UNIVERSIDAD DE LA RIOJA

TESIS DOCTORAL

Título
Detección y caracterización de péptidos con actividad antimicrobiana en aislados de *Staphylococcus*. Aplicación al ámbito agroalimentario y en salud pública
Autor/es
Rosa Fernández Fernández
Director/es
Carmen Torres Manrique y Carmen Lozano Fernández
Facultad
Facultad de Ciencia y Tecnología
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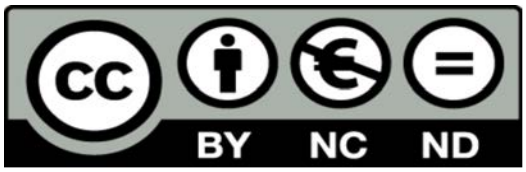

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Detection and characterization of peptides with antimicrobial activity in *Staphylococcus* isolates. Application to the agro-food and public health.

Tesis doctoral con Mención Internacional

Rosa Fernández Fernández

Logroño 2023

UNIVERSIDAD DE LA RIOJA

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Departamento de Agricultura y Alimentación
Área de Bioquímica y Biología Molecular
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Programa de Doctorado en Ciencias Biomédicas y Biotecnológicas

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Detection and characterisation of peptides with antimicrobial activity in *Staphylococcus* isolates. Application to agro-food and public health

Memoria presentada por **ROSA FERNÁNDEZ FERNÁNDEZ** para optar al título de Doctora con la Mención Internacional por la Universidad de La Rioja

Directora: Carmen Torres Manrique
Directora: Carmen Lozano Fernández

Logroño, octubre de 2023

UNIVERSIDAD DE LA RIOJA

Departamento de Agricultura y Alimentación
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Grupo OneHealth-UR

Dra. **CARMEN TORRES MARIQUE,** Catedrática del Área de Bioquímica y Biología Molecular de la Universidad de La Rioja.

Dra. **CARMEN LOZANO FERNÁNDEZ**, Profesora Titular del Área de Bioquímica y Biología Molecular de la Universidad de La Rioja.

Por la presente declaran que,

La memoria titulada "Detección y caracterización de péptidos con actividad antimicrobiana en aislados de *Staphylococcus*. Aplicación al ámbito agroalimentario y en salud pública" que presenta Dña. **ROSA FERNÁNDEZ FERNÁNDEZ**, Graduada en Biología por la Universidad Pública del País Vasco, ha sido realizada en el Área de Bioquímica y Biología Molecular de la Universidad de La Rioja, bajo su dirección, y reúne las condiciones exigidas para optar al grado de Doctor.

Lo que hace constar en Logroño, a 4 de octubre de 2023.

Fdo.: Carmen Torres Manrique

Fdo.: Carmen Lozano Fernández

Si quieres rápido, camina solo
Si quieres llegar rápido, camina acompañado

La suerte es donde confluyen el esfuerzo y la oportunidad

El único modo de hacer un gran trabajo es amar lo que haces
"Steve Jobs"

Agradecimientos

Me parece increíble estar escribiendo estas líneas, con el corazón lleno de emociones. Hace poco más cinco años comenzó mi andadura en el 229, aunque ya mucho antes y sin saberlo una voz interior me indicaba que ese era mi camino. Lo que no lograba imaginar es que las puertas de la Universidad se iban a abrir para mí con tantas gratas sorpresas y experiencias que jamás olvidaré. Todo ello, sin duda, es gracias a mis directoras de tesis "las Cármenes". Sé que me caracterizo por mis habilidades comunicativas, pero no tengo palabras para agradecer todo lo que habéis hecho por mí.

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A mi familia

Esta tesis se presenta como compendio de 6 publicaciones. A continuación, se citan dichas publicaciones, así como sus datos bibliométricos:

Artículo 1:

Bacteriocin-Like Inhibitory Substances in Staphylococci of Different Origins and Species With Activity Against Relevant Pathogens**. Fernández-Fernández, Rosa;** Lozano, Carmen; Eguizábal, Paula; Ruiz-Ripa, Laura; Martínez-Álvarez, Sandra; Abdullahi Nasir, Idris; Zarazaga, Myriam; Torres, Carmen. Frontiers in Microbiology, 2022, 13, 870510. doi: 10.3389/fmicb.2022.870510

Factor de impacto de la revista (JCR 2022): 5.2

Área: MICROBIOLOGY, Cuartil Q2, Posición en el área: 38/135 (Edición: SCI)

Artículo 2:

Antimicrobial Resistance and Antimicrobial Activity of *Staphylococcus lugdunensis* Obtained from Two Spanish Hospitals. **Fernández-Fernández, Rosa;** Lozano, Carmen; Ruiz-Ripa, Laura; Robredo, Beatriz; Azcona-Gutiérrez, José Manuel; Alonso Carla, Andrea; Aspiroz, Carmen; Zarazaga, Myriam; Torres, Carmen**.** Microorganisms, 2022, 10, 1480. doi:10.3390/microorganisms10081480

Factor de impacto de la revista (JCR 2022): 4.5

Área: MICROBIOLOGY, Cuartil Q2, Posición en el área: 47/135 (Edición: SCI)

Artículo 3:

Comprehensive Approaches for the Search and Characterization of Staphylococcins. **Fernández-Fernández, R.** Lozano, C. Reuben, R.C. Ruiz-Ripa, L. Zarazaga, M. Torres, C. Microorganisms, 2023, 11, 1329. doi:10.3390/ microorganisms11051329

Factor de impacto de la revista (JCR 2022): 4.5

Área: MICROBIOLOGY Cuartil: Q2, Posición en el área: 47/135 (Edición: SCI)

Artículo 4:

Detection and evaluation of the antimicrobial activity of micrococcin P1 isolated from commensal and environmental staphylococcal isolates against MRSA. **Fernández-Fernández, Rosa**; Lozano, Carmen; Fernández-Pérez, Rocío; Zarazaga, Myriam; Peschel, Andreas; Krismer, Bernhard; Torres, Carmen. International Journal of Antimicrobial Agents, 2023 (in press)

Factor de impacto de la revista (JCR 2022): 10.8

Área: MICROBIOLOGY Cuartil: Q1 Posición en el área: 15/135 (Edición SCI)

Artículo 5:

Detection of antimicrobial producing *Staphylococcus* from migratory birds: Potential role in nasotracheal microbiota modulation. **Fernández-Fernández, Rosa**; Abdullahi Nasir, Idris; González-Azcona, Carmen; Ulloa, Adriana; Martínez, Agustí; García-Vela, Sara; Höfle, Ursula; Zarazaga, Myriam; Lozano, Carmen; Torres, Carmen. Frontiers in Microbiology, 2023. 14,1144975. doi:10.3389/fmicb.2023.1144975

Factor de impacto de la revista (JCR 2022):5.2

Área: MICROBIOLOGY Cuartil: Q2 Posición en el área: 38/135 (Edición: SCIE)

Artículo 6:

Citizen Contribution for Searching for Alternative Antimicrobial Activity Substances in Soil. **Fernández-Fernández**, **Rosa**; Robredo, Beatriz; Navajas, Enrique; Torres, Carmen. Antibiotics, 2022. 12, 57. doi: 10.3390/antibiotics12010057

Factor de impacto de la revista (JCR 2022): 4.8

Área: PHARMACOLOGY & PHARMACY Cuartil: Q1, Posición en el área: 66/277 (Edición: SCI)

Asimismo, se incluyen en esta tesis los resultados recopilados en dos trabajos recientemente publicados o en vías de publicación en revista JCR.

Genomic analysis of bacteriocin-producing staphylococci: high prevalence of lanthipeptides and the micrococcin P1 biosynthetic gene clusters. **Fernández-Fernández, Rosa**; Elsherbini, Ahmed MA; Lozano, Carmen; Martínez, Agustí; de Toro, María; Zarazaga, Myriam; Peschel, Andreas; Krismer, Bernhard; Torres, Carmen. Probiotic and Antimicrobial Proteins, 2023. doi: 10.1007/s12602-023-10119-w

Bacteriocin-producing *Staphylococcus* for agro-food applications: relevance of micrococcin P1. **Fernández-Fernández, Rosa**; Lozano, Carmen; Campaña, Allelen; Fernández-Pérez, Rocío; Peña, Raquel; Zarazaga, Myriam; Carrasco, Jaime; Torres, Carmen. (submitted for publication).

Abreviaturas

AA Actividad Antimicrobiana

ABC ATP Binding Cassette

AC Asociado a Comunidad

ADN Ácido desoxiribonucleico

AG Asociado al Ganado

Agr Accessory gene regulator

AH Ámbito Hospitalario

ARG Adquisición de genes de resistencia

ARN Ácido ribonucleico

ATP Adenosine Triphosphates

ATCC American Type Culture Collection

Bac+ Bacterias productoras de bacteriocinas

BAL Bacterias Lácticas

BHI Brain Heart Infusion

BLAST Basic Local Alignment Search Tool

BLIS Bacteriocin-Like Inhibitory Substances

BT Butanol

CAESAR Central Asia and Eastern European Surveillance of Antimicrobial Resistance

CC Complejo Clonal

CECT Colección Española de Cultivos Tipo

CMI Concentración mínima inhibitoria

CLSI Clinical and Laboratory Standards Institute

Da Dalton

Dha Didehidroalanina

Dhb Didehidrobutirina

dNTP Deoxynucleotide Triphosphates Cys Cisteína

EARS-Net European Antimicrobial Resistance Surveillance Network

ECDCP European Centre for Disease Control and Prevention

EEE Espacio Económico Europeo

EFSA European Food Safety Authority

EGM Elemento Genético Móvil

ERV Enterococo resistente a vancomicina

ESKAPE	*Enterococcus spp., Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Organización de las Naciones Unidas para la Agricultura y Alimentación
GBD	Global Burden of Disease
Gram+/-	Gram positivo/negativo
GRAS	Generally Recognized as Safe
h	hora
IEC	Immune Evasion Cluster
IPPB	Infección de Piel y Partes Blandas
Kb	Kilobases
KDa	Kilodalton
Lan	Lantionina
LPV	Leucocidina de Panton Valentine (PVL en inglés)
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time of Flight
Man-PTS	Sistema transportador de manosa fosfotransferasa
MDR	Multirresistencia
MeLan	Metillantionina
Min	minutos
mL	mililitro
MLST	Multilocus Sequence Type
mg	miligramo
MP1	Micrococcina P1
NaCl	Cloruro sódico
NCBI	National Center of Biotechnology Information
Nº/n=	Número
NRP	Non Ribosomal Peptides
OCB/OCE	Operón codificante de Bacteriocinas/Estafilococcinas
OMS/WHO	Organización Mundial de la Salud/World Health Organization
ONT	Oxford Nanopore
PAM	Péptidos Antimicrobianos
Pb	pares de bases
PCR	Polymerase Chain Reaction (Reacción en cadena de la polimerasa)
QPS	Qualified Presumption of Safe

RAM Resistencia a antibióticos

RiPP Ribosomal Peptides Postraslationally modified

RM Resistente a meticilina

rpm revoluciones por minuto

S. *Staphylococcus*

SARM Staphylococcus aureus resistente a meticilina

SASM *Staphylococcus aureus* sensible a meticilina

SCC*mec* *Staphylococcal* Cassette Chromosome *mec* (casete cromosómico estafilocócico del gen *mec*)

SCoN *Staphylococcus* coagulasa negativo

SCoP *Staphylococcus* coagulasa positivo

SERM *Staphylococcus epidermidis* resistente a meticilina

SLC Sobrenadante libre de células

SM Sensible a meticilina

SOL *Spot-on-lawn*

spa *Staphylococcus* protein A gene (gen de la proteína A de *Staphylococcus*)

Spp. especies

SPRM *Staphylococcus pseudintermedius* resistente a meticilina

ST Secuencia tipo

SWI Small World Initiative

TSA Tryptic Soy Agar

TSB Tryptic Soy Broth

UE Unión Europea

UFC Unidad formadora de colonias

uHPLC Ultra High Perfomance Liquid Chromatography

UR Universidad de La Rioja

UV Ultravioleta

WGS Secuenciación masiva del genoma

µg microgramo

ºC grados centígrados

Antibióticos: PEN, penicilina; OXA; oxacilina; FOX, cefoxitina; ERY, eritromicina; CLI, clindamicina; CLI^{I}, clindamicina inducicle; TOB, tobramicina; STR, estreptomicina; TET, tetraciclina; CIP, ciprofloxacino; CHL, cloranfenicol; SXT, trimetoprim–sulfametoxazol; FA, ácido-fusídico; LZD, linezolid.

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Resumen/Abstract

Las bacteriocinas son objeto de estudio en la búsqueda de herramientas efectivas frente a la problemática de la resistencia a los antibióticos. Staphylococcus es un género bacteriano que incluye microorganismos comensales de la microbiota nasal y de la piel de animales y personas, aunque algunas especies también pueden ser patógenos oportunistas, especialmente *S. aureus*. Su interés como microorganismo productor de bacteriocinas está cobrando especial interés en los últimos años, sobre todo en especies coagulasa negativas.

En esta tesis se ha evaluado la actividad antimicrobiana (AA) de 1205 cepas de estafilococos, tanto coagulasa-positivos (SCoP, n=284) como -negativos (SCoN, n=921), de 5 orígenes y 29 especies diferentes frente a bacterias indicadoras de relevancia en salud pública y seguridad alimentaria, incluyendo bacterias multiresistentes (MDR) y zoonóticas, por el método *spot-on-lawn*. Se han detectado 92 cepas de estafilococos de diversos orígenes con AA (nº cepas productoras/Nº cepas evaluadas): humanos (24/83), animales de vida libre (34/662), mascotas (15/50), alimentos (17/278) y medio ambiente (2/132). Cabe destacar que solo se detectó AA frente a bacterias indicadoras Gram-positivas (G+). Se seleccionaron 37 estafilococos productores (cepas Bac+) para su caracterización en base a su especie, origen, características fenotípicas/genotípicas y su perfil de actividad inhibitoria. Se evaluó la AA en sobrenadantes libres de células (SLC) y en extractos obtenidos con butanol (BT). Nueve de los 37 (24%) estafilococos presentaron AA en los SLC mientras que más del 90% de los extractos BT reflejaron AA frente al menos uno de los indicadores analizados.

Por medio de secuenciación masiva del genoma (WGS) y espectrometría de masas se identificó la bacteriocina micrococcina P1 (MP1) en cuatro cepas, 3 SCoN recuperados de carne y agua, de especies infrecuentemente implicadas en infecciones (2 S. *sciuri*, 1 *S. hominis*) y 1 SCoP (*S. aureus*) procedente de agua superficial. La bacteriocina MP1 presentó gran actividad (tanto en SLC como en BT) frente a cepas de muchos géneros y especies diferentes de bacterias G+, algunas de ellas de gran relevancia en biomedicina, veterinaria y seguridad alimentaria. Asimismo, se identificaron otras bacteriocinas relevantes (principalmente lantibióticos), como epilancina 15X, BSA y bacCH91 (previamente descritos en la bibliografía) y bacteriocinas desconocidas potencialmente lantibióticos en tres aislados de SCoN procedentes de alimentos. En cuanto a los estudios por WGS, se detectaron genes codificantes de bacteriocinas de Clase II tales como bacSp2222 (2 *S. pseudintermedius*, 1 humano/1 perro), lactococcina 972 presente en gran frecuencia en esta colección; bacteriocinas circulares en 3 cepas productoras (2 SCoN y 1 SCoP) y lugdunina, péptido antimicrobiano no-ribosomal detectado en 2 cepas de *S. lugdunensis* (de origen humano). Atendiendo a los estafilococos productores de MP1, cabe destacar su gran actividad antimicrobiana frente a *S. aureus* resistente a meticilina (SARM) confirmada en estudios

de competencia, así como de sus extractos MP1-prepurificados donde se ha evidenciado su potencial inhibitorio y bacteriostático en medio líquido y en combinación con antibióticos (sinergia con clindamicina, oxacilina y cloranfenicol).

Por otro lado, se ha evaluado la posible modulación de la microbiota nasotraqueal por estafilococos con AA utilizando el modelo de cigüeña. Para ello, se ha analizado la AA de los estafilococos detectados en la microbiota nasotraqueal de estos animales frente a los aislados G+ presentes en la misma muestra y frente a una colección representativa de bacterias G+ de la microbiota nasotraqueal de cigüeñas. Cabe destacar la detección de 9 aislados Bac+, especialmente SCoN (n=7), con elevado interés por su AA y su potencial en la modulación microbiana. Si bien es necesario profundizar en la caracterización y purificación de las bacteriocinas detectadas, estos trabajos abren la puerta al interés de las cepas SCoN productoras de bacteriocinas o extractos de las mismas en distintas aplicaciones, especialmente en la modulación de la microbiota nasal como una novedosa aplicación en la prevención de enfermedades causadas por agentes patógenos de interés, como es el caso de SARM.

Finalmente, en esta tesis se ha estudiado la actividad antimicrobiana de 15 de las cepas de estafilococos productoras de bacteriocinas frente a 27 microorganismos patógenos adicionales utilizados como indicadores y distribuidos en categorías en base a sus potenciales áreas de aplicación: mastitis e industria láctea, patógenos aviares y agentes zoonóticos, zoonosis porcinas, seguridad alimentaria, acuicultura, industria vitivinícola y cultivo de hongos. Estos estudios evidenciaron la potente AA frente a indicadores G+ (≥60% de los indicadores inhibidos) mientras que no se detectó capacidad inhibitoria frente a los indicadores G-. Cabe destacar la mayor AA detectada en los extractos BT comparada con la identificada en *spot-on-lawn* y en los SLC, tanto en el porcentaje de indicadores inhibidos como en la intensidad de los halos. Por otro lado, se han identificado posibles consorcios bacterianos para su aplicación conjunta frente a un determinado patógeno. Con todo ello y teniendo en cuenta los resultados de hemólisis, actividad gelatinasa, resistencia a antibióticos y virulencia de las cepas de estafilococos Bac+ incluidas en este trabajo, se proponen las cepas productoras de la MP1 (especialmente las 3 SCoN) como potenciales candidatas a estudio para aplicaciones futuras.

Resumen/Abstract

Bacteriocins have been considered an effective tool to address the problem of antibiotic resistance. *Staphylococcus* is normally found as commensal microorganisms of the nasal and skin microbiota of animals and humans, although some species can be opportunistic pathogens, especially *S. aureus*. Currently, *Staphylococcus* is gaining attention as a bacteriocin-producing genus, especially coagulase-negative species.

In this work, the antimicrobial activity (AA) of 1205 staphylococcal isolates, both coagulase-positive (CoPS, n=284) and -negative (CoNS, n=921), from 5 different origins and 29 different species against indicator bacteria of public health and food safety relevance, including multi-resistant (MDR) and zoonotic bacteria, were by the *spot-on-lawn* method. Thus, 92 antimicrobial-producing staphylocci (Bac+ isolates) were detected from various sources (nº of producing isolates/nº of tested isolates): humans (24/83), wild-life animals (34/662), pets (15/50), food (17/278) and environment (2/132). Notably, AA was only detected against Gram-positive (G+) indicator bacteria. Then, 37 Bac+ staphylococci were selected for characterisation based on their species, origin, phenotypic/genotypic characteristics, and inhibitory activity profile. AA was assessed in cell-free supernatants (CFS) and butanol extracts (BT). Nine out of 37 (24%) staphylococci showed AA in CFS while more than 90% of BT extracts revealed AA against at least one of the indicators tested.

Moreover, the bacteriocin micrococcin P1 (MP1) was identified by whole genome sequencing (WGS) and mass spectrometry, in 3 CoNS recovered from meat and water, from species infrequently implicated in infections (2 *S. sciuri*, 1 *S. hominis*) and 1 SCoP (*S. aureus*) from surface water. This bacteriocin showed high activity (both in CFS and BT) against indicators of many different genera and species of G+ bacteria, some of them of great relevance in biomedicine, veterinary medicine, and food safety. In addition, relevant bacteriocins mainly lanthipeptides were detected including epilancin 15X, BSA and bacCH91 (previously described in the literature) and other unknown lanthipeptides identified in three CoNS isolates from food. On the other hand, WGS studies allowed the detection of Class II bacteriocin gene clusters such as bacSp2222 (2 *S. pseudintermedius*, 1 human/1 dog), lactococcin 972 present in high frequency in this collection; circular bacteriocins in 3 producing isolates (2 CoNS and 1 CoPS) and lugdunin, a non-ribosomal antimicrobial peptide detected in 2 *S. lugdunensis* isolates (recovered from human). As for MP1-producing staphylococci, it is to highlight the antimicrobial activity detected against methicillin-resistant *S. aureus* (MRSA) both in competition studies as well as their MP1-prepurified extracts (BT), which showed potent inhibitory and bacteriostatic activity in liquid medium and in combination with antibiotics (synergy with clindamycin, oxacillin, and chloramphenicol) has been demonstrated.

On the other hand, the potential of antimicrobial-producing staphylococci in the modulation of the nasotracheal microbiota has been evaluated using the stork model. For this purpose, the AA of the *Staphylococcus* was analysed against the G+ isolates present in the same sample and against a representative collection of G+ bacteria from the nasotracheal microbiota of storks. It is worth mentioning the detection of 9 isolates, especially CoNS (n=7), which are of great interest due to their AA and their potential for microbial modulation. Although further characterisation and bacteriocin purification is necessary, this work opens the door to the interest of CoNS isolates producing bacteriocins or extracts in the modulation of the nasal microbiota as a novel application in the prevention of diseases caused by relevant pathogens such as MRSA.

Finally, in this thesis, the antimicrobial activity of 15 bacteriocin-producing staphylococcal isolates was studied against 27 pathogenic microorganisms used as indicators and distributed in categories based on their potential areas of application: a) mastitis and dairy industry; b) poultry pathogens and zoonotic agents; c) swine zoonoses; d) food safety; e) aquaculture; f) wine industry; and g) mushroom cultivation. These studies showed potent AA against G+ indicators (≥60% of the indicators were inhibited) while no inhibitory capacity was detected against G- indicators. It is worth noting the increase observed in the AA of the BT extracts, compared with *Spot-on-lawn* and CFS, both considering the percentage of inhibited indicators and the intensity of the halos. On the other hand, possible bacterial combinations have been identified for synergic applications against a specific pathogen. Based on all the above, the results of hemolysis, gelatinase activity, antibiotic resistance and virulence of the bacteriocin-producing staphylococcal isolates included in this work, the three MP1-producing CoNS isolates can be proposed as potential candidates for future applications.

Mapa Conceptual

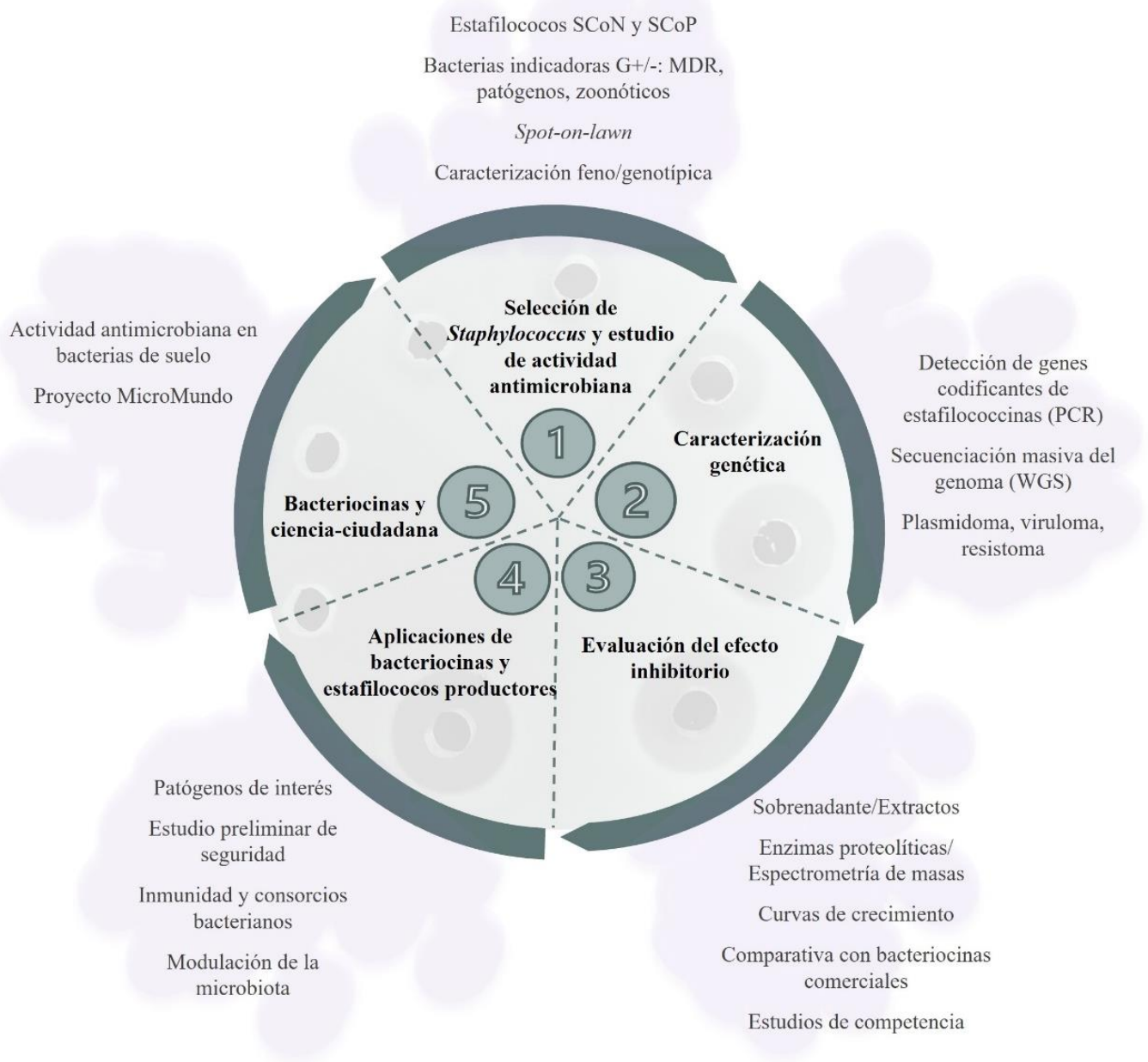
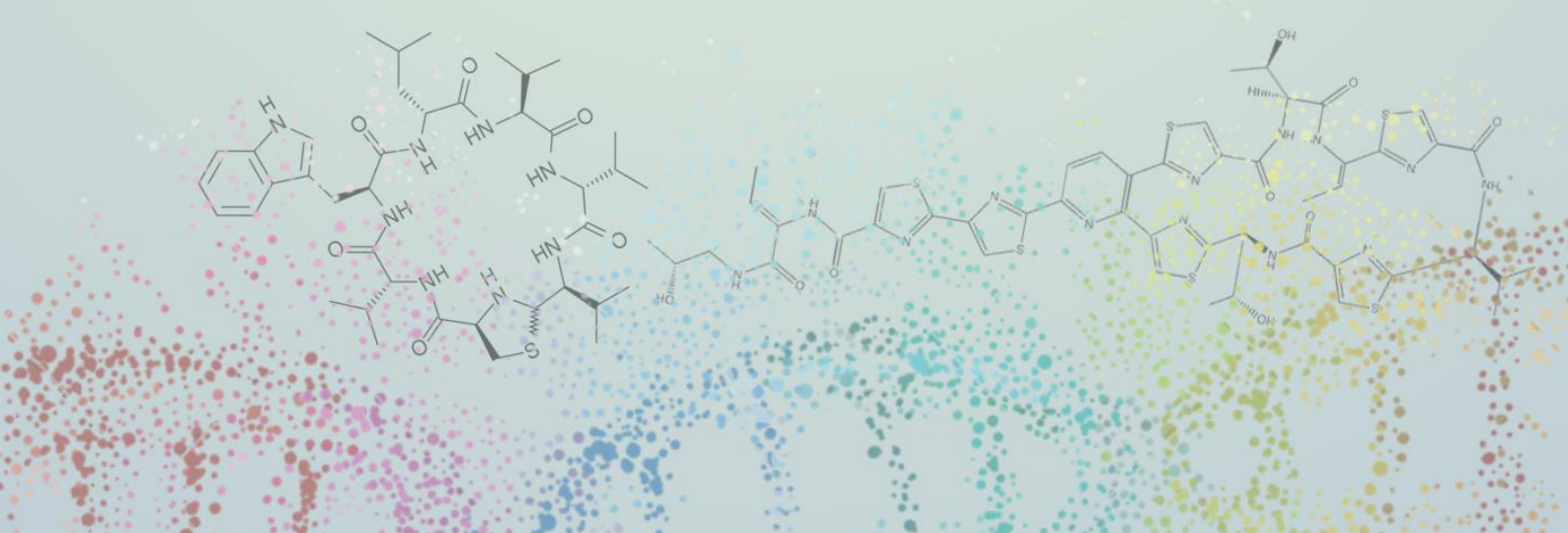


Figura 1 Resumen gráfico de la estructura seguida en esta tesis doctoral elaborada por compendio de publicaciones donde se presentan los trabajos derivados que conforman los 5 capítulos (elaboración propia).

Índice

INTRODUCCIÓN



Introducción

1 Problemática de la resistencia a antibióticos (RAM)

Desde el descubrimiento de la penicilina en 1928 por Alexander Fleming surgieron nuevos antibióticos que se fueron incluyendo en el arsenal terapeútico lo que permitió grandes avances dando lugar a la actual medicina moderna **(Sengupta et al. 2013; Piddock, 2012).** Gracias a ello, se han salvado millones de vidas facilitando intervenciones de alto riesgo (operaciones quirúrgicas y trasplantes) o tratando infecciones tanto severas como cotidianas (infección urinaria o de piel) **(Gould y Bal, 2013; Ventola, 2015).** Desde finales de la década de 1960 hasta principios de la década de 1980 se descubrieron multitud de nuevos antibióticos, aunque la aparición de bacterias resistentes a los mismos no tardó en ser un problema.

La resistencia a los antibióticos (RAM) es la respuesta evolutiva natural de las bacterias a la fuerte presión selectiva que ejercen estos fármacos. En este contexto, las bacterias han evolucionado gradualmente siendo capaces de evadir la acción de los antibióticos bien por el desarrollo de mutaciones en genes importantes para su acción **(McEwen y Collignon, 2018**), o por la adquisición de genes de resistencia a antibióticos (ARG); dichos genes de resistencia pueden evolucionar desde su presencia en el cromosoma de determinadas bacterias hasta su movilización y transferencia a otras bacterias, incluido patógenos **(Larsson y Flach, 2022).**

En el proceso evolutivo escalonado de los ARG, el primer paso suele consistir en la capacidad de un ARG para desplazarse por el genoma mediante inserciones **(Razavi et al. 2020; Partrige et al. 2018)**, casetes genéticos o integrones **(Gillings et al. 2008; Razavi et al. 2017).** El segundo paso implica la reubicación del ARG a un elemento de transferencia autónoma como un plásmido o elementos conjugativos. El tercer paso es la transferencia horizontal de un ARG a un patógeno y finalmente, la cuarta etapa, que consiste en la transferencia física de la bacteria portadora del ARG a la microbiota humana o de animales domésticos **(Figura 2)**. Esta fase puede transcurrir en cualquier momento y se conoce como conectividad ecológica **(Baquero et al. 2019)**. Todos estos pasos, aunque ocurren de forma natural pueden verse favorecidos por el uso de antibióticos **(Larsson y Flach, 2022)** de manera que nos podemos encontrar con reservorios de resistencia, desde el entorno hospitalario y la comunidad, hasta los animales de producción, la fauna silvestre y muchos otros nichos ecológicos, incluido el medio ambiente **(Marti et al. 2014; Huijbers et al. 2015).**

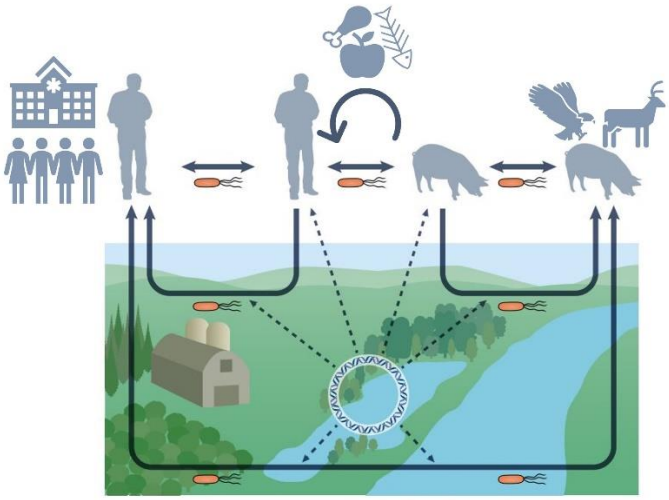


Figura 2 Ejemplo gráfico representativo de las vías de transmisión de patógenos bacterianos y reclutamiento de genes de resistencia **(adaptada de Larsson y Flach, 2022).**

El abuso y el mal uso de los antibióticos sumado a factores que favorecen la propagación de bacterias resistentes como son: la transmisión de enfermedades, el diagnóstico lento y erróneo de la enfermedad, la escasez de vacunas o la contaminación ambiental han hecho que la problemática de la resistencia a antibióticos sea considerada de alta prioridad por las organizaciones sanitarias **(Wright, 2010).** Tanto es así que la resistencia a los antibióticos se ha convertido en un problema sanitario global de primera línea de acción **(Govindaraj Vaithinathan y Vanitha, 2018; Gil-Gil et al. 2019)**.

En este sentido, es importante abordar la problemática de la resistencia a los antibióticos desde la perspectiva "One Health", un mundo una salud, incluyendo el ámbito humano y animal, así como también el medioambiente **(Figura 3, Hernando-Amado et al. 2019).** Asimismo, para garantizar la "salud global" y comprender la propagación de la RAM es importante delimitar las vías de transmisión **(Gil-Gil et al., 2019)**, entre las que destaca el incremento de los viajes a nivel mundial o los movimientos migratorios de la fauna silvestre, entre otros **(Hernando-Amado et al. 2019).**

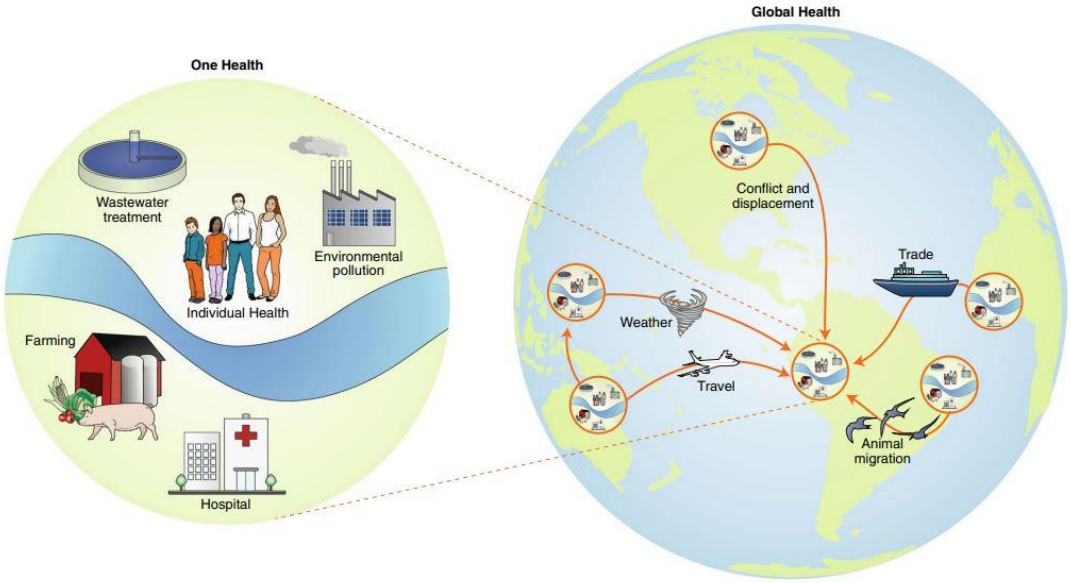


Figura 3 Ámbitos implicados en el concepto "Una sola salud" y "Salud mundial", propuesto como estrategia para el estudio de la resistencia a los antibióticos **(Hernando-Amado et al. 2019).**

Las redes de vigilancia epidemiológica en Europa y Asia [European Antimicrobial Resistance Surveillance Network **(-EARS-Net);** Central Asia and Eastern European Surveillance of Antimicrobial Resistance **(CAESAR);** European Centre for Disease Control and Prevention, **(ECDCP)**] han documentado que las bacterias resistentes a los antibióticos se han vuelto mucho más frecuentes durante la última década **(Antoñanzas et al. 2015; Murray et al. 2022)**. En este sentido, las estimaciones de muertes atribuidas a la resistencia a los antibióticos han sido superadas pasando de predecir unos 10 millones de muertes anuales para 2050 **(O'Neill 2014)** a los últimos estudios llevados a cabo en 2019 que alertan de que el número de muertes asociadas a bacterias resistentes a antibióticos puede ascender a 4,5 millones a nivel global **(Murray et al. 2022).** Entre estas estimaciones, se ha demostrado con un 95% de confianza que en 2019 el número de muertes directamente atribuidas a la RAM ascendió a 1,27 millones **(Murray et al. 2022).** Por otro lado, el número de muertes bien atribuidas o asociadas a la resistencia no se encuentran homogéneamente distribuidas en todas las regiones del mundo, sino que se acumulan en determinadas zonas geograficas. A nivel regional, se estima que la tasa de muerte atribuida a la resistencia a los antibióticos alcanza los valores máximos en África subsahariana y mínimos en Australasia **(Murray et al. 2022).** En la Unión Europea, aproximadamente 111.000 muertes al año se deben, directa o indirectamente, a infecciones hospitalarias, y muchas de estas infecciones están causadas por bacterias resistentes a los antimicrobianos (alrededor de 25.000 muertes en Europa), incluido las causadas por *Staphylococcus aureus* resistente a la meticilina (SARM) **(Antoñanzas et al. 2015).** Solo SARM ha ocasionado más de 100.000 muertes mundiales en 2019 directamente atribuidas a este microorganismo **(Figura 4).**

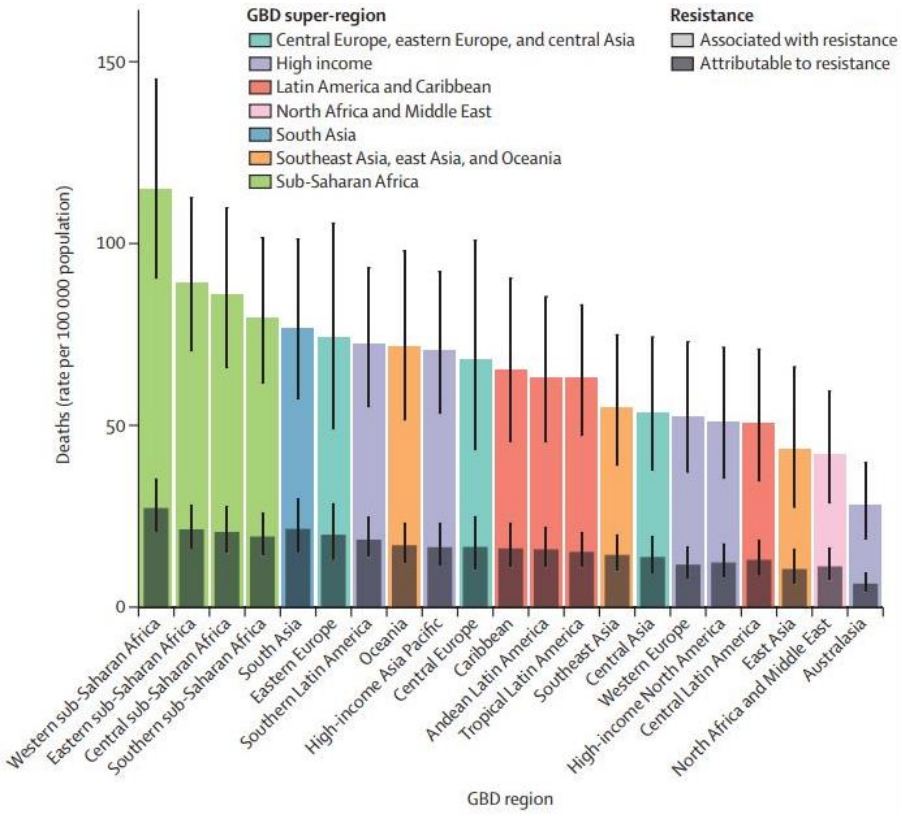


Figura 4 Estimación de la tasa de muertes por cada 100000 habitantes en cada región en función del estudio epidemiológico mundial GBD, Global Burden of Disease **(Murray et al. 2022).**

A nivel global, existen seis patógenos que preocupan enormemente y están asociados con el fenómeno de la resistencia a antibióticos y estos son *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii,* y *Pseudomonas aeruginosa*, los cuales son responsables de 929.000 de los 1,27 millones de muertes atribuidas a la RAM y de 3,57 de los 4,95 millones de las muertes asociadas a la resistencia reportada a nivel global en 2019 **(Murray et al. 2022)**. Algunos de estos organismos se incluyen en el conjunto de bacterias denominadas ESKAPE: *Enterococcus spp., Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* y *Escherichia coli* **(Zhen et al. 2019)**, y han sido incluidos por la Organización Mundial de la Salud (OMS) en una lista prioritaria mundial de los patógenos mayoritariamente implicados en enfermedades infecciosas en humanos con el objetivo de orientar la investigación, y el desarrollo de nuevos antibióticos **(WHO, 2019).**

Las bacterias ESKAPE con frecuenca presentan mecanismos de resistencia de gran relevancia clínica y constituyen una amenaza crítica para la salud pública **(Pendleton et al. 2013),** afectando especialmente a personas inmunodeprimidas y en estado crítico. Por ello, es importante centrar la atención en estos patógenos para tratar de reducir las probabilidades de recibir un tratamiento antibiótico inadecuado, lo que se traduce en mayores tasas de mortalidad y en la posibilidad de propagación a otros pacientes **(Pogue et al. 2015; Bodro et al. 2013)**.

