2, mrr(A)1, mph(C)1 | -- |
| S. xylosus               | 3                  | 0                                                      | PEN-I-FA-01 | blaZ1 | -- |
| S. hominis               | 1                  | 1                                                      | Susceptible | -- | -- |
| S. hyicus                | 1                  | 1                                                      | Susceptible | -- | -- |
| S. simulans              | 1                  | 0                                                      | TET-I-FA-01 | tetK1 | -- |
| Total CoPS               | 21                 | 1                                                      | PEN12-0X1.0-ERY8-CL5.0-17-STR-0T5.0-CHL3.0-SXT-FA-01 | blaZ12, mecA1, ermB1, mrr(A)1, erm(T)1, mrr(A)1, aacD4, tetM6, catGC221.0, aacC2-0, ina7, ant(B)-la1 | lukSF-I-F16, sec15, seA12, sec12, expB1, lukED1, etD21 |
| Total CoNS               | 39                 | 12                                                     | PEN8-FOX2-ERY7-CL5.1-13-TO5.0-0T6.0-CIP-0ST7-FA-11 | blaZ2, mecA1, ermB1, mrr(A)1, mph(C)1, saI(A)13, nua(A)1, tetK1, tetM2, tetL1 | -- |

a PEN, penicillin; OX, oxacillin; FOX, cefoxitin; ERY, erythromycin; CL5, clindamycin; CL5, clindamycin inducible; TOB, tobramycin; STR, streptomycin; TET, tetracycline; CIP, ciprofloxacin; CHL, chloramphenicol; FUS, fusidic acid; SXT, trimethoprim–sulfamethoxazole.

b The number in superscripts indicate the number of isolates when not all isolates of the group had the same characteristics.

Corynebacterium, Bacillus, Clostridioides, Klebsiella, and Neisseria, among others (de Freire Bastos et al., 2020). This might indicate that this genus needs bacteriocins as a strategy to be maintained in complex ecosystems in different hosts (Jame et al., 2016).

All Gram-positive indicator bacteria were inhibited by at least one of the BLIS+ staphylococci, but this did not happen with Gram-negative indicator bacteria. It was especially relevant to the antimicrobial activity detected against S. delphini, S. pseudintermedius, C. perfringens, and M. luteus. Notably, C. perfringens is an important pathogen in poultry production (Nhung et al., 2017), and S. pseudintermedius is an emerging zoonotic pathogen in humans, especially in those with close contact with dogs/cats (Lозано et al., 2017).

Interestingly, we detected 7 BLIS+ isolates of the species S. aureus, S. pseudintermedius, S. sciuri, and S. hominis, which presented antimicrobial activity against > 70% of the indicator bacteria, including MRSP, MRSA, VRE, L. monocytogenes, C. perfringens, or S. suis, among others. These BLIS+ isolates are of interest for further in-depth characterization.

It is known that staphylococcal strains can carry one or more gene clusters responsible for bacteriocin production (de Freire Bastos et al., 2020). In our study, 16 of the 60 BLIS+ isolates showed one of the bacteriocin genes analyzed. Many Staphylococcus isolates are producers of lantibiotics, and a variety of these bacteriocins have been described (Neubauer et al., 1999). Significantly, putative lantibiotic-biosynthetic gene clusters have been recently found in the genome of S. capitis, which share homology with the biosynthetic systems of epidermin/gallidermin and the non-lantibiotic bacteriocin epidermin (Kumar et al., 2017). According to our results, structural genes encoding putative lantibiotic-like bacteriocins were detected by PCR and sequencing in 11 isolates, both CoNS (n = 5) and CoPS (n = 6). Moreover, the gene of the recently described bacteriocin BacSp222 (Wladyka et al., 2015) was detected in three of the BLIS+ S. pseudintermedius isolates, and interestingly, all showed H-Act; nevertheless, this gene was not found in the other staphylococci species tested. In addition, it is worth highlighting the detection of the gene encoding a putative circular bacteriocin (uberoysin-like) in two of our S. chromogenes BLIS+ isolates tested. The circular bacteriocins have been commonly described in Bacillus and have been rarely identified in Staphylococcus species, excepting the Aureocycin 4185 described in S. aureus (Potter et al., 2014). The detection of this putative circular bacteriocin in two S. chromogenes isolates would be the first report of this CoNS species, indicating the possible transfer of genetic material between staphylococcal species or the possible detection of a new bacteriocin. Notably, four of our isolates with high (X3041, X3011, and C5835) or medium (X2969) antimicrobial activity lacked all the 23 bacteriocin encoding genes tested. Further studies will be performed with these isolates to determine if they produce new bacteriocins that could be of interest.

According to the AMR phenotype and genotype and the virulence content of the BLIS+ isolates, it was observed in this study that 30.7% of BLIS+ CoNS and 19% of CoPS were susceptible to all antibiotics tested; nevertheless, 12.8% and 33.3% of BLIS+ CoNS and CoPS isolates, respectively, were
MDR. Moreover, all the CoNS lacked the virulence genes tested, while different virulence gene profiles were observed among CoPs. Currently, the use of bacteriocins or bacteriocin-producing bacteria as an alternative to antibiotics needs to consider strains that meet all safety, efficacy, and viability criteria to be used in consortium formulations, which are being proposed as the next generation of probiotics (Eveno et al., 2021). In this context, isolates lacking acquired AMR genes or virulence genes are of special relevance.

**CONCLUSION**

Bacteriocin production is a defense strategy or adaptive mechanism that contributes to the success of niche colonization. In this study, 6.7% of the staphylococci tested were BLIS\(^7\), showing a wide variety of inhibition patterns in relation to the species of producer isolates and their origins. It is worth noting the interest of CoNS isolates, due to their high antimicrobial activity profiles confirmed by the detection of bacteriocin encoding genes and the lack of relevant acquired AMR or virulence genes. These results leave a gateway for further biochemical and genetic characterization of high relevance for these BLIS\(^7\) isolates that can be considered as excellent candidates for a further in-depth study. To conclude, the OneHealth approach is important to better understand the interactions between colonizing bacteria in specific niches and their spread to other environments, considering the molecular ecology of AMR and other mechanisms of bacterial fitness.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

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**AUTHOR CONTRIBUTIONS**

CT, MZ, and CL contributed to the design and the general supervision of the study. RF-F developed most of the experimental work and the first version of the manuscript. CT made the first revision of the manuscript. PE, LR-R, and IA contributed to some experimental laboratory work related to AMR genes and molecular typing. CT and MZ contributed to project funding. All authors revised the different versions of the manuscript, read, and agreed to the submitted version of the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.870510/full#supplementary-material

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