Además de las indudables consecuencias sanitarias, las infecciones debidas a bacterias ESKAPE provocan hospitalizaciones de mayor duración y suponen una carga económica significativa para los sistemas sanitarios nacionales **(Christaki et al. 2020).** Concretamente, SARM implica costes significativos en su tratamiento comparado con cepas sensibles a meticilina (SASM). Si comparamos a los pacientes con SARM y con SASM, la duración de la estancia incrementa de 2 a 10 días, y los costes directos son entre 1,5 y 3 veces mayores (del orden de 1.200-50.000€) **(Antoñanzas et al. 2015).**

En cuanto a los costes derivados de la problemática de la RAM a nivel global, los países de la Unión Europea (UE) y del Espacio Económico Europeo (EEE), se estima que los costes sanitarios adicionales y la pérdida de productividad superarían en Europa los 1.100 millones de euros anuales entre 2015 y 2050 si no se encuentran alternativas eficaces **(OECD, 2019).** A escala mundial, se estiman pérdidas de aquí a 2050 de entre el 1,1 y el 3,8% del producto interior bruto anual **(Jonas et al. 2017).** En definitiva, un total de 24 millones de personas se verán obligadas a caer en la pobreza extrema debido a la RAM en 2030, especialmente en los países de renta baja **(Jonas et al. 2017).**

2 Necesidad de nuevas alternativas frente a la RAM

Las infecciones bacterianas se han convertido de nuevo en una gran amenaza por lo que es necesario encontrar alternativas eficaces y abordar esta problemática de salud mundial desde un enfoque global coordinado y multisectorial. En los últimos años se han desarrollado pocos agentes antimicrobianos nuevos, insuficientes para hacer frente al problema de la RAM. Todo ello, nos lleva a plantear la búsqueda de nuevas alternativas de forma urgente.

Por un lado, se han propuesto numerosas recomendaciones generales para optimizar el uso de antimicrobianos, preservar su eficacia y retrasar la emergencia de bacterias resistentes. Entre ellas, se aboga por la mejora de las prácticas de diagnóstico y prescripción, la reducción del uso de antimicrobianos en el ámbito humano, la ganadería, la cría de animales, la agricultura y la exposición medioambiental en general, el desarrollo de nuevos antimicrobianos, la garantía de acceso a medicamentos esenciales de calidad garantizada y la mejora de la vigilancia. Asimismo, se han enunciado alternativas de uso específico frente a enfermedades infecciosas como son: i) mantener la heterogeneidad de los agentes antimicrobianos; ii) estudiar el efecto de antimicrobianos previamente aprobados, pero no utilizados; iii) garantizar el uso de concentraciones séricas del fármaco adecuadas y, iv) emplear terapias combinadas **(Holmes et al. 2016).**

En la **Tabla 1** se presenta un resumen de nuevas estrategias terapéuticas en el tratamento de infecciones, incluyendo alternativas naturales, sintéticas y con base biotecnológica **(Ghosh et al. 2019)**. Entre ellas nos encontramos con sustancias antimicrobianas de origen natural como las bacteriocinas, péptidos principalmente de síntesis ribosomal, con actividad antimicrobiana producidos por bacterias como respuesta ante múltiples situaciones de estrés, como la competencia por la supervivencia. Existen ciertas características diferenciales entre los antibióticos convencionales y las bacteriocinas que resultan beneficiosas. Ambos tienen en común la capacidad de inhibir el desarrollo de determinados microorganismos, pero difieren en su composición, síntesis, espectro de acción, modo de acción, mecanismos de inmunidad, resistencia y/o toxicidad. En este sentido, las bacterias lácticas han sido las más estudiadas como productoras de bacteriocinas y existe una extensa bibliografía en este campo. *Staphylococcus* es también un género conocido como productor de bacteriocinas que presenta una serie de características que hacen de él un grupo microbiano adecuado para ser explorado en prometedoras aplicaciones en muchos campos, especialmente contra patógenos Gram-positivos (G+) resistentes a antibióticos. No obstante, el estudio de bacteriocinas producidas por estafilococos es aún limitado, constituyendo un prometedor campo de estudio. Además, las técnicas biotecnológicas o de síntesis química de bacteriocinas pueden ser herramientas importantes para mejorar la actividad antimicrobiana de las bacteriocinas, modificar sus propiedades fisicoquímicas o reducir sus costes de producción.

Tabla 1 Nuevas estrategias frente a la RAM **(adaptada de Ghosh et al. 2019).**

Estrategia	Ventajas	Posibles desventajas
Fagos	Productos farmacéuticos autorreplicantes Selectivos para cepas específicas de bacterias Adaptables a la ingeniería genética	Inmunogenicidad Farmacocinética Liberación de endotoxinas bacterianas Preparados inadecuados - no se eliminan las endotoxinas y sustancias pirógenas Desarrollo de resistencias
Lisinas	Adaptables a la ingeniería genética Selectiva para cepas específicas de bacterias No es propenso a desarrollar resistencias	Producción Falta de conocimientos suficientes
Crispr/cas9	Puede ajustarse para una variedad de aplicaciones antimicrobianas Reducción del uso de antibióticos Especificidad frente a cepas patógenas	Producción costosa a gran escala Toxicidad
Péptidos antimicrobianos*	Desarrollo de resistencias poco frecuente Actividad de amplio/estrecho espectro en función del péptido Especificidad frente a cepas patógenas Resistencia al calor y a los rayos UV Facilidad de síntesis	Producción costosa a gran escala Sensible a la proteólisis Toxicidad Vía de administración

*Se incluyen en esta clasificación las bacteriocinas y los péptidos sintéticos.

Tabla 1 Continuación.

Estrategia	Ventajas	Posibles desventajas
Péptidos regulatorios de la defensa innata	Actúan modulando el sistema inmunitario No se desarrollan resistencias al no tener actividad antimicrobiana directa	Producción costosa a gran escala Sensible a la proteólisis
Probióticos	Fácil disponibilidad	Infecciones intestinales y vaginales
Anticuerpos	Selectivo hacia cepas específicas de bacterias No dañan la microbiota	Alto coste de producción Periodo de conservación reducido

A continuación, se abordarán las estrategias basadas en el uso de péptidos antimicrobianos, atendiendo especialmente a las bacteriocinas, su combinación con otros agentes y al uso de cepas productoras de dichos compuestos como probióticos o cultivos protectores, siendo de especial relevancia el género *Staphylococcus*.

2.1 Péptidos antimicrobianos y bacteriocinas

Los péptidos antimicrobianos (PAM), con independencia de su origen, poseen principalmente un dominio cargado positivamente que participa en interacciones electrostáticas con la superficie celular bacteriana cargada negativamente, y un dominio hidrofóbico que interactúa con los lípidos de la membrana bacteriana. El resultado es la desintegración de la membrana celular y, finalmente, la muerte bacteriana **(Mahlapuu et al. 2016; Bechinger, 2015).** Sin embargo, la desintegración de la membrana celular bacteriana es tan desfavorable en términos energéticos para las bacterias que estas no son capaces de desarrollar resistencia fácilmente **(Andersson et al. 2016; Guilhelmelli et al. 2013).** Varios PAMs están siendo sometidos a ensayos clínicos, aunque la toxicidad *in vivo* y la difícil escalabilidad industrial han obstaculizado la traslación clínica de esta clase de agentes antimicrobianos alternativos **(Greber y Dawgul, 2016).**

Por otro lado, se encuentran las bacteriocinas, también péptidos antimicrobianos, pero en este caso sintetizados por bacterias por síntesis ribosomal para evitar la competencia y aumentar la supervivencia. Las bacteriocinas pueden ofrecer más ventajas que otros PAM ya que pueden ser selectivas frente a determinadas cepas bacterianas. Por ejemplo, la bacteriocina turicina no afecta a las bacterias comensales, pero mata a *Clostridium difficile* **(Rea et al. 2011).** Además, la resistencia frente a las bacteriocinas es un proceso lento e infrecuentemente observado. Entre otras ventajas destacadas de las bacteriocinas se encuentra su resistencia a las duras condiciones de calor y exposición a los rayos UV, lo que les aporta un gran potencial como alternativas de tratamiento de infecciones y en la industria alimentaria **(Cotter et al. 2013).**

2.2 Terapias combinadas

En general, las bacteriocinas muestran una fuerte actividad contra las cepas diana, a menudo en el rango nanomolar, lo que las hace más potentes que muchos antibióticos **(Mathur et al. 2013;**

Ming et al. 2015). Además, se puede plantear el uso conjunto de bacteriocinas y antibióticos con el fin de obtener una acción sinérgica que pueda acelerar los efectos letales de cada compuesto, reduciendo así la probabilidad de desarrollo de resistencias. La combinación de bacteriocinas y antibióticos puede reducir la concentración requerida de antibiótico, disminuyendo así la probabilidad de sus efectos secundarios adversos **(Mendes et al. 2009; Abdelraouf et al. 2012).** Por último, la sinergia entre bacteriocinas y antibióticos también pueden reducir los costes y ampliar el espectro de actividad, lo que puede ser útil en el tratamiento de infecciones clínicas de etiología desconocida.

Varios estudios han demostrado que el uso de mezclas de bacteriocinas de diferentes clases, especialmente con distintos mecanismos de acción, aumenta su actividad antimicrobiana y amplía su espectro de actividad **(Vignolo et al. 2000; Turgis et al. 2016)**, al tiempo que reduce la resistencia de las cepas bacterianas a estas sustancias **(de Freire Bastos et al. 2015).**

2.3 Probióticos y cultivos protectores

La Organización mundial de la Salud (OMS, WHO en inglés) y la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO, food and agriculture organization) describen los probióticos como "microorganismos vivos que, cuando se administran en cantidades adecuadas, confieren un beneficio para la salud del huésped" **(FAO/WHO 2001; Hill et al. 2014).**

Los probióticos tienen como función principal proteger contra los microorganismos patógenos mediante múltiples mecanismos y deben cumplir varios criterios básicos **(Goldin, 1998):** ser preferiblemente de origen humano, ser capaces de colonizar temporalmente y sobrevivir en el nicho de interés y adherirse a la mucosa epitelial. Además, los probióticos no pueden ser patógenos ni mostrar toxicidad **(Hatakka y Saxelin, 2008).**

Dado que los probióticos se ingieren principalmente por vía oral, el tracto gastrointestinal es el principal órgano diana de los microorganismos probióticos. Sin embargo, sus efectos en la prevención de infecciones no residen exclusivamente en el intestino. Los ámbitos de investigación más prometedores, además de los efectos gastrointestinales, son las infecciones orales, respiratorias y urogenitales **(Hatakka y Saxelin, 2008; Marcotte et al. 2019; Pendharkar et al. 2015).**

Asimismo, se ha acuñado el término “cultivo protector” para referirse a bacterias que han sido específicamente seleccionadas por su capacidad para inhibir el crecimiento de organismos patógenos o agentes microbiológicos de deterioro, aunque no se haya verificado su efecto probiótico **(Young y O’Sullivan, 2011).** La **Figura 5** representa a modo de ejemplo la actuación protectora de estas bacterias que han de mantener el estatus **GRAS** (generalmente consideradas seguras, inglés Generally Recognized as Safe) **o QPS** (presunción cualificada de seguridad, en

inglés Qualified Presumption of Safe) de manera que no presenten riesgos a la salud humana, animal ni al medio ambiente tras su inclusión en la cadena alimentaria. Los microorganismos pueden ser considerados como QPS en función de su clasificación taxonómica, del grado de exposición humana/animal, su proximidad filogenética a patógenos conocidos y la seguridad sobre el medio ambiente **(EFSA, 2023)**. En cuanto a la seguridad de las cepas consideradas GRAS o QPS, se debe asegurar que no tengan repercusiones negativas en la salud (patógenos oportunistas o intoxicación alimentaria, entre otros) y que no porten factores de virulencia ni mecanismos de resistencia adquiridos.

En este sentido, la Agencia Europea de Seguridad Alimentaria (EFSA, European Food Safety Authority) ha reconocido el uso de 692 bacterias como aditivos alimentarios con diferentes funciones. Por otro lado, hasta la fecha, la EFSA sólo permite el uso de bacterias lácticas (BAL) como probióticos destacando los géneros *Lactobacillus* y *Bifidobacterium.*

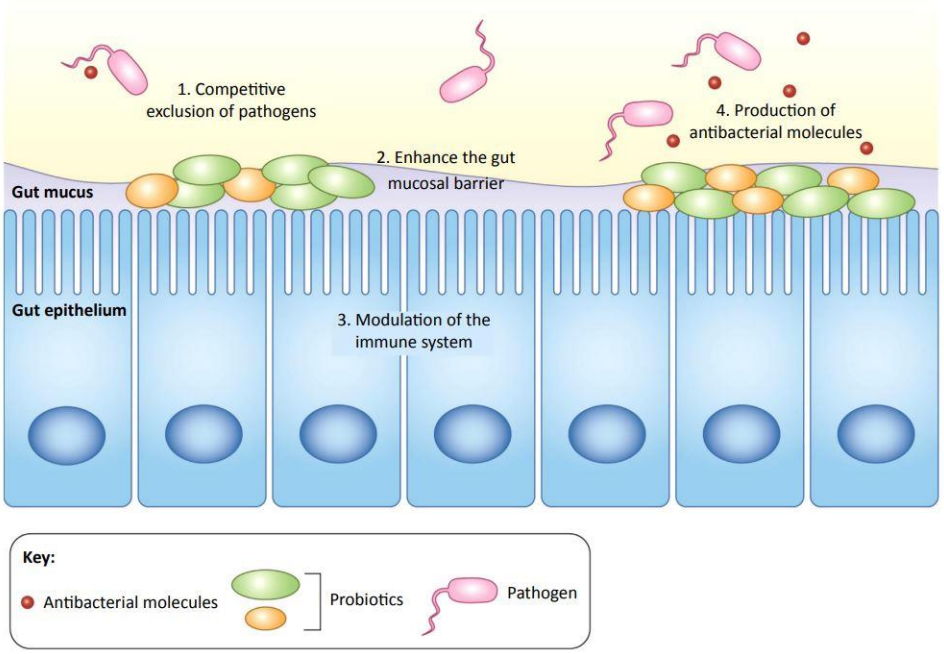


Figura 5 Ejemplo gráfico del mecanismo de protección de los microorganismos frente infecciones **(Ghosh et al. 2019).**

Por último, cabe destacar una novedosa aplicación de microorganismos, en este caso con la finalidad de reconstruir la microbiota en pacientes con graves infecciones bacterianas u otros casos de disbiosis gastrointestinal **(Aroniadis y Brandt, 2014).** El tratamiento denominado transplante fecal consiste en la introducción del microbioma de un donante sano en el intestino enfermo, de especial importancia en el caso de la enterococlitis por *C. difficile*. Asimismo, esta alternativa terapéutica es muy prometedora para eliminar la colonización por *Enterobacteriaceae* multirresistentes a los antibióticos como *E. coli, Salmonella, Klebsiella pneumoniae, Acinetobacter baumannii,* SARM y enterococos resistentes a la vancomicina **(Ghosh et al. 2019).**

Sin embargo, la eficacia del trasplante fecal frente a este tipo de patógenos en humanos es limitada y actualmente sólo se ha aprobado para tratar colonización e infección por *C. difficile*.

3 Bacteriocinas

Se ha descubierto gran diversidad de bacteriocinas que pueden diferenciarse en función de su mecanismo de síntesis: i) péptidos de síntesis ribosomal sin modificaciones postraduccionales, ii) péptidos de síntesis ribosomal y modificados postraduccionalmente (RiPP) y iii) péptidos de síntesis no ribosomal, producidos por sintetasas peptídicas (NRP) **(Polikanov et al. 2018).** La mayoría de las bacteriocinas son RiPP y en general son péptidos termo-tolerantes y de espectro de acción reducido, que pueden actuar sobre las células diana mediante diversos mecanismos **(James et al. 1996).** A continuación, se describen algunas de sus características principales:

3.1 Operón codificante de bacteriocinas (OCB)

A nivel genético, las bacteriocinas se codifican a partir de grupos de genes organizados en operones que residen en el cromosoma, plásmidos u otros elementos genéticos móviles (EGMs). Estos operones codificantes de bacteriocionas están constituidos por un conjunto de genes estructurales, reguladores, de transporte o secreción y de autoinmunidad **(Mak, 2018).** La mayoría de las bacteriocinas requieren de modificaciones post-traduccionales para ser activas, por lo que su síntesis está ligada a la expresión de enzimas de modificación. Además, estos operones son generalmente inducibles y requieren acumulación extracelular de péptidos **(Noda et al. 2018).**

3.2 Mecanismo de acción

La acción antimicrobiana de las bacteriocinas producidas por bacterias G+ suele asociarse a una alteración de la integridad de la membrana bacteriana **(Cavera et al. 2015)** que en definitiva conduce a la muerte celular. Este proceso puede deberse a gran diversidad de mecanismos **(Figura 6).** En la **Figura 6a** se representa la formación de poros en la membrana celular **(Cotter et al. 2013; Bali et al. 2016).** Relacionado con este mecanismo, se han descrito otros asociados al sistema transportador manosa fosfotransferasa (Man-PTS) y posterior inserción en la membrana citoplasmática, provocando la permeabilización y despolarización de la membrana por la fuga de iones y material intracelular **(Álvarez-Sieiro et al. 2016)**. **En la Figura 6b** se muestra la inhibición enzimática de la biosíntesis de peptidoglicano por unión de la bacteriocina al lípido II **(Castiglione et al. 2008)**. Se han descrito también mecanismos que provocan la muerte celular mediante la hidrólisis de la pared celular como se muestra en la **Figura 6c** (un ejemplo es la lisostafina) **(Bali et al. 2016)**. Por último, algunas bacteriocinas actúan a nivel ribosomal interviniendo en el proceso de síntesis proteica como por ejemplo la bacteriocina micrococcin P1 (tiopéptido) **(Ciufolini y Lefranc, 2010).** En definitiva, las bacteriocinas que actúan sobre la

membrana de una forma u otra son bactericidas (con mayor grado de actividad lítica) aunque se han descrito numerosas bacteriocinas con otros mecanismos de acción y con efecto bacteriostático como es el caso de la micrococcina P1.

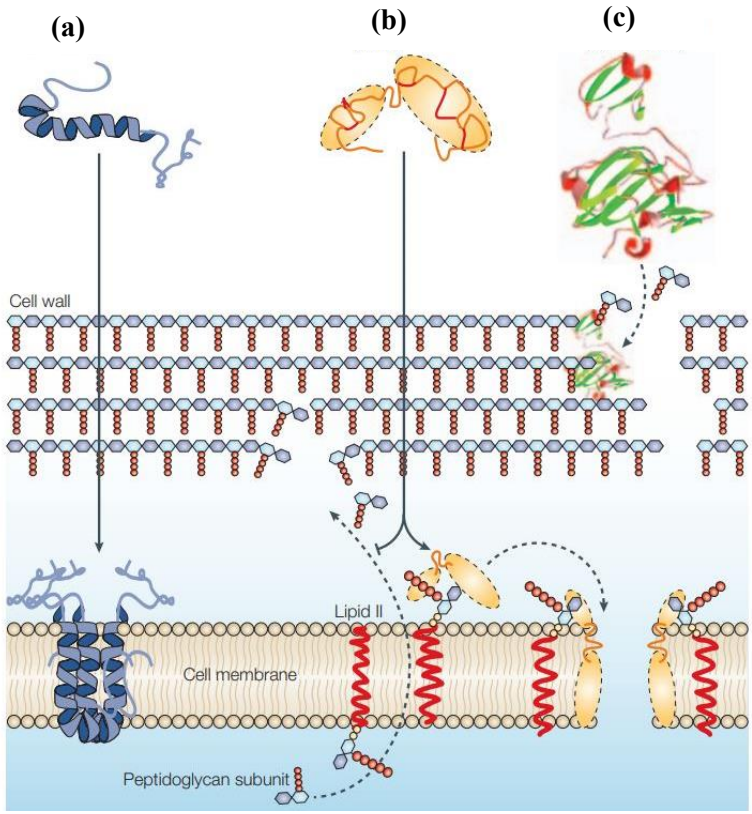


Figura 6 Ejemplo gráfico de los mecanismos de acción de las bacteriocinas más comunes: (a) formación de poros en la membrana celular, (b) inhibición de la síntesis del peptidoglicano; (c) hidrólisis de la pared **(Cotter et al. 2005).**

3.3 Autoinmunidad e inmunidad cruzada

La autoinmunidad se describe como el mecanismo de autoprotección que posee una bacteria para evitar la muerte celular debida a la acción de la propia bacteriocina específica que sintetiza **(Draper et al. 2008).** Las cepas productoras de bacteriocinas contienen genes codificantes de proteínas de autoinmunidad capaces de proteger al organismo productor de la actividad de sus propios productos **(Bierbaum y Sahl, 2009; Heng et al. 2007; Nissen-Meyer et al. 2009; van Belkum et al. 2011).** En muchos casos, los sistemas de inmunidad consisten en una única proteína pequeña, localizada generalmente en la membrana celular bien en su superficie **(Dubois et al. 2009; Heidrich et al. 1998)** o incrustadas **(Bierbaum y Sahl, 2009; Coelho et al. 2014).** Asimismo, se ha descrito frecuentemente la inmunidad de algunas bacterias a la acción de bacteriocinas producidas por otros microorganismos, fenómeno conocido como inmunidad cruzada derivado de la presencia de elementos genéticos implicados en la defensa celular **(Teso-Pérez et al. 2021).**

3.4 Resistencia

La resistencia es el fenómeno por el cual una bacteria deja de ser inhibida por la acción de un agente antimicrobiano, bien antibiótico o bacteriocina. Se distinguen dos mecanismos principales implicados en la resistencia **(Collins et al. 2012)**: i) resistencia adquirida (la desarrollada por una cepa anteriormente sensible) y ii) resistencia innata (la que se encuentra intrínsecamente en determinados géneros o especies con variaciones entre las distintas cepas bacterianas) **(Katla et al. 2003)**. Las alteraciones celulares responsables de la resistencia tanto innata como adquirida, se deben a determinantes genéticos que en muchos casos son desconocidos y despiertan gran interés a la comunidad científica para la optimización del uso de bacteriocinas **(Tessema et al., 2011).**

La resistencia adquirida a bacteriocinas es multifactorial y puede ser debida a mutaciones espontáneas de genes implicados en la expresión de receptores específicos de la envoltura celular bacteriana, en la síntesis de la pared celular, en la regulación transcripcional o en el metabolismo, o en el transporte de energía que en definitiva afecta a una reducción de la permeabilidad de la membrana **(Gravesen et al. 2002; Katla et al. 2003; Cotter et al. 2013; de Freire Bastos et al. 2015).** Por otro lado, las mutaciones espontáneas son muy poco frecuentes y resultan de la exposición a altas concentraciones de bacteriocinas y/o durante periodos prolongados **(Pessione 2014).** Asimismo, se sabe que la escisión proteolítica de las bacteriocinas por proteasas, peptidasas extracelulares o gelatinasa **(Nes et al. 2015; Sedgley et al. 2009)**, es otro mecanismo a través del cual podría producirse la resistencia a las bacteriocinas.

Finalmente, cabe mencionar el fenómeno de resistencia cruzada, definido como la resistencia de una cepa bacteriana a bacteriocinas diferentes **(Teso-Pérez et al. 2021).** Este fenómeno es habitual entre bacteriocinas altamente relacionadas si bien se ha descrito en cepas productoras de bacteriocinas de diferentes clases **(de Freire Bastos et al. 2015).** En este sentido, se ha descrito como mimetismo inmunitario, el proceso por el cual las bacterias expresan homólogos funcionales de los sistemas inmunitarios de las bacteriocinas **(Draper et al. 2012).**

3.5 Elementos de adquisición y diseminación de operones codificantes de bacteriocinas

Debido a las posibles aplicaciones biotecnológicas de las bacteriocinas, es importante conocer las bases genéticas de estas sustancias. La producción de bacteriocinas es ubicua y su diseminación se ha visto probablemente facilitada por la localización de los determinantes genéticos responsables de la producción de estas en EGMs, como plásmidos conjugativos o transposones **(Jack et al. 1995).**

Centrándonos en los mecanismos descritos en microorganismos G+, las bacterias lácticas han sido las más estudiadas, concretamente el lantibiótico nisina descrito por primera vez en una cepa de *Lactococcus lactis* **(Rogers y Whittier, 1928).** Los estudios sobre el entorno genético de la nisina revelaron que el gen estructural precursor de la nisina *nisA* **(Buchman et al. 1988; Dodd et al. 1990; Kaletta y Entian, 1989)** se vehiculiza en un transposón conjugativo de 70 kb, designado como Tn*5301* y Tn*5276* **(Dodd et al. 1991; Rauch et al. 1991).** Además, se ha visto que genes estructurales que codifican bacteriocinas no-lantibióticos también están localizados en plásmidos **(Jack et al. 1995).**

Por otro lado, estudios comparativos de los perfiles plasmídicos de cepas productoras y no productoras de bacteriocinas del género *Staphylococcus* han demostrado la localización plasmídica de varias de las bacteriocinas descritas.

Por ejemplo, la cepa productora de bacteriocina *Staphylococcus epidermidis* 5, alberga cinco plásmidos, y uno de ellos (plásmido pED503 de 18,6 Kb) porta los determinantes genéticos necesarios para la producción del lantibiótico Pep5. Del mismo modo, se ha confirmado que la bacteriocina epidermina está codificada en un operón inserto en el plásmido de 54 Kb (pTü32) identificado en la cepa productora *Staphylococcus epidermidis* Tü3298 **(Augustin et al. 1992; Schnell et al. 1991; Schnell et al. 1992; Schnell et al. 1988).** Recientemente se han caracterizado dos plásmidos presentes en *S. epidermidis* portadores de las bacteriocinas epidermina (pEpi56) y nukacina (pNuk650) cuyas secuencias completas han sido depositadas en la base de datos NCBI (National Center for Biotechnology Information) con los números de acceso OK031036 y OK031035, respectivamente **(Nakazono et al. 2022).** La **Figura 7** muestra el plásmido pEpi56 portador de los genes codificantes de epidermina.

Además, se ha descrito una elevada frecuencia de plásmidos portadores de genes implicados en la producción e inmunidad de bacteriocinas (pequeños 8-10,4 kb y grandes 40 kb) en aislados de *S. aureus* con actividad antimicrobiana **(Giambiagi-de Marval et al. 1990)**.

Finalmente, presenta gran interés la detección en *S. aureus* del plásmido pRJ6, primer plásmido conjugativo portador de bacteriocinas **(Coelho et al. 2009)** y se sugirió que dicho plásmido pRJ6 fue transferido desde SCoN a *S. aureus*, dato que se confirma con la detección de plásmidos portadores de bacteriocinas, idénticos a los descritos previamente en *S. aureus*, en especies de SCoN como *S. arlettae* y *S. saprophyticus* **(Oliveira et al. 1998; Gamon et al. 1999; Nascimiento et al. 2006).**

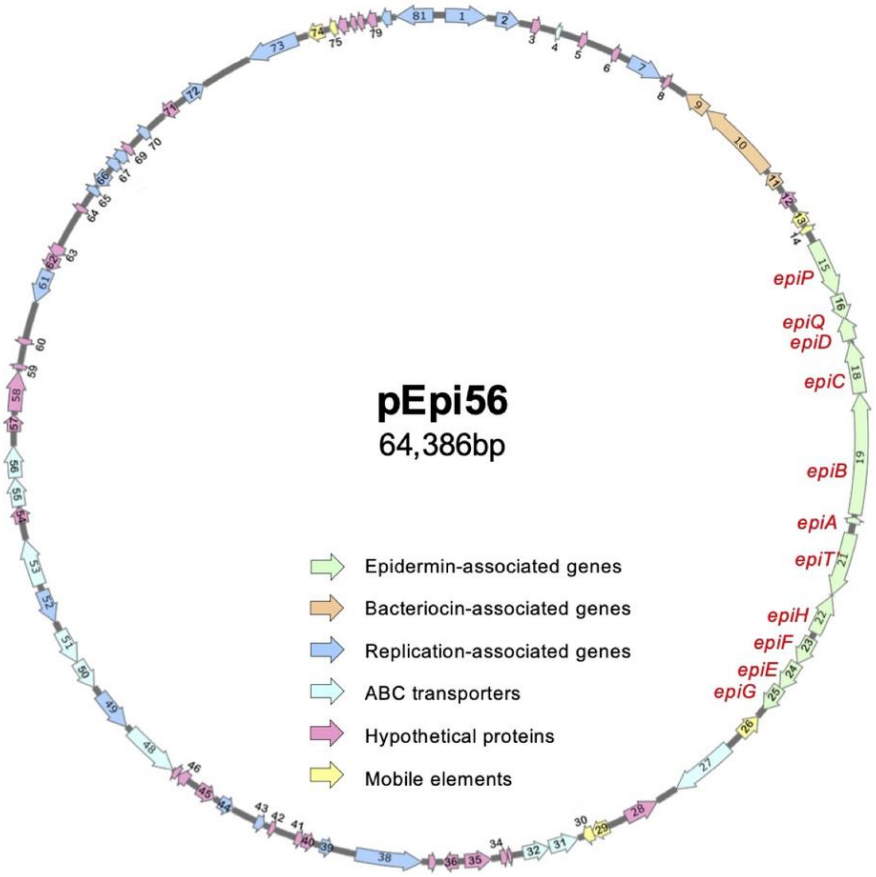


Figura 7 Secuencia completa del plásmido pEpi56 portador de los genes codificantes de epidermina **(adaptada de Nakazono et al. 2022).**

4 Clasificación de las bacteriocinas

Se distinguen cuatro clases principales de bacteriocinas dependiendo de las características estructurales y físico-químicas **(Figura 8) (Newstead et al. 2020; de Freire Bastos et al. 2020; Torres-Salazar el al. 2021; Heilbronner et al. 2021)**: Clase I) péptidos pequeños termoestables y modificados post-traduccionalmente, conocidos como lantibióticos (<5 kDa, 19-37 aminoácidos) **(Bierbaum y Sahl, 2009)**; Clase II) péptidos pequeños sin modificación post-traduccional y termoestables (<10 kDa) **(Nissen-Meyer et al. 2009)**; Clase III) péptidos grandes (>30 kDa) y termolábiles, subdivididos en bacteriocinas líticas y no líticas **(Heng et al. 2007)**; Clase IV) péptidos cíclicos formados por un enlace covalente postraduccional que normalmente conforman estructuras circulares **(van Belkum et al. 2011).**

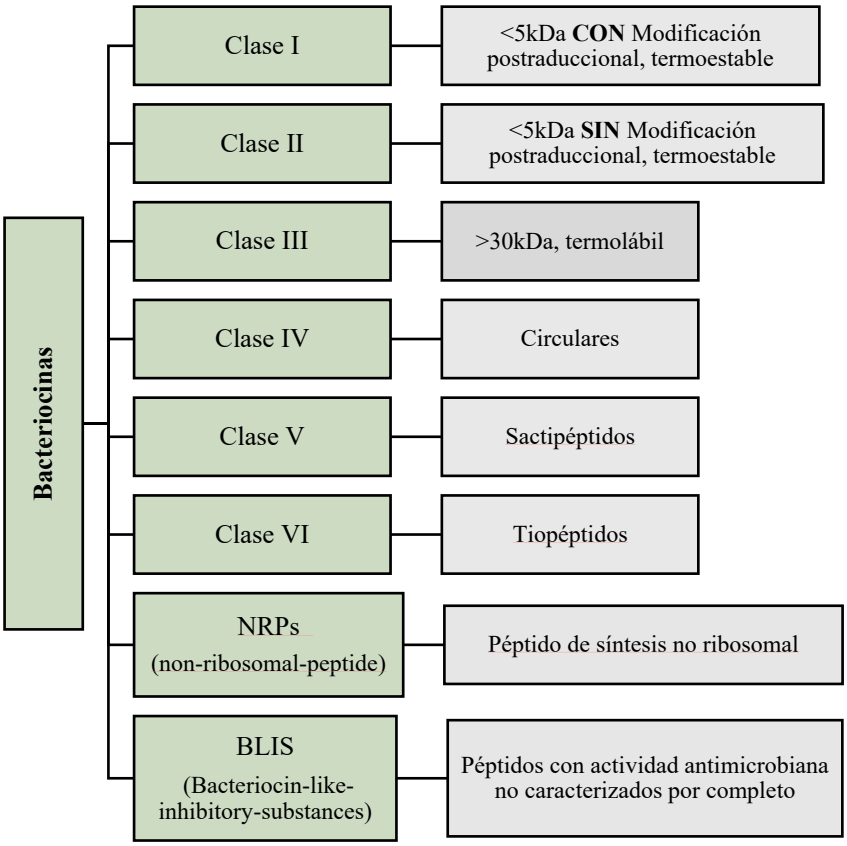


Figura 8 Clasificación general de las bacteriocinas Gram positivas en función de sus características generales, atendiendo especialmente a aquellas producidas por estafilococos (elaboración propia).

Revisiones recientes han propuesto la inclusión de dos nuevas clases de bacteriocinas para comprender mejor las características de cada grupo de péptidos antimicrobianos **(Coelho et al. 2017; Zheng et al. 2017; de Freire Bastos et al. 2020).** Así, se consideran la Clase V (sactibióticos) y la Clase VI (tiopéptidos, como la Micrococcina P1).

Además, los péptidos antimicrobianos con síntesis no ribosomal (NRPs, non-ribosomal-peptides) como la lugdunina **(Zipperer et al. 2016)** y péptidos con actividad antimicrobiana no

caracterizados por completo (BLIS, bacteriocin-like-inhibitory-substances) **(James y Tagg, 1991; Mak, 2018)**, son considerados de forma independiente en el conjunto de bacteriocinas conocidas. A pesar de las características poco claras de BLIS, y la dificultad de producción de NRPs, estos compuestos despiertan interés por su potencial en estudios aplicados avanzados frente a bacterias patógenas (**Mak, 2018).**

4.1 Bacteriocinas producidas por cepas de *Staphylococcus*: estafilococcinas

Las bacteriocinas producidas por cepas de estafilococos se denominan estafilococcinas, y constituyen un limitado grupo de compuestos antimicrobianos. Hasta la fecha, se han identificado y caracterizado 47 estafilococcinas de todas las clases de bacteriocinas conocidas hasta el momento y descritas tanto en especies de estafilococos -coagulasa positivos como -coagulasa negativos (SCoP y SCoN, respectivamente) **(Tabla 2)**. En cuanto a las estafilococcinas producidas por aislados SCoP, se han descrito un total de 20 péptidos con actividad antimicrobiana incluidas en las clases I, II IV y diversos BLIS producidos por *S. aureus* (aureocinas), *S. pseudintermedius* y *S. agnetis* (Hicinas/Agneticinas) **(Tabla 2).** En cuanto a las bacteriocinas producidas por aislados SCoN, la **Tabla 2** muestra las 27 estafilococcinas descritas hasta la fecha, que en su mayoría pertenecen a la Clase I (lantibióticos), aunque también se incluyen bacteriocinas de las Clases II, III, NRP y 4 BLIS**.**

Tabla 2 Bacteriocinas descritas en SCoP y SCoN en función de su clase (clase I, II, III, IV, V, VI, NRPs y BLIS) **(adaptada de Fernández-Fernández et al., 2023a).**

Clasificación[a]	Bacteriocina	Especie (cepa productora)	Tipo de estafilococo	Origen (cepa productora)	Actividad antimicrobiana	
					G +	G−
Clase I	BacCH91	*S. aureus* (CH-91)	SCoP	Aviar (DSM26258)	+	−
	Bsa	*S. aureus* (MW2)		SARM-AC	+	−
	Hyicina/Agneticina 3682	*S. hycius/S. agnetis* (3682)	SCoP/SCoN*	Leche (vaca)	+	−
	NisinJ	*S. capitis* (APC2923)	SCoN	Humano (piel)	+	−
	Nukacina L217	*S. chromogenes* (L217)		Vaca (ubre)	+	−
	Estafilococcina T (StT)	*S. cohnii* (T)		Humano	+	+
	Nukacina IVK45	*S. epidermidis* (IVK45)		Humano (nasal)	+	-
	Pep5	*S. epidermidis* (5)		Clínica	+	−
	Epicidina 280	*S. epidermidis* (BN 280)		Clínica	+	−
	Epilancina K7	*S. epidermidis* (K7)		Laboratorio	+	−
	Epidermina	*S. epidermidis* (Tü 3298)		Clínica	+	−
	Epilancina 15X	*S. epidermidis* (15X154)		Clínica	+	−
	Gallidermina	*S. gallinarum* F16/P57 Tü3928		Pollo	+	+
	Hominicina	*S. hominis* (MBBL 2-9)		Humano	+	+
	Nukacina KQU-131	*S. hominis* (KQU-131)		Pescado (fermentado)	+	−
	Homicorcina	*S. hominis* (MBL_AB63)		Semillas	+	
	Nukacina 3299 Simulancina 3299	*S. simulans* (3299) *S. simulans* (Ec105)		Mastitis (vaca)	+	−
	Warnericina RB4	*S. warneri* (RB4)		Rice	+	−
	SWLP1	*S. warneri* (DSM 16081)		Human skin	+	-
	Nukacina ISK-1	*S. warneri* (ISK-1)		Arroz (fermentado)	+	−

Tabla 2 Continuación

Clasificación[a]	Bacteriocina	Especie (cepa productora)	Tipo de estafilococo	Origen (cepa productora)	Actividad antimicrobiana	
					G +	G −
Clase II	Estafilococcina C55	*S. aureus* (C55)	SCoP	Humano (piel)	+	+
	Aureocina A70	*S. aureus* (A70)		Leche	+	−
	Aureocina 4181	*S. aureus* (4181)		Mastitis (vaca)	+	−
	Aureocina A53	*S. aureus* (A53)		Leche	+	−
	BacSp222	*S. pseudintermedius* (222)		Perro (piel)	+	+
	Capidermicina	*S. capitis* (CIT060)	SCoN	Humano (piel)	+	−
	Epidermicina NI01	*S. epidermidis* (224)		Clínica	+	−
Clase III	Endopeptidasa ALE-1	*S. capitis* (EPk1)	SCoN	Clínica	+	−
	Lisostafina	*S. simulans biovar* (NRRL B-2628; ATCC1362)		Laboratorio	+	−
Clase IV (cíclicas)	Aureocyclicina 4185	*S. aureus* (4185)	SCoP	Mastitis (vaca)	+	−
Clase V (sactipéptido)	Hyicina/Agneticina 4244	*S. hycius/S. agnetis* (4244)	SCoP/SCoN*	Mastitis (vaca)	+	−
Clase VI (tiopéptido)	Micrococcina P1	*S. equorum* (WS 2733)	SCoN	Queso	+	-
NRPs	Lugdunina	*S. lugdunensis* (N920143)	SCoN	Humano (nasal)	+	−
BLIS	Estafilococcina BacR1	*S. aureus* (UT0007) *S. aureus* (UT0002)	SCoP	Clínica	+	+
	Aureocina 215FN	*S. aureus* (215FN)		Vaca (nasal)	+	−
	Estafilococcina 414	*S. aureus* (414)		Aviar	+	−
	Estafilococcina 462	*S. aureus* (462)		Visón	+	−
	Estafilococcina IYS2	*S. aureus* (IYS2)		Humano (saliva)	+	−
	Estafilococcina Au-26	*S. aureus* (26)		Humano (vagina)	+	+
	Bac 1829	*S. aureus* (KSI1829)		Laboratorio (RN4220)	+	+
	Bac 201	*S. aureus* (AB201)		Humano (herida)	+	+
	Estafilococcina 188	*S. aureus* (188)		Clínica	+	+
	Estafilococcina D91	*S. aureus* (D91)		Clínica	+	+
	TE8	*S. capitis* (TE8)	SCoN	Humano (piel)	+	−
	Estafilococcina 1580	*S. epidermidis* (1580)		Laboratorio	+	−
	Hogocidina-α/ Hogocodina-β	*S. hominis* (A9)		Humano (piel)	+	−
	Warnericina RK	*S. warneri* (RK)		Medio ambiente	+	+

[a]Abreviaturas: NRPs, péptido de síntesis no ribosomal (por sus siglas en ingles Non-Ribosomal-Peptide); BLIS, péptido antimicrobiano no caracterizado (por sus siglas en inglés Bacteriocin-Like-Inhibitory-Substances).
*SCoP/SCoN: especies de estafilococos consideradas como coagulasa variable.

A continuación, se muestran las características más relevantes de las clases de bacteriocinas descritas hasta el momento detallando su operón codificante, autoinmunidad y modo de acción.

4.1.1 Clase I

Los lantibióticos son los péptidos antimicrobianos más frecuentemente descritos en la clase I y se caracterizan por poseer modificaciones postraduccionales complejas que dan lugar a la formación de lantionina (Lan) y/o βmetilantionina (MeLan), enlaces tioéter entre residuos de cisteína (Cys)

y el β-carbono de los aminoácidos aceptores didehidroalanina (Dha) y didehidrobutirina (Dhb), respectivamente **(Bierbaum y Sahl 2009).** Estas modificaciones postraduccionales son el resultado de la actividad de enzimas codificadas en el mismo operón **(Dischinger et al. 2014; Ongey y Neubauer 2016).**

Los lantibióticos son potentes agentes antimicrobianos, así pues, la cepa productora debe ser inmune a la acción de su propia bacteriocina específica para evitar la muerte celular. Entre los sistemas de autoprotección, la **Figura 9** muestra la acción defensiva de tres mecanismos distintos basados en: proteínas de inmunidad individuales (LanI), un transportador ABC (normalmente compuesto por dos o tres subunidades, LanFE(G)) **(Aso et al. 2005)** y un tercer miembro denominado factor accesorio LanH **(Draper et al. 2008).** Los sistemas LanI y LanFE(G) pueden actuar solos o combinados mientras que LanH, actúa como proteína auxiliar para el ensamblaje de un transportador ABC funcional. Los genes de estos factores accesorios no están presentes en la mayoría de los operones asociados a lantibióticos y, por lo tanto, no son necesarios para el funcionamiento de la mayoría de los mecanismos de inmunidad lantibiótica **(Draper et al. 2008).**

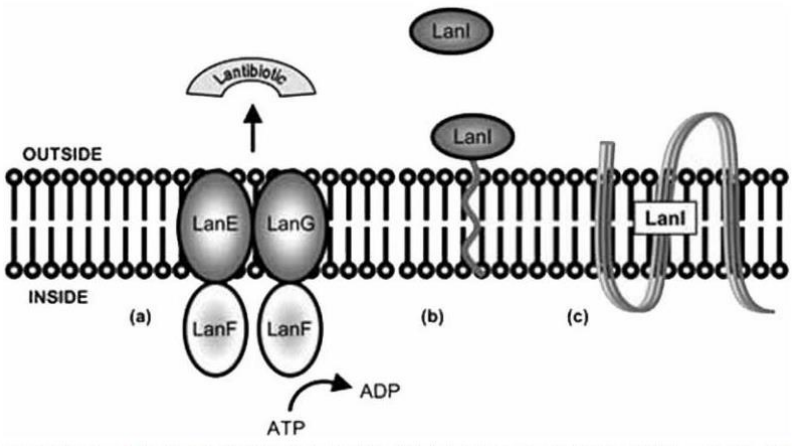


Figura 9 Ejemplo de sistema de autoinmunidad en lantibióticos **(Draper et al. 2008).**

4.1.2 Clase II

Se incluyen en la clase II bacteriocinas no modificadas postraduccionalmente. Los operones codificantes de estafilococcinas (OCE) de este tipo son más simples ya que carecen de genes implicados en la modificación post-traduccional y la maduración ocurre generalmente durante el proceso de transporte **(Nishie et al. 2012),** donde se lleva a cabo la escisión del péptido señal, lo que resulta en la producción del péptido bioactivo maduro.

En el caso de algunas bacteriocinas de clase II, la proteína autoinmune suele ser una metaloproteasa de la familia Abi **(Kjos et al., 2010),** la cual actúa formando un complejo fuerte

con la bacteriocina unida a su receptor, impidiendo la muerte de las células **(Diep et al., 2007).** Otro mecanismo de inmunidad descrito en bacteriocinas de clase II, depende de la actividad de proteínas transportadoras que actúan como bombas de expulsión de la bacteriocina al exterior del citoplasma celular **(Gajic et al., 2003).**

4.1.3 Clase III

La lisostafina y la endopeptidasa ALE1 son las únicas estafilococcinas descritas en la clase III, destacando la lisostafina por ser una de las bacteriocinas más investigadas debido a su elevado potencial en aplicaciones clínicas **(Schindler y Schuhardt 1964).** Atendiendo al OCE de la lisostafina, cabe destacar que está compuesto por el gen estructural y dos genes accesorios, *lss* y *lif*, que codifican la endopeptidasa y la proteína relacionada con la inmunidad, respectivamente **(Heath et al. 1987, 1989).** Si bien estos genes de inmunidad no son específicos, las cepas productoras de lisostafina cuentan con sistemas de protección exclusivos debido a la composición de su pared bacteriana. En este caso, el peptidoglicano presenta una alta resistencia a la hidrólisis debido a la incorporación de residuos de serina en las posiciones tercera y quinta de los puentes cruzados pentapéptidos de la pared celular **(Ehlert et al., 2000; Thumm y Götz, 1997).**

4.1.4 Clase IV

Pertenecen a la clase IV los péptidos antimicrobianos con estructura circular, debido a un enlace amida entre los extremos N y C. Entre las características distintivas de los péptidos circulares de clase IV, destaca su ruta de biosíntesis en tres pasos: 1) traducción, 2) escisión del péptido señal, 3) unión de los extremos N- y C- del prepéptido antimicrobiano, y la secreción del péptido maduro **(Gabrielsen et al. 2014; Pérez et al. 2018).** El operón de las bacteriocinas circulares incluye además un gen que codifica una proteína de membrana perteneciente a la superfamilia conocida como SpoIIM, posiblemente implicada en la circularización de la bacteriocina madura **(Pérez et al. 2018).**

En cuanto a las proteínas implicadas en la inmunidad a las bacteriocinas circulares (clase IV), éstas están probablemente asociadas a la presencia de proteínas de membrana y transportadores ABC, necesarios para la inmunidad completa en este tipo de péptidos complejos **(van Belkum et al., 2011).**

4.1.5 Sactipéptidos

Los sactipéptidos o sactibióticos son péptidos lineales o circulares caracterizados por la presencia de enlaces tioéter entre el grupo tiol de los residuos Cys y el carbono α de los aminoácidos aceptores antes de la eliminación del péptido líder **(Mathur et al. 2015; Coelho et al. 2017).** Dos enzimas importantes se ven implicadas en las modificaciones postraduccionales, aquellas que conducen a la formación de enlaces tioéter (enzima radical S-adenosilmetionina) y peptidasas

específicas que contribuyen al procesamiento de los péptidos precursores modificados y su circularización.

4.1.6 Tiopéptidos

Los tiopéptidos son péptidos macrocíclicos pequeños, ricos en azufre que, debido a su complejidad estructural y a su compleja biosíntesis, se asignaron a una nueva clase, la clase VI. Para la obtención del péptido maduro, los péptidos precursores sufren diversas modificaciones postraduccionales que generalmente resultan en la formación de un anillo central nitrogenado, y una cola de longitud variable, con grupos azoles y residuos de aminoácidos altamente modificados, entre otros **(Zheng et al. 2017).**

Su estructura altamente compleja, está codificada en un operón que incluye los genes relacionados con los péptidos precursores, enzimas modificadoras, de escisión y de inmunidad **(Zhang y Liu, 2013).** El representante más conocido, también descrito en aislados de estafilococos, es la bacteriocina micrococcin P1 (MP1). La producción de MP1 es debida a un clúster de 11 genes (*tclESQIJKLMNPU*) donde se incluyen el gen estructural, *tclE*, que codifica el péptido precursor de 49 aminoácidos, (14 aa forman el péptido estructural) y otros ocho genes codifican enzimas implicadas en la maduración e inmunidad del tiopéptido **(Bennallack et al. 2014).**

4.2 Operones codificantes de estafilococcinas (OCE)

Se han descrito multiples OCE que en su mayoría mantienen la estructura típica representada en la **Figura 10** donde se incluye el gen estructural además de genes relacionados con la maduración, secreción e inmunidad **(Tabla 3)**. Asimismo, en la **Figura 11** se muestran los operones más frecuentes descritos en estafilococos para cada una de las diferentes clases de bacteriocinas descritas.

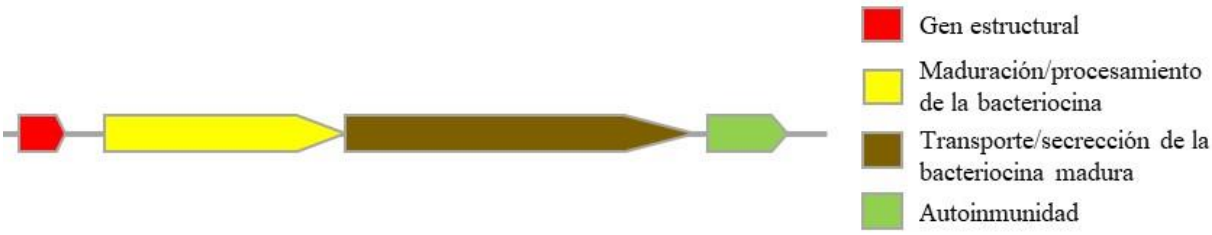


Figura 10 Ejemplo gráfico de los componentes principales de un operón codificante de bacteriocinas **(adaptada de Teso-Pérez et al. 2021).**

Tabla 3 Funcionalidad y clases de bacteriocinas en las que se encuentran los genes que conforman el operon codificante de las estafilococcinas **(adaptada de Freire Bastos et al. 2020).**

Gen	Función	Clases de bacteriocinas en las que los genes están presentes
Estructural	Péptido precursor inactivo o bacteriocina madura	Todas
Inmunidad/Resistencia	Proteína de defensa celular con actividad sobre su propia bacteriocina	Todas
Modificaciones	Conjunto de genes implicados en modificaciones postraduccionales	Lantibióticos, sactipéptidos y tiopéptidos
Proteasa	Liberación del péptido precursor	Lantibióticos y sactipéptidos
Transporte	Transportador de tipo ABC involucrado en la externalización de la bacteriocina y también en el procesamiento proteolítico del péptido o en la inmunidad	Lantibióticos, clase II y IV y sactipéptidos
Regulación	Proteínas reguladoras de la producción de la bacteriocina	Lantibióticos, clase II y tiopéptidos
Circularización*	Formación del enlace amida en el péptido estructural	Clase IV y sactipéptidos

*Aunque se ha postulado su presencia, aún no se han identificado las enzimas implicadas en esta reacción.

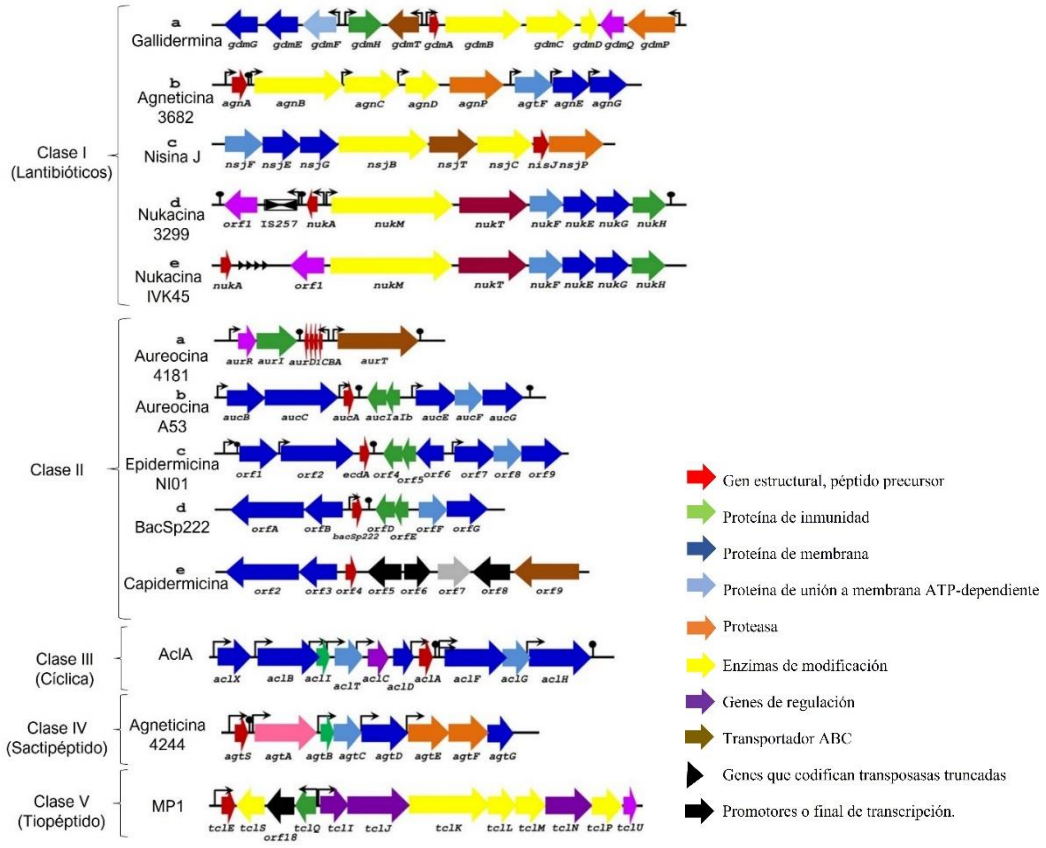


Figura 11 Operón codificante de estafilococcinas. Los colores indican la función de cada uno de los genes y/o el tipo de proteínas que codifican. Los genes no están representados en escala proporcional **(adaptada de Freire Bastos et al. 2020).**

4.3 Mecanismos de acción de las estafilococcinas

Atendiendo a su mecanismo de acción **(Figura 12),** la mayoría de las estafilococcinas provocan la muerte celular actuando en la membrana de los microorganismos diana **(de Freire Bastos et al. 2020; Pieta et al. 2016).** Por ejemplo, las bacteriocinas Pep5, BacSp222 o la lugdunina provocan poros que causan la disipación del potencial de membrana. Otros péptidos antimicrobianos (por ejemplo, Epi/Gdm) inhiben la biosíntesis de peptidoglicano y del ácido teicoico **(Ebner et al. 2018).** Finalmente, cabe destacar el modo de acción de la estafilococcina micrococcina P1, tiopéptido que actúa de forma bacteriostática deteniendo el proceso de elongación de proteínas a través de la translocación del ribosoma a lo largo del ARNm molde **(Ciufolini y Lefranc 2010).**

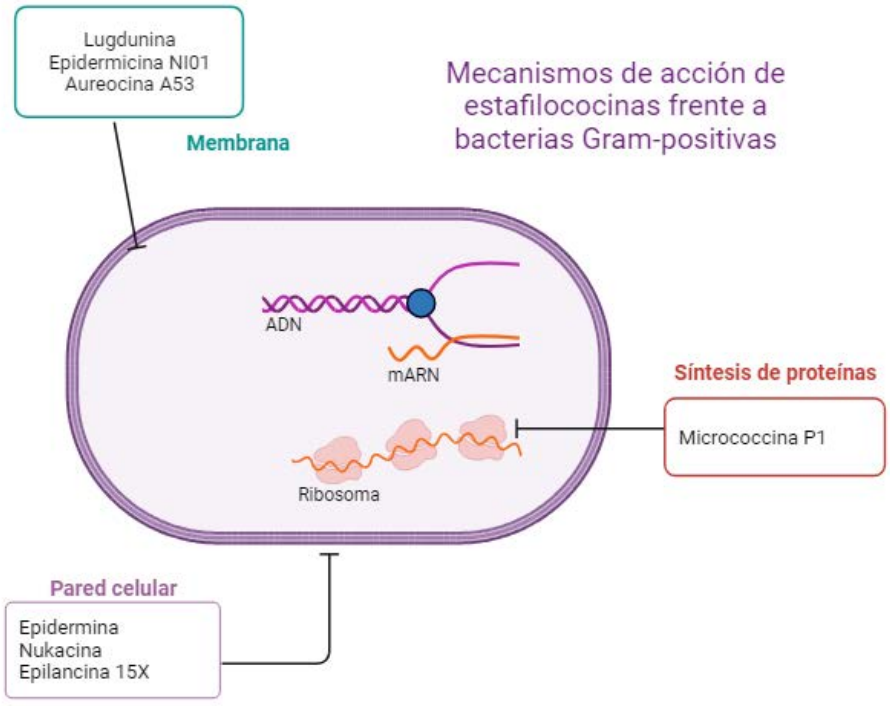


Figura 12 Mecanismos de acción de las estafilococcinas **(adaptada de Heilbronner et al. 2021).**

5 Descripción del género *Staphylococcus*: estafilococos coagulasa positivos y negativos

Staphylococcus es un género bacteriano G+, inmóvil, con forma esférica (coco) que frecuentemente se encuentra formando racimos (*staphile* en griego), lo que le dio el nombre al género **(Figura 13)**. En cuanto a su metabolismo, *Staphylococcus* es anaerobio facultativo, catalasa positiva, DNAasa positivo y no forma esporas. Asimismo, aparte de los hallazgos y clasificaciones filogenéticos, en medicina humana se sigue un esquema simplificado, basado principalmente en su capacidad para coagular el plasma sanguíneo diferenciando entre SCoP y SCoN **(Becker et al. 2014).**

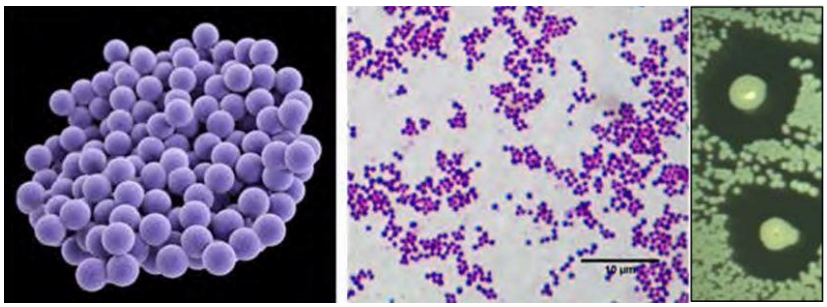


Figura 13 Imágenes de la especie *Staphylococcus aureus* al microscopio y ejemplo de actividad antimicrobiana por la técnica *spot-on-lawn.*

El género *Staphylococcus* consta de 47 especies y 23 subespecies válidamente descritas de las cuales 38 fueron clasificadas como SCoN **(Becker et al. 2014).** Recientemente, algunas especies de estafilococos han sido reasignados a otros géneros tras la evaluación de genomas a nivel filogenético. Un ejemplo es el grupo de especies *Staphylococcus sciuri,* considerados actualmente en un nuevo género, *Mammaliicoccus*, con *Mammaliicoccus sciuri* como especie tipo **(Chun et al., 2018; Madhaiyan et al., 2020).** El nombre actual del género hace referencia al nicho ecológico en el que suelen aislarse estas bacterias, que es una amplia variedad de mamíferos de granja y salvajes, así como productos derivados de los mismos **(Van der Veken et al. 2020).**

Normalmente, el género *Staphylococcus* y especialmente las especies SCoN son miembros comensales de la microbiota de la piel y superficies mucosas en humanos y también colonizan con frecuencia animales, se encuentran en alimentos y están dispersos en el medio ambiente **(Ikhimiukor et al. 2023).** Sin embargo, *Stathylococcus* puede actuar como patógeno oportunista siendo en este caso la especie *S. aureus* (principal representante SCoP) la más asociada con eventos clínicos **(Becker et al. 2014).**

5.1 ¿Por qué estudiar bacteriocinas producidas por los estafilococos?

La escasez de nuevos y potentes agentes antimicrobianos ha llevado a la comunidad científica a explorar alternativas no convencionales contra la RAM, entre ellos los microorganismos productores de sustancias antimicrobianas y las bacteriocinas como posibles agentes terapéuticos.

Diversos estudios han señalado el interés de los aislados de *Staphylococcus* como productores de bacteriocinas. Además, la naturaleza ubicua de los estafilococos, su metabolismo flexible, y su versatilidad, les permite sobrevivir y habitar nichos muy diversos (superficies bióticas y abióticas, animales, seres humanos, plantas, alimentos etc.).

Por otro lado, los estafilococos son resistentes a condiciones adversas como el pH ácido, la presencia de péptidos antimicrobianos del hospedador, la exposición a la radiación UV, la sequedad y las perturbaciones ambientales, entre otros **(Vermassen et al. 2016; Byrd et al. 2018).** Además, la mayoría de los estafilococos suelen ser halotolerantes (toleran concentraciones de sal de hasta el 21%), resistentes al metabolismo del nitrógeno y a las presiones oxidativas **(Vermassen et al. 2014).** Asimismo, estudios recientes han informado de sus actividades lipolíticas y proteolíticas **(Müller et al. 2016; Stavropoulou et al. 2018).**

Asimismo, recientes estudios sobre la comunicación bacteriana en la cavidad nasal, han demostrado que las cepas de estafilococos productoras de bacteriocinas compiten por un nicho ecológico frente a indicadores y se mantienen con éxito a lo largo del tiempo desplazando al resto de las cepas cohabitantes **(Thomas, 2023).**

Manteniendo un enfoque evolutivo y/o ecológico se ha evaluado la presencia/ausencia y abundancia de bacteriocinas y cepas en determinadas comunidades microbianas, para identificar asociaciones negativas entre competidores a nivel de especie **(Yan et al. 2013; Zipperer et al. 2016)**. En cuanto a los estudios sobre bacteriocinas naturales, es importante analizar la actividad antimicrobiana de dichos aislados productores frente a un conjunto de cepas competidoras coexistentes en un mismo entorno ecológico.

Por todo ello, el género *Staphylococcus* y sus bacteriocinas despiertan interés por sus aspectos tecnofuncionales **(Pantůček et al. 2018**), presentan un gran potencial biotecnológico y resultan relevantes como moduladores de la microbiota, especialmente nasal y cutánea.

6 Nicho ecológico del género *Staphylococcus* y estafilococcinas detectadas en función del origen

6.1 Especies de estafilococos detectadas en humanos y sus estafilococcinas

El género *Staphylococcus* es un constituyente habitual de la microbiota nasal y de piel en humanos **(Figura 14).** Entre ellos destacan las especies de SCoN, como *S. epidermidis, S. capitis, S. hominis, S. cohnii, S. warneri y S. lugdunensis,* entre otras **(Edslev et al. 2021; Laux et al. 2019).**

Se sabe que *S. epidermidis* es un miembro esencial de la microbiota tanto de la piel como de la nariz. *S. warneri* se ha encontrado en un porcentaje menor que otros SCoN, aunque también está presente en las fosas nasales y en la piel (**Laux et al. 2019**). Destaca también *S. lugdunensis*, especie de SCoN aislado frecuentemente en muestras nasales humanas (10 al 26% de incidencia en personas sanas) (**Laux et al. 2019**). En cuanto a la presencia de SCoP, se ha reportado que el 20-30% de las fosas nasales anteriores están colonizadas por *S. aureus* **(Krismer et al. 2017; Newstead et al. 2020).** La presencia de este patógeno oportunista se ha relacionado con una reducción de la diversidad bacteriana, una exacerbación de los síntomas de la enfermedad y, con frecuencia, la colonización precede a la infección **(Edslev et al. 2021; von Eiff et al. 2001; Laux et al. 2019).**

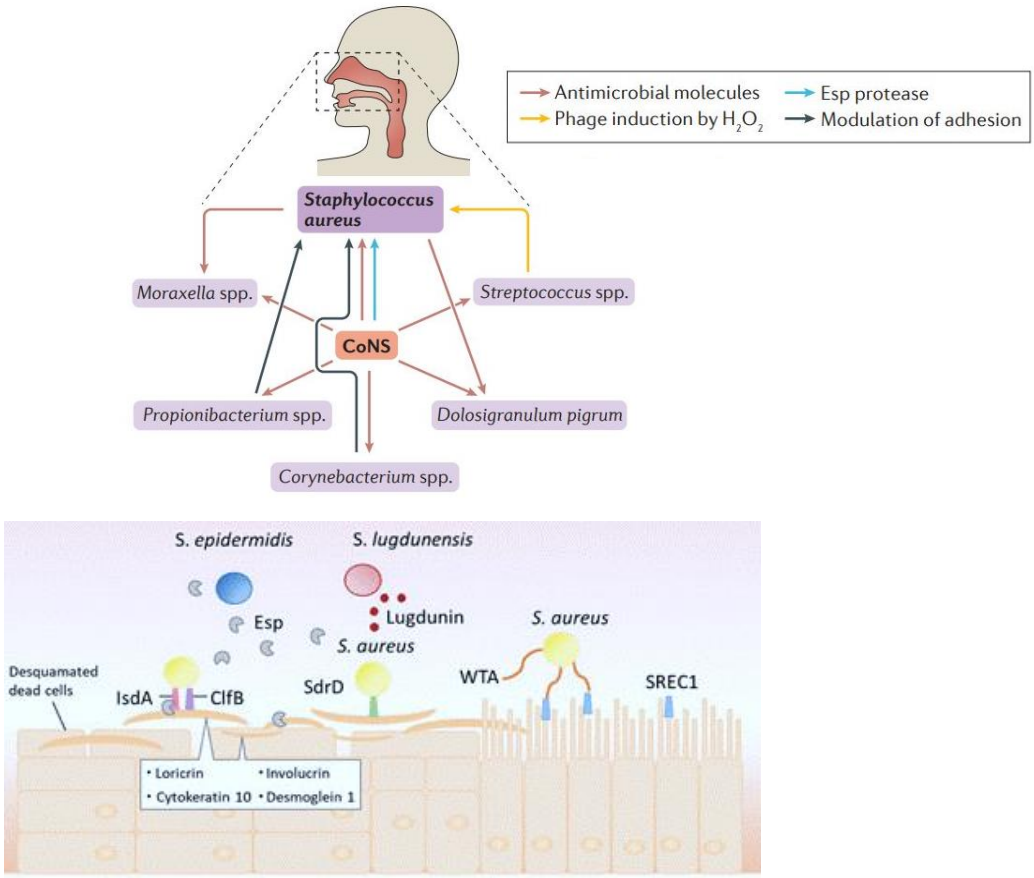


Figura 14 Especies de estafilococos frecuentes en la microbiota nasal humana e interacción entre sí y con las células epiteliales **(adaptada de Laux et al. 2019 y Krismer et al. 2017).**

Se ha evaluado la posible asociación negativa entre especies de una comunidad bacteriana observando que, en el caso de la cavidad nasal humana, la colonización con *S. lugdunensis* se asocia con una reducción de la portación nasal de *S. aureus.* Además, se ha evidenciado que cepas de la especie *S. lugdunensis* producen la bacteriocina lugdunina, que puede inhibir a *S. aureus* **(Zipperer et al. 2016).** Del mismo modo, se ha observado que cepas de *S. hominis* productoras de bacteriocinas inhiben a *S. aureus* y protegen de su infección en personas con piel atópica y dermatitis **(Nakatsuji et al. 2017).** Todo ello muestra el importante papel de los estafilococos productores de bacteriocinas como agentes moduladores de la microbiota en estado basal o en casos de infección. Atendiendo a la especie *S. pseudintermedius,* se ha observado un aumento en el diagnóstico de infecciones humanas derivadas de la colonización de dicho SCoP y muy especialmente en personas en contacto con perros **(Lozano et al., 2017; Somayaji et al. 2016),** aunque su incidencia en la microbiota nasal humana es menor que la descrita para *S. aureus.*

Por otro lado, se ha evidenciado que el porcentaje de portadores de *S. aureus* y *S. pseudintermedius* podría verse afectado por factores de riesgo como el contacto laboral con animales de granja **(Abdullahi et al. 2021)** o por la transmisión zoonótica entre perros y sus dueños **(Lozano et al. 2017; Pires Dos Santos et al. 2016; Somayaji et al. 2016),** respectivamente. De este modo, el estudio de la microbiota y la caracterización de posibles agentes patógenos y/o zoonóticos es importante para garantizar la salud global.

Se han descrito diversas estafilococcinas producidas por cepas comensales aisladas de humanos: estafilocina C55, Bsa, Capidermicina, Endopeptidasa ALE-1, Nisina J, Nukacina IVK45**,** Pep5, Epidermicina NI01, Epilancina 15X, estafilocina T (StT)**,** Hominicina, Lugdunina y SWLP1**.** Además, se han aislado bacteriocinas a partir de muestras clínicas humanas, como Epidermina y Epicidina 280 así como otras sustancias inhibidoras similares a las bacteriocinas, pero no completamente caracterizadas (BLIS) como las estafilococcinas BacR1, IYS2, Au-26, Bac 201, 188, D91**,** TE8, y hogocidina α/β **(Tabla 2).**

6.2 Especies de estafilococos detectadas en animales y sus estafilococcinas

Staphylococcus es un organismo muy ubicuo que también coloniza un amplio número de hospedadores animales, especialmente mamíferos, pero también aves y peces. Si bien múltiples estudios han evidenciado que los SCoN forman parte de la microbiota cutánea/nasal de los animales, las especies de SCoP patógenas como *S. aureus* y *S. pseudintermedius* han sido las más analizadas a lo largo del tiempo. Así, por ejemplo, se ha observado que el ganado es un reservorio frecuente de *Staphylococcus aureus* resistente a meticilina (SARM) con diferentes porcentajes de colonización en función de la especie: cerdos (16%), ovejas (3,7%) y aves de corral (5,8%) **(Abdullahi et al. 2023a)**. Algunos de los subgrupos más relevantes como el complejo clonal (CC)

398 **(van Cleef et al. 2010; Ceballos et al. 2019)** tienen una gran distribución mundial entre distintas especies animales, especialmente en ganado porcino, y constituyen un grave problema para la salud pública **(Gómez-Sanz et al., 2010; Petinaki y Spiliopoulou, 2012).** Además, el cerdo es un animal altamente asociado a *S. aureus*, especie estafilocócica presente en la microbiota nasal del 65% de los cerdos **(Abdullahi et al. 2023c).** Asimismo, otras especies de SCoN como *S. equorum, S. schlifieri, S. cohinii, S. chromogenes, S. haemolyticus, S. hyicus y S. microti,* son muy frecuentes en la microbiota cutánea y nasal de estos animales **(Strube et al. 2018; Schlattmann et al. 2020; Slifierz et al. 2015; Abdullahi et al. 2023b,c)**. Ciertos estudios sobre la microbiota nasal porcina han revelado que la colonización por *S. aureus* también está relacionada con la ausencia de *S. sciuri, S. cohnii* o *S. saprophyticus* **(Verstappen et al. 2017).** Sin embargo, parece que *S. sciuri* es más frecuente en animales en los que *S. aureus* es menos frecuente en la microbiota nasal, aunque ambas especies se han encontrado colonizando al mismo tiempo a estos animales **(Abdullahi et al. 2023b,c).**

Por otro lado, estudios recientes realizados en España y Portugal **(Ruiz-Ripa et al. 2020; Sousa et al. 2016)** han confirmado la elevada frecuencia de colonización de estafilococos en animales salvajes. Concretamente, el 60-75% de las aves y el 38% de los mamíferos silvestres poseen aislados de estafilococos SCoN **(Mama et al. 2019a).** En este sentido, *S. sciuri* fue el colonizador SCoN más común de animales salvajes sanos seguido de la especie *S. lentus* también detectada en animales de granja y en personas con exposición profesional al ganado **(Zhang et al. 2009; de Martino et al. 2010; Ugwu et al. 2015).** *S. xylosus* y *S. chromogenes* fueron detectados con frecuencia en jabalíes **(Mama et al. 2019a).** Asimismo, un estudio reciente de Estados Unidos ha descrito una alta presencia de SCoN en diversas especies animales (gatos, perros, vacas, caballos, entre otros). Se identificaron un total de 15 especies SCoN siendo las más frecuentes *S. felis, S. simulans, S. xylosus* y *S. epidermidis* **(Figura 15, Ikhimiukor et al. 2023).**

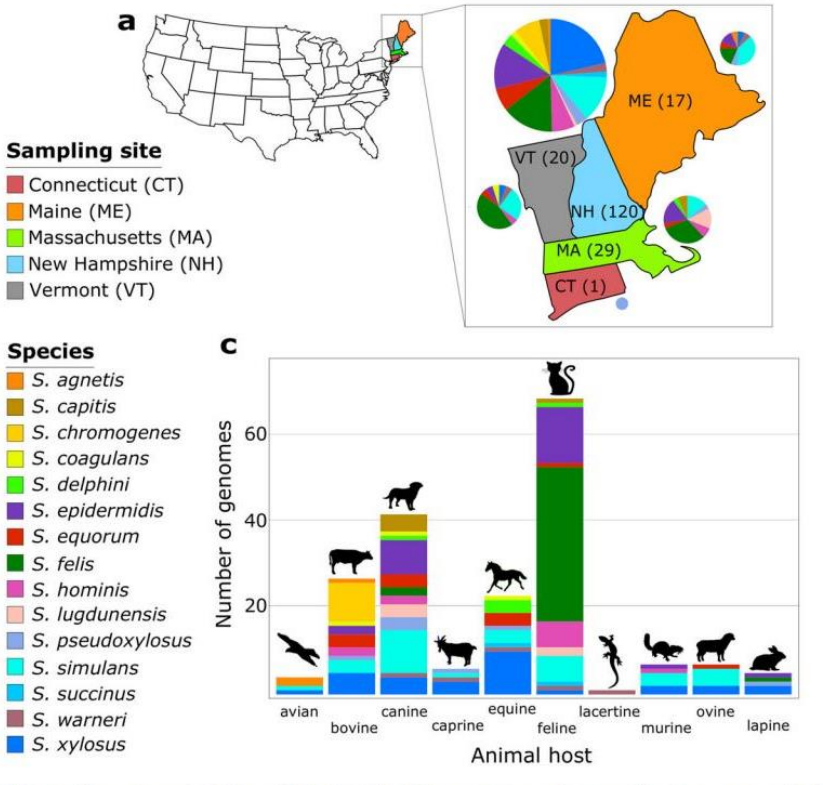


Figura 15 Diversidad de especies de SCoN detectadas en animales de vida libre en una región de Estados Unidos **(Ikhimiukor et al. 2023).**

Las especies de SCoP también se encontraron con frecuencia en muestras nasofaríngeas y rectales de mamíferos en libertad recuperados en España **(Ruiz-Ripa et al. 2019; Mama et al. 2019b)**. Algunas especies de mamíferos, como los jabalíes, los muflones y los ciervos, están frecuentemente colonizadas por *S. pseudintermedius y S. hyicus* **(Ruiz-Ripa et al. 2019)**. Cabe destacar que *S. aureus* y especialmente el linaje genético CC398, fue la especie de SCoP detectada con mayor frecuencia (**Mama et al. 2019b)**. Por otro lado, se ha detectado un posible reservorio SARM-*mec*C en los erizos y los conejos silvestres, lo que podría suponer un riesgo para la salud humana **(Ruiz-Ripa et al. 2019; Rasmussen et al. 2019; Bengtsson et al. 2017; Sahin-Tóth et al. 2022)**.

En cuanto a la presencia de estafilococos en animales de compañía, se sabe que este género se encuentra entre los principales componentes de la microbiota nasal de perros sanos. Teniendo en cuenta los métodos de cultivo tradicionales, la especie *S. intermedius* ha sido frecuentemente aislada en muestras procedentes del tracto respiratorio superior de perros, que, aunque se considera patógena se encuentra con frecuencia constituyendo la microbiota normal de los perros **(Tress et al. 2017).** Por otra parte, un análisis comparativo realizado en 2019 por Gómez-Sanz y colaboradores mostró que, aunque los SCoP eran predominantes en propietarios y mascotas, los SCoN resistentes a meticilina (SCoN-RM), especialmente *S. epidermidis* resistente a la meticilina (SERM), son colonizadores comunes de propietarios y mascotas sanos **(Gómez-Sanz et al. 2019).**

El transporte conjunto de SCoP y SCoN-RM pone de relieve la importancia de los animales de compañía como reservorios de importantes patógenos oportunistas multirresistentes, que pueden transferirse a las personas en contacto.

Finalmente, atendiendo a los SCoP, *S. pseudintermedius* es el principal colonizador de la piel y membranas mucosas de perros, aunque también ha sido descrito en otras especies animales como jabalíes y burros (**Gharsa et al. 2015; Mama et al. 2019b).** Éste se considera uno de los patógenos bacterianos más frecuentemente aislados de muestras clínicas en estos animales (10%) **(van Duijkeren et al. 2011; Bannoehr y Guardabassi, 2012).** Esta especie también se encuentra implicada frecuentemente en infecciones oportunistas, siendo el principal agente etiológico de infecciones de piel y partes blandas, otitis e infecciones del tracto urinario en perros **(Bergot et al. 2018; Pires Dos Santos et al. 2016).** Además, destaca la aparición de *S. pseudintermedius* resistente a la meticilina (SPRM) **(Gómez-Sanz et al. 2011)** como un importante problema sanitario **(van Duijkeren et al. 2011).**

En cuanto a las bacteriocinas detectadas en animales **(Tabla 2)**, se han descrito varias estafilococcinas producidas por estafilococos recuperados de mastitis bovina (Aureocina 4181**,** Aureocyclicina 4185**,** Hyicina/Agneticina 4244, Nukacina L217 y Simulancina 3299) así como de estafilococos procedentes de muestras nasales de ganado vacuno y de aves de corral como la aureocina 215FN**,** la BacCH91 y las estafilococcinas 414 y 462**.** Atendiendo a las estafilococcinas detectadas en animales de compañía, únicamente se han descrito dos bacteriocinas en aislados de perros (BacSp222, *S. pseudintermedius*) y gatos (Micrococcina P1, *S. felis*) **(Wladyka et al. 2015; O'Neill et al. 2021).** Sin embargo, los estudios centrados en evaluar la producción de bacteriocinas en animales salvajes son muy limitados (**Poeta et al. 2007; Almeida et al. 2011: Prichula et al. 2021**) y todos los trabajos encontrados se centran en el género *Enterococcus.*

6.3 Especies de estafilococos detectadas en alimentos y sus estafilococcinas

El género *Staphylococcus* puede estar presentes en productos alimentarios, principalmente en aquellos derivados de origen animal. Se ha descrito una amplia lista de especies de SCoN en carne fermentada, salchichas, pescado fermentado, leche, queso y, más recientemente, en soja fermentada **(Khusro et al. 2022)** y se ha informado de la presencia de otras especies del género *Staphylococcus* en carne cruda de cerdo, pollo y vacuno **(Van Reckem et al. 2021).** Sin embargo, la presencia de *S. aureus* en alimentos se relaciona con posibles procesos de toxiinfección debido a su capacidad de producir enterotoxinas **(Irlinger, 2008; Okafor et al. 2022).**

En los últimos años, los estafilococos han atraído la atención de investigadores debido a su relevante papel en la mejora de las propiedades organolépticas (textura, acidez y sabor) de los

productos alimentarios fermentados **(Chen et al. 2021).** Asimismo, varias especies de SCoN (*S. xylosus* y *S. carnosus*) se han utilizado como cultivos iniciadores para estandarizar la producción e inhibir los patógenos transmitidos por los alimentos **(Khusro et al. 2022; Lee et al. 2018a)**. Uno de los beneficios más destacados de los estafilococos en los alimentos es la capacidad de estabilización del color rojo de los productos cárnicos, derivada de la producción de nitrato reductasa, una acción que también inhibe a algunos patógenos transmitidos por los alimentos **(Hammes et al. 2012).**

La presencia de estafilococos productores de bacteriocinas en alimentos ha sido ampliamente reportada en la literatura, especialmente en leche y alimentos fermentados: Hyicina 3682, Aureocina A70, Aureocina A53, Gallidermina, Micrococcin P1, Nukacina ISK-1, Nukacina KQU131, Warnericina RB4 y la recientemente descubierta Homicorcina **(Tabla 2).**

6.4 Especies de estafilococos detectadas en muestras ambientales y sus estafilococcinas

Las bacterias son microorganismos altamente ubicuos presentes en el medio ambiente: agua, aire y suelo, entre otros. Se sabe que el género *Staphylococcus* tiene una gran capacidad de adaptación a la desecación siendo capaz de sobrevivir en ambientes acuáticos durante varios días **(Ashfaq et al. 2022)**. Además, se ha descrito la presencia de especies de estafilococos en suelo (**Pantůček et al. 2018**), en el aire **(Sanz et al. 2021)** o incluso en minerales de alto valor como el oro (**Maliničová et al. 2020**).

Entre todos estos ambientes, despierta gran interés la matriz agua, por ser un elemento conector entre las superficies agrícolas, los terrenos naturales y las zonas urbanas. En base a ello, se han llevado a cabo estudios para conocer la diversidad de especies, así como la diversidad de líneas genéticas, y la resistencia a antimicrobianos y virulencia en *Staphylococcus spp.* de muestras de aguas por ser nichos ambientales receptores de gran cantidad de residuos de origen antropogénico como son las sustancias antimicrobianas utilizadas en medicina humana o producción animal. **Gómez y colaboradores (2016 y 2017)** evidenciaron la alta prevalencia de *Staphylococcus* en aguas urbanas y superficiales portadoras de gran diversidad de especies SCoN así como de aislados SARM CC398 multiresistentes.

Por otro lado, estudios sobre la microbiota en aire han señalado que los estafilococos tienen una gran resistencia ambiental y son, por tanto, uno de los géneros frecuentemente encontrados en el aire. Además, la diseminación aérea de estafilococos ha sido ampliamente referenciada e incluso se ha sugerido a este género como indicador de la emisión bacteriana aérea en superficies de producción animal **(Seedorf et al. 2005; Schulz et al. 2004; Sanz et al. 2021)**. En este sentido, estudios recientes han confirmado la alta prevalencia del género *Staphylococcus* en aire, siendo

la mayor parte de los aislados recogidos *S. saprophyticus* (39.4%) **(Omeira et al. 2006; Devrieseet al. 1991; Xiao et al. 2017).**

Si nos centramos en la producción de estafilococcinas obtenidas de muestras ambientales, destaca Warnericina RK, producida por *S. warneri* procedente de una muestra de suelo que posee actividad anti *Legionella* (**Héchard et al. 2005)**.

6.5 *Staphylococcus* como patógeno oportunista

Sin embargo, dentro de este género nos encontramos con especies que pueden ocasionar infecciones de diversa gravedad siendo importantes patógenos oportunistas. *S. aureus* es capaz de producir un gran arsenal de factores de virulencia que contribuyen a la severidad de la enfermedad. En este sentido destaca la producción de toxinas relacionadas con el síndrome del shock tóxico, intoxicaciones alimentarias y el síndrome de piel escaldada estafilocócica, o la toxina de Panton Valentine, entre otros **(Lee et al. 2018b).**

Dentro del género *Staphylococcus* preocupa, además, su capacidad para desarrollar mecanismos de resistencia a antibióticos y, entre ellos, destaca la resistencia a meticilina. La primera detección de cepas SARM fue en un hospital de Inglaterra en 1961 **(Jevons, 1961)** y surgió tras el uso de este tipo de beta-lactámicos. La resistencia en este caso es mediada por genes *mec* (mayoritariamente *mec*A, aunque tambien en algunos casos *mec*C o *mec*B) que provocan la modificación de la diana (Penicillin-Binding-Protein-PBP) **(Köck et al. 2010; García Álvarez et al. 2011; Gómez et al. 2014).** Las cepas SARM presentan en ocasiones resistencia a otras familias de antibióticos, y frecuentemente muestran fenotipos de multirresistencia. Se han descrito distintas líneas genéticas de SARM y se han distinguido tres grandes grupos de clasificación epidemológica que incluyen: SARM asociado al ámbito hospitalario (AH), asociado a la comunidad (AC), y asociado al ganado (AG)**.**

Actualmente, las infecciones producidas por SARM-AC tienen un gran impacto clínico (**Khan et al. 2018)**. Poco a poco se han desdibujado las diferencias fenotípicas entre SARM-AC y SARM-AH **(Bal et al. 2016)**. Sin embargo, estos dos grupos se pueden distinguir muchas veces por sus características moleculares, las infecciones asociadas que producen y los grupos de riesgo **(Junie et al. 2018).**

Además, en las últimas décadas se ha incrementado la preocupación por la emergencia y diseminación de una nueva variante de SARM asociado al ganado, el linaje CC398, el cual se encuentra principalmente en cerdos **(Gómez-Sanz et al. 2010)** y coloniza a personas en contacto profesional con ganado porcino. Asimismo, la presencia del clon SARM-CC398 se ha descrito en menor frecuencia en otros animales de producción, o en alimentos derivados de los mismos (especialmente de origen porcino) (**Gómez-Sanz et al. 2010; Khanna et al. 2008; Lozano et al.**

2011; Lozano et al. 2012; Wulf et al. 2008). Por otro lado, cada vez son más frecuentes las infecciones causadas por SARM-CC398, apareciendo principalmente en hospitales localizados en regiones con alta densidad de ganado porcino **(Ceballos et al. 2019)**. Por otro lado, el nivel de colonización nasal por SARM-CC398 en personas en contacto directo con ganado pocino es muy elevada, y la frecuencia depende del nivel de contacto y si los animales están vivos o ya muertos a nivel de matadero **(Quero et al. 2023, Abdullahi et al. 2023c).**

7 Seguridad en el uso y aplicaciones de bacteriocinas

Las bacteriocinas, suelen considerarse antimicrobianos y sustancias terapéuticas seguras **(Soltani et al. 2021).** Sin embargo, no sólo es importante descubrir nuevas bacteriocinas y evaluar su capacidad inhibitoria, sino también realizar pruebas de toxicidad para demostrar su uso seguro.

En gran medida la citotoxicidad de las bacteriocinas para las células eucariotas depende de la concentración, la pureza, la composición y el tipo de línea celular eucariótica utilizada **(Favaro y Todorov, 2017; Cavicchioli et al. 2018).** Asimismo, se ha señalado que el uso de moléculas bioactivas con respuestas inmunitarias indeseables podría ser perjudicial para el huésped **(Sahoo et al. 2017; Scholl y Martin, 2008; McCaughey et al. 2016)**. Por lo tanto, la evaluación del perfil de inmunogenicidad de las bacteriocinas, sobre todo cuando se piensa en su posible utilización en la industria alimentaria o como posibles bioterapéuticos en humanos o animales, es un punto clave para garantizar su seguridad.

Para permitir el uso de una bacteriocina, es necesario validar su actividad y sus efectos *in vitro* antes de utilizarlos en ensayos clínicos, tanto en modelos animales como en seres humanos. A este respecto, deben tenerse en cuenta los parámetros farmacocinéticos del huésped **(Meade et al. 2020),** la toxicidad potencial inducida por la bacteriocina **(Gupta et al. 2014)** y la vía de administración. Recientemente, se han realizado ensayos *ex vivo* con estafilococcinas como Aureocina A53 y Aureocina A70 **(de Freire Bastos et al. 2020).** Existen pocos estudios sobre el uso *in vivo* de estafilococcinas, aunque algunas ya han sido probadas en animales (**de Freire Bastos et al. 2020),** como el caso del uso de modelos murinos para analizar el posible papel de la estafilococcina 1580 en la inhibición de la caries **(Fitzgerald et al. 1986),** el uso de la lisostafina para el tratamiento de heridas, neumonía y/o infecciones sistémicas producidas por SARM **(Desbois et al. 2010; Blazanovic et al. 2015; Chen et al. 2014)** o como alternativa para la mastitis producida por *S. aureus* (modelo animal de mastitis murina) **(Schmelcher et al. 2012),** y la MP1 para las infecciones cutáneas producidas por SARM **(Ovchinnikov et al. 2020).** La epidermicina NI01 se ha probado en el modelo de larvas de *Galleria mellonella* **(Gibreel y Upton, 2013)** y para erradicar la colonización nasal de SARM en ratas **(Halliwell et al. 2016)** con resultados muy prometedores. Otros estudios realizados en humanos han demostrado que la cepa de *S. hominis*

ShA9 productora de bacteriocinas es una buena alternativa para controlar a *S. aureus* durante la disbiosis cutánea y otras enfermedades como la dermatitis atópica **(Nakatsuji et al. 2017, 2021).**

8 Otras aplicaciones de las bacteriocinas

La producción de bacteriocinas ha sido descrita en el género *Staphylococcus*, reflejando amplios perfiles de actividad antimicrobiana frente a diversos patógenos, principalmente G+. Además, cabe destacar otras acciones potencialmente relevantes detectadas en aislados de origen humano y animal en este género, como la actividad antituberculosa, anticancerígena, antiviral, antiinflamatoria e inmunomoduladora señalando algunos estudios **(Lee et al. 2019; Lee et al. 2018a; Carson et al. 2017; Kanjan et al. 2020; Khusro et al. 2019; Sánchez-Mainar et al. 2016; Van der Veken et al. 2020; Jo et al. 2022; Khusro et al. 2020a; Song et al. 2019; Kaur y Kaur, 2015; Khusro et al. 2018; Khusro et al. 2020b) (Tabla 4).** Además, estudios recientes han evidenciado el uso potencial de estafilococcinas o de aislados productores de estafilococos como bioconservantes en la carne para garantizar la vida útil microbiana del producto **(Xu et al. 2021)** o como antifúngicos para prevenir la aparición de mohos toxigénicos **(Cebrián et al. 2020).**

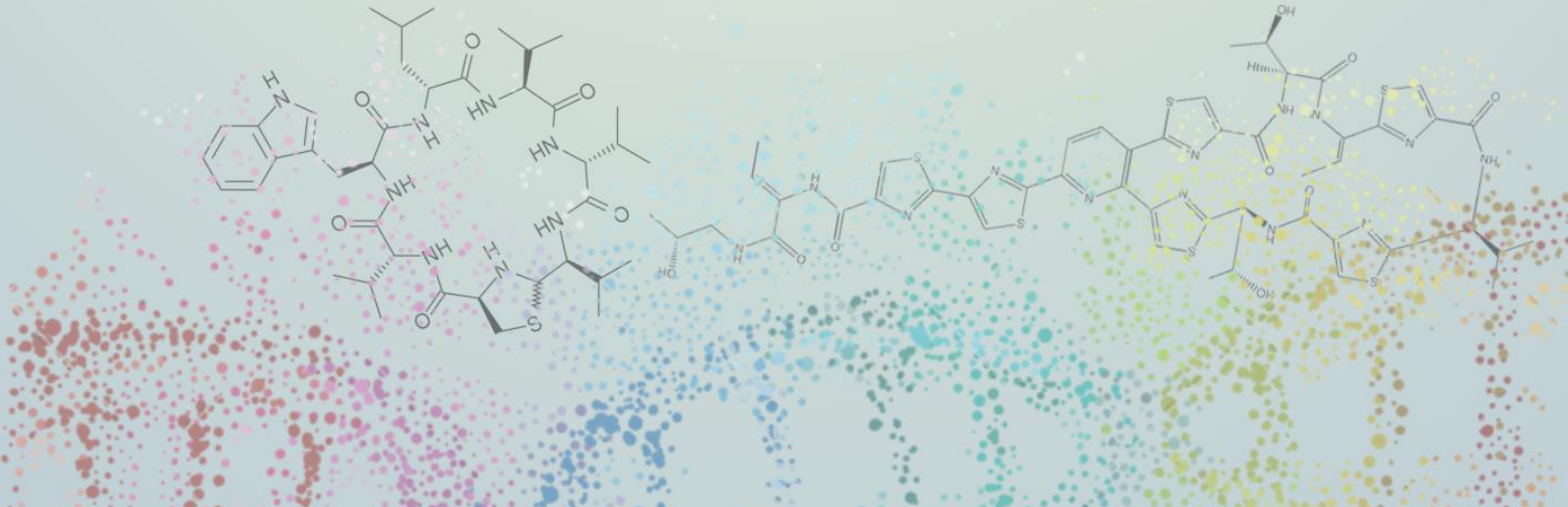
Tabla 4 Potenciales aplicaciones de los estafilococos productores de sustancias antimicrobianas.

Área de aplicación	Tipo de actividad	Cepa de estafilococo productor	Patógeno diana o enfermedad	Referencias
Sector agro-alimentario	Antimicrobiana	*S. chromogenes, S. capitis, S. hominis, S. epidermidis*,	Bacterias multirresistentes	Carson et al. 2017
		S. simulans PMRS35,	*E. coli* TISTR 780, *S. aureus* TISTR 1466, *Salmonella typhimurium* DMST 562, *B. cereus* DMST 5040	Kanjan and Sakpetch, 2020
		S. succinus AAS2	*E. coli, Proteus mirabilis, Pseudomonas aeruginosa, S. aureus, K. pneumoniae, S. saprophyticus*	Khusro et al. 2019
		S. sciuri I20-1, S. equorum SE3, S. equorum KS1039	*Clostridium botulinum, Listeria monocytogenes, S. aureus*	Sánchez Mainar et al. 2016 y 2017; Lee et al. 2018a
		S. sciuri IMDO-S72	*S. aureus, Listeria monocytogenes, C. botulinum, Bacillus cereus*	Van der Veken et al. 2020
	Bioconservante	*S. xylosus, S. carnosus*	*Brochothrix thermosphacta, Enterobacteriaceae*, *Pseudomonas* spp.	Xu et al. 2021
	Antifungico	*S. xylosus* Sx8	*Aspergillus flavus, A. parasiticus, Penicillium griseofulvum P. nordicum*	Cebrián et al. 2020

Tabla 4 Continuación.

Área de aplicación	Tipo de actividad	Cepa de estafilococo productor	Patógeno diana o enfermedad	Referencias
Clínica	Antituberculosa	*S. hominis* MANF2	*Mycobacterium tuberculosis*	Khusro et al. 2018; Khusro et al. 2020a
	Anticancerosa	*S. hominis* MANF2, *S. xylosus* VITURAJ10	Células cancerígenas de mama, colon y pulmón	Khusro et al. 2020b; Mangrolia y Osborne, 2020
	Antiviral	*S. epidermidis*	Virus Influenza A	Jo et al. 2022
	Antioxidante and anti-inflammatoria	*S. succinus* 14BME20, *S. saprophyticus*	-	Song et al. 2019
	Modulador de la microbiota	*S. lugdunensis, S. warneri, S. epidermidis, S. hominis, S. cohnii, S. capitis*	Desequilibrio de la microbiota piel-nasal	Laux et al. 2019; Nakatsuji et al. 2017; Edslev et al. 2021; Krismer et al. 2017

JUSTIFICACIÓN DE LA UNIDAD TEMÁTICA HIPÓTESIS



Justificación de la Unidad Temática e Hipótesis

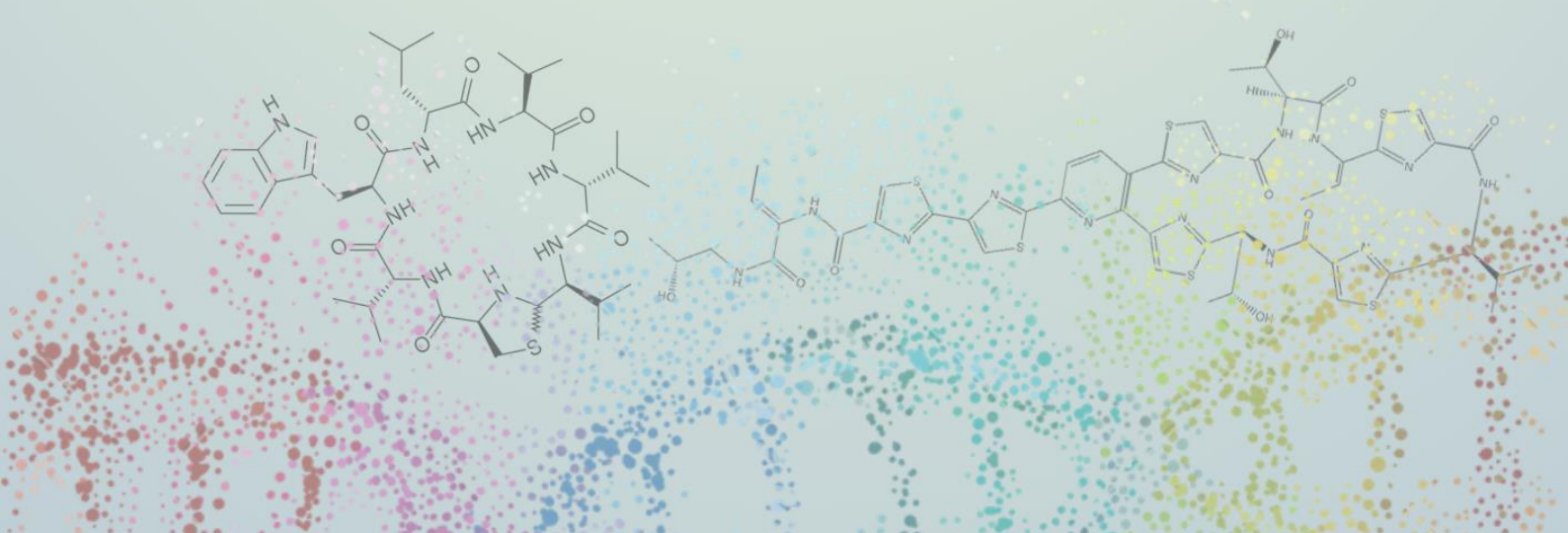
Ante la grave problemática de la resistencia a los antibióticos, es cada vez más evidente la necesidad de nuevas estrategias para combatir las bacterias patógenas y mejorar los ecosistemas. La comunidad científica aboga por la búsqueda de compuestos naturales como agentes terapéuticos de nueva generación entre los que cabe destacar las bacteriocinas, péptidos con actividad antimicrobiana producidos por bacterias que constituyen un mecanismo de adaptación a entornos hostiles. Generalmente son sintetizados a nivel ribosomal, son resistentes a la temperatura y al calor, aunque no a la proteólisis y tienen un espectro de acción reducido y específico.

Dentro de los microorganismos productores, las especies del género *Staphylococcus* pueden tener un especial interés por las bacteriocinas que producen y por su importante papel como moduladores de la microbiota, especialmente nasal o cutánea. Asimismo, los estafilococos presentan una gran ubicuidad y versatilidad pudiendo encontrarse en el ser humano, los animales, el medio ambiente e incluso en los alimentos. Se distingue entre SCoP y SCoN, siendo de especial interés la detección de cepas de SCoN productoras de estafilococcinas por presentar baja asociación con procesos infecciosos.

Si bien existen estudios sobre bacteriocinas detectadas en estafilococos, éstos son todavía muy escasos sobre todo atendiendo a cepas de SCoN ambientales. Queda pendiente profundizar en el análisis de muchos aspectos importantes acerca de su potencial aplicación. Esta tesis parte de la hipótesis de que las cepas de SCoN productoras de bacteriocinas y los extractos obtenidos de las mismas, pueden suponer una fuente importante de sustancias antimicrobianas con amplia aplicación en salud pública y en agroalimentación. Por ello, es de especial relevancia llevar a cabo un amplio muestreo de bacteriocinas en cepas de estafilococos de muy diversos orígenes y estudiar su capacidad de producción frente a múltiples indicadores. Asimismo, es importante evaluar las características de las bacteriocinas producidas y los determinantes genéticos que las codifican, su posible actividad combinada con antibióticos y aplicaciones en diversos ámbitos.

En base a ello, se presenta esta tesis como compendio de publicaciones donde se abordan todos los aspectos anteriormente planteados en diferentes capítulos para, en su conjunto, tratar de completar los escasos conocimientos actuales sobre la producción de bacteriocinas y más concretamente de estafilococcinas. En definitiva, esta tesis aporta evidencias prometedoras del uso de las bacteriocinas como nuevas alternativas frente al uso de antibióticos reflejando que las bacterias no sólo son el problema, sino que pueden ser una solución efectiva frente a la resistencia a los antibióticos.

OBJETIVOS
OBJECTIVES



Objetivos/Objectives

1. Detectar y seleccionar bacterias productoras de sustancias antimicrobianas (Bac^+) en una colección de cepas de *Staphylococcus* de diferentes especies y orígenes y llevar a cabo una caracterización feno/genotípica preliminar de las mismas.
2. Estudiar genéticamente las cepas Bac^+ con el fin de evaluar los determinantes genéticos asociados a la producción de bacteriocinas, su clasificación y diferenciación, así como también determinar los posibles mecanismos de resistencia a antibióticos, virulencia y la presencia de elementos genéticos móviles.
3. Caracterizar la actividad antimicrobiana de las cepas Bac+ seleccionadas y estudiar la naturaleza peptídica de las sustancias producidas:
    3.1 Pre-purificar los péptidos antimicrobianos de las cepas Bac+ seleccionadas y analizar su actividad antimicrobiana.
    3.2 Realizar estudios de competencia entre cepas Bac+ y otras cepas de *Staphylococcus* de interés.
    3.3 Realizar una comparativa con la actividad antimicrobiana de bacteriocinas comerciales (nisina) frente a patógenos de interés como SARM-CC398.
    3.4 Estudiar la actividad combinada de bacteriocinas pre-purificadas en este estudio y antibióticos de uso en clínica.
4. Evaluar las posibles aplicaciones tanto de las bacterias productoras como de sus extractos prepurificados frente a patógenos de interés en el sector agroalimentario y en salud pública y como moduladores de la microbiota nasal utilizando como organismo modelo la cigüeña.
5. Estudiar la actividad antimicrobiana en otros géneros bacterianos a partir de muestras de suelo obtenidas por medio de un proyecto de aprendizaje-servicio.

Objetivos/Objectives

1. To detect and select antimicrobial-producing bacteria (Bac+) in a collection of *Staphylococcus* isolates of different species and origins and to carry out a preliminary phenotypic/genotypic characterization of these isolates.

2. To characterize the producing isolates at genetic level in order to evaluate the genetic determinants associated with the production of bacteriocins, their classification and differentiation, as well as to detect the possible mechanisms of antibiotic resistance, virulence and the presence of mobile genetic elements.
3. To evaluate the antimicrobial activity of the selected isolates and study the peptide nature of the substances produced:

    3.1 To pre-purify antimicrobial peptides from selected Bac+ isolates and to evaluate their antimicrobial activity.

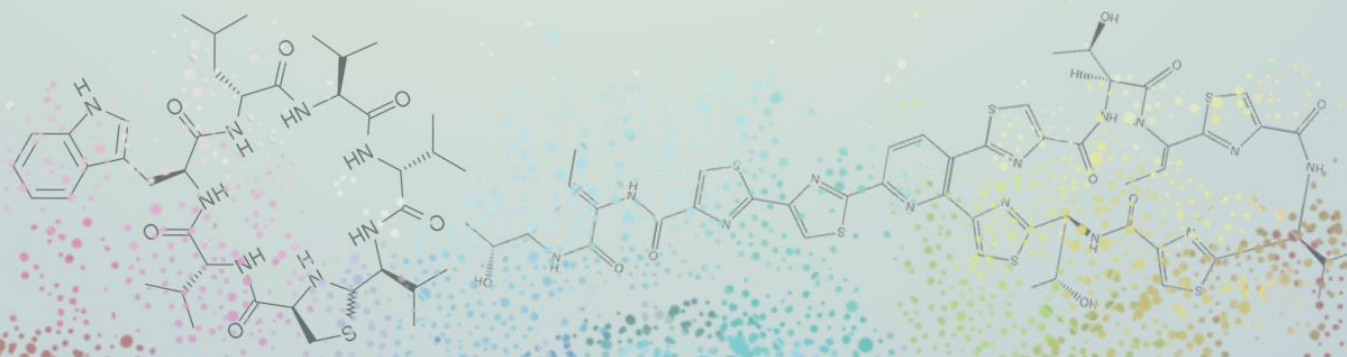
    3.2 To carried out competition studies between Bac+ isolates and other *Staphylococcus* isolates of interest.

    3.3 To perform a comparison with the antimicrobial activity of commercial bacteriocins (nisin) against pathogens of interest such as MRSA-CC398.

    3.4 To study the combined activity of pre-purified bacteriocins in this study and antibiotics used in the clinic.

4. To evaluate the potential applications of both the producing bacteria and their prepurified extracts against pathogens of interest in the agri-food sector and in public health and as modulators of the nasal microbiota using the stork as a model organism.
5. To study the antimicrobial activity in other bacterial genera from soil samples obtained through a service-learning project.

METODOLOGÍA



Metodología

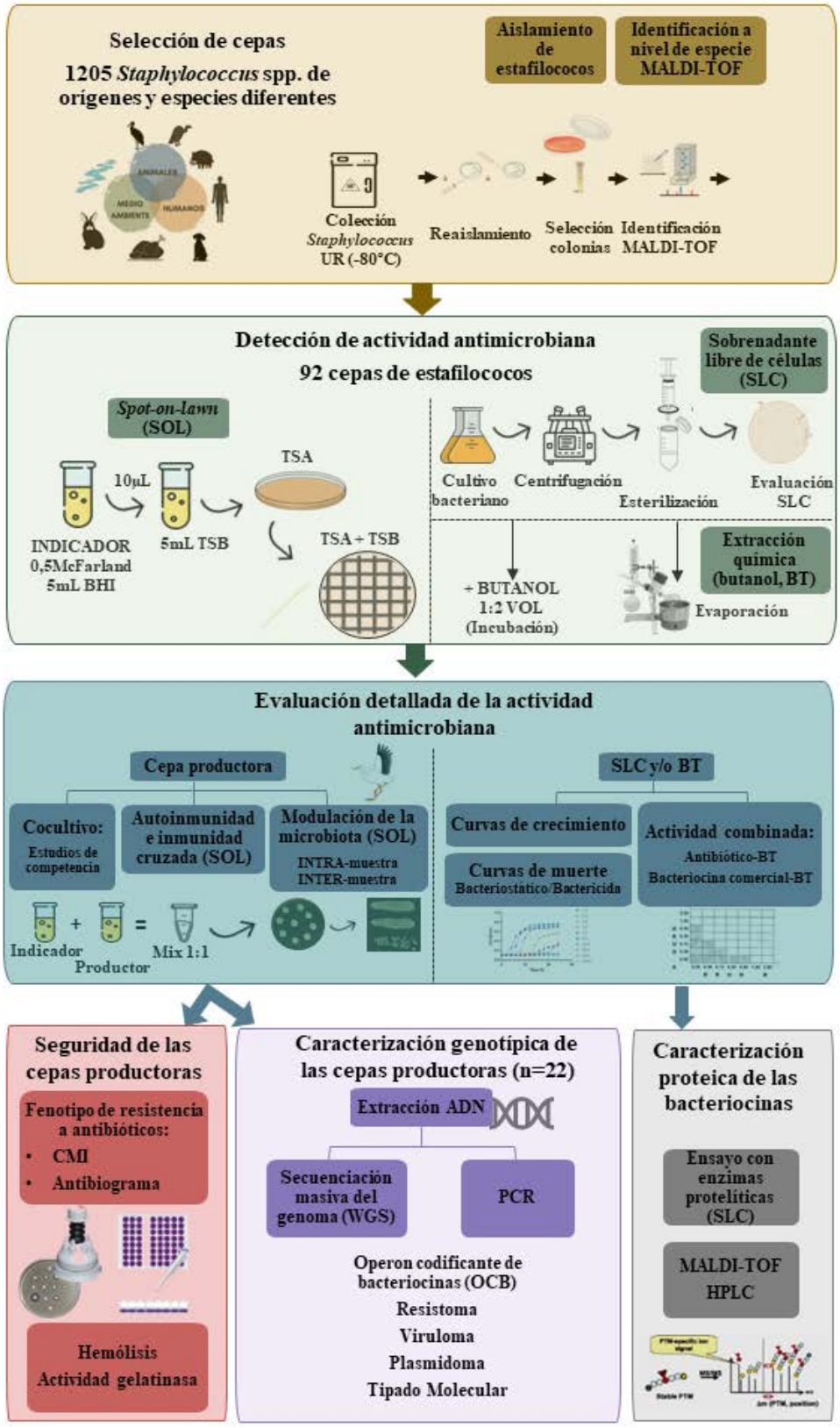


Figura 16 Resumen gráfico de los métodos llevados a cabo en esta tesis doctoral de forma secuencial (elaboración propia).

Metodología

Con el objetivo de facilitar la comprensión del lector y de ayudar a la reproducibilidad de la metodología seguida en esta tesis, se incluyen en este apartado cada una de las técnicas indicando el apartado correspondiente a los artículos en los cuales dichas técnicas fueron utilizadas (veáse apartado de resultados)

I. Identificación a nivel de especie por espectrometría de masas MALDI-TOF……1.1, 1.2

II. Detección de actividad antimicrobiana

Spot-on-lawn …… 1.1, 1.2, 3.1, 4.1, 4.2, 5.1

Sobrenadantes libres de células crudos (SLC) …… 3.1, 4.1 y 4.2

Extracción química con butanol (BT) ……3.1, 4.1 y 4.2

III. Análisis de inmunidad cruzada……4.2

IV. Caracterización fenotípica de las cepas productoras

Resistencia a antibióticos

Difusión disco placa o antibiograma……1.1, 1.2, 4.1 y 5.1

Concentración mínima inhibitoria (CMI)……3.1

V. Aspectos de seguridad

Actividad hemolítica ……4.2

Actividad gelatinasa……4.2

VI. Caracterización genotípica de las cepas productoras

Extracción de ADN……1.1, 1.2

Análisis del genotipo de resistencia……1.1, 1.2

Estudio de genes de virulencia……1.1, 1.2

Estudio de genes codificantes de estafilococcinas……1.1, 1.2 y 4.1

Tipado molecular……1.1

Análisis de secuenciación masiva del genoma……2.2

Resistoma

Viruloma

Plasmidoma

Operones codificantes de bacteriocinas

VII. Determinación de la naturaleza proteica del compuesto con actividad antimicrobiana

Tratamiento con enzimas proteolíticas……4.1

Identificación de la bacteriocina por espectrometría de masas (MALDI-TOF o uHPLC)…… 3.1

VIII. Análisis de actividad antimicrobiana frente a bacterias patógenas…… 3.1

Curvas de crecimiento (con SLC y BT)

Determinación de la actividad bacteriostática o bactericida de los extractos

Actividad combinada de bacteriocina y antibiótico (método checkedboard)

Actividad comparada con bacteriocinas comerciales

Estudios de competencia entre cepas productoras de bacteriocinas y patógenos

IX. Actividad antifúngica……4.2

X. Herramientas bioinformáticas y bases de datos……2.2

NCBI

antiSMASH

BAGEL4

Center of Genomic Epidemiology: ResFinder, VirulenceFinder y PlasmidFinder

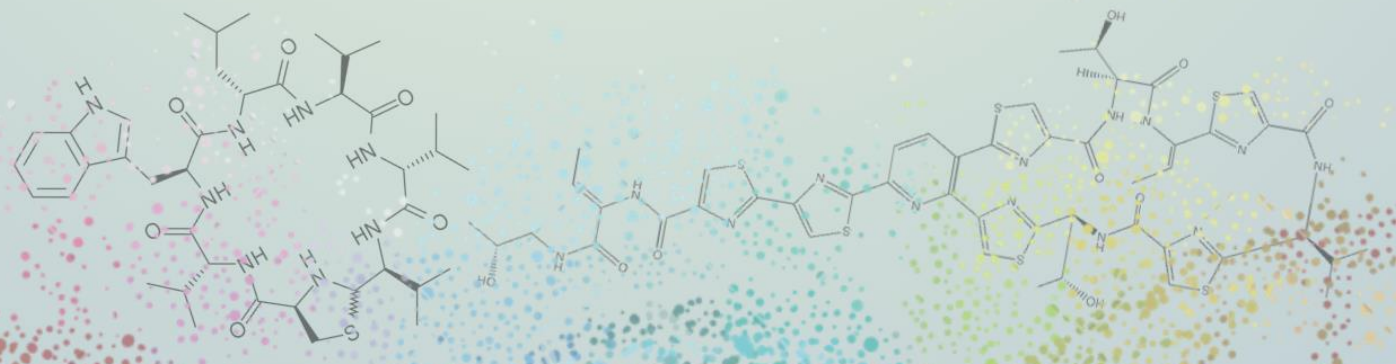
Blast

EmbossNedlee

PubMLST

RidomSPA
ClustalW2
Jalview
Clinker
MAFFT
Mauve
Geneious

RESULTADOS



Capítulo 1

Resultados

1. Capítulo 1: Detección de cepas de *Staphylococcus* con actividad antimicrobiana y caracterización preliminar de las mismas

Las bacterias se establecen en nichos complejos por medio de relaciones entre especies y con el ambiente y poseen la capacidad de adquirir y transferir mecanismos de adaptación. El éxito de su supervivencia puede incluir capacidades como una mayor especificidad a un número limitado de sitios de fijación del hospedador, una mayor capacidad para absorber y metabolizar micronutrientes o la producción de sustancias antimicrobianas, como las bacteriocinas. Éstas, son mayoritariamente péptidos sintetizados por síntesis ribosomal con propiedades antimicrobianas que desde un punto de vista ecológico se sintetizan para conferir una ventaja selectiva al productor. En este sentido, la cavidad nasal puede ser un ejemplo de nicho estresante ya que presenta dificultades para la supervivencia de los microorganismos debido a su alta salinidad o a la limitación de nutrientes. Por ello, lamicrobiota nasal es una comunidad idónea para la detección de bacterias productoras de sustancias antimicrobianas, normalmente adaptadas a condiciones de estrés ambiental específicas como la liberación de peróxido de hidrógeno y la limitación de hierro.

El género *Staphylococcus* se caracteriza por su metabolismo flexible, su versatilidad y su gran capacidad para desarrollarse en todo tipo de ambientes tales como superficies bióticas y abióticas, animales, seres humanos, plantas, alimentos etc. Así encontramos estafilococos como comensales de animales y humanos, en el medio ambiente y en alimentos, entre otros ambientes, aunque también pueden actuar como patógenos oportunistas.

Dentro del género *Staphylococcus*, se han identificado aislados de estafilococos tanto SCoP como SCoN, productores de bacteriocinas. Cabe destacar la especie de SCoN *Staphylococcus lugdunensis* componente habitual de la microbiota normal de la piel humana y de la cavidad nasal. Estudios recientes han detallado que *S. lugdunensis* puede producir un nuevo péptido cíclico antimicrobiano denominado lugdunina, que se incluye en una nueva clase de antibacterianos debido a su síntesis no ribosomal.

Debido al creciente interés por la identificación y caracterización de nuevos agentes antimicrobianos, en este capítulo se aborda el primer objetivo planteado en esta tesis doctoral que consiste en la detección de cepas de *Staphylococcus* productoras de sustancias antimicrobianas pertenecientes a diferentes especies y orígenes frente a bacterias indicadoras G+/- relevantes en seguridad alimentaria y en salud pública **(Figura 17)**, incluyendo bacterias multirresistentes y organismos zoonóticos. Asimismo, se lleva a cabo un estudio preliminar de genes estructurales

de estafilococcinas en cepas de *Staphylococcus* de interés seleccionadas como productoras de bacteriocinas (Bac+) **(Tabla 5).**

En el marco de esta tesis, se ha evaluado la producción de sustancias antimicrobianas por el método de *spot-on-lawn* en un total de 946 aislados de estafilococos (238 SCoP y 708 SCoN) de 29 especies y distintos orígenes frente a una amplia selección de bacterias indicadoras (diferentes géneros/especies), incluidos microorganismos multirresistentes. Asimismo, los aislados productores (Bac+) se han caracterizado por PCR para determinar la presencia de genes que codifican bacteriocinas. Se ha analizado también el fenotipo y genotipo de resistencia a antibióticos, así como la presencia de genes de virulencia.

Los ensayos realizados han permitido detectar 83 estafilococos Bac+ **(Tabla 6)**, 21 SCoP y 62 SCoN, con actividad antimicrobiana frente a bacterias indicadoras G+, incluyendo *Staphylococcus* coagulasa positivos sensibles y resistentes a meticilina. Las cepas Bac+ pertenecen a 11 especies diferentes de estafilococos, mayoritariamente SCoN, y han sido identificadas en su gran mayoría en animales de vida libre (n=25), seguido de humanos (n=24), alimentos (n=17), mascotas (n=15), y medio ambiente (n=2). Entre ellos, destacan 8 cepas Bac+ con alta actividad antimicrobiana (inhibieron >70% de los indicadores estudiados), 23 cepas Bac+ con actividad antimicrobiana moderada (entre 20% y 70% de los indicadores fueron inhibidos). No obstante, la gran mayoría (52 aislados Bac+) fueron productores con baja actividad (inhibieron <20% de los indicadores analizados).

En cuanto a la caracterización genética de los 83 aislados Bac+ se han observado los siguientes resultados: (a) 11 cepas portaban genes estructurales que codificaban potencialmente lantibióticos (bacteriocinas de clase I); (b) 3 cepas de *S. pseudintermedius* albergaban el gen estructural codificante de la bacteriocina BacSp222; (c) 2 cepas de *S. chromogenes* portaban el gen estructural de una bacteriocina cíclica recientemente descrita (similar a la uberolisina), siendo el primer informe en esta especie de SCoN especie; (d) 23 aislados de *S. lugdunensis* fueron positivos para el gen *lug*D codificante de la bacteriocina cíclica de síntesis no ribosomal lugdunina.

Por otro lado, las pruebas de sensibilidad a antibióticos y se ha observado que el 31,5% de los estafilococos Bac+ fue sensible a todos los antibióticos estudiados (principalmente SCoN), y además carecían de los genes de virulencia estudiados. Estos SCoN Bac+ resultaron muy buenos candidatos para nuevos estudios de caracterización.

1.1 Artículo 1 Bacteriocin-like inhibitory substances in staphylococci of different origins and species with activity against relevant pathogens

1.2 Artículo 2 Antimicrobial resistance and antimicrobial activity of *Staphylococcus lugdunensis* obtained from two Spanish hospitals

1.1 Artículo 1: Bacteriocin-like inhibitory substances in staphylococci of different origins and species with activity against relevant pathogens

En este estudio se analizó la producción de sustancias antimicrobianas por el método de *spot-on-lawn* en una colección de 890 estafilococos de distintos orígenes (humanos, animales, alimentos y medio ambiente), y especies, tanto coagulasa positivos (SCoP, 238 cepas de 3 especies) como coagulasa negativos (SCoN, 652 cepas de 26 especies). Posteriormente, se evaluó la presencia de genes codificantes de estafilococcinas por PCR/secuenciación en las cepas productoras seleccionadas (Bac+) en las cuales se estudió también el perfil de resistencia a antibióticos tanto a nivel fenotípico (difusión en disco) como los determinantes genéticos asociados a mecanismos de resistencia y virulencia (PCR).

De los 890 estafilococos, 60 cepas (6,7%) mostraron actividad antimicrobiana contra al menos una de las 25 bacterias indicadoras probadas. Entre las cepas productoras de bacteriocinas (Bac+), el 8,8% fueron SCoP y en el 6,0% SCoN. Cabe destacar 13 aislados Bac+ con un amplio espectro de actividad antimicrobiana, y 7 de ellos (de las especies *S. aureus, S. pseudintermedius, S. sciuri y S. hominis*) demostraron actividad antimicrobiana frente a más del 70% de las bacterias indicadoras ensayadas. Además, la mayoría de las cepas Bac+ fueron aisladas de animales domésticos, salvajes y alimentos. En cuanto a la caracterización genética (por PCR y secuenciación) de los 60 aislados Bac+, se detectaron genes codificantes de estafilococcinas (lantibióticos, BacSp222 y una bacteriocina cíclica similar a la uberolisina). Finalmente, en las pruebas de sensibilidad antimicrobiana, se evidenció que un tercio de los aislados SCoN mostraron sensibilidad a todos los antibióticos analizados y no portaron ninguno de los genes de virulencia estudiados.

1.2 Artículo 2: Antimicrobial resistance and antimicrobial activity of S*taphylococcus lugdunensis* obtained from two Spanish hospitals

En este trabajo se quiso evaluar la resistencia, virulencia y producción de bacteriocinas en una colección clínica de cepas *S. lugdunensis* ya que esta especie de SCoN ha ganado especial atención en la salud pública como patógeno humano y también como bacteria productora de bacteriocinas. De este modo, se caracterizaron 56 aislados de *S. lugdunensis* recuperados de muestras humanas en dos hospitales españoles. La producción de actividad antimicrobiana (AA) se evaluó en todos ellos por el método de *spot-on-lawn* frente a 37 bacterias indicadoras,

incluyendo MDR, y se determinó por PCR la presencia del gen *lug*D, que codifica para la bacteriocina no ribosomal lugdunina.

El ensayo de actividad antimicrobiana reveló 23 aislados Bac+ con inhibición relevante frente a *S. aureus* sensible y resistente a meticilina, entre otros. El gen *lug*D se detectó en el 84% de los 56 aislados de *S. lugdunensis* (23 cepas Bac+ y 24 cepas Bac-), lo que sugiere diferentes niveles de expresión de dicho gen. Finalmente, 21 cepas de *S. lugdunensis* mostraron sensibilidad a los 20 antibióticos probados (37,4%) mientras que los porcentajes de resistencia detectados fueron los siguientes (% de resistencia/genes detectados): penicilina (44,6%/*blaZ*), oxacilina (1,8%/*mec*A en SCC*mecV*), eritromicina-clindamicina inducible (7,1%/*erm*(C), *msrA*), tetraciclina (5,3%/*tetK*), gentamicina y/o tobramicina (3,6%/*ant*(40)-Ia, *acc*(6)-*aph*(2")), y fosfomicina (21,4%). El 5% de los aislados presentó fenotipo de multirresistencia.

1.3 Resumen gráfico del Capítulo 1

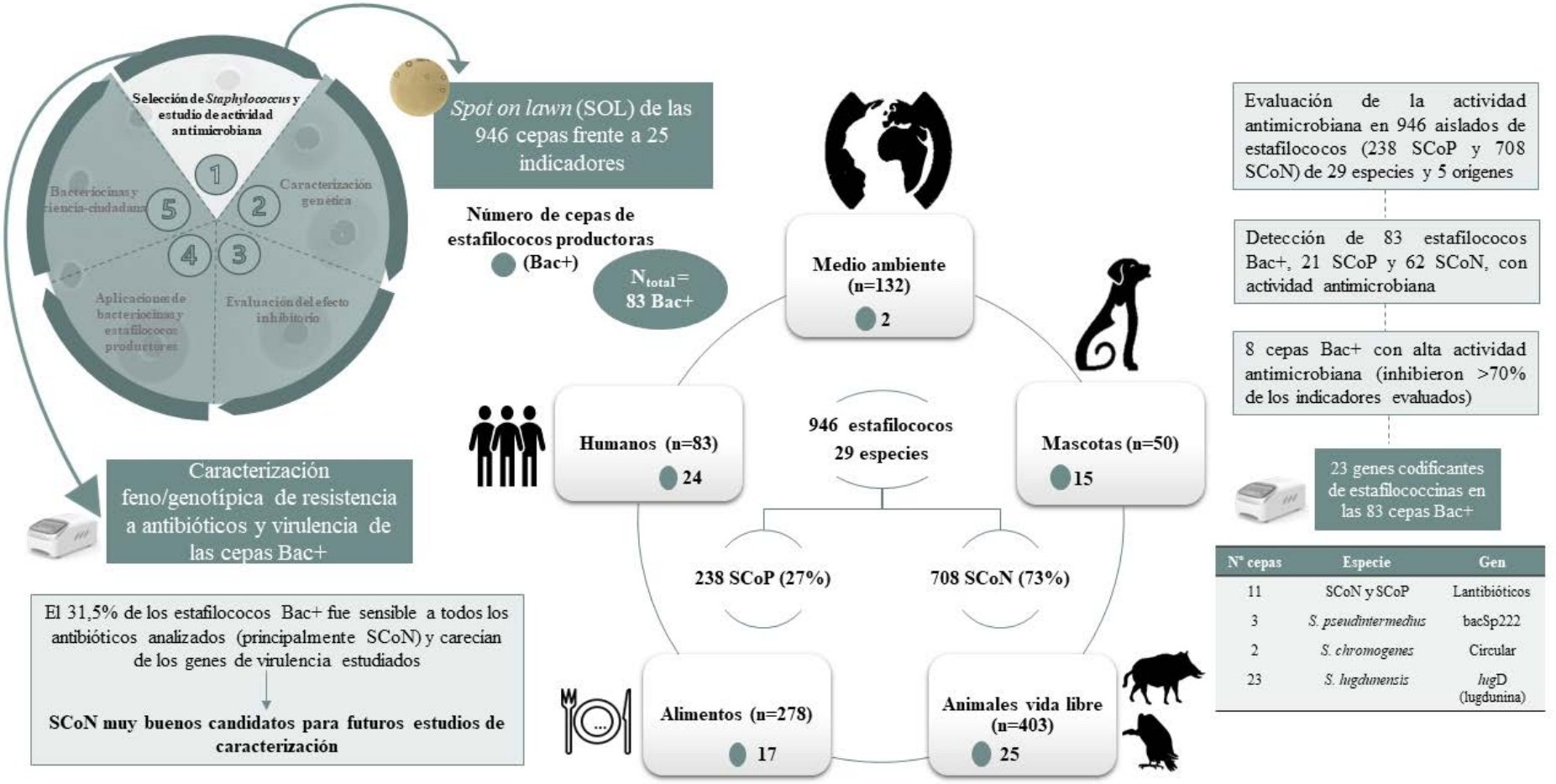


Figura 17 Resumen gráfico de la metodología y resultados obtenidos en el primer capítulo referente a la detección de cepas de *Staphylococcus* productoras de sustancias antimicrobianas (elaboración propia).

Artículo 1

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

*Rosa Fernández-Fernández, Carmen Lozano, Paula Eguizábal, Laura Ruiz-Ripa, Sandra Martínez-Álvarez, Idris Nasir Abdullahi, Myriam Zarazaga and Carmen Torres**

Area of Biochemistry and Molecular Biology, OneHealth-UR Research Group, University of La Rioja, Logroño, Spain

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***Correspondence:**
Carmen Torres
carmen.torres@unirioja.es

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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 activiy 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 fibupeptide lugdunin (a major non-ribosomal peptide, NRP) described by Zipperer et al. (2016). Moreover, bacteriocins previously described in other genera, such as the thiopeptide Micrococcin 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*, *Clostridium*, *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
	S. delphini	31/0	0	19/0	12/0	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%)	4/0	17/4 (23.5%)	5/0	0	12/0
	S. fleurettii	29/0	0	14/0	15/0	0	0
	S. chromogenes	26/10 (38.5%)	0	7/3 (42.8%)	17/7 (41.17%)	0	2/0
	S. warneri	26/6 (23.1%)	1/0	24/6 (25%)	0	0	1/0
	S. vitulinis	24/0	0	5	19	0	0
	S. simulans	22/1 (4.5%)	0	16/0	3/1 (33.3%)	0	3/0
	S. arlettae	21/0	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%)	1/0	3/0	1/0	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	4/1 (25%)	0	0
	S. succinus	6/0	0	0	5/0	0	1/0
	S. haemolyticus	5/0	0	0	2/0	0	3/0
	S. nepalensis	5/0	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	1/0	0	0	0	0	1/0
	S. felis	1/0	0	0	1/0	0	0
	S. lugdunensis	1/0	1/0	0	0	0	0
	S. simiae	1/0	0	0	1/0	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 lysostaphin (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*, *epiA*, *sacaA/sacbA*, *gdmA*, *bacSp222*, *nsj*, *hyiA*, *hycS*, *bacCH91*, *bsaA2*, *lugD*, *acIA*, *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, streptomycin, 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: *bla*Z, *mec*A, *mec*B, *mec*C, *erm*(A), *erm*(B), *erm*(C), *erm*(T), *msr*(A), *mph*(C), *Inu*(A), *Inu*(B), *vga*(A), *sal*(A), *aac(6′)-Ie-aph(2″)-Ia*, *ant*(4′)-Ia, *str*, *ant*(6), *tet*(L), *tet*(M), *tet*(K), *fex*(A), *fex*(B), cat_{pC194}, cat_{pC221}, cat_{pC223}, *fus*B, *fus*C, *fus*D, *dfr*A, *dfr*D, *dfr*G, and *dfr*K (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 *luk*SF-PV, *luk*M, *luk*ED, and *luk*PQ, 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 *luk*S/F-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 SCC*mec* 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).

TABLE 2 | Indicator bacteria used in the antimicrobial test, differentiated in terms of genera and species.

Type of bacteria	Genera	Indicator Bacteria (n = 25)[a]	UR-Reference
Gram+	*Staphylococcus*	MRSA (2)	C1570, C5313
		MSSA	C411
		MRSP	C2381
		MSSP	C3468
		S. delphini	C9459
		S. epidermidis	C2663
		S. haemolyticus	C2709
		S. lugdunensis	C10107
		S. sciuri	C9780
	Enterococcus	*E. casseliflavus*	C1232
		E. durans vanA	C1433
		E. faecalis	C410
		E. faecium vanA	C2321
		E. gallinarum	C2310
		E. hirae vanA	C1436
	Micrococcus	*M. luteus*	C157
	Listeria	*L. monocytogenes*	C137
	Streptococcus	*S. suis* (4)	X2057, X2058, X2060, X2061
	Clostridium	*C. perfringens*	X2967
Gram−	*Escherichia*	*E. coli*	C408
	Pseudomonas	*P. aeruginosa*	C3282

[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.*

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 $p \leq 0.05$.

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%)

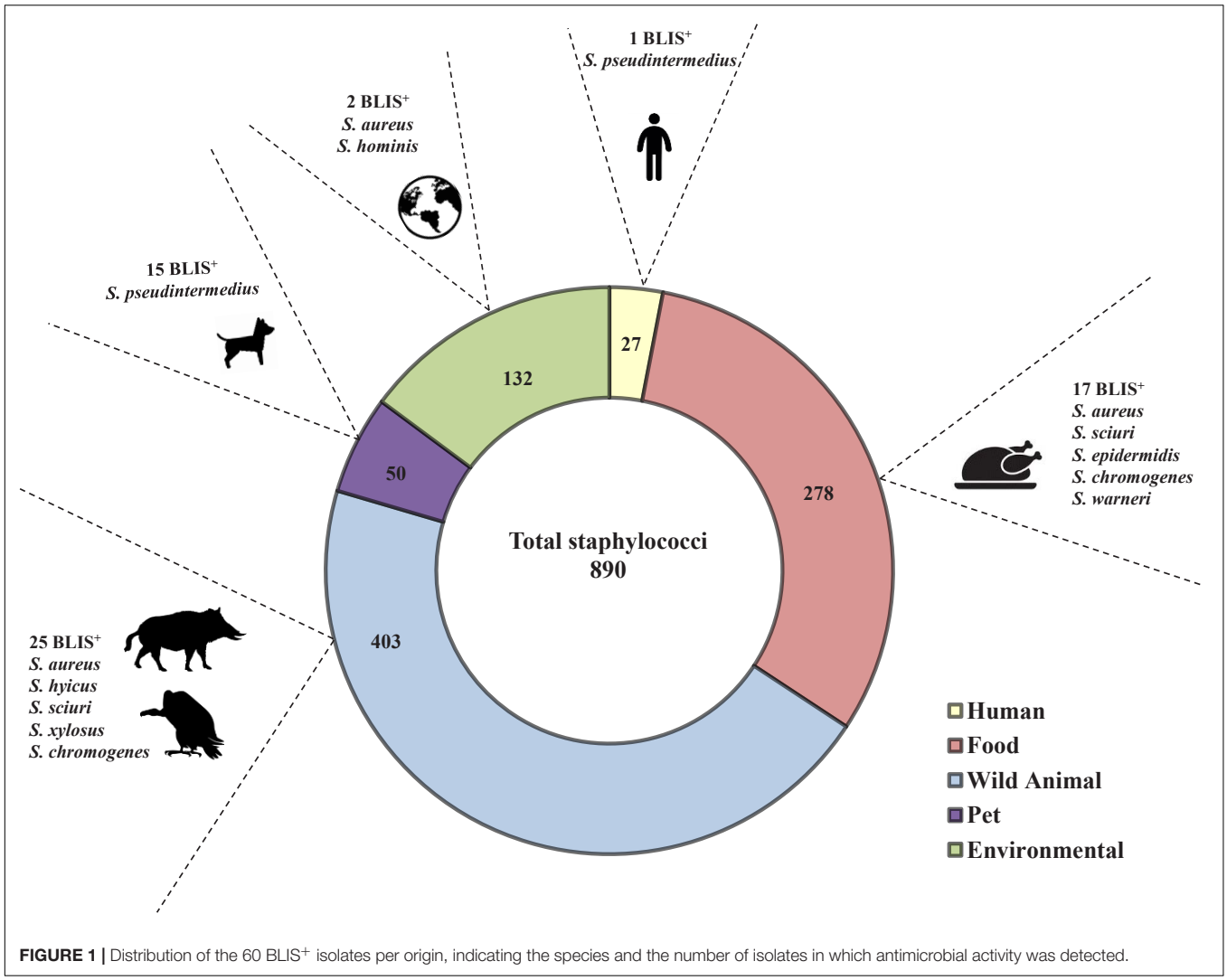

FIGURE 1 | Distribution of the 60 BLIS$^+$ isolates per origin, indicating the species and the number of isolates in which antimicrobial activity was detected.

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 ($p \leq 0.05$). 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

TABLE 3 | Antimicrobial activity of the 60 bacteriocin-like inhibitory substances producer (BLIS$^+$) isolates against 25 indicator bacteria.

Indicator bacteria (No isolates)[a]	**No of BLIS$^+$ isolates[b]**										
	***S. pseudintermedius* (16)**	***S. aureus* (5)**	***S. sciuri* (13)**	***S. chromogenes* (10)**	***S. warneri* (6)**	***S. epidermidis* (4)**	***S. xylosus* (3)**	***S. hominis* (1)**	***S. hyicus* (1)**	***S. simulans* (1)**	**No/%[c]**
MRSA (2)	10	2	2	2	0	0	0	1	0	1	18/30
MSSA	0	2	2	2	0	0	0	1	0	1	8/13
MRSP	7	1	2	0	1	1	0	1	0	0	13/22
MSSP	9	2	12	6	1	1	0	1	0	1	33/55
S. epidermidis	6	1	2	0	0	1	0	1	1	1	13/22
S. delphini	10	4	13	6	0	1	0	1	1	1	37/62
S. haemolyticus	0	0	0	0	1	1	0	0	0	1	3/5
S. lugdunensis	5	1	11	0	0	0	0	1	1	1	20/33
S. sciuri	3	2	2	6	0	1	0	1	0	1	16/27
E. casseliflavus	3	1	2	1	0	0	0	1	1	0	9/15
E. durans vanA	3	2	2	1	0	0	0	1	0	0	9/15
E. faecalis	7	1	2	0	0	0	0	1	0	0	11/18
E. faecium vanA	3	1	2	2	0	0	0	1	1	0	10/17
E. gallinarum	8	1	2	2	0	0	0	1	1	0	15/25
E. hirae vanA	6	2	2	2	0	0	0	1	1	0	14/23
L. monocytogenes	4	1	2	2	0	0	0	1	0	0	10/17
M. luteus	4	2	2	7	1	2	3	1	1	0	23/38
S. suis (4)	12	4	2	1	0	0	0	0	0	0	19/32
C. perfringens	5	3	2	1	6	2	0	1	1	0	21/35
E. coli/P. aeruginosa	0	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.*

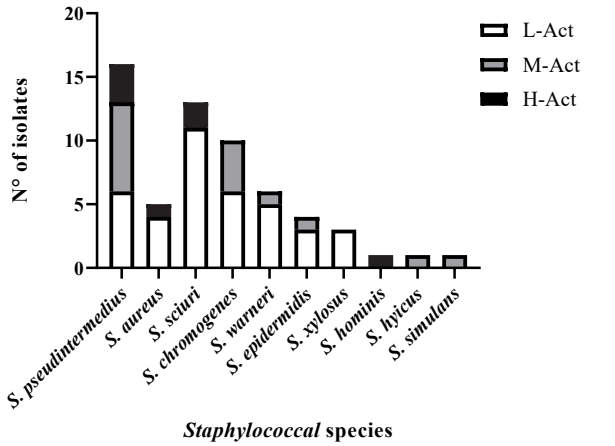

FIGURE 2 | Antimicrobial activity level [low (L-Act), medium (M-Act), or high (H-Act)] of the 60 BLIS$^+$ isolates (of 10 staphylococcal species) according to the percentage of indicator bacteria inhibited (L-Act: <20%; M-Act: 20–70%; H-Act: >70%).

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 specie *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 *inu*(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 pandemy, 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 staphylococcins in strains from food products, such as milk (Hyicin 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) (Cundliffe 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	**Pet**		**Environmental**	**Food**		**Environmental**	**Wild animal**		**Food**	**Food**	**Wild animal**	**Wild animal**
MR-*Staphylococcus* (3)	3	3	3	3	3	3	3	0	0	2	2	0	3
MS-*Staphylococcus* (7)	3	3	3	6	6	6	6	3	0	2	5	3	7
Total *Staphylococcus spp.* (10)	6	6	6	9	9	9	9	3	0	2	7	3	10
VR-*Enterococcus* (3)	3	3	3	3	3	3	3	3	2	0	0	2	0
VS-*Enterococcus* (3)	3	3	3	3	3	3	3	2	0	0	0	2	0
Total *Enterococcus* spp. (6)	6	6	6	6	6	6	6	5	2	0	0	4	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	0	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.*

TABLE 5 | Characteristics of the BLIS$^+$ isolates that carry bacteriocin encoding genes.

Species	Strain	Origin	Antimicrobial level[a]	Bacteriocins detected	Antimicrobial resistance phenotype-[genotype][b]	Virulence content	*spa*-MLST-CC/*agr*
S. pseudintermedius	C4502	Pet	M-Act	Lantibiotic-like	SXT-[*dfr*G]	*se-int, sea1, sed1 siet, luk*S/F-I	ST160/III
	C8189	Human	H-Act	Bacsp222	ERY-CLI-[*erm*(B)]	*luk*S/F-I,*siet*	ST241/III
	C8478	Pet	H-Act	Bacsp222	ERY-CLI-[*erm*(B)]	*luk*S/F-I,*siet*	ST241/III
	C8479	Pet	H-Act	Bacsp222	ERY-CLI-[*erm*(B)]	*luk*S/F-I,*siet*	ST241/III
S. aureus	C5802	Environment	H-Act	Lantibiotic-like	PEN-[*blaZ*]	*luk*MF′,*luk*ED, *etD2*	t843-ST130-CC130/III
	C6770	Wild animal	L-Act	Lantibiotic-like	Susceptible	–	t1125-CC5/II
	C8609	Wild animal	L-Act	Lantibiotic-like	Susceptible	–	t11225-CC425/II
	X3410	Food	L-Act	Lantibiotic-like	Susceptible	–	t10234/I
S. chromogenes	C9838	Wild animal	M-Act	Uberolysin-like	Susceptible	–	NS[c]
	C9581	Wild animal	M-Act	Uberolysin-like	Susceptible	–	NS[c]
	C9727	Wild animal	M-Act	Lantibiotic-like	Susceptible	–	NS[c]
	C9726	Wild animal	M-Act	Lantibiotic-like	Susceptible	–	NS[c]
S. epidermidis	X3009	Food	M-Act	Lantibiotic-like	ERY-FA-[*msr*(A), *mph*(C)]	–	ST1025
S. xylosus	C9255	Wild animal	L-Act	Lantibiotic-like	PEN-[*blaZ*]	–	NS[c]
S. hyicus	C9585	Wild animal	M-Act	Lantibiotic-like	Susceptible	–	NS[c]
S. simulans	C9832	Wild animal	M-Act	Lantibiotic-like	FA-TET-[*tet*(K)]	–	NS[c]

[a]*Antimicrobial activity levels against the indicator bacteria tested (L-Act, low <20%; M-Act, medium, 20–70%; H-Act, high >70%).*
[b]*CLI, clindamycin; ERY, erythromycin; FA, fusidic acid; PEN, penicillin; SXT, trimethoprim sulfamethoxazole; TET, tetracycline.*
[c]*NS, non-studied.*

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, NisinJ, and Staphylococcin C55) (Dajani and Wannamaker, 1969; Lynch et al., 2019; O'Sullivan et al., 2020) and nose (Nukacin IVK45 and Lugdunin) (Janek et al., 2016; Zipperer et al., 2016).

In this study, production of BLIS was found in 6.7% of isolates of a large collection of staphylococci (both CoPS and CoNS) of many different origins (wild animal, food, pet, environment, and human) that were tested against a wide series of indicator bacteria, including MDR bacteria, and also bacteria with relevant mechanisms of AMR or with zoonotic interest. According to our results, the species with the highest percentages of BLIS$^+$ isolates were *S. chromogenes* (38.5%), *S. pseudintermedius* (26.7%), and *S. warneri* (23.1%), which highlights the capacity of CoNS, usually considered as common colonizers and non-pathogenic bacteria, to compete against other bacteria, including pathogens (Janek et al., 2016; O'Sullivan et al., 2019). The percentages of BLIS$^+$ isolates in other staphylococcal species were lower: *S. hyicus* (16.7%), *S. hominis* (12.5%), *S. epidermidis* (10.5%), *S. xylosus* (6.25%), *S. sciuri* (6.1%), and *S. aureus* (3.4%). *S. epidermidis* has been described as one of the most frequent bacteriocin-producing CoNS species, mainly among human isolates (Bastos et al., 2009; Janek et al., 2016). Although all human *S. epidermidis* isolates tested in our study were BLIS-negative, *S. epidermidis* was the fifth CoNS species with higher antimicrobial activity rates in our study, although all the BLIS$^+$ *S. epidermidis* isolates were recovered from food.

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 staphylococcins, a wide variety of antimicrobial profiles against pathogens have been described depending on the peptide (*Streptococcus*, *Enterococcus*,

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	PEN^{10}-OXA^{1}-ERY^{7}-CLI^{6}-STR^{5}-TET^{6}-CHL^{3}-SXT^{6}-FA^{1}	*blaZ*^{10}, *mecA*^{1}, *erm*(B)^{7}, *aadE*^{4}, *tet*(M)^{6}, *cat*$_{pC221}$^{3}, *dfrG*^{6}, *lnu*(A)^{1}, *ant*(6)-Ia^{1}	*lukS/F-I*^{16}, *siet*^{16}, *se-int*^{12}, *sec1*^{1}, *expB*^{1}
S. aureus	5	3	PEN^{2}-ERY^{1}-CLI^{I} ^{1}	*blaZ*^{2}, *msr*(A)^{1}, *erm*(C)^{1}, *erm*(T)^{1}, *lnu*(A)^{1}	*lukMF'*^{1}, *lukED*^{1}, *etD2*^{1}
S. sciuri	13	0	ERY^{2}-CLI^{I} ^{13}-FA^{6}-TOB^{1}-CIP^{2}-SXT^{1}	*erm*(B)^{3}, *msr*(A)^{1}, *sal*(A)^{13}, *lnu*(A)^{4}, *ant*(4')-Ia^{1}, *dfrA*^{1}	–
S. chromogenes	10	8	CIP^{1}-TET^{2}	*tet*(M)^{2}, *tet*(K)^{1}, *tet*(L)^{1}	–
S. warneri	6	2	PEN^{3}-ERY^{2}-CIP^{1}-TET^{2}	*blaZ*^{3}, *tet*(K)^{2}, *erm*(B)^{2}	–
S. epidermidis	4	0	PEN^{2}-FOX^{2}-ERY^{3}-SXT^{1}-TET^{1}-FA^{2}	*mecA*^{2}, *msr*(A)^{2}, *mph*(C)^{1}	–
S. xylosus	3	0	PEN^{1}-FA^{2}	*blaZ*^{1}	–
S. hominis	1	1	Susceptible	–	–
S. hyicus	1	1	Susceptible	–	–
S. simulans	1	0	TET^{1}-FA^{1}	*tet*(K)^{1}	–
Total CoPS	21	1	PEN^{12}-OXA^{1}-ERY^{8}-CLI^{I} ^{17}-STR^{5}-TET^{6}-CHL^{3}-SXT^{6}-FA^{1}	*blaZ*^{12}, *mecA*^{1}, *erm*(B)^{7}, *erm*(C)^{1}, *erm*(T)^{1}, *msr*(A)^{1}, *aadE*^{4}, *tet*(M)^{6}, *cat*$_{pC221}$^{3}, *dfrG*^{6}, *lnu*(A)^{2}, *ant*(6)-Ia^{1}	*lukS/F-I*^{16}, *siet*^{16}, *se-int*^{12}, *sec1*^{1}, *expB*^{1}-*lukMF'*^{1}, *lukED*^{1}, *etD2*^{1}
Total CoNS	39	12	PEN^{6}-FOX^{2}-ERY^{7}- CLI^{I} ^{13}-TOB^{1}-TET^{6}-CIP^{4}-SXT^{2}-FA^{11}	*blaZ*^{3}, *mecA*^{2}, *erm*(B)^{5}, *msr*(A)^{3}, *mph*(C)^{1}, *sal*(A)^{13}, *lnu*(A)^{4}, *tet*(K)^{4}, *tet*(M)^{2}, *tet*(L)^{1}, *ant*(4')-Ia^{1}, *dfrA*^{1}	–

[a] *PEN, penicillin; OXA, oxacillin; FOX, cefoxitin; ERY, erythromycin; CLI, clindamycin; CLI^{I}, 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 (Janek 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 (Lozano 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 epidermicin (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 (uberolysin-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 Aureocyclin 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$^+$, 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$^+$ 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.

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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Artículo 2

microorganisms

MDPI

Article

Antimicrobial Resistance and Antimicrobial Activity of *Staphylococcus lugdunensis* Obtained from Two Spanish Hospitals

Rosa Fernández-Fernández [1], Carmen Lozano [1,*], Laura Ruiz-Ripa [1], Beatriz Robredo [2], José Manuel Azcona-Gutiérrez [3], Carla Andrea Alonso [3], Carmen Aspiroz [4], Myriam Zarazaga [1] and Carmen Torres [1,*]

[1] Área Bioquímica y Biología Molecular, OneHealth-UR Research Group, Universidad de La Rioja, 26006 Logroño, Spain; rosa.fernandez.1995@gmail.com (R.F.-F.); laura_ruiz_10@hotmail.com (L.R.-R.); myriam.zarazaga@unirioja.es (M.Z.)
[2] Área Didáctica de las Ciencias Experimentales, OneHealth-UR Research Group, Universidad de La Rioja, 26006 Logroño, Spain; beatriz.robredo@unirioja.es
[3] Servicio de Microbiología, Hospital San Pedro, 26006 Logroño, Spain; jmazcona@riojasalud.es (J.M.A.-G.); caalonso@riojasalud.es (C.A.A.)
[4] Servicio de Microbiología, Hospital Royo Villanova, 50015 Zaragoza, Spain; caspirozs@gmail.com
* Correspondence: carmen.lozano@unirioja.es (C.L.); carmen.torres@unirioja.es (C.T.)

Abstract: *Staphylococcus lugdunensis* is a coagulase-negative-staphylococci (CoNS) that lately has gained special attention in public health as a human pathogen and also as a bacteriocin-producer bacteria. In this study, we characterized 56 *S. lugdunensis* isolates recovered from human samples in two Spanish hospitals. Antimicrobial susceptibility testing was performed and antimicrobial resistance and virulence genotypes were determined. Antimicrobial activity (AA) production was evaluated by the *spot-on-lawn* method against 37 indicator bacteria, including multidrug-resistant (MDR) isolates, and the presence of the *lug*D gene coding for lugdunin bacteriocin was analyzed by PCR. The antibiotic resistance detected was as follows (% resistance/genes detected): penicillin (44.6%/*bla*Z), oxacillin (1.8%/*mecA* on SCC*mec*-V), erythromycin-clindamycin inducible (7.1%/*erm*(C), *msrA*), tetracycline (5.3%/*tetK*), gentamicin and/or tobramycin (3.6%/*ant*(4′)-Ia, *acc*(6′)-*aph*(2″)), and fosfomycin (21.4%). A MDR phenotype was detected in 5% of isolates. Twenty-one of the *S. lugdunensis* isolates showed susceptibility to all 20 antibiotics tested (37.5%). The screening for AA revealed 23 antimicrobial producer (AP) isolates with relevant inhibition against coagulase-positive-staphylococci (CoPS), including both methicillin-susceptible and –resistant *S. aureus*. The *lug*D gene was detected in 84% of the 56 *S. lugdunensis* isolates. All of the AP *S. lugdunensis* isolates (n = 23) carried the *lug*D gene and it was also detected in 24 of the non-AP isolates, suggesting different gene expression levels. One of the AP isolates stood out due to its high antimicrobial activity against more than 70% of the indicator bacteria tested, so it will be further characterized at genomic and proteomic level.

Keywords: *S. lugdunensis*; coagulase-negative-staphylococci; antibiotic resistance; bacteriocins

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1. Introduction

Coagulase-negative staphylococcal species (CoNS) are commensal bacteria in humans and animals. *Staphylococcus lugdunensis* belongs to the CoNS group, and it is part of the normal human skin microbiota that also has been found in the nasal cavity [1]. Some CoNS, such as *S. lugdunensis*, are considered as significant opportunistic pathogens due to their implication in different human infections [2], going from skin and soft tissue infections (SSTI) to invasive diseases such as infective endocarditis, bone and joint infections, prosthetic joint-infections, vascular catheter-related infections and abscesses, among others [1,2].

Notably, most bacterial infections are caused by pathogens from the human microbiota. Individuals colonized with multidrug resistant (MDR) microorganisms are exposed

to higher risks of invasive infections with more difficult treatment in surgery or immunosuppression cases [3]. In this sense, the current antibiotic resistance problem represents an important health emergency, being the major cause of morbidity and mortality associated with infections worldwide including in developed countries [4,5].

Fortunately, *S. lugdunensis* usually remains susceptible to many antibiotics [6]. However, although *S. lugdunensis* strains (as other CoNS) lack many of the common virulence factors of *S. aureus,* other virulence mechanisms have been identified in this species [7]. In this respect, *S. lugdunensis* has lately been recognized as a pathogenic microorganism and should be considered between one of the most clinical relevant CoNS.

On the other hand, recent studies have reported that *S. lugdunensis* can produce a novel cyclic antimicrobial peptide named lugdunin, which is included in a new class of antibacterials due to its non-ribosomal synthesis. Lugdunin displays a potent antimicrobial activity against a wide range of Gram-positive bacteria including methicillin resistant *S. aureus* (MRSA) [3]. In this sense, human microbiota should be considered as a source for new antimicrobial substances [8,9].

The objective of this study was to characterize a collection of *S. lugdunensis* isolates recovered at two hospitals located in different Spanish regions and determine the phenotypes and genotypes of antibiotic resistance, the virulence content, and the production of antimicrobial compounds against a wide selection of indicator bacteria (different genera/species), including MDR microorganisms.

2. Material and Methods

2.1. Bacterial Collection

This retrospective study included 56 *S. lugdunensis* isolates recovered during a five-year period (2013–2018) from patients of two Spanish hospitals: 48 isolates from Hospital San Pedro (HSP) of Logroño and eight from Hospital Royo Villanova (HRV) of Zaragoza. These isolates were obtained from the following type of samples: skin and soft-tissue infections (SSTI, n = 23), catheter (n = 13), blood (n = 8), urine (n = 7), genital exudates (n = 4), and epidemiological samples (n = 1) (Supplementary Table S1). Antimicrobial resistance, virulence content and bacteriocin production capacity were characterized in these isolates.

2.2. Antimicrobial Resistance Phenotype and Genotype

The susceptibility testing for antimicrobial agents was performed by the commercialized broth microdilution method (Microscan, Beckman Coulter, Brea, CA, USA). Twenty antimicrobial agents were tested: penicillin, oxacillin, cefoxitin, ceftaroline, gentamicin, tobramycin, ciprofloxacin, levofloxacin, erythromycin, clindamycin, pristinamycin, linezolid, fosfomycin, mupirocin, tetracycline, trimethoprim-sulfamethoxazole, vancomycin, teicoplanin, quinupristin/dalfopristin, and daptomycin. The antimicrobial resistance phenotype was evaluated according to the European Committee on Antimicrobial Susceptibility Testing criteria [10].

Based on the resistance phenotype, the presence of the following antimicrobial resistance genes was investigated by PCR: *bla*Z, *mec*A, *mec*C, *tet*(L), *tet*(K), *tet*(M), *msr*(A), *erm*(A), *erm*(B), *erm*(C), *acc*(6′)-*aph*(2″), *ant*(4′)-Ia, *mup*(A), and *mup*(B) [11,12]. The methicillin resistant *S. lugdunensis* isolates were subjected to SCC*mec*-typing [13].

2.3. Virulence Content

The presence of the following virulence genes was tested by PCR: leukocidin genes (*luk*SF-PV, *luk*M, *luk*ED, and *luk*PQ), the toxic shock syndrome toxin 1 (*tst*), and the exfoliative toxins A, B, D (*eta, etb,* and *etd*) [12].

Positive and negative control strains from the collection of the Universidad de La Rioja were included in all PCR assays for antimicrobial resistance genotype and virulence content.

2.4. Antimicrobial Activity

The screening of antimicrobial activity (AA) production was performed for the 56 *S. lugdunensis* isolates by the *spot-on-lawn* method using 37 indicator bacteria (including diverse genera and species, as well as MDR bacteria and relevant pathogens). The characteristics of the indicator bacteria are included in Supplementary Table S2. Bacteria were grown in brain heart infusion (BHI) agar (Condalab, Spain) for 24 h at 37 °C. In order to prepare test plates, 5 mL of sterile semisolid Tryptic Soy Broth (SS-TSB) (BD, Difco, France) supplemented with 0.3% yeast extract and 0.7% agar was maintained at 45 °C, inoculated with 10 µL of a 0.5 MacFarland BHI broth dilution of each indicator strain and poured and spread as a lawn onto yeast extract-supplemented solid Tryptic Soy Agar (TSA) (BD, Difco, France) plates. A single colony of each *S. lugdunensis* isolate to be tested for AA production was transferred with a sterile toothpick to the agar plate seeded with the indicator. Plates were incubated at 37 °C for 24 h to evaluate the halo of inhibition growth (in mm) [14]. Isolates were considered antimicrobial producers (AP) when they showed a clear inhibition zone against at least one of the 37 indicator isolates.

Moreover, the presence of the *lug*D gene was taken as a reference to identify the genetic cluster associated with the production of the non-ribosomal peptide (NRP), lugdunin (GenBank accession number NC_017353.1). For that, the *lug*D amplicon (189 pb) was identified using the following primers and PCR conditions: F-TTCGGGAACTACTGGAATGC (Tm = 60.1 °C), R-AAATGCAATGTCCCTCCAAC (Tm = 59.8 °C); 1 cycle at 94 °C for 7 min, 30 cycles at 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 10 min [15]. Subsequently, *lug*D amplicons were confirmed by Sanger sequencing.

2.5. Statistical Analysis

The Pearson's chi-square test was used to explore significant differences between the isolates tested. Analyses were carried out using SPSS statistical software version 26.0 (IBM®, SPSS Inc., Chicago, IL, USA) and significance was set at $p \leq 0.05$.

3. Results

S. lugdunensis represented 2.4% of the infections caused by CoNS in the two tested hospitals. The collection of 56 *S. lugdunensis* isolates included in this study represented approximately 35% of the total *S. lugdunensis* recovered in the period 2013–2018 in those hospitals, (the remaining isolates were not maintained and they could not be analyzed); they were obtained from a wide diversity of origins (mostly implicated in infections, 42 out of the 56 total isolates): SSTI (41%), catheter (23%), blood (14.3%) urine (12.5%), genital exudate (7.1%) and epidemiological samples (1.8%) (Table 1).

3.1. Phenotype and Genotype of Antimicrobial Resistance

The phenotypes and genotypes of antimicrobial resistance of the 56 *S. lugdunensis* isolates included in this study are shown in Table 1. In this respect, 62.5% of the isolates showed resistance to at least one of the antimicrobial agents tested: penicillin (44.6%), oxacillin (1.8%), fosfomycin (21.4%), erythromycin-clindamycin (7.1%), tetracycline (5.3%), tobramycin (3.5%), gentamicin (1.8%) and mupirocin (1.8%). No isolate showed resistance for the remaining tested antibiotics. Three isolates (5%) were MDR (showing resistance to three or more families of antimicrobial agents) (Table 1). Focusing on the sample origin, the rates of resistant isolates (for at least one tested antibiotic) were as follows: epidemiological sample (100%, one isolate), blood (87.5%), urine (71.4%), SSTI (65.2%), genital exudate (50%) and catheter (42.9%). All 25 penicillin-resistant isolates carried the *bla*Z gene; in addition, genes implicated in the macrolide/lincosamide [*erm*(C), *msr*(A)] and aminoglycoside [*aac*(6′)-*aph*(2″), *ant*(4′)-Ia] resistances were also detected. Tetracycline and mupirocin resistances were rarely found in our collection and were mediated by the *tet*(K) and *mup*(A) genes, respectively. With respect to the methicillin resistance, it was confirmed that the *S. lugdunensis* strain C9897 carried the *mec*A gene within the SCC*mec* type V element (Table 2).

Moreover, none out of the 56 isolates carried any of the virulence genes studied.

Table 1. Origin, antimicrobial resistance phenotype and genotype, antimicrobial activity production (AP) and bacteriocin genes of the 56 *S. lugdunensis* isolates included in this study.

Origin [a]	Number of Isolates	Antimicrobial Resistance		Antimicrobial Activity	
		Phenotype [b,c]	Genotype	AP [d]	*lug*D
Catheter	5	Susceptible	NT	+	+
	1	Susceptible	NT	-	-
	1	Susceptible	NT	-	+
	1	Susceptible	NT	+	+
	1	PEN	*blaZ*	+	+
	3	PEN	*blaZ*	-	+
	1	FOS	NT	+	+
Epidemiological	1	PEN	*blaZ*	-	+
Blood	4	PEN	*blaZ*	-	+
	1	Susceptible	NT	-	+
	2	PEN	*blaZ*	+	+
	1	PEN-TET	*blaZ*, *tet*(K)	-	-
Genital Exudate	2	Susceptible	NT	-	+
	1	FOS	NT	-	-
	1	PEN-FOS	*blaZ*	-	+
SSTI	3	Susceptible	NT	-	-
	3	Susceptible	NT	-	+
	2	Susceptible	NT	+	+
	1	PEN	*blaZ*	-	-
	4	PEN	*blaZ*	+	+
	2	FOS	NT	-	-
	1	FOS	NT	+	+
	1	PEN-FOS	*blaZ*	+	+
	1	ERY-CLIind	*msr*(A)	+	+
	1	PEN- ERY-CLIind	*blaZ*, *erm*(C), *msr*(A)	-	+
	1	PEN-OXA-TOB	*blaZ*, *mec*A, *ant*(4′)-Ia	+	+
	1	ERY-CLIind-TET-FOS	*erm*(C), *msr*(A), *tet*(K)	-	+
	1	ERY-CLIind-MUP-FOS	*erm*(C), *msr*(A), *mup*(A)	-	+
	1	GEN-TOB-FOS	*ant*(4′)-Ia, *acc*(6′)-*aph*(2″)	-	+
Urine	1	Susceptible	NT	-	+
	1	Susceptible	NT	+	+
	1	PEN	*blaZ*	-	+
	1	PEN	*blaZ*	+	+
	1	FOS	NT	-	+
	1	PEN-TET	*blaZ*, *tet*(K)	-	+
	1	PEN-FOS	*blaZ*	+	+

[a] Origin: SSTI: skin and soft tissue infection. [b] Abbrevations: PEN: penicillin; ERY: erythromycin; CLIind: clindamycin inducible; OXA: methicillin/cefoxitin; GEN: gentamicin; TOB: tobramycin; TET: tetracycline; FOS: fosfomycin; MUP: mupirocin. [c] Susceptible to all antimicrobial tested. [d] AP: antimicrobial producer; +/−: positive/negative; NT: non tested.

Table 2. Antimicrobial resistance phenotypic and genotypic correlation for all the antibiotics tested.

Antibiotic	No of Resistant Isolates	Antimicrobial Resistance Genes (No of Isolates)
Penicillin	25	*blaZ* (25)
Oxacillin	1	*mec*A included in SCC*mec*-V (1)
Fosfomycin	12	non studied
Erythromycin-Clindamycin inducible	4	*msr*(A) (1), *msr*(A) + *erm*(C) (3)
Tetracycline	3	*tet*(K) (3)
Tobramycin	2	*ant*(4′)-Ia (2)
Gentamicin	1	*acc*(6′)-*aph*(2″) (1)
Mupirocin	1	*mup*(A) (1)

3.2. Antimicrobial Activity

Twenty-three antimicrobial producer (AP) isolates (41%) with activity against at least one of the 37 indicator bacteria tested were found in this study. They were identified by the *spot-on-lawn* method, including indicator bacteria of the following relevant genera (number of isolates): staphylococci (26), enterococci (7), and *Listeria* (1), among others (Table 3 and Supplementary Tables S2 and S3). The AP isolates were recovered mainly from samples of SSTI (43%), but also from samples of catheter, urine and blood (Table 1). The 23 AP isolates could be differentiated in the following categories: (i) nine isolates showed a broad interspecific activity (InterA-AP), because indicators of at least two different genera were inhibited by the producer isolate; (ii) 12 AP isolates showed broad intraspecific activity (IntraA-AP) because the activity was only detected against indicator bacteria of the same genera as the producer one (*Staphylococcus*), but was of several species; (iii) two isolates considered as moderate antimicrobial producers due to their reduced (RA-AP) spectrum of activity (Table 2).

Moreover, three levels of antimicrobial activity were established based on the percentage of indicator bacteria inhibited by each AP: *S. lugdunensis* isolate: high activity (H-Act, activity against >70% of the indicator bacteria tested), medium activity (M-Act, 20–70%), and low activity (L-Act, <20%) (Figure 1).

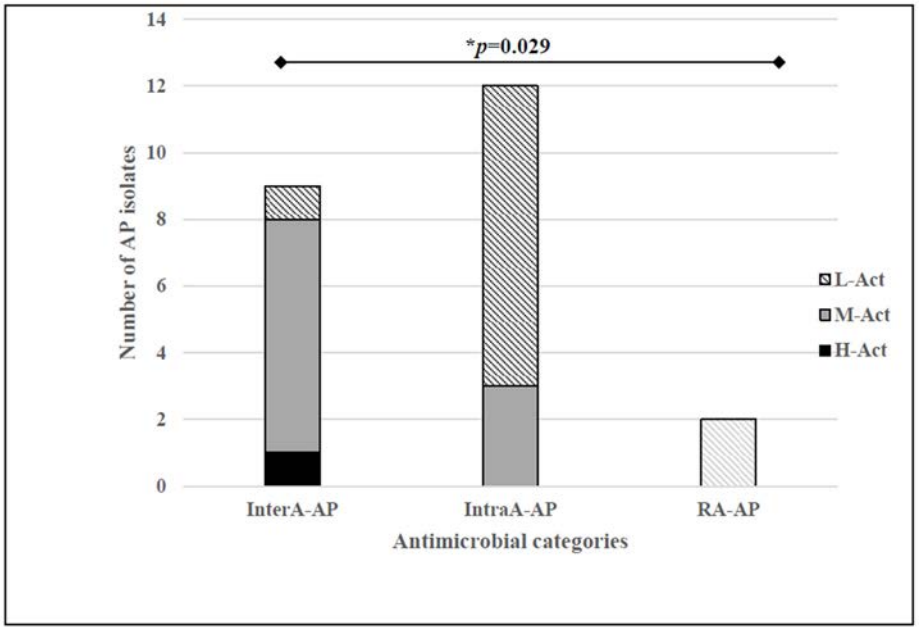

Figure 1. Number of antimicrobial producer (AP) isolates of each established antimicrobial categories (InterA-AP, IntraA-AP and RA-AP) that present high, medium and low antimicrobial activity (H-Act, M-Act and L-Act, respectively). * Statistically significant differences were observed ($p \leq 0.05$).

The 9 *S. lugdunensis* isolates classified in the broad InterA-AP category were recovered from blood ($n = 1$), catheter ($n = 4$), SSTI ($n = 2$) and urine ($n = 2$). The antimicrobial profiles of these isolates are summarized in Figure 2, showing an interesting inhibition capacity against more than three relevant indicator bacteria species such as coagulase positive staphylococci (CoPS), CoNS, *Enterococcus*, *Micrococcus luteus* and *Listeria monocytogenes*. In terms of antimicrobial activity levels, only one InterA-AP isolate (C9954) showed high activity against >70% of the indicators tested, and the rest showed medium ($n = 7$) or low ($n = 1$) antimicrobial activity. As for the IntraA-AP and RA-AP isolates, only three IntraA-AP *S. lugdunensis* isolates were considered moderate producers because they inhibit 30% of the indicators tested, while the others only showed antimicrobial activity against less than 5% of the indicators (Figure 1).

Table 3. Antimicrobial activity of the 23 *S. lugdunensis* isolates characterized as bacteriocin producers against the 37 indicator bacteria.

Indicator Bacteria (nº Isolates) [b]		Antimicrobial Activity of the Bacteriocin Producer Isolate against Indicator Bacteria (Number of Indicator Bacteria Inhibited)																						
		InterA-AP [a]												IntraA-AP [a]									RA-AP [a]	
		C9954	C9161	C9148	C10107	C9894	C9980	C9893	C9159	C9145	C9892	C10052	C9890	C9911	C10343	C9142	C9146	C9147	C9151	C10320	C10341	C10511	C9897	C9342
Gram +	**MR-CoPS (6)**	6	3	3	4	2	1	-	4	1	1	2	2	-	-	1	1	1	-	-	-	-	-	-
	MS-CoPS (11)	11	10	10	8	9	9	8	7	5	9	8	8	1	1	-	-	-	-	-	-	-	-	-
	CoNS (9)	5	3	3	1	2	1	1	2	-	2	1	2	-	1	-	-	-	1	1	1	1	-	-
	***Enterococcus vanA/vanB2* (4)**	2	1	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Other *enterococci* (3)	2	2	-	3	1	-	2	1	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-
	Total *staphylococci* (26)	22	16	16	13	13	11	9	13	6	12	11	12	1	2	1	1	1	1	1	1	1	-	-
	Total *enterococci* (7)	4	3	-	3	1	-	3	1	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-
	***L. monocytogenes* (1)**	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
	***M. luteus* (1)**	1	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gram −	***E. coli* (1)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	***P. aeruginosa* (1)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Categories of antimicrobial activity: InterA-AP, Interespecific Activity (antimicrobial activity against different groups of bacteria belonging to different genera, in addition to staphylococci); IntraA-AP, Intraspecific Activity (antimicrobial activity against different species of staphylococci, but not against other genera); RA-AP, Reduced Activity (antimicrobial activity against one bacterial group, genera or species). [b] Abbreviations: MR, methicillin resistant; MS, methicillin susceptible; CoPS, coagulase-positive *Staphylococcus*; CoNS, coagulase-negative *Staphylococcus*.

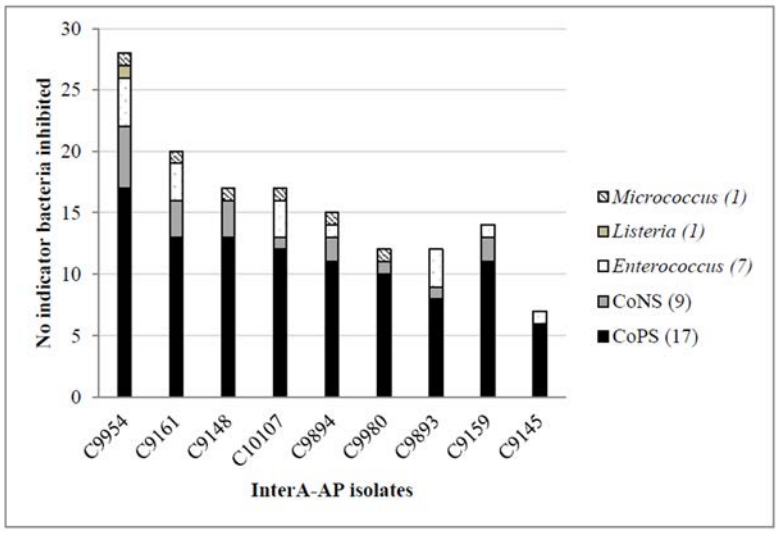

Figure 2. Antimicrobial profile summary of the 9 high antimicrobial producers *S. lugdunensis* isolates with a broad Interspecific Activity (InterAAP). Abbreviations: CoPS, coagulase-positive *Staphylococcus*; CoNS, coagulase-negative *Staphylococcus*.

Moreover, PCR and sequencing analysis confirmed that all 23 of the AP isolates, and 24 of the 33 non-AP isolates carried the gene *lugD*, which codes for a protein implicated in the synthesis of the NRPS lugdunin (Table 1). Only nine *S. lugdunensis* isolates were negative for antimicrobial activity by the *spot-on-lawn* method and did not carry the *lugD* gene.

3.3. Antibiotic Resistance Phenotype versus Antimicrobial Activity

The antimicrobial resistance phenotype of the 56 *S. lugdunensis* isolates compared to that of the 23 AP and the 33 Non-AP isolates is shown in Figure 3. Similar resistance rates were found when all *S. lugdunensis* or only AP isolates were considered (total%/AP%): in this sense, 62.9%/60.9% of the *S. lugdunensis* isolates showed resistance to at least one of the antimicrobials tested, and penicillin was the most frequently observed, with a rate of 44.6%/47.8%, followed by fosfomycin (21.4%/17.3%). The rate of resistance to oxacillin, tobramycin, erythromycin and clindamycin was lower (<5%). Focusing on Non-AP *S. lugdunensis* isolates, the antimicrobial resistance rate was lower (36.4%) and the following resistance percentages were detected: penicillin (42.4%), fosfomycin (24.2%), erythromycin-clindamycin and tetracycline (9.1%), and tobramycin, gentamicin and mupirocin (3%).

Based on the antimicrobial activity categories, Table 4 summarizes the origin, type of sample, antimicrobial resistance phenotype/genotype and bacteriocin genes of the 23 AP isolates. Focusing on InterA-AP isolates, 33% of them (n = 3) were susceptible to all the antimicrobials tested, including the two isolates with a higher inhibition profile (C9954 and C9161). However, four isolates showed resistance exclusively to penicillin, one isolate showed resistance to penicillin and fosfomycin, and the other was resistant to erythromycin-clindamycin^{Inducible}. As for the antimicrobial resistance profile of IntraA-AP and RA-AP isolates, 42.8% of them showed susceptibility to all of the antimicrobials tested. Among the resistant isolates, four showed resistance exclusively to penicillin, two exclusively to fosfomycin, one isolate was resistant to penicillin and fosfomycin, and other one showed resistance to penicillin, oxacillin and tobramycin.

Non-statistically significant differences were found when comparing the origin of the isolates, the antimicrobial activity, and also the established categories (Inter-AP, Intra-AP and RA-AP), and their antimicrobial resistance phenotype. However, the correlation between categories of antimicrobial production and the antimicrobial activity levels revealed statistically significant values (p = 0.029) (Figure 1). Moreover, focusing on categories, the antimicrobial activity against *Enterococcus* and *Micrococcus* was also statistically significant (p = 0.034 and p = 0.046, respectively).

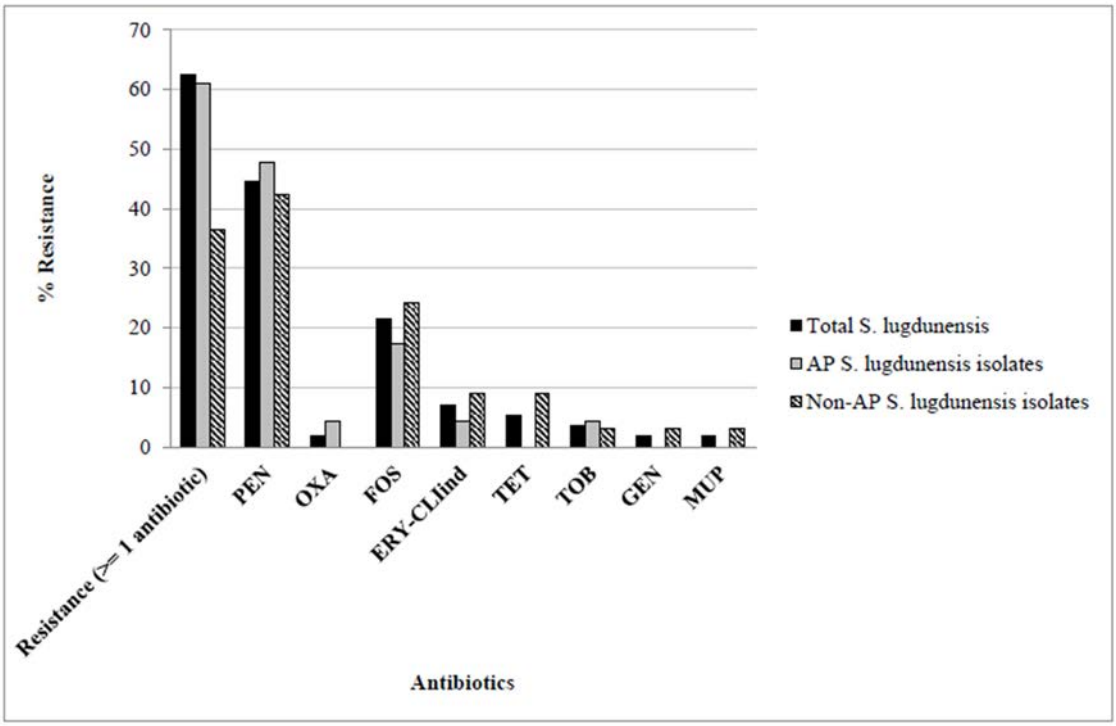

Figure 3. Antimicrobial resistance phenotype of the 56 *S. lugdunensis* isolates versus the 23 AP and the 33 non-AP *S. lugdunensis* isolates. Abbreviations: PEN, penicillin; OXA, oxacillin; FOS, fosfomycin; ERY-CLInd, erythromycin-clindamycin inducible; TET, tetracycline; TOB, tobramycin; GEN, gentamycin; MUP, mupirocin. Non-statistically significant differences ($p \leq 0.05$) were observed.

Based on the antimicrobial activity categories, Table 4 summarizes the origin, type of sample, antimicrobial resistance phenotype/genotype and bacteriocin genes of the 23 AP isolates. Focusing on InterA-AP isolates, 33% of them (n = 3) were susceptible to all the antimicrobials tested, including the two isolates with a higher inhibition profile (C9954 and C9161). However, four isolates showed resistance exclusively to penicillin, one isolate showed resistance to penicillin and fosfomycin, and the other was resistant to erythromycin-clindamycin. As for the antimicrobial resistance profile of IntraA-AP and RA-AP isolates, 42.8% of them showed susceptibility to all of the antimicrobials tested. Among the resistant isolates, four showed resistance exclusively to penicillin, two exclusively to fosfomycin, one isolate was resistant to penicillin and fosfomycin, and other one showed resistance to penicillin, oxacillin and tobramycin.

Table 4. Origin, type of sample, antimicrobial resistance phenotype/genotype and bacteriocin genes of the 23 AP isolates based on the antimicrobial activity categories.

Antimicrobial Activity [a]	Number of Isolates	Origin [b,c]	Antimicrobial Resistance Phenotype [b,d]	Antimicrobial Resistance Genotype [b,d]	No of Isolates Carring *lugD*
InterA-AP	9	Blood[1]-Catheter[4]-SSTI[2]-Urine[2]	Susceptible[3]-PEN[5]-FOS[1]-(ERY-CLInd)[1]	*blaZ*[5] *mecA*[1]	9
IntraA-AP	12	Blood[1]-Catheter[4]-SSTI[6]-Urine[1]	Susceptible[6]-PEN[5]-FOS[1]	*blaZ*[5]	12
RA-AP	2	SSTI[2]	PEN[1]-FOS[1]-OXA[1]-TOB[1]	*blaZ*[1], *mecA*[1], *ant(4')(4'')*[1]	2

[a] Categories of antimicrobial activity: InterA-AP, Interespecific Activity (antimicrobial activity against different groups of bacteria belonging to different genera, in addition to staphylococci); IntraA-AP, Intraspecific Activity (antimicrobial activity against different species of staphylococci, but not against different genera); RA-AP, Reduced Activity (antimicrobial activity against one bacterial group). [b] A number in superscript indicates the total isolates with the indicated characteristic. [c] Origin: SSTI: skin and soft tissue infection. [d] Abbreviations: PEN: penicillin; OXA: methicillin/cefoxitin; FOS: fosfomycin; ERY- CLInd: erythromycin-clindamycin inducible; GEN: gentamicin; TOB: tobramycin.

Table 4. Origin, type of sample, antimicrobial resistance phenotype/genotype and bacteriocin genes of the 23 AP isolates based on the antimicrobial activity categories.

Antimicrobial Activity [a]	Number of Isolates	Origin [b,c]	Antimicrobial Resistance Phenotype [b]	Antimicrobial Resistance Genotype [b,d]	No of Isolates Carring *lugD*
InterA-AP	9	Blood[1]-Catheter[4]-SSTI[2]-Urine[2]	Susceptible[3]-PEN[5]-FOS[1]-(ERY- CLInd)[1]	*blaZ*[5] *mecA*[1]	9
IntraA-AP	12	Blood[1]-Catheter[4]-SSTI[6]-Urine[1]	Susceptible[6]-PEN[5]-FOS[1]	*blaZ*[5]	12
RA-AP	2	SSTI[2]	PEN[1]-FOS[1]-OXA[1]-TOB[1]	*blaZ*[1], *mecA*[1], *ant(4')(4'')*[1]	2

[a] Categories of antimicrobial activity: InterA-AP, Interespecific Activity (antimicrobial activity against different groups of bacteria belonging to different genera, in addition to staphylococci); IntraA-AP, Intraspecific Activity (antimicrobial activity against different species of staphylococci, but not against different genera); RA-AP, Reduced Activity (antimicrobial activity against one bacterial group). [b] A number in superscript indicates the total isolates with the indicated characteristic. [c] Origin: SSTI: skin and soft tissue infection. [d] Abbreviations: PEN: penicillin; OXA: methicillin/cefoxitin; FOS: fosfomycin; ERY- CLInd: erythromycin-clindamycin inducible; GEN: gentamicin; TOB: tobramycin.

4. Discussion

S. lugdunensis is a component of the human microbiome and its role in a wide spectrum of diseases has been recently demonstrated [16]. It has been estimated that *S. lugdunensis* physiological colonization affects the 20%–50% of patients [17,18]. *S. lugdunensis* has low presence in human clinical samples, ranging from 0.5% to 9% in CoNS-positive samples [19,20]. However, recent studies have reported that the proportions of CoNS identified as *S. lugdunensis* and their isolation frequency have steadily increased, although susceptibility rates were not substantively modified during the studied time [21].

In our study, *S. lugdunensis* represented approximately 2.4% of the total CoNS isolated from several origin samples during a five-year period, which reveal a low implication rate

with respect to the total CoNS. A relevant percentage of the samples were obtained from SSTI (41%), followed by those associated with catheters (23%). Moreover, isolates obtained from blood cultures (14%), urine (13%), genital exudates (7%) or epidemiological isolates (2%) were also detected. Although many of the *S. lugdunensis* isolates are not especially pathogenic and commonly act as colonizer bacteria, these CoNS should not be undervalued.

S. lugdunensis has been referred to in the literature as a remarkably susceptible CoNS specie for most of antibiotics [1]. In this study, 21 out of the 56 isolates (37.5%) were susceptible to all groups of antibiotics tested. Different penicillin resistance rates have been detected among *S. lugdunensis* isolates worldwide, from 15–25% in Sweden and Denmark [6,22,23] to 87% in Taiwan [24]. Our penicillin resistance results (44.6%) were similar to those found in previous studies carried out in the USA [21,25]. Significantly, a perfect concordance between resistance phenotype/genotype for penicillin was detected in our study using Microscan. This resistance was mediated in 100% of the penicillin resistant isolates by the expression of the *bla*Z gene. However, other studies have noted a phenotype-genotype discrepancy in relation to penicillin resistance detection when other commercial microdilution methods were used [26].

As for methicillin resistance, only one *S. lugdunensis* isolate was identified as methicillin-resistant which carried the *mec*A gene. Similar results were published by [25], revealing that 3% of the 36 isolates tested were oxacillin resistant and displayed the *mecA* gene. Although there is incomplete information about the SCC*mec* types present in methicillin-resistant *S. lugdunensis* isolates, it has been reported some isolates carriers of elements that were variants of SCC*mec* type V [27]. Therefore, a comprehensive analysis of the SCC*mec* types is required to better understand the acquisition and spread of resistance to beta-lactams [1]. It is to highlight the low beta-lactam resistance detected in the *S. lugdunensis* studied isolates within more than half of the isolates were susceptible to penicillin and oxacillin resistance was rarely detected. As already suggested by others [21], the possibility of using narrow-spectrum beta-lactam agents must be strongly considered in the treatment of infections for this CoNS species.

Resistance to macrolide/lincosamide antibiotics, such as erythromycin and clindamycin, is overall very low [23,25], representing only a 7.1% of the total isolates tested in our work. Moreover, aminoglycoside, tetracycline and mupirocin resistances were also detected, but in low percentages.

The high frequency of fosfomycin resistance detected in our collection of *S. lugdunensis* isolates (21.4%) is of interest; very few studies focused on this antimicrobial agent, although high resistance levels have been reported in some of them (>50%) [28].

As for the virulence content of CoNS, *S. lugdunensis* has been recognized as a CoNS species with a considerable pathogenic potential [7]. Our isolates lacked all the virulence genes tested, previously described in *S. aureus* as being more associated with this species.

Antimicrobial resistance is becoming a severe public health problem and CoNS species deserve special attention due to their significant impact on the clinical and food fields. A better understanding of the processes governing bacterial fitness, competition, and bacteria dissemination is needed. In this sense, it is well known that human skin is populated by a complex microbiota [29,30] that protect us from pathogen colonization thanks to the release of specific antimicrobial peptides termed bacteriocins. *S. lugdunensis* usually acts as a human skin commensal, and recent studies highlight this specie due to its ability to produce lugdunin, a novel antibiotic compound that inhibits the growth of *S. aureus*, other Gram-positive bacteria, and even vancomycin-resistant enterococci [3].

In this study, 23 *S. lugdunensis* AP isolates were identified, differentiating between isolates with broad InterA-AP (n = 9) and those with IntraA-AP or RA-AP (n = 12 and n = 2, respectively). It is worth highlighting the 9 AP isolates with high antimicrobial activity against CoPS, relevant indicator bacteria such as MSSA and MRSA, *Enterococcus vanA/vanB2*, *Micrococcus luteus* and *Listeria monocytogenes*. One of these AP isolates (C9954) showed high inhibitory activity against more than 70% of the indicator bacteria, including MDR, so it will be an interesting candidate for a further in-depth characterization.

In addition, PCR and sequencing analysis confirmed the presence of *lug*D in 86% of the *S. lugdunensis* isolates studied. This gene is the precursor of lugdunin bacteriocin and conforms the NRP operon with the other four genes named *lug*A, B, C, and D. Zipperer et al. described in 2016 that this NRP operon was found in all *S. lugdunensis* genomes available in the databases. In the present study, the *lug*D gene was detected in all the 23 AP-positive isolates, but 24 of the 33 non-AP isolates were positive for the *lug*D gene. The lack of *lug*D in the other 9 non-AP *S. lugdunensis* isolates could be due to mutations on the primer region. In this respect, further genomic studies will be carried out in order to confirm the presence of the complete lugdunin operon in the tested isolates and to analyse the differences with those previously described.

In conclusion, in the present study, the 37.5% of *S. lugdunensis* isolates were susceptible to all tested antibiotics. More than half of the isolates were penicillin susceptible and only one was identified as methicillin-resistant. The low beta-lactam resistance detected in the *S. lugdunensis* studied isolates corroborates the possibility of using narrow-spectrum beta-lactam agents in the treatment of *S. lugdunensis* infections.

Twenty-three *S. lugduunensis* isolates showed antimicrobial activity, nine of them with high activity against CoPS, and one isolate with high inhibitory activity against more than 70% of the indicator bacteria. Its role in the modulation of microbiota in which this species is present seems to be of great relevance. Finally, most of the isolates contained the gene *lug*D, although this gene was not identified in 9 isolates. The relation among the presence/expression of this operon and the antimicrobial activity of *S. lugdunensis* isolates should be analyzed in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10081480/s1: Table S1: Clinical characteristics of the *S. lugdunensis* isolates included in the study; Table S2: Characteristics of the 37 indicator bacteria used in this study for the screening of antimicrobial activity production in the collection of 56 *S. lugdunensis* isolates; Table S3: Antimicrobial activity profile of the 23 *S. lugdunensis* isolates characterized as antimicrobial producers against the 37 indicator bacteria.

Author Contributions: C.T., M.Z. and C.L. designed the study; R.F.-F., L.R.-R. and B.R., performed the experiments and analyzed the data. C.A., C.A.A. and J.M.A.-G., recovered the isolates and performed the susceptibility testing analysis. R.F.-F. and C.T. made the first analysis of the data and prepared the draft of the paper. All authors have read and agreed to the published version of the manuscript.

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Supplementary material

Table S1. Clinical characteristics of the *S. lugdunensis* isolates included in the study.

Isolate	Origin[a]	Sex[b]	Age	Service	Hospital[c]
C10342	Blood	M	-	Infectious disease service	HSP
C10108	Blood	M	55	Cardiology	HRV
C10107	Blood	M	82	Internal Medicine/Traumatology	HRV
C10109	Blood	M	51	Emergency service	HRV
C9143	Blood	F	81	Emergency service	HSP
C9142	Blood	F	34	Emergency service	HSP
C9912	Blood	M	82	Emergency service	HSP
C9144	Blood	M	69	Emergency service	HSP
C9151	Catheter	M	57	Nephrology	HSP
C9148	Catheter	M	69	Nephrology	HSP
C9147	Catheter	M	53	Nephrology	HSP
C9161	Catheter	M	69	Nephrology	HSP
C9954	Catheter	M	57	Nephrology	HSP
C9982	Catheter	M	74	Nephrology	HSP
X700	Catheter	M	51	Nephrology	HSP
C9145	Catheter	F	73	Nephrology	HSP
X312	Catheter	M	57	Nephrology	HSP
X536	Catheter	F	75	Nephrology	HSP
C10341	Catheter	M	74	Nephrology	HSP
X313	Catheter	F	45	Home hospitalization	HSP
C9146	Catheter	M	54	Nephrology	HSP
C9925	Epidemiological	F	66	Internal Medicine/Traumatology	HRV
C10513	Genital Exudate	F	39	Primary care	HSP
C9149	Genital Exudate	F	41	Gynaecology	HSP
C9150	Genital Exudate	M	25	Primary care	HSP
X314	Genital Exudate	F	29	Gynaecology	HSP
C9343	SSTI	F	2	Emergency service	HSP
X463	SSTI	M	70	General Surgery	HRV
C10052	SSTI	F	93	Primary care	HSP
C10320	SSTI	F	58	Gynaecology	HSP
C9896	SSTI	M	76	Traumatology	HSP
C10511	SSTI	F	43	Gynaecology	HSP
C9894	SSTI	M	55	Traumatology	HSP
C10343	SSTI	M	50	Angiology and vascular surgery	HSP
C9342	SSTI	F	49	Primary care	HSP
C9895	SSTI	F	89	Internal Medicine	HSP
C10042	SSTI	F	38	Gynaecology	HSP
C10248	SSTI	F	46	Gynaecology	HSP
C10510	SSTI	M	40	Primary care	HSP
X462	SSTI	M	43	Emergency service	HRV
C9890	SSTI	M	67	Primary care	HSP
C10053	SSTI	F	24	Primary care	HSP

C9892	SSTI	F	51	Primary care	HSP
C9893	SSTI	M	72	Primary care	HSP
C9897	SSTI	M	72	Nephrology	HSP
C10112	SSTI	M	73	Primary care	HSP
C9891	SSTI	M	73	Emergency service	HSP
C9927	SSTI	F	54	Traumatology	HRV
C10113	SSTI	F	31	Emergency service	HSP
X508	Urine	M	89	Primary care	HSP
C10247	Urine	F	36	Primary care	HSP
C9980	Urine	F	81	Primary care	HSP
C9981	Urine	F	26	Primary care	HSP
C9889	Urine	M	76	Emergency service	HSP
C9159	Urine	M	89	Emergency service	HRV
C9911	Urine	F	25	General surgery	HSP

[a]Origin: SSTI: skin and soft tissue infection.
[b]Sex: M, male; F, female.
[c]Hospital: HSP, San Pedro Hospital; HRV, Royo Villanova Hospital.

Table S2. Characteristics of the 37 indicator bacteria used in this study for the screening of antimicrobial activity production in the collection of 56 *S. lugdunensis* isolates.

Specie (no of strains)	Strain	Origin	Relevant Antimicrobial Resistance Phenotype/Genotype
***S. aureus* (4)**	C1570	Human	Methicillin/*mecA*[a]
	C7246	Human	Methicillin/*mec*C[a]
	C5313	Human	Methicillin/*mecA*
	ATCC29213 (C411)		
***S. delphini* (1)**	C9459	Wild animal	
***S. pseudintermedius* (12)**	C2381	Pet	Methicillin/*mecA*
	C3930	Pet	Methicillin/*mecA*
	C2382	Pet	Methicillin/*mecA*
	C8187	Human	MDR
	C2915	Human	
	C2912	Pet	
	C8189	Human	
	C3917	Human	
	C8188	Human	
	C8368	Human	
	C3007	Pet	
	C3468	Pet	
***S. epidermidis* (1)**	C2663	Human	Methicillin/*mecA* and Linezolid
***S. haemolyticus* (1)**	C2709	Human	Linezolid
***S. lugdunensis* (6)**	C9927	Human	
	C9981	Human	
	C9159	Human	
	C10107	Human	
	C9954	Human	
	C9980	Human	
***S. sciuri* (1)**	C9780	Wild animal	
***Enterococcus casseliflavus* (1)**	C1232		
***E. durans* (1)**	C1433		Vancomycin*/vanA*
***E. faecalis* (1)**	C3735		Vancomycin*/vanB2*
***E. faecalis*(1)**	ATCC29212 (C410)		
***E. faecium* (1)**	C2321		Vancomycin*/vanA*
***E. gallinarum* (1)**	C2310		
***E. hirae* (1)**	C1436		Vancomycin*/vanA*
***Listeria monocytogenes* (1)**	CECT4032 (C137)		
Micrococcus luteus	C157		
***Escherichia coli* (1)**	ATCC25922 (C408)		
***Pseudomonas aeruginosa* (1)**	C3282		

[a]The C1570 isolate belonged to ST398 and the C7246 isolates belonged to ST1945.

Table S3. Antimicrobial activity profile of the 23 *S. lugdunensis* isolates characterized as antimicrobial producers against the 37 indicator bacteria.

		Antimicrobial activity of the antimicrobial producer isolates[a]																						
		InterA-AP[b]													IntraA-AP[b]								RA-AP[b]	
Specie of indicator bacteria[c]	**UR[d]-reference**	**C9954**	**C9161**	**C9148**	**C10107**	**C9894**	**C9980**	**C9893**	**C9159**	**C9145**	**C9892**	**C10052**	**C9890**	**C9911**	**10343**	**C9142**	**C9146**	**C9147**	**C9151**	**10320**	**10341**	**10511**	**C9897**	**C9342**
MR-SA	**C1570**	2	0	0	1	0	0	0	1	0	1	1	2	0	0	2	1	1	0	0	0	0	0	0
	C7246	2	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	C5313	3	0	0	2	0	2	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MS-SA	**C411**	4	2	2	0	1	1	2	0	1	2	1	0	1	1	0	0	0	0	0	0	0	0	0
S. delfini	**C9459**	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MR-SP	**C2381**	2	2	2	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C3930	2	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C2382	2	2	2	2	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
MS-SP	**C8187**	3	3	3	3	3	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
	C2915	3	3	3	3	3	3	1	1	1	1	1	3	0	0	0	0	0	0	0	0	0	0	0
	C2912	3	3	3	3	3	3	1	1	1	1	1	3	0	0	0	0	0	0	0	0	0	0	0
	C8189	3	3	3	3	3	3	1	1	1	1	1	3	0	0	0	0	0	0	0	0	0	0	0
	C3917	3	3	1	3	2	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
	C8188	3	3	3	3	3	3	1	1	0	1	1	2	0	0	0	0	0	0	0	0	0	0	0
	C8368	2	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C3007	3	3	3	1	2	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
	C3468	3	3	3	3	2	3	3	3	2	1	1	3	0	0	0	0	0	0	0	0	0	0	0
S. epidermidis	**C2663**	3	0	1	0	2	0	1	1	0	2	3	2	0	0	0	0	0	0	0	0	0	0	0
S. haemolyticus	**C2709**	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2	1	1	1	0	0
S. lugdunensis	**C9927**	2	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C9981	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C9159	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	C10107	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C9954	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C9980	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S.sciuri	**C9780**	2	2	0	2	2	2	0	2	0	2	0	1	0	0	0	0	0	0	0	0	0	0
Enterococcus . casseliflavus	**C1232**	0	0	0	2	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. durans vanA	**C1433**	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. faecalis vanB2	**C3735**	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. faecalis	**C410**	2	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0
E.faecium vanA	**C2321**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. gallinarum	**C2310**	2	2	0	1	0	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
E. hirae vanA	**C1436**	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Listeria monocytogenes	**C137**	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Micrococcus luteus	**C157**	4	2	2	1	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Escherichia coli	**C408**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa	**C3282**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a]The numbers indicate the size of the inhibition halo against each indicator bacteria: 4, halo>10mm; 3, 9>halo>6 mm; 2, 5>halo>3, 1, halo<3; 0, no halo.

[b]Categories of antimicrobial activity: **InterA**, Interespecific Activity (antimicrobial activity against different groups of bacteria belonging to different genera, in addition to staphylococci); **IntraA,** Intraespecific Activity (antimicrobial activity against different species of staphylococci, but not against other genera); **RA**, Reduced Activity (antimicrobial activity against one bacterial group).

[c]Abbreviations: MR, methicillin resistant; MS, methicillin susceptible; CoPS, coagulase-positive *Staphylococcus*; CoNS, coagulase-negative *Staphylococcus*; SA: *S. aureus;* SP: *S. pseudintermedius.*

[d]UR: University of La Rioja.

1.4 Anexo al Capítulo 1

Tabla 5 Secuencias de cebadores y condiciones de PCR usadas en este estudio para la detección de genes estructurales codificantes de bacteriocinas y familias de bacteriocinas **(Fernández-Fernández et al. 2022a**).

Bacteriocina	Gen	Secuencia de cebadores (5′→3′)	Tª hibridación (ºC)[b]	Tamaño de amplicón (bp)
Aureocina A70	*aurA*	F-CCTTATAACTTCGAATGCT R-AAATATTAACAAGAGAAA	50	525
Aureocina A53	*aucA*	F-GAAGTTGTGAAAACTATTA R-CATAAAACAAAGAGCCAAAG	50	322
Aureociclina 4185	*acIA*	F-ATGTTGTTAGAGTTAACAGG R-TTAAAAAGCAATACCTGCTTTTTTTCC	55	195
BacCH91	*bacCH91*	F-TTAGTGAAAATAAATAGTA R-CATTTGTAAGCACCTCAC	42[a]	380
BsaA2	*bsaA2*	F-TTAACAGCAGAAGCTATTAAAACTACCAG R-ATGGAAAAAGTTCTTGATTTAGACG	50	144
[a]BS (BsaA1/A2 BacCH91)	Familia	F-GTTCTTGATTTAGACGTRCAAG R-CAGAAGCTRTTAAAACTACCMGTC	50	ND[c]
BacSp222	*bacSp222*	F-AAAAGGTTAGGGGGAGCCA R-CCGCACACTTCTCTCCACTT	55	818
Capidermicina	*orf4*	F-CCGCTCACAAAGCTTAAAC R-CAAAATACGGTCCAAAAGCTG	57	185
Endopeptidasa ALE-1	*ale-1*	F-AGGACCATTATGGGGAACAA R-AACGCGTGGTTAAGGGTATG	57	177
Epidermina	*epiA*	F-GGAGTGTTTAAAATGGAAGC R-CCTTTTCCCAGTCTATTTTG	55	431
Epidicina 280	*eciA*	F-CGGAGGGATATATTATGG R-CAATCACTACTATTGACAATCAC	50	195
Epilancina 15X	*elxA*	F_ATGAATAACGAATTATT(C/T)(A/G)ATTTGGATC R-CTTTGTAGAGGATTTACACTAACTTG	52	139
Epilancina K7	*elkA*	F-CTCAAAAGAGTGATTTAAGTCCGC R-CCACCAGTAATATTGCAACCGC	50	115
Epidermicina NI01	*ecdA*	F-GGCAGCATTTATGAAGTTAATTCAG R-ACCGGCGTTAATCCATTTTA	57	106
Estafilococcina C55	*sacaA/ sacbA*	F-AGCGTGGTGATTCTTATG R-TCTGATTTATTTAGTTCTGGATA	50	499
Gallidermina/Estafilococcina T (StT)	*gdmA*	F-AGGAGTGTTTAAAATGGAAGCA R-GAAGCTACCTGTTTTGGCACA	50	154

Tabla 5 Continuación

Bacteriocina	Gen	Secuencia de cebadores (5′→3′)	Tª hibridación (ºC)[b]	Tamaño de amplicón (bp)
[a]GEST (Gallidermina, Estafilococcina T (StT), Epidermina)	Familia	F-RATCTTGAYGTTAAAGTWAATGC R-TACCTGTTTTKGCACATCCA	52	ND[c]
Hyicina/Agneticina 3682	*hyiA*	F-TATAGGAGGTTTTTTGCTATGG R-CAACAATAACTATTGAAGCTACC	50	158
Hyicina/Agneticina 4244	*hycS*	F-TGGAAGTCATACATGAGAGGAGG R-ACCGAATGTACCTGTGATACCC	50	150
Lugdunina	*lugD*	F-TTCGGGAACTACTGGAATGC R-AAATGCAATGTCCCTCCAAC	57	189
Lisostafina	*lss*	F-CTGAGTACATTTGCCGC R-GACCGTAACCATATCCT	50	756
Nisina J	*nsj*	F-ACTTTATAACTAAGATTAGC R-TCGCTTTATTATTTAGTATGCACG	52	182-216
Nukacina IVK45	*nuk*A	F-AAAGGGGGTATTATAATGGAAAA R-TCCTTGCATGATTTTATCCACA	57	202
Nukacina KQU-131	*nkqA*	F-GGAGGTAACAAACATGGAAAATTC R-GCCATAATATTAGTATCATGTTAATC	50	259
Nukacin ISK-1	*nukA*	F-AGGAGGTAACAAACATGG R-CCCCTTTTTATGAACAACAAG	50	195
Nukacina 3299	*nukA*	Nukacin ISK-1	50	176
[a]NUK (Nukacina KQU-131, Nukacina 3299, Nukacina ISK1)	Familia	F-TGAAGGACATTGAAGTAGCAAA R-TTCATATGGCAATCGTGTGAC	57	121
Pep5	*pepA*	F-AGAGGAGGTGGTTATATATG R-TGAGTTCCATGCCCAGTG	50	427

[a]BS, GEST y NUK son las familias de bacteriocinas descritas en este estudio.
[b]Condiciones específicas de PCR: 1 ciclo a 94 °C 2 min,30 ciclos a 94 °C 30 s, 42 °C 30 s, y 72 °C 45 s. El resto de PCR se realizaron bajo condiciones estándar usando las temperaturas de hibridación indicadas para cada cebador: 1 ciclo a 94 °C 7 min,30-35 ciclos a 94 °C 1 m, Tº hibridación (°C) 1 m, 72 °C 1 m, y 72 °C 10 m.
[c]ND: no definido.

Tabla 6 Número de cepas productoras detectadas por *spot-on-lawn*, sus especies y orígenes divididos por cada uno de los trabajos incluidos en el primer capítulo de esta tesis.

	Especies	**Total**	**Humanos**	**Alimentos (pollo crudo)**	**Animales de vida libre (aves y mamíferos)**	**Mascotas (perro)**	**Medio ambiente (aguas superficiales)**
Artículo 1	*S. aureus*	5		2	2		1
	S. pseudintermedius	16	1			15	
	S. sciuri	13		2	11		
	S. xylosus	3			3		
	S. epidermidis	4		4			
	S. chromogenes	9		3	6		
	S. warneri	6		6			
	S. simulans	1			1		
	S. hominis	1					1
	S. hyicus	2			2		
Artículo 2	*S. lugdunensis*	23	23				
Total		83	24	17	25	15	2

Capítulo 2

2. Capítulo 2: Detección y caracterización genética de bacteriocinas

Las bacteriocinas son péptidos con actividad antimicrobiana codificados en operones formados por genes estructurales y genes accesorios que codifican enzimas implicadas en la modificación postraduccional, en el transporte o proteínas de autoinmunidad. Comúnmente, se ha recurrido a la detección de los genes estructurales de bacteriocina mediante PCR y/o hibridación ADN/ADN. Sin embargo, debido a que cada cepa productora puede tener ligeras variaciones en sus secuencias genéticas, es necesario optimizar tanto las condiciones de amplificación del gen en estudio como sus pares de cebadores para evitar inespecificidades y aumentar el éxito de detección.

Afortunadamente, desde la aparición de los métodos de detección *in-silico*, la identificación de operones codificantes de bacteriocinas se ha reducido considerablemente en términos de tiempo y coste. En este sentido, se ha descrito una amplia variedad de herramientas bioinformáticas y bases de datos de bacteriocinas como son: BACTIBASE, antiSMASH, BAGEL, APD3, ANTIMIC, DRAMP, o URMITE, entre otros. Sin embargo, cabe destacar que albergar los grupos de genes de una estafilococcina no implica necesariamente su producción; por lo tanto, la producción de bacteriocinas se debe confirmar siempre mediante ensayos *in vitro*.

Las bacteriocinas se han clasificado en tres grandes clases en función de su composición aminoacídica, estructura química, complejidad y pasos implicados en su producción: Clase I, péptidos pequeños modificados postraduccionalmente; Clase II, bacteriocinas no modificadas; y Clase III, péptidos más grandes y termolábiles. A pesar de esta gran variedad de clases de bacteriocinas, no existe un consenso claro sobre los criterios a seguir para asignar una bacteriocina a una determinada clase por lo que revisiones recientes han propuesto la inclusión de la clase IV (Circulares), clase V (Sactipéptidos) y clase VI (Tiopéptidos) y péptidos no ribosomales (NRP) entre las bacteriocinas detectadas en bacterias G+.

En este contexto, en el capítulo 2 se completa el segundo objetivo de la tesis planteando el análisis genómico de las cepas productoras de interés **(Figura 18)**. De este modo, se incluyen en esta tesis 22 genomas de cepas de estafilococos previamente confirmadas como productoras de bacteriocinas en los que se ha analizado la presencia de operones codificantes de bacteriocinas, su resistoma y plasmidoma. Por otro lado, se ha desarrollado un nuevo sistema de clasificación de estafilococcinas en base a su relación filogenética a nivel de genes y de proteínas estructurales. Además, se presenta una guía completa y actualizada para la identificación de secuencias nucleotídicas y aminoacídicas de las estafilococcinas registradas en las bases de datos del NCBI.

2.1 Artículo 3 Comprehensive approaches for the search and characterization of staphylococcins

2.2 Genomic analysis of bacteriocin-producing staphylococci: high prevalence of lanthipeptides and the micrococcin P1 biosynthetic gene clusters

2.1 Artículo 3..... Comprehensive approaches for the search and characterization of staphylococcins

Dentro de esta revisión bibliográfica se recogen las bacteriocinas detectadas en aislados de estafilococos (actualizado en enero de 2023) indicando sus números de acceso de genes y proteinas del NCBI, la posición y tamaño génico (pb) y la masa esperada de la bacteriocina (Da).

Además, se presenta una herramienta novedosa para la identificación y clasificación de bacteriocinas en base a la secuencia genética y aminoacídica del gen estructural. Se realizó un análisis filogenético basado en la homología de 34 secuencias de aminoácidos y 33 de genes estructurales codificantes de estafilococcinas el cual nos permitió distinguir seis grupos de estafilococcinas que denominamos familias: 1) BS, que incluye las bacteriocinas Bsa, Hyicina 3682 y BacCH91; 2) EP5, formada por Epidicina 280/Homicorcina y Pep5; 3) NUK, que comprende todas las nukacinas (Nukacina IVK45, Nukacina KQU-131, Nukacin 3299 y Nukacina ISK-1); 4) GEST, que incluye Gallidermina, Estafilococcina T (StT) y Epidermina; 5) la familia de bacteriocinas denominada EPI, formada por Epilancina 15X y Epilancina K7, y, por último, 6) la familia CAPSP, que incluye las bacteriocinas Capidermicina y BacSp222. El operón que codifica la Aureocina A70 (*aur*A, B, C, D) no se consideró como una familia porque sólo codifica una única bacteriocina.

El sistema de clasificación que se presenta se corresponde con la mayoría de los reportados recientemente y proporciona más detalles de las similitudes de las estafilococcinas incluidas en cada una de las 6 clases propuestas por este trabajo. Concretamente, dentro de la Clase I (Lantibióticos), nuestras familias revelaron mayores similitudes entre las bacteriocinas de tipo Nukacina (NUK familia), Gallidermina, Estafilococcina T (StT), y Epidermina (familia GEST); y Bsa, Hyicina 3682 y BacCH91, considerados como familia BS. En cuanto a la Clase II, propusimos la familia CAPSP (conformada por Capidermicina y BacSp222) debido a sus mayores similitudes en la secuencia de nucleótidos y aminoácidos. Esta información es de gran utilidad tanto para la detección de estafilococcinas mediante PCR y/o secuenciación masiva, así como para su verificación mediante espectrometría de masas y ha sido utilizada en varios trabajos incluidos en esta tesis.

2.2 Genomic analysis of bacteriocin-producing staphylococci: high prevalence of lanthipeptides and the micrococcin P1 biosynthetic gene clusters

En este trabajo se incluyeron 22 genomas de cepas comensales de *Staphylococcus* tanto SCoP como SCoN previamente descritos como productores de bacteriocinas en el capítulo 1. Las cepas pertenecían a 11 especies y eran de diferentes orígenes (alimentos, medio ambiente, animales de vida libre, mascotas y humanos). El objetivo de este estudio fue la caracterización genómica para el análisis *in silico* de la presencia de operones codificantes de estafilococcinas (OCE). También se estudiaron el resistoma y el plasmidoma. Para ello, se llevó a cabo la extracción del genoma completo de las 22 cepas que fue secuenciado por metodología Illumina, ensamblado con Spades y anotado con Prokka. Para la detección de OCE se usó antiSMASH y BAGEL mientras que para el análisis del plasmidoma y resistoma se recurrió a la base de datos on-line "center of genomic epidemiology".

Se detectaron cinco tipos de OCE entre 16 de los 22 estafilococos productores de bacteriocinas clasificados dentro de las siguientes clases: clase I (lantipéptidos), clase II (lactococcin972, bacsp222 y blp), bacteriocinas circulares (uberolisina y aureocyclicina 4185), el péptido no ribosomal lugdunina y el tiopéptido (micrococcina P1, MP1). Cabe destacar la alta frecuencia de lantipéptidos detectados en la colección: OCE variantes de las bacteriocinas BSA, bacCH91, epilancina15X, hominicina y dos OCE codificantes de lantibióticos potencialmente nuevos en cepas de estafilococos de alimentos y animales salvajes. Además, es de gran interés la detección de OCE codificantes de MP1 en 4 de los genomas incluidos en este estudio (un *S. aureus* y un *S. hominis*, de origen ambiental; dos *S. sciuri*, de origen alimentario). Asimismo, para investigar la semejanza entre los OCE detectados entre los genomas incluidos en este estudio y otros previamente descritos, se realizaron análisis comparativos con las secuencias cargadas en las bases de datos utilizando herramientas como el visualizador Jalview (para alineamientos) o Clinker (para comparativas de entornos genéticos). Por otra parte, siete de los 22 genomas (~32%) carecían de genes relacionados con mecanismos de resistencia adquirida a antibióticos o a desinfectantes. Además, se identificaron varias proteínas Rep (~73% de las cepas) relacionadas con la presencia de plásmidos que pueden actuar como elementos de adquisición y diseminación de genes.

En conclusión, estos trabajos han revelado la gran abundancia y diversidad de OCE, incluyendo sistemas únicos y poco frecuentes entre los genomas de estafilococos de diversos orígenes. En este sentido, el género *Staphylococcus* y especialmente los aislados de SCoN se han confirmado como una valiosa fuente de nuevas estructuras peptídicas con funcionalidades prometedoras para el tratamiento y la prevención de enfermedades infecciosas.

2.3 Resumen gráfico del Capítulo 2

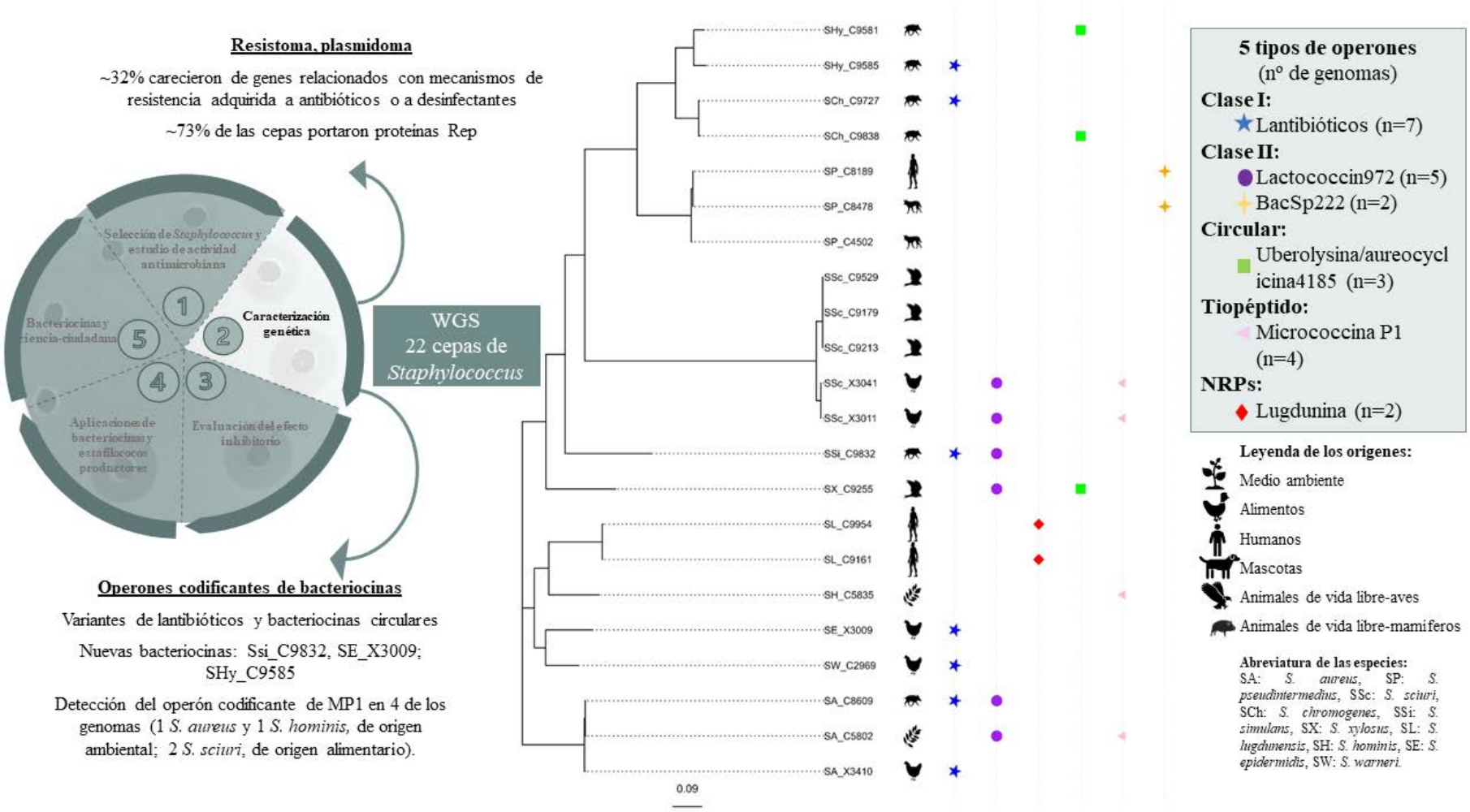

Figura 18 Resumen gráfico de la metodología y resultados obtenidos en el segundo capítulo referente a la detección y caracterización genética de bacteriocinas (elaboración propia).

Artículo 3

microorganisms

MDPI

Article

Comprehensive Approaches for the Search and Characterization of Staphylococcins

Rosa Fernández-Fernández, Carmen Lozano *, Rine Christopher Reuben, Laura Ruiz-Ripa, Myriam Zarazaga and Carmen Torres

Area of Biochemistry and Molecular Biology, OneHealth-UR Research Group, University of La Rioja, 26006 Logroño, Spain

* Correspondence: carmen.lozano@unirioja.es; Tel.: +34-941299752

Abstract: Novel and sustainable approaches are required to curb the increasing threat of antimicrobial resistance (AMR). Within the last decades, antimicrobial peptides, especially bacteriocins, have received increased attention and are being explored as suitable alternatives to antibiotics. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria as a self-preservation method against competitors. Bacteriocins produced by *Staphylococcus*, also referred to as staphylococcins, have steadily shown great antimicrobial potential and are currently being considered promising candidates to mitigate the AMR menace. Moreover, several bacteriocin-producing *Staphylococcus* isolates of different species, especially coagulase-negative staphylococci (CoNS), have been described and are being targeted as a good alternative. This revision aims to help researchers in the search and characterization of staphylococcins, so we provide an up-to-date list of bacteriocin produced by *Staphylococcus*. Moreover, a universal nucleotide and amino acid-based phylogeny system of the well-characterized staphylococcins is proposed that could be of interest in the classification and search for these promising antimicrobials. Finally, we discuss the state of art of the staphylococcin applications and an overview of the emerging concerns.

Keywords: *Staphylococcus*; staphylococcin; applications; antimicrobial-resistance alternatives

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1. Introduction

Antimicrobial resistance (AMR) is considered one of the most relevant threats affecting not only human and animal health but also environmental health and food security. Unless AMR's spread and associated infections are globally prioritized and mitigated, health and economic burdens across the world will continue to worsen. Therefore, the sustainable prevention of human and animal infections and the reduction of the transmission of foodborne and zoonotic pathogens is necessary for ensuring food safety and public health. The frequent detection of top-priority antibiotic-resistant pathogens, especially methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium*, carbapenem-resistant and extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*, carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, and fluoroquinolone-resistant *Campylobacter* spp. and *Salmonella* [1] within the human, animal, and environment/food systems pose a significant threat which could be exacerbated if urgent measures are not explored. In this respect, it has been reported that infections caused by MRSA are more difficult to treat and costlier for healthcare systems [2].

Within the last decades, several emerging therapeutic alternatives, including antimicrobial peptides, bacteriophages, nanomedicines, probiotics, phytochemicals, photodynamic light therapy, etc., are being explored as suitable alternatives to antibiotics [3–6]. Bacteriocins are antimicrobial peptides produced by bacteria, mainly of ribosomal synthesis [7] that have recently attracted immense interest due to their high antimicrobial activities and stability [8]. Bacteriocin production seems to be a common characteristic among microorganisms, and it has been reported that most bacteria synthesize at least one antimicrobial

compound [9]. Moreover, bacteriocin production is considered a self-preservation mechanism that allows bacteria to outcompete other members of the community, interfere in communication with the host and/or other bacteria, and prevent colonization, setting up very robust communities [10–12].

Staphylococcus is a Gram-positive commensal bacterial genus of humans and animals and can also be found in diverse environments and food [13–16]. Some staphylococcal species, including coagulase-positive (CoPS) and coagulase-negative staphylococci (CoNS), have been described as bacteriocin producers, commonly termed as staphylococcins [17–19]. Interestingly, CoNS species are frequently found as commensals of humans and animals, being infrequently associated with infections. These characteristics make them excellent candidates for the research and development of safe and economical antimicrobial substances against antibiotic-resistant pathogens.

In recent times, increasing research focusing on the characterization, mechanisms, activity (mostly against *S. aureus*), safety evaluation (including cytotoxicity), and regulations of bacteriocins detected in staphylococci have been reported [7,19]. Recently, we identified and characterized some relevant bacteriocin-producing staphylococci from animal, human, and environmental sources in our laboratory [20–22].

In this review, a comprehensive and technical dossier for the search for and characterization of bacteriocins detected in staphylococci is presented as an interesting source of novel natural antimicrobial compounds with relevant interest to deal with the AMR problem. Furthermore, an extensively up-to-date classification of staphylococcal bacteriocins is provided, as well as a novel phylogeny classification of staphylococcin structural genes and proteins. Finally, a brief overview of possible bacteriocin applications is included.

2. Classification and Niche of *Staphylococcus*

Many staphylococcal species, both CoPS and CoNS, are found in humans, animals, and food [23]. They normally interact as commensal bacteria, although they can also act as opportunistic pathogens [24], especially CoPS, with *S. aureus* and *S. pseudintermedius* as the most reported cause of infections [25]. *S. aureus* is commonly related to skin and soft tissue infections in humans and cow mastitis [26]. As for companion animals, *S. pseudintermedius* is frequently isolated from pyoderma and postoperative dermatological infection cases in dogs [27]. Staphylococci are also common contaminants of animal-derived foods, such as raw meats or milk-derived products, and are responsible for most food toxi-infections in humans [23,24].

Staphylococci inhabit a wide diversity of polymicrobial environments and often compete for resources. Some commensal staphylococcal species can prevent the colonization of other pathogenic ones. Bacteriocin production is regarded as one of the defense mechanisms developed for self-preservation. Since bacteriocins, both CoPS and CoNS, are being considered as a new strategy to combat bacterial infections and the problem of antibiotic resistance, it is important to study their respective niche microbiota.

2.1. Staphylococci in Skin/Nasal Microbiota of Humans

Long-term bacterial residents isolated from the human skin microbiota include those from the genus *Staphylococcus*. It is estimated that 20–30% of the anterior nares are colonized by *S. aureus* [11,18], and the presence of this opportunistic pathogen has been linked to reduced bacterial diversity, exacerbated disease symptoms, and frequently precede infection [28–30]. This percentage of *S. aureus* carriers could be affected by risk factors such as occupational contact with farm animals [31].

Various CoNS species are known to inhabit the human skin microbiota, including *S. epidermidis*, *S. capitis*, *S. hominis*, *S. cohnii*, and *S. warneri*, among others [28,30]. *S. epidermidis* is known to be a core microbiome member of both the skin and nose, typically, while *S. warneri* has been found at a lower percentage than other CoNS but also in the nares and on the skin [30]. *S. lugdunensis* has been isolated from the human nose at an incidence rate of 10 to 26% [30]. Interestingly, some strains of these species have been found

to negatively impact *S. aureus* viability, thus, preventing nasal *S. aureus* colonization or infections [11,30,32–34].

Over time, bacteriocin-producing staphylococcal isolates have been recovered from human skin and nasal microbiota. Among the well-described staphylococcins, we can highlight the Staphylococcin C55 [35,36], Bsa [37,38], Capidermicin [8], Endopeptidase ALE-1 [39], NisinJ [32,40], Nukacin IVK45 [41], Pep5 [42–44], Epidermicin NI01 [45], Epilancin 15X [46,47], Staphylococcin T (StT) [48], Hominicin [49,50], Lugdunin [51], and SWLP1 [19] bacteriocins. Moreover, the bacteriocins Epidermin [52–56] and Epicidin 280 [57] have been isolated from human clinical samples and an extensive number of bacteriocin-like-inhibitory-substances have also been detected in *Staphylococcus* of human origin, such as Staphylococcin BacR1 [58], Staphylococcin IYS2 [59], Staphylococcin Au-26 [60], Bac 201 [61], Staphylococcin 188 [62], Staphylococcin D91 [63], TE8 [64], and Hogocidinα/β [34] (Tables 1 and 2).

Finally, several bacteriocins included in Tables 1 and 2 are produced by isolates re-covered from environmental samples (Warnericin RK [65]) or laboratory strains (Staphylococcin 1580 [66], Bac 1829 [67], Lysostaphin [68,69] and Epilancin K7 [70]).

Table 1. Bacteriocins described in coagulase-positive staphylococci (CoPS) and coagulase-variable staphylococci (*S. hyicus*/*S. agnetis*).

Bacteriocin	Producer (Strain)	Origin	Activity against *		Classification [a]	References
			Gram (+)	Gram (−)		
Staphylococcin C55	*S. aureus* (C55)	Human skin	*S. aureus*, streptococci, pneumococci, *Corynebacterium*, *Enterococcus*	*Neisseria*	Class II	[35,36]
Staphylococcin BacR1	*S. aureus* (UT0007) *S. aureus* (UT0002)	Clinical	*Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Enterococcus*, *Bacillus*	*Neisseria*, *Haemophilus*, *Moraxella*, *Bordetella*, *Pasteurella*	BLIS	[58]
Aureocin A70	*S. aureus* (A70)	Milk	*Listeria monocytogenes*, *Staphylococcus*	–	Class II	[71,72]
Aureocin 4181	*S. aureus* (4181)	Bovine mastitis	*Staphylococcus*, *Streptococcus*	–	ClassII	[73]
Aureocin A53	*S. aureus* (A53)	Milk	Lactic acid bacteria, *L. monocytogenes*, *S. aureus*, *Mycobacterium bovis*	–	Class II	[74]
Aureocin 215FN	*S. aureus* (215FN)	Cow nare	*Corynebacterium*, *Streptococcus*, *L. monocytogenes*, *Bacillus*, *Lactobacillus*	–	BLIS	[75,76]
Staphylococcin 414	*S. aureus* (414)	Turkey	*Staphylococcus*, *Micrococcus*, *Bacillus*, *Lactobacillus*, *Streptococcus*	–	BLIS	[77]
Staphylococcin 462	*S. aureus* (462)	Mink	*S. aureus*	–	BLIS	[78]
Staphylococcin IYS2	*S. aureus* (IYS2)	Human saliva	*S. aureus*, *Streptococcus*, *Propionibacterium*, *L. monocytogenes*, *Corynebacterium*, *Actinomyces*	–	BLIS	[59]
Staphylococcin Au-26	*S. aureus* (26)	Human vagine	*Staphylococcus*, *Lactobacillus*, *Micrococcus*, *Streptococcus*	*Neisseria*	BLIS	[60]
Bac 1829	*S. aureus* (KSI1829)	Laboratory isolate *S. aureus* (RN4220)	*S. aureus*, *Streptococcus*, *Enterococcus*, *Corynebacterium*	*Haemophilus*, *Moraxella*, *Bordetella*, *Pasteurella*	BLIS	[67]
Bac 201	*S. aureus* (AB201)	Wound	*Staphylococcus*, *Streptococcus*, *Enterococcus*	*Neisseria*, *Acinetobacter*	BLIS	[61]
Staphylococcin 188	*S. aureus* (188)	Clinical	*Staphylococcus*, *Micrococcus*, *Streptococcus*, *Corynebacterium*, *Mycobacterium tuberculosis*	*Escherichia coli*, *Salmonella*, *Shigella*,	BLIS	[62]

Table 1. *Cont.*

Bacteriocin	Producer (Strain)	Origin	Activity against *		Classification [a]	References
			Gram (+)	Gram (−)		
Staphylococcin D91	*S. aureus* (D91)	Clinical	*Staphylococcus*, *Streptococcus*	*Proteus*, *E. coli*, *Pseudomonas*	BLIS	[63]
BacCH91	*S. aureus* (CH-91)	Poultry (DSM26258)	*Staphylococcus*, *Streptococcus*, *Micrococcus*	–	Class I	[79]
Bsa	*S. aureus* (MW2)	MRSA community-acquired (ST8, ST80)	*Staphylococcus*, *Micrococcus*	–	Class I	[37,38]
Aureocyclicin 4185	*S. aureus* (4185)	Bovine mastitis	*Listeria monocytogenes*, *Micrococcus*, *Bacillus*	–	Class IV	[80]
Hyicin/Agneticin 3682	*S. hycius*/*S. agnetis* (3682)	Bovine milk	*Staphylococcus*, *Listeria*, *Streptococcus*	–	Class I	[81]
Hyicin/Agneticin 4244	*S. hycius*/*S. agnetis* (4244)	Bovine mastitis	*Staphylococcus*, *Listeria* Anti-biofilm against *S. aureus*	–	Sactipeptide	[82]
BacSp222	*S. pseudintermedius* (222)	Dog skin	*Staphylococcus*, *Micrococcus*, *Streptococcus*, *Bacillus*	+	Class II	[83]

[a] Staphylococcin classification in 6 groups (Class I–IV) also considering sactipeptides, thiopeptides, NRPs, and BLIS. * Antimicrobial activity: (+) positive; (−) negative.

Table 2. Bacteriocins described in coagulase-negative staphylococci (CoNS).

Bacteriocin	Producer (Strain)	Origin	Activity against *		Classification [a]	References
			Gram (+)	Gram (−)		
Capidermicin	*S. capitis* (CIT060)	Human skin	*Bacillus*, *Enterococcus*, *Lactococcus*, *Micrococcus*, *Staphylococcus*	–	Class II	[8]
Endopeptidase ALE-1	*S. capitis* (EPk1)	Clinical sample	+	–	Class III	[39]
NisinJ	*S. capitis* (APC2923)	Human skin	*Listeria*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Enterococcus*	–	Class I	[32,40]
TE8	*S. capitis* (TE8)	Human skin	*S. aureus*	–	BLIS	[64]
Nukacin L217	*S. chromogenes* (L217)	Bovine teat apices	*Staphylococcus*, *Streptococcus*	–	Class I	[84]

Table 2. *Cont.*

Bacteriocin	Producer (Strain)	Origin	Activity against *		Classification [a]	References
			Gram (+)	Gram (−)		
Staphylococcin T (StT)	*S. cohnii* (T)	Healthy human	*Staphylococcus*, *Streptococcus*, *Micrococcus*, *Listeria*	*Neisseria*	Class I	[48]
NukacinIVK45	*S. epidermidis* (IVK45)	Human nasal	*Micrococcus*, *Corynebacterium*, *Streptococcus*, *Dolosigranulum pigrum*	–	Class I	[41]
Pep5	*S. epidermidis* (5)	Clinical	*Staphylococcus*, *Micrococcus*, *Corynebacterium*	–	Class I	[42–44]
Epicidin 280	*S. epidermidis* (BN 280)	Clinical	*Staphylococcus*	–	Class I	[57]
Epilancin K7	*S. epidermidis* (K7)	Laboratory strain	+	–	Class I	[70]
Epidermin	*S. epidermidis* (Tü 3298)	Clinical	+	–	Class I	[52–56]
Epidermicin NI01	*S. epidermidis* (224)	Clinical	*S. aureus*, *Enterococcus* Anti-biofilm against *S. epidermidis*	–	Class II	[45]
Epilancin 15X	*S. epidermidis* (15X154)	Clinical	*Staphylococcus*, *Enterococcus*	–	Class I	[46,47]
Staphylococcin 1580	*S. epidermidis* (1580)	Laboratory strain	*Staphylococcus*, *Streptococcus*, *Bacillus*, *Corynebacterium*, *Listeria*, *Acinetobacter*	–	BLIS	[66]
Micrococcin P1	*S. equorum* (WS 2733)	Cheese	*S. aureus*, *Enterococcus*, *Listeria*	–	Thiopeptide	[85]
Gallidermin	*S. gallinarum* F16/P57 Tü3928	Chicken	*Propionibacterium*, *Staphylococcus*, *Streptococcus*, *Micrococcus* Anti-biofilm against *S. aureus*	*Neisseria*, *Moraxella*	Class I	[86–90]
Hominicin	*S. hominis* (MBBL 2-9)	Healthy human	*S. aureus*, *Micrococcus*, *Bacillus*, *Lactobacillus*	+	Class I	[49,50]
Nukacin KQU-131	*S. hominis* (KQU-131)	Thai fermented fish Pla-ra	Lactic acid bacteria, *Micrococcus*, *Bacillus*	–	Class I	[91]
Hogocidin-α Hogocodin-β	*S. hominis* (A9)	Human skin	*S. aureus*	–	BLIS	[34]

Table 2. *Cont.*

Bacteriocin	Producer (Strain)	Origin	Activity against *		Classification [a]	References
			Gram (+)	Gram (−)		
Homicorcin	*S. hominis* (MBL_AB63)	Seeds	*Staphylococcus*, *Micrococcus luteus*, *Bacillus subtilis*, *Lactococcus lactis*		Class I	[92]
Lugdunin	*S. lugdunensis* (N920143)	Human nasal	*S. aureus*, *Enterococcus*	–	NRPs	[51]
Nukacin 3299 Simulancin 3299	*S. simulans* (3299) *S. simulans* (Ec105)	Bovine mastitis	*Staphylococcus*, *S. agalactiae*, *Corynebacterium*	–	Class I	[93]
Lysostaphin	*S. simulans biovar staphylolyticus* (ATCC1362)	NRRL B-2628	*Staphylococcus*	–	Class III	[68,69]
Warnericin RB4	*S. warneri* (RB4)	Rice	*Thermo-acidophiles*, *Alicyclobacillus*, *Micrococcus*	–	Class I	[94]
Warnericin RK	*S. warneri* (RK)	Environmental	+	*Legionella*	BLIS	[65]
SWLP1	*S. warneri* (DSM 16081)	Human skin	+	–	Class I	[19]
Nukacin ISK-1	*S. warneri* (ISK-1)	Fermented rice bran "Nukadoko"	*Staphylococcus*, *Streptococcus*, *Micrococcus*, *Lactococcus*, *Bacillus*	–	Class I	[95–99]

[a] Staphylococcin classification in 6 groups (Class I–IV) also considering sactipeptides, thiopeptides, NRPs, and BLIS. * Antimicrobial activity: (+) positive; (−) negative.

2.2. Staphylococci in Skin/Nasal Microbiota of Animals

Animal nasal and skin microbiota has been broadly analyzed, mostly for their carriage of pathogenic CoPS species such as *S. aureus* and *S. pseudintermedius*. On the other hand, several other studies also focused on multiple members of the animal skin/nasal microbiota, including CoNS.

Livestock often acts as a reservoir for livestock-associated MRSA (LA-MRSA), a variant of *S. aureus* with worldwide distribution among different animal species, which remains a serious public health threat [100]. The most relevant subgroup of LA-MRSA, the clonal complex (CC) 398, is known to colonize livestock, especially pigs, as well as humans in contact with pigs. A high prevalence of LA-MRSA CC398 is usually detected in regions with intensive pig farming [101,102]. *Staphylococcus* is very frequent in the skin and nasal microbiota of pigs, and *S. aureus is* commonly detected, together with other CoNS species such as *S. equorum*, *S. schlifieri*, *S. cohinii*, *S. chromogenes*, *S. haemolyticus*, *S. hyicus*, and *S. microti*, among others [103–106].

Moreover, nasal microbiota plays an important role in individual predisposition to *S. aureus* nasal carriage in pigs [107]. In this respect, studies evaluating the pig microbiota revealed that *S. aureus* colonization is also linked with the absence of *S. sciuri*, *S. cohnii*, or *S. saprophyticus* [108]. Although both *S. aureus* and *S. sciuri* have been found colonizing pigs [106], it seems that *S. sciuri* is more frequent in animals where *S. aureus* is less frequent in the nasal microbiota [109].

Recent studies have confirmed the high frequency of colonization of staphylococci in wild animals. CoPS species were commonly found in nasopharynx and rectal samples of free-ranging mammals recovered in Spain [110,111]. Moreover, CoNS isolates have been detected among 60–75% of wild birds, according to studies performed in Spain and Portugal [112,113], and in 38% of wild mammals [114].

Some mammal species, such as wild boars, mouflons, and deers, are frequently colonized by MSSA, *S. pseudintermedius*, and *S. hyicus* staphylococcal species [110]. *S. aureus*, especially the LA-MRSA CC398 genetic lineage, was the most frequent CoPS species detected [111]. Remarkably, hedgehogs and wild rabbits could be reservoirs of MRSA carrying the *mecC* gene, which could be a risk to human health [110,115–117].

According to CoNS, *S. sciuri* was the most common colonizer of healthy wild animals. With respect to wild birds, *S. lentus* was the second most frequently recovered species and has also been detected in farm animals and people with professional exposure to livestock [118–120]. *S. xylosus* and *S. chromogenes* were also frequently detected in wild boars [114].

Focusing on companion animals, *S. pseudintermedius* is a CoPS commonly found in the normal nasal and skin microbiota of dogs and is considered one of the most frequent bacterial pathogens isolated from clinical samples in these animals [27]. Moreover, it highlights the emergence of methicillin-resistant *S. pseudintermedius* (MRSP) [121] as a significant health problem [122]. Studies assessing the commensal staphylococci in pets revealed CoNS as the predominant (89%) microbial group of the bacterial community of the nasal cavity of healthy dogs [123,124].

On the other hand, a comparative analysis performed in 2019 by Gómez–Sanz and collaborators showed that although CoPS was predominant in owners and pets, MRCoNS, especially methicillin-resistant *S. epidermidis* (MRSE), are common colonizers of healthy owners and pets [125]. The co-carriage of CoPS and MRCoNS highlights the relevance of companion animals as reservoirs of important multidrug-resistant opportunistic pathogens, which can be transferred to in-contact individuals.

As for bacteriocin production, several staphylococcins have been reported among staphylococci of livestock (Tables 1 and 2), frequently in those recovered from bovine mastitis (Aureocin 4181 [73], Aureocyclicin 4185 [80], Hyicin/Agneticin 4244 [82], Nukacin L217 [84], and Simulancin 3299 [93]). Moreover, several staphylococcins such as Aureocin 215FN [75,76], Staphylococcin 414 [77], Staphylococcin 462 [78], and BacCH91 [79] have also been detected in cow nares, turkey, mink, and poultry, respectively. On the other

hand, the detection of bacteriocin-producing staphylococci in pets is very limited, and as far as we know, only two bacteriocins have been reported in isolates of dogs (BacSp222, *S. pseudintermedius*) and cats (Micrococcin P1, *S. felis*) [83,126]. There is little information about the detection of staphylococcins in staphylococci of wild animals. However, in a recent study, antimicrobial substances of *Staphylococcus* from migratory birds were detected and their potential role in nasotracheal microbiota modulation was analyzed [20].

2.3. Staphylococcus in Food

Staphylococcus isolates can be present in animal-derived food products. *S. aureus* has been related to food poisoning due to the production of enterotoxins, while CoNS species are not commonly involved in any case of staphylococcal infection [127,128]. However, CoNS can also carry enterotoxin genes, such as *S. saprophyticus* and *S. epidermidis* species, considered opportunistic pathogens [129].

A wide list of CoNS species has been well described in fermented meat, sausages, fermented fish, milk, cheese, and, more recently, in fermented soybean [16]. Moreover, *S. pasteuri* has been found in a large percentage (65.7%) of drinking water [23], and other species of the genus *Staphylococcus* have been reported in raw pork, chicken, and beef meat [130]. In recent years, *Staphylococcus* spp. have attracted the attention of worldwide researchers due to their relevant role in improving the organoleptic properties (texture, acidity, and flavor) of fermented food products [131]. One of the most remarked benefits of staphylococci in food is the stabilization capacity of the red color of meat products, derived through the production of nitrate reductase, an action that also inhibits foodborne pathogens [132].

Several CoNS species have typically been associated with fermented foods (sausages and meat-based items) and used as starter cultures [133]. Among them, *S. xylosus* and *S. carnosus* are the CoNS species most frequently applied as starter cultures to standardize production and inhibit foodborne pathogens [16].

Staphylococcin-producing isolates in food have been widely reported in the literature, especially in milk and fermented food (Hyicin 3682 [81], Aureocin A70 [71,72], Aureocin A53 [74], Gallidermin [86–90], Micrococcin P1 [85], Nukacin ISK-1 [95–99], Nukacin KQU-131 [91], Warnericin RB4 [94] and the recently discovered Homicorcin [92]) (Tables 1 and 2). Moreover, recent works have reported the presence of bacteriocin-producing CoNS isolates in chicken-derived food that can act as protectors against other contaminant or pathogenic bacteria. The detection of two *S. sciuri* isolates with high antimicrobial activity is notable [21].

3. The Rationale for Exploring Bacteriocin-Producing *Staphylococci*: Beneficial and Functional Properties

The dearth of production of new and potent antimicrobial agents has led the scientific community to explore creative and unconventional remedies for AMR, including microorganisms and their products. Different studies have reported interest in bacteriocin-producing *Staphylococcus* isolates as noticeable sources of therapeutic agents.

Moreover, the ubiquitous nature of staphylococci, added to their flexible, multifaceted, and versatile metabolism, allow them to survive and inhabit highly diverse and distinct niches ranging from biotic and abiotic surfaces, environments, animals, humans, plants, (fermented) food, etc. Unlike other microbial groups, staphylococci are robust to environmental stresses, for example, acidic pH, the presence of the host's antimicrobial peptides, regular UV radiation exposure, dryness, constant environmental changes, and perturbations, among others [134,135].

Strains from different staphylococcal species, such as *S. xylosus*, *S. simulans*, and *S. equorum*, can tolerate harsh environmental stresses, such as high concentrations of salts (up to 21%) thanks to the possession of membrane pumps, voltage-gated channels, and accumulation of glycine betaine [134,136,137]. Additionally, most staphylococci are often robust toward nitrogen metabolism and oxidative pressures [138].

On the other hand, recent studies have reported their lipolytic and proteolytic activities [139,140]. Some strains of staphylococci often degrade amino acid-derived biogenic amines [141], and depending on the species and strain, staphylococci disintegrate fatty acids, resulting in the formation of methyl ketones [142]. With respect to the metabolism of carbohydrates, several staphylococcal strains usually produce organic acids depending on oxygen availability [133,143]. In this respect, *Staphylococcus* is a suitable microbial group to be explored for techno-functional aspects, including bacteriocins and ecological interests [144].

4. Bacteriocins: Promising Antimicrobial Substances

Currently, many natural peptidic antimicrobials have been discovered, and they usually fall into one of these three classes: ribosomally-synthesized peptides (RSAPs), ribosomally-synthesized and post-translationally modified peptides (RiPPs) or peptides produced by non-ribosomal peptide synthetases (NRPs) [145]. Most of the bacteriocins are ribosomally-synthesized and have been generally described as small, heat-tolerant, broad-spectrum proteinaceous substances that may act on target cells in a variety of different mechanisms [146].

4.1. Staphylococcins: Classes and Diversities

Staphylococcus is a well-known bacteriocin-producing genus [17], and staphylococcins constitute a relatively narrow group of compounds. Staphylococcins are defined as antimicrobial peptides or proteins produced by staphylococci [19].

According to the classical bacteriocin classification, staphylococcins have been commonly divided into four groups of peptides and proteins (Class I–IV) [18,19]: (A) Class I bacteriocins, heat-stable and post-translationally modified small peptides known as lantibiotics (<5 kDa, 19–37 amino acids) [147]; (B) Class II bacteriocins, non modified post-translationally and heat-stable and small peptides (<10 kDa) [148]; (C) Class III bacteriocins, large (>30 kDa) and heat-labile peptides subdivided as lytic and non-lytic bacteriocins [149]; (D) Class IV bacteriocins, cyclic peptides formed by the post-translationally covalent linkage [150]. Moreover, other bacteriocins of the sactipeptides and thiopeptides groups have been recently discovered [151,152]. In this sense, recent reviews proposed to divide staphylococcins into six classes to better understand the characteristics of each group of antimicrobial peptides. These consider placing Aureocyclicin 4185 into Class IV, the first cyclic bacteriocin described in *Staphylococcus*; sactibiotics as Hyicin/Agneticin 4244 into Class V; and thiopeptides, as Micrococcin P1, into Class VI [19].

In addition to these bacteriocin classes, there are other types of NRP antimicrobials peptides with non-ribosomal synthesis (NRPs) [153] and among them, lugdunin pro-duced by *S. lugdunensis* is worth noting as an NRP produced by *S. lugdunensis* [51]. Moreover, there is another group of antimicrobial substances that act as bacteriocins but are neither obtained in pure form nor fully characterized. These substances known as Bacteriocin-Like Inhibitory Substances (BLIS) have been reported in the literature since 1991 [154]. Despite the unclear characteristics of BLIS, it should be noted that the unclear chemical structure of these compounds often does not limit even advanced applicative studies on these substances [155]. The presence of BLIS-producing staphylococcal isolates was recently reported among 60 of 890 staphylococcal isolates (6.7%) of different species and origins [21].

4.2. Biochemical and Genetic Characterization of Staphylococcins

To date, 47 staphylococcins have been fully identified and characterized depending on whether the producing *Staphylococcus* strain is considered CoPS or CoNS, respectively (Tables 1 and 2).

Regarding CoPS, 20 staphylococcins have been reported, including those produced by *S. aureus* (aureocins of Class I, II, IV or BLIS), *S. pseudintermedius* (BacSp222, Class II), or the coagulase-variable staphylococci *S. agnetis* (Hyicins/Agneticins of Class I or Class V) (Table 1). Aureocins such as BacCH91 and Bsa, considered lantibiotics, are generally

included in Class I and are mostly active against Gram-positive pathogens. Nevertheless, aureocins have also been described in Class II (Staphylococcin C55, Aureocin A70, the newly described variant Aureocin 4181 and Aureocin A53) and Class IV (Aureocyclicin 4185). Other aureocins without a completely determined gene or protein sequence are considered BLIS, and their characteristics are also shown in Table 1.

The 27 CoNS bacteriocins presented in Table 2 are mostly lantibiotics (Class I), but also bacteriocins of Classes II (Epidermicin NI01 and Capidermicin), III (Lysostaphin and Endopeptidase ALE-1), NRPs (Lugdunin), and 4 BLIS (TE8, Hogocidinα/β, Staphylococcin 1580, and Warnericin RK).

5. Bacteriocin Detection and Characterization Methods

To succeed in the detection of novel antimicrobial peptides, a rational selection of the environmental source of potential producers is a crucial step. Moreover, culture conditions and nutrient requirements should be carefully considered before the screening process. In this respect, it is known that changes in the natural environment of the producing isolate affect antimicrobial peptide synthesis, especially under in vitro conditions. In addition to the fact that bacteriocin production tends to occur against a narrow spectrum of bacteria, the detection and identification of the producing strains could be difficult. Here, we present a summary of the most common methodologies used to search for new antimicrobial compounds and suggestions for bacteriocin detection.

5.1. Phenotypic Methods

Multiple techniques have been used to identify and screen bacterial isolates for bacteriocin production in vitro. Frequently, agar diffusion assays are employed to evaluate the antimicrobial activity of potentially producing isolates using spot-on-lawn [156,157].

Moreover, diffusion assays are performed to evaluate the bioactivity of antimicrobial agents prior to and after the pre-purification process by comparing the zones of activity obtained with cell-free supernatants as well as the whole-cell extracts obtained after chemical extraction procedures against indicator bacteria [157]. In this respect, the extract of the producing strain is aseptically applied to blank discs (about 6 mm in diameter) or wells (diameter of 6 to 8 mm) and then introduced onto a plate previously seeded with the indicator microorganism (target). Multiple variations using specific culture media and various incubation conditions could be followed [158].

Moreover, other diffusion methods have been reported to screen the antimicrobial activity of extracts, fractions, or pure substances or to investigate the antagonism between microorganisms. Among these techniques, the agar plug diffusion and cross streak methods are the most commonly used [158].

However, all these methods have limitations because they cannot discriminate between inhibitory activity caused by bacteriocins or other antimicrobial substances [17]. Therefore, for a deeper physicochemical characterization of the antimicrobial agent's nature, an overnight culture of the bacteria could be prepared, and later, a cell (or viable cell) free extract could be obtained and characterized. The effect of proteolytic enzymes, different temperatures and times of incubation, and several ranges of pH values are commonly evaluated [159,160]. Other studies have also tested the effect of organic solvents (alcohols, phenols) and salts [161].

5.2. Genotypic Methods

Genes encoding bacteriocins, as well as those genes encoding a set of immunity proteins and other accessory proteins, are arranged in operon clusters that reside in either chromosomes, plasmids, or other mobile genetic elements. The ribosomal synthesis and the presence of a self-defense immunity system distinguish bacteriocins from secondary metabolites that also exhibit antimicrobial activity [162]. Commonly, the detection of bacteriocin structural genes has been carried out through PCR and/or DNA/DNA hybridization [163,164].

The bacteriocin PCR matrix is based on known bacteriocin-related genes from the databases. To date, this method is actively used for screening bacteria that produce lantibiotics. However, due to each producing strain usually carrying different gene sequences or slight variations, PCR analysis and primer pairs should be optimized to avoid unspecificities [165].

Although a wide variety of bacteriocin genes have been described, there is no method based on PCR that allows the detection of several staphylococcins from a preliminary screening. Thankfully, since the advent of in-silico screening, this process of bacteriocin discovery has been significantly reduced in terms of time and cost. Moreover, the new genome mining tools offer an important technological resource in the discovery of novel natural products based either on the detection of bacteriocin structural genes or other bacteriocin-associated genes [166]. A wide variety of bioinformatic tools such as BACTIBASE, antiSMASH, BAGEL, APD3, ANTIMIC, DRAMP, or URMITE have been described [167–171]. However, it is noteworthy that harboring the staphylococcin gene clusters does not necessarily imply peptide production; thus, bacteriocin production should be confirmed by the antagonism assays explained above after finding those genes in the genomes [19].

5.3. Protein Methods

For bacteriocin detection and purification, it is important to verify the optimal conditions of production, and it is recommended to test the resulting eluents to verify their antimicrobial activity. Since bacteriocins form an extremely heterogeneous group of substances, specific purification protocols generally need to be designed. Three major bacteriocin purification methods can be distinguished according to the biochemical structure. (1) Subsequent ammonium sulfate precipitation, ion exchange, hydrophobic interaction, gel filtration, and reversed-phase high-pressure liquid chromatography [172], (2) a protocol based on simple three-step phases starting with ammonium sulfate precipitation, continuing with chloroform/methanol extraction/precipitation, and finishing with reversed-phase high-pressure liquid chromatography, as the sole chromatographic step involved [173], and (3) bacteriocin isolation through a unique unit operation. This last protocol can distinguish between the expanded bed adsorption method [174] and the use of organic solvents such as butanol [51].

After purification, MALDI-TOF mass spectrometry can be used for quick bacteriocin detection, and chromatograms should be examined for the identification of a known bacteriocin. Moreover, the presence of multiple peaks may indicate the presence of more than one peptide [157]. Thus, a combination of reverse-phase high-performance liquid chromatography (HPLC) and MALDI-TOF mass spectrometry can be used to determine if a purified substance obtained from the pooled active fractions contains a single, active bacteriocin or if multiple peptides are present [157].

In conjunction with peptide purification, genomic analysis for the identification of bacteriocin gene clusters is required to determine the novelty of the recovered antimicrobial peptide, and for the identification of new bacteriocins, ultra-HPLC coupled with mass spectrometry is recommended.

As a help for researchers, we present in this review a complete guide for staphylococcin identification. Table 3 shows a total of 27 structural bacteriocin nucleotidic and amino acid sequences registered on the NCBI databases until January 2023 and summarizes their associated genes, their accession GenBank number and GenePept sequences, their gene position, gene size (bp), and bacteriocin masses (Da). This information is of great use for staphylococcin detection both with PCR and whole genome analysis but also for their verification with mass spectrometry analysis.

Table 3. Description of the staphylococcal bacteriocin sequences used in this study [a].

Bacteriocin	Gene [a]	GenBank Accession No.	GenePept Accession No. [a]	UniProt	Gene Size (bp)	Protein Size (Da)
Staphylococcin C55	*sacaA*	AF147744	AAD47011	Q9S4D3	188	3339
	sacbA		AAD47012	Q9S4D2	203	2993
Aureocin A53	*aucA*	AF447813	AAN71834	Q8GPI4	142	6012.5
Aureocin A70/ Aureocin 4181	*aurD*	AF241888*/ MK796167	AAK73555		95	3147.7 ± 1.5
	aurC		AAK73554		95	2983.6 ± 1.5
	aurB		AAK73553		92	2824.4 ± 1.5
	aurA		**AAK73552**		95	2951.5 ± 1.5
BacCH91	*bacCH91*	JQ655767	AFN42846	I6XG59	144	2074.9
Bsa	***bsaA2***		**BAB95630**	A0A0H3K3P8	143	2089
	bsaA1	BA000033	BAB95631	A0A0H3JXA5	143	2281
Aureocyclin 4185	*acIA*	KF836421	ATV90647		195	
BacSp222	*bacSp222*	CP011490	ALI97662	A0A0P0C3P7	150	5921.92
Hyicin/Agneticin 3682	*hyiA*	KY021154	ARD24445	A0A1V0JZL0	144	2139
Hyicin 4244	*hycS*	KY887472	ASL69762	A0A221C8V1	128	3274
Capidermicin	*orf4*	MN234131	QFR37570	A0A5P8N9U9	153	5438
Endopeptidase ALE-1	*ale-1*	D86328	BAA13069	O05156	1089	39,350
NisinJ	*nsj*	NZ_MN602039	QGN18867		183	
Gallidermin/ Staphylococcin T (StT)	*gdmA*	U61158 [b]	AAB61135 [b]	P21838	159	2165.6
Epidermin	*epiA*	X62386	P08136	P08136	156	2151
Epidermicin NI01	*ecdA*	JQ025382	AFD03077	H9BG66	390	6074
Epicidin 280/Homicorcin	*eciA*	Y14023	CAA74348	O54220	90	3133 ± 1.5 and 3136 ± 1.5
Pep5	*pepA*	Z49865	CAA90023	P19578	183	6575.4 ± 1.7
Epilancin 15X	*elxA*	JQ979180	P86047	P86047	168	3173
Epilancin K7	*elkA*	U20348	AAA79236	Q57312	165	3032 ± 1.5
NukacinIVK45	*nukA*	KP702950	AKQ51579		173	2940
Nukacin KQU-131	*nkqA*	AB432987	BAG70955	B5MFD0	173	3003.97
Nukacin 3299	*nukA*	GQ380548	ACU82391	E0WX65	173	2957.3
Nukacin ISK-1	*nukA*	AB125341	BAD01007	Q9KWM4	173	783 (g/mol)
Lugdunin	*lugA*	CP020406	ARB77241		7124	
	lugC		ARB77243		8813	
	lugD		**ARB77244**		1739	
Lysostaphin	*lss*	U66883	P10547	P10547	1482	
Hominicin	- [c]	- [c]	WP_152903494		- [c]	2038.4
Micrococcin P1 [d]	*tclE*	KM613043.1	AIU53942.1	Q9F9L4	150	1144.4

[a] In the cases in which several genes for a bacteriocin have been identified, the coding genes are marked in bold. [b] Bacteriocins with high similarities between their coding gene sequence as the case of Gallidermin and Staphylococcin T (StT) or Epicidin 280 and Homicorcin. [c] The nucleotide sequence for Hominicin is not available. [d] The reference accession number is the one of *Micrococcus caseolyticus*.

5.4. Universal Nucleotide and Amino Acid-Based Staphylococcin Phylogenetics

Here, we present a novel tool for bacteriocin detection in order to help researchers in their search and characterization. All structural genes and coding amino acids of well-described staphylococcins were selected (Table 3) (consult entry databases), and a

phylogenetic analysis was conducted in MEGA X [175] based on the maximum likelihood homology of the sequences included (Figure 1). Thus, 34 amino acids and 33 gene sequences were used. The relationships were inferred using the neighbor-joining method [176]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [177]. The tree is drawn to scale 0.5, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [178]. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Those genes or protein sequences with more than 50 substitutions per site were considered unrelated bacteriocins.

Thus, phylogenetic analysis both at genetic and protein levels revealed six bacteriocin groups that we refer to as families (Figure 1): BS, including the bacteriocins Bsa, Hyicin 3682 and BacCH91; EP5, formed by Epidicin 280/Homicorcin and Pep5; NUK, which comprises all the nukacins (Nukacin IVK45, Nukacin KQU-131, Nukacin 3299, and Nukacin ISK-1); GEST, including Gallidermin, Staphylococcin T (StT), and Epidermin; the bacteriocin family named EPI, formed by Epilancin 15X and Epilancin K7, and finally, the family CAPSP, including Capidermicin and BacSp222 bacteriocins. The gene cluster encoding the Aureocin A70 (*aurA*, *B*, *C*, *D*) was not considered as a family because it only codifies for a unique bacteriocin and showed high similarity only comparing the amino acid sequence.

Notably, our arrangement corresponds to a majority of those recently reported [19] but provides more details of the similarities of staphylococcins included in each of the 6 classes proposed by this work. Concretely, within Class I Lantibiotics, our families revealed higher similarities between nukacins (NUK family), Gallidermin, Staphylococcin T (StT), and Epidermin (GEST family) and Bsa, Hyicin 3682 and BacCH91 bacteriocins, considered as BS family. As for Class II, we proposed the family CAPSP (conformed by Capidermicin and BacSp222) due to their higher similarities in nucleotide and amino acid sequence. These bacteriocin families have been used in a previous work of our group for designing PCR primers in order to detect possible bacteriocin genes [21].

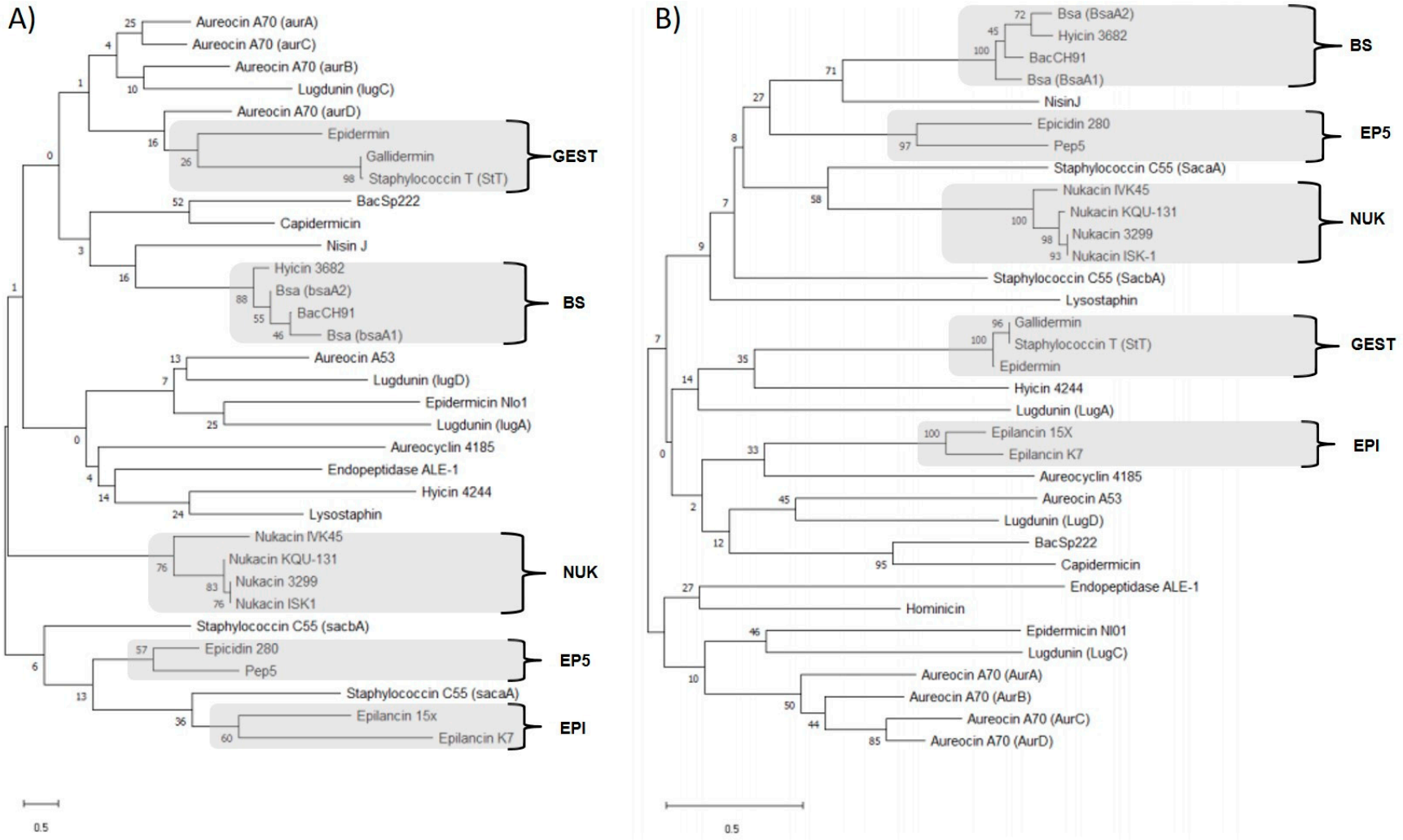

Figure 1. Bacteriocin similarities and staphylococcin families determined by the phylogenetic analysis carried out with the program MegaX: (**A**) Nucleotide-based phylogenetic tree and (**B**) Amino acid-based phylogenetic tree. In grey are marked the proposed bacteriocin families.

6. Applications of Bacteriocin-Producing *Staphylococcus* Isolates or Their Staphylococcins

Based on the rise of antibiotic-resistant bacteria, a complementary approach in searching for novel drug formulations is demanded. In this respect, purified or partially purified bacteriocins hold great promise and may ultimately be employed as pharmabiotics and/or novel alternatives to existing antibiotics [179]. Moreover, the activity of conventional antimicrobials can be enhanced when combined with novel and often naturally derived antimicrobials [17,180]. It is to note the antimicrobial properties of some staphylococcins alone or in combination with other antimicrobials with high interest to be used in the clinical field, both veterinary and human medicine [7,19,180].

There are few studies concerning the use of staphylococcins carried out in vivo, having a need for in vitro validation before their use in clinical trials both in animal models and in humans. In this respect, the pharmacokinetic parameters of the host [181], the potential bacteriocin-induced toxicity [182], and the route of administration must be considered.

Several studies have tested some staphylococcins in animals. Murine models have been used to analyze the possible role of Staphylococcin 1580 for inhibition of caries [183], Lysostaphin for treating MRSA wounds, pneumonia, and/or systemic infections [184–186], or as an alternative for mastitis produced by *S. aureus* [187] and MP1 for skin infections produced by MRSA [188]. Epidermicin NI01 has been tested in the *Galleria mellonella* larvae model [189] and for eradicating nasal colonization of MRSA in rats [190] with very promising results. Human studies have also been performed, and the bacteriocin-producing *S. hominis* ShA9 has been reported as a good alternative to control *S. aureus* during skin dysbiosis and other diseases such as atopic dermatitis [34,191].

Skin infections, especially skin and soft tissue infections (SSTIs) caused by *S. aureus*, are among the most common infections in the world and have been one of the most studied. In the same way, it has been reported that *S. aureus* nasal carriers suffer from infective processes or present a low richness of species in their nasal microbiota, which can precede disease. Moreover, MRSA and specific genetic lineages of *S. aureus* (MRSA-CC398) present emerging antibiotic resistance determinants of special interest. Therefore, being able to control the high prevalence of this multidrug-resistant microorganism in the skin and nasal microbiota of pigs and in-contact humans is a public health challenge.

One new and emerging potential application of staphylococcins or bacteriocin-producing staphylococci is their interesting role in human microbiota modulation [192]. One applied example consists of the balance of skin microbiome in atopic dermatitis, made by CoNS strains to compete or limit *S. aureus* growth, including MRSA [34]. In this respect, several bacteriocin-producing CoNS isolates of skin and mucous tissues with interesting antimicrobial activities commonly against potentially pathogenic Gram-positive and, in a few cases, also in Gram-negative microorganisms, have been reported [49,51,193]. Due to the great potential of bacteriocins, especially those produced by commensal isolates, the identification and characterization of novel antimicrobial peptides should be a clear goal [34,188,191].

On the other hand, bovine mastitis is one of the most persistent and economically significant diseases affecting dairy cattle worldwide. *S. aureus* and *Streptococcus* spp. are the most common etiologic agents involved in bovine mastitis [194,195]. In recent years, the emergence of resistance and the increasingly strict regulations on dairy farms regarding the use of these drugs in animal production has forced the development of alternatives, such as bacteriocins, for the control and prevention of this disease [196]. As mentioned before, Lysostaphin has been tested in a murine mastitis model [187]. Recently, ex vivo and in vitro assays have been carried out with Lysostaphin and with other staphylococcins such as Aureocin A53 and Aureocin A70 [19].

Apart from the antimicrobial activity of bacteriocins produced by *Staphylococcus* described over time, anti-virus, anti-inflammatory, and immunomodulation activities have also been recently reported [133,197–204]. In this respect, one of the most intriguing new fields of investigation is the study of bacteriocins as potential anti-cancer and anti-tubercular agents [205–207]. Moreover, recent studies have shown the potential use of staphylococcins

or *Staphylococcus*-producing isolates as bio preservatives in meat to assure the microbial shelf-life of the product [208] or as anti-fungic agents to prevent toxigenic molds [209].

However, it is important not only to discover new bacteriocins and antagonism activities but also to test for toxicity to prove their safe use in a preclinical phase (in vivo antimicrobial and/or toxicity effects) as candidates for therapeutic processes.

To this end, biotechnological techniques such as bioengineering or chemical synthesis of bacteriocins can be important tools to improve the antimicrobial activity of bacteriocins, change their physicochemical properties, or reduce the cost of production.

7. Emerging Concerns Associated with the Use of Staphylococcins

Apart from the antimicrobial properties exhibited by bacteriocins that make them suitable antimicrobial agents, various concerns associated with their applications in human and animal medicine, food production, and industries have emerged. Among the emerging concerns associated with staphylococcin application, here we will give an overview of their safety in host cells as their toxicity, immunogenicity, bioavailability and absorption, exposure and development of resistance, and the legal framework.

Staphylococcins, as well as other bacteriocins, are generally considered safe antimicrobials and therapeutic substances. However, some staphylococcins have been reported to be cytotoxic to mammalian cell lines, usually at high concentrations [7]. To a large extent, bacteriocins' cytotoxicity to eucaryotic cells depends on the concentration, purity, composition, and type of eucaryotic cell line used [210,211]. Therefore, since the cytotoxicity of bacteriocins is often evaluated using different concentrations and types of eucaryotic cell lines, it is difficult to generalize and/or compare the cytotoxicity levels. For a comparative safety evaluation, it is necessary to have a consensus on the type of assay, the concentration of staphylococcin, the composition, and the type of cell line to be used.

Regarding the safety and immunogenicity of staphylococcins, it has been reported that the use of bioactive molecules with undesirable immune responses could be detrimental to the host [212–214]. Therefore, the assessment of bacteriocins' immunogenicity profile should be considered crucial and necessary, especially when they are intended for use in the food industry or as biotherapeutics in humans or animals.

Another concern associated with the use of staphylococcins in the food industry or as a therapeutic strategy in humans or animals is the potential risk for the development of resistance upon prolonged exposure to the target or spoilage of pathogenic microorganisms. The resistance development to bacteriocins has been reported to be either (1) intrinsically (innate) within specific genera or related strains or (2) acquired, i.e., resistance developed from a previously susceptible strain [215,216]. However, our understanding of the potential for bacteriocin resistance development has been revealed primarily from experiments performed under laboratory conditions [217]. In this respect, the bacteriocins most studied for the development of resistance are nisin, lacticin 3147, and pediocin-like bacteriocins [216], and regarding staphylococccin resistance, lysostaphin is the most studied so far [218].

Finally, there is a lack of a universal consensus on the legal and regulatory aspects of the use of bacteriocins. Although several bacteriocin-producing microorganisms have attained the 'generally regarded as safe (GRAS)' status, it is generally necessary to achieve the guidelines for the approval of bacteriocins either as food additives/preservatives, technological or therapeutic agents depending on their intended use and the subsisting laws of the particular country.

8. Conclusions

Bacteriocin-producing staphylococci, especially the commensal CoNS of human and animal microbiota, provide an excellent model to find bacteriocins that could be promising candidates to combat AMR and to compete against pathogens or protect against infections. Moreover, staphylococcins have steadily shown great potential and are being considered for potential applications in clinical, veterinary, food, and biotechnology.

However, characterizing and mining staphylococci for staphylococcins could be demanding. Therefore, this review provides comprehensive and up-to-date approaches for the search, characterization, and evaluation of staphylococcins from staphylococci of different origins. For the first time, we developed a universal nucleotide and amino acid-based phylogeny of all the fully characterized and known staphylococcins. We believe that these resources will undoubtedly help and spur researchers' interest in exploring staphylococci and advancing the science and application of staphylococcins.

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Genomic analysis of bacteriocin-producing staphylococci: high prevalence of lanthipeptides and the micrococcin P1 biosynthetic gene clusters

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Genomic analysis of bacteriocin-producing staphylococci: high prevalence of lanthipeptides and the micrococcin P1 biosynthetic gene clusters

Rosa Fernández-Fernández[1†], Ahmed M. A. Elsherbini[2,3,4†], Carmen Lozano[1], Agustí Martínez[1], María de Toro[5], Myriam Zarazaga[1], Andreas Peschel[2,3,4], Bernhard Krismer[2,3,4] and Carmen Torres[1*]

Author information:

[1]Area of Biochemistry and Molecular Biology of the University of La Rioja, Logroño, Spain
[2]University of Tübingen, Interfaculty Institute of Microbiology and Infection Medicine, Department of Infection Biology, 72076 Tübingen, Germany
[3]Cluster of Excellence EXC 2124 Controlling Microbes to Fight Infections, Tübingen, Germany
[4]German Center for Infection Research (DZIF), partner site Tübingen, Tübingen, Germany
[5]Genomics and Bioinformatics Core Facility, Center for Biomedical Research of La Rioja, Logroño, Spain

[†], contributed equally to this work

[*]**Corresponding author:** Prof. Carmen Torres
University of La Rioja, Area of Biochemistry and Molecular Biology,
OneHealth-UR Research Group, 26006 Logroño, Spain,
Phone. 34941299750; Email: carmen.torres@unirioja.es

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Abstract

Bacteriocins are antimicrobial peptides produced by bacteria, considered as promising alternatives to antibiotics. This study aimed to *in-silico* analyse the presence of bacteriocin gene clusters (BGCs) among the genomes of 22 commensal *Staphylococcus* isolates from different origins (environment, human, food, pet, and wild animals) previously identified as bacteriocin producers. The resistome and plasmidome were also studied in all isolates. Potential BGC were analysed among the 22 genomes and five types of BGCs were detected in 18 bacteriocin-producing staphylococci: class I (Lanthipeptides), class II, circular-bacteriocins, the non-ribosomal-peptide lugdunin and thiopeptides (micrococcin P1, MP1). The analysis highlights specifically the high frequency of lanthipeptides detected in the collection: BGC variants of BSA, bacCH91 and epilancin15X were identified in two *Staphylococcus aureus* and one *Staphylococcus warneri* isolates from food and wild animals. Moreover, two potentially new lanthipeptide-like BGCs with no identity to database entries were found in *Staphylococcus epidermidis* and *Staphylococcus simulans* from food and wild animal, respectively. Interestingly,

four isolates (one *S. aureus* and one *Staphylococcus hominis*, environmental origin; two *Staphylococcus sciuri*, food) carried the MP1 BGC with differences to those previously described. On the other hand, seven of the 22 genomes (~32%) lacked known genes related with antibiotic or disinfectant acquired resistance mechanisms. Moreover, the potential carriage of plasmids as elements of gene acquisition and dissemination was evaluated and several Rep proteins were identified (~73% of strains). In conclusion, a wide variety of BGCs has been observed among the 22 genomes and an interesting relationship between related *Staphylococcus* species and the type of bacteriocin has been revealed. Therefore, bacteriocin-producing *Staphylococcus* and especially coagulase-negative staphylococci (CoNS) can be considered good candidates as a source of novel bacteriocins.

Keywords: bacteriocins, BGC, CoNS, lanthipeptides, micrococcin P1

Introduction

Antimicrobial resistance (AMR) represents one of the biggest challenges of modern medicine worldwide [1]. This AMR global problem requires novel antimicrobial agents and strategies to overcome the threat by various pathogens, including multidrug-resistant (MDR) and zoonotic bacteria [2, 3]. About 35% of all drugs and 60-80% of all antimicrobial products have originated from natural products, and the development of innovative products derived from natural substances is gaining attention for pharmaceutical and therapeutic uses [4,5,6]. However, financial pressure from drug companies as well as difficulties in the isolation and identification of new natural compounds have severely limited the discovery rate from these important sources [5,6].

Typically, bacteriocins are ribosomally synthesized peptides, encoded by operons including the structural genes whose products are often post-translationally modified by specific enzymes encoded by adjacent genes. In these genetic systems regulatory genes, immunity genes, transporters and other genes that encode accessory proteins are also found.

[7,8,9]. Traditionally, the identification of novel bacteriocins has used cultivation-based approaches that involved screening of numerous isolates for antimicrobial activity, followed by long-term biochemical characterization [10,11]. More recently, genome mining has become an important methodology in the discovery of novel natural products with antimicrobial activity [7]. In this respect, ribosomally synthesized and post-translationally modified peptides (RiPPs) of bacterial origin are natural compounds that are highly attractive candidates for antibacterial prevention and therapy [7,12]. Nevertheless, other types of bacteriocins can be also very relevant such as Aureocins or Epidermicin NI01 [13].

Bacteriocins have been divided in three major classes depending on their amino acid

composition, chemical structure, complexity, mode of action and steps involved in their production (synthesis, transport, and immunity): class I, small post-translationally modified peptides; class II, unmodified bacteriocins; and class III, larger and thermo-labile peptides [14,15]. However, recent reviews have proposed the inclusion of additional classes among Gram-positive bacteriocins: class IV (circular), class V (sactipeptides), class VI (thiopeptides) and non-ribosomal peptides (NRP) [13,14,16, 17, 18, 19, 20, 21,22].

Within the last few years, increasing numbers of bacteriocins have been isolated and identified from Gram-positive microorganisms [15] and the lanthipeptides (class I) belong to the most frequently found [23]. These peptides represent a promising type of natural antibacterial molecules, active against many Gram-positive pathogens, including antibiotic-resistant strains such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) [24]**.**

Environmental members of the phylum Actinobacteria and Firmicutes including bacilli and lactic acid bacteria, and many Proteobacteria such as *Escherichia coli* are well-known as producers of biologically active substances [25,26,27]. In addition, *Staphylococcus* species, in particular CoNS isolated from a wide variety of natural sources have also been found to be interesting sources of BGCs for bacteriocins [28,29,30].

In previous studies carried out by our group [31,32,33], a collection of 92 bacteriocin-producing staphylococci was detected in a screening approach performed with 1205 staphylococci of different species and origins (~7.7% were bacteriocin producers) (Table S1). Twenty-two of these isolates of 11 species, both coagulase-positive and negative staphylococci (CoPS and CoNS, respectively), were included in this study for further characterization. Isolates were selected under a OneHealth perspective based on their antimicrobial activity profile (high production) and their origin. The specific objectives of this study were **(1)** to predict and analyse the presence of BGCs in the genomes of the 22 selected bacteriocin-producing *Staphylococcus* isolates and **(2)** to investigate the novelty or the homology between our BGCs after comparison with those previously reported in databases.

Material and methods

Isolates Included in the Study

The genomes of twenty-two *Staphylococcus* isolates, both CoPS and CoNS, of eleven different species and six origins, were included in this study for genomic comparison. The bacteriocin-producing isolates were (number of isolates, identification code): a) *S. aureus* from food (n=1, X3410), environment (n=1, C5802), and wild mammal (n=1, C8609); b) *Staphylococcus pseudintermedius* from dogs

(n=2, C4502, C8478) and human (n=1, C8189); c) *Staphylococcus chromogenes* isolates from wild mammals (n=2, C9838, C9727); d) *Staphylococcus hyicus* isolates from wild mammals (n=2, C9581, C9585); e) *S. sciuri* isolates [recently reclassified as *Mammaliicoccus sciuri* [34,35] were recovered from wild birds (n=3, C9213, C9179, C9529) and food (n=2, X3011, X3041); f) *Staphylococcus lugdunensis* isolates from humans (n=2, C9161, C9954); g) *S. hominis* of environmental origin (n=1, C5835); h) *S. warneri* (n=1, X2969) from food; i) *S. simulans* recovered from a wild mammal (n=1, C9832); j) *S. epidermidis* (n=1, X3009), from food and k) *Staphylococcus xylosus* (n=1, C9255) isolated from wild birds. The characteristics of the 22 isolates analysed in this study are indicated in **Table S2**.

DNA Extraction, Amplification, and Sequencing

The NucleoSpin microbial DNA Kit (Macherey and Nagel, Germany) was used for DNA extraction. For it, 2 ml of a 24-hour grown culture in normal BM medium at 37°C, were pelleted (40 mg) by centrifugation (10 min at 10000 rpm) and the FastPrep-24 Classic homogenizer (MP Biomedicals) was used for mechanical disruption. Genomic DNA was subsequently purified according to the manufacturers' instructions.

The genomic libraries for eight *Staphylococcus* isolates (X2969, X3009, X3041, C8609, C9832, C9581, C9585 and C9838) were prepared with the TruSeq DNA PCR-Free Kit (Illumina) by the Genomics and Bioinformatics Core Facility (CIBIR), whereas the genomic libraries of the other isolates (C4502, C9161, C9179, C9213, C9255, C9529, C9727, C9954, X3011 and X3410) were prepared with the Nextera XT library preparation kit (Illumina) by the NGS Competence Center Tübingen (NCCT). In both cases, libraries were sequenced in different runs on an Illumina MiSeq platform with the MiSeq Reagent Kit v3 (Illumina, 150 -cycle). The genomes of the isolates C5802, C5835, C8478, C8189 were previously obtained in collaborative studies of the OneHealth-UR research group and all isolates belong to the strain collection of the University of La Rioja.

The Whole Genome project has been deposited at GenBank under the accession PRJNA974190 and the respective accession numbers are included in Table S2.

Data Analysis

Raw reads quality was checked using FastQC (v0.11.8) [36]. Trimming of raw reads, *de-novo* assembly and assembly polishing were performed with Trimmomatic (v0.39) [37], SPAdes (v3.15.5) and Pilon (v1.24), respectively [38,39], through the Shovill pipeline [40]. Then, assembled genomes were functionally annotated using Prokka (v1.14.6) [41].

Using protein files exported from the Prokka annotations in FASTA format, a maximum likelihood tree was computed via the RaxML program (v8.2.12) [42], applied inside PhyloPhlAn (v3.0.60) pipeline using the PhyloPhlAn protein markers database and with the default diversity parameter for genus/family level phylogenies [43]. The tree was visualized using the ggtree package (v3.4.4) of R (v4.2.1) [44,45].

Resistome and Plasmidome

The resistance genotypes and the presence of plasmid replication (*rep*) genes were studied through ResFinder and PlasmidFinder web tools, respectively [46]**.** In all cases, the default parameters with relaxed mode were used and *S. aureus* was selected as reference species.

Bacteriocin Gene Clusters Prediction

To predict and analyse homologous bacteriocin gene clusters (BGCs) in the 22 genomes, all annotated genomes in GenBank format were uploaded to antiSMASH (v6.1.1) [47] and BAGEL4 [48] with all parameters with a relaxed mode. For BGC prediction, the genomes were considered as a pool of contigs both including chromosome and plasmid regions.

To extract homologous BGCs of MP1 from *Macrococcus caseolyticus,* pBac115 plasmid sequence (accession number KM613043.1) was used as reference, and the protein sequences identified by antiSMASH were blasted against the NCBI protein database using the Cblaster (v1.3.15) tool with default parameters [49]. To simplify the output, unique non-redundant homologous BGCs per species were aligned against the *M. caseolyticus* BGC and the MP1 genomes from this study using the Clinker (v0.0.25) tool with the default parameters [50].

To investigate whether all identified micrococcin P1 (MP1) BGCs were encoding the identical thiopeptide, the structural protein sequences were extracted from all BGCs of interest. Then, they were mutually aligned by MAFFT (v7.310) and visualized with Jalview (v2.11.2.0) according to ClustalX colour scheme [51].

Moreover, identity comparisons between the MP1 plasmid from *S. hominis* S34-1 (CP040733.1) and the MP1 producing isolates (*S. sciuri* X3041 and X3011, *S. hominis* C5835, *S. aureus* C5802) were performed with Clustal W2 and Emboss Needle [52], and Clinker (v0.0.25) tools [50].

Results

The complete genomes of 22 bacteriocin-producing *Staphylococcus* spp. isolates, both CoPS and CoNS, were obtained by WGS and annotation. Phenotypic and molecular characteristics of the isolates as well as results of resistome and plasmidome analyses are presented in Tables S2 and S3. Several bacteriocin encoding BGCs were identified, which are summarized in Table 1.

According to the analysis of the 22 genomes included in this study, seven out of 22

(~32%) isolates lacked known antibiotic or disinfectant resistance-related genes. The remaining 15 isolates (~68%) carried at least one gene associated with antibiotic resistance (beta-lactams, macrolides, lincosamides, streptogramins and aminoglycosides (LSA), tetracyclines, phenicol, fusidic acid and Fosfomycin) or disinfectant agents. Regarding the plasmidome, a wide variety of Rep protein genes were identified in 16 of the studied genomes. Among these genomes, eight carried *rep* and antimicrobial resistance genes and the other eight only carried *rep* genes (Table S2). Interestingly, isolates C9727, C9954 and C9161 did not carry any potential antimicrobial resistance gene, disinfectant related resistance mechanism or plasmids (lack of Rep protein genes) (Table S2, S3).

The use of antiSMASH and BAGEL4 software revealed the presence of a large number and wide diversity of BGCs in the various genomes (Table 1). Comparison of all predicted BGCs identified five types of BGCs in 18 of the 22 genomes of bacteriocin-producing isolates from different species and origins included in this study (Figure 1). The BGCs from this study were assigned to class I (lanthipeptides), class II (bacSp222, lactococcin972 and blp), circular bacteriocins, NRPs (lugdunin) and thiopeptide (MP1), as follows:

1) **Class I:** Seven out of the 22 genomes (~32%) carried a lanthipeptide-like BGCs with identities to database entries. The isolates were recovered from wild-animals (*S. aureus* C8609 and *S. simulans* C9832) or food (*S. aureus* X3410, *S. warneri* X2969, *S. epidermidis* X3009). Interestingly, two of these isolates (C9832 and X3009) seemed to carry currently unknown lanthipeptide-encoding BGCs. Moreover, a type V lanthipeptide was detected in *S. hyicus* isolate C9585 recovered from a wild animal (wild boar) and strong identity was found between the BGC predicted in *S. chromogenes* C9727 (wild boar) and the gene cluster for the lanthipeptide hominicin.
2) **Class II:** Genes for lactococcin972-like peptides were detected amongst 8 out of 22 genomes (~36%) from: a) three isolates recovered from wild-animals (*S. aureus* C8609, *S. xylosus* C9255 and *S. simulans* C9832); b) two *S. sciuri* isolates from food samples (X3041 and X3011); and c) one *S. aureus* C5802 isolate recovered from an environmental sample. Moreover, part of the bacSp222 bacteriocin gene cluster was detected in two *S. pseudintermedius* (C8478 and C8189) isolates recovered from the same dog-human zoonosis case and a *blp*-like bacteriocin operon was identified in *S. xylosus* C9255.
3) BGCs for **circular bacteriocins** were identified in three isolates recovered from wild-animals (*S. chromogenes* C9838, *S. hyicus* C9581 and *S. xylosus* C9255). The predicted BGCs revealed

high identity with uberolysin/circularinA and aureocyclicin 4185 described in the databases for other species.

4) **Non-Ribosomal-Peptide-Synthetase genes (NRPS)** coding for lugdunin were detected in two *S. lugdunensis* isolates (C9161 and C9954) recovered from humans.
5) Four isolates (~18%) carried a BGC coding for the **thiopeptide** micrococcin P1: *S. aureus* C5802 and *S. hominis* C5835 from environmental samples and two *S. sciuri* from food X3041 and X3011.

Class I: Lanthipeptides

The incidence of lanthipeptides was particularly noteworthy and detected in ~32% of the evaluated bacteriocin-producing isolates (n=7) (Figure 1). A comparison between the lanthipeptide BGC operons was performed following the same criteria in all the used sequences. Figure 2 represents the genetic environment of the BGCs detected in this study (*S. aureus* X3410 and C8609, *S. hyicus* C9585, *S. warneri* X2969, *S. epidermidis* X3009, *S. simulans* C9832 and *S. chromogenes* C9727) and those previously described (accession number): bacCH91 (JQ655767), hyicin3682 (KY021154), epidermin (X62386), BSA (BA000033.2), pep5 (Z49865) and epilancin15X (JQ979180).

The *S. aureus* X3410 BGC revealed a high degree of identity to the bacCH91 bacteriocin (JQ655767), epidermin (X62386) and hyicin3682 (KY021154). On the other hand, the *S. warneri* X2969 BGC showed a close identity with the coding operon of epilancin15X (JQ979180) also related with pep5 BGC (Z49865). Regarding *S. epidermidis* X3009 BGC, slight identities were detected between the predicted structural genes of X2969 (this study) and hyicin3682 (KY021154) and the encoding operons. Finally, the *S. simulans* C9832 and *S. hyicus* C9585 lanthipeptide operons were classified separately from the rest of BGCs (Figure 2).

Finally, the BGC predicted on the *S. chromogenes* C9727 genome was identified as hominicin. Since only the partial amino acid sequence of this bacteriocin has been reported [53], further studies will be performed to determine their identity.

Comparative analysis presented in Figure 3 revealed 100% amino acid identity between the bacteriocin structural peptide of *S. aureus* X3410 and the BacCH91 structural peptide. Moreover, only slight differences were observed when comparing the bacteriocin structural pre-peptides of *S. aureus* C8609 with the BsaA2 (83% of identity) and BsaA1 (92%) amino acid sequences and the one predicted for *S. warneri* X2969 with the Epilancin15X structural pre-peptide (82% of identity). Moreover, antiSMASH analysis allowed us to identify a lanthipeptide V-like BGC in the *S. hyicus* C9585 genome. Noteworthy, the

BGCs of bacteriocin-producing *S. epidermidis* X3009 and *S. simulans* C9832 did not show homologues in the database, indicating the detection of two putatively new lanthipeptides (Figure 3).

Class II Bacteriocins

Genome analysis also led to the identification of two types of class II bacteriocins. The BGC for lactotococcin972 was predicted in six genomes but after genomic environment comparisons, all but one potential lactococcin972 BGCs predicted in this revealed identity with the structural gene of the *L. lactis*_pBL1_NC004955.1 reference sequence (Figure S1). Moreover, high identities were observed when considering the lactococcin972 BGC predicted among the genomes included in this study, specially between *S. aureus* C8609 and C5802 and *S. sciuri* X3041 and X3011. Contrary, the BGC predicted to code for lactococcin972 in the *S. simulans* C9832 genome, did not show relevant identity neither with the reference sequences considered nor the rest of the predicted operons.

We also detected the identical structural gene of the bacSp222 bacteriocin in the genomes of the two *S. pseudintermedius* isolates recovered from the same human-pet zoonosis case. This bacteriocin was described for the first time in the plasmid p222 of *S. pseudintermedius,* used as reference (CP011490.1) [54]. Finally, a Class II type BGC was predicted in *S. xylosus* C9255 which was related to the blp bacteriocin family [55].

Circular Bacteriocins

In the present study, three putative novel circular BGCs have been identified in *S. hyicus* C9581, *S. xylosus* C9255 and *S. chromogenes* C9838 isolates recovered from wild animals. The detected coding genes showed identity with the conserved domain of uberolysin, a circular bacteriocin firstly detected in *Streptococcus uberis* [56]. Comparative analysis of the BGCs of the potential circular bacteriocin carriers predicted in the present study and those used as references (*S. aureus*_WH39_CP060492; *S. uberis*_DQ650653) was performed. A high identity was exhibited between one of the BGC predicted in the *S. chromogenes* C9838 and the one of *S. xylosus* C9255 genomes with the operon coding for the circular bacteriocin described for *S. aureus* WH39. Additionally, this BGCs were closely related with the aureocyclicin 4185 BGC and the strongest identity was detected when compared with the BGC predicted in the *S. hyicus* C9581 genome. Finally, the other BGC predicted to code for a circular bacteriocin in *S. chromogens* C9838 could be considered as an unrelated genetic system (Figure S2).

Non-Ribosomal Peptides

Regarding NRPs, operons related with this type of secondary metabolites were predicted in six genomes from a wide variety of species and origins (Table 1). Genome

analysis revealed high diversity between them and no bacteriocins were detected except for the two clinical *S. lugdunensis* genomes (9%), carrying the lugdunin BGC. Interestingly, comparisons with the lugdunin coding operon revealed high identity >95% with the first discovered lugdunin producer (*S. lugdunensis*_IVK28_CP063143.1) (Figure S3) [57].

Thiopeptides

Previous studies by our group confirmed the production of MP1 bacteriocin by environmental staphylococcal isolates using mass-spectrometry analysis [58]. Based on this finding, we decided to further characterize these isolates by genome-mining tools. Thus, the BGC for MP1 thiopeptide production were detected in sequences of the four bacteriocin-producing isolates included in this study (*S. aureus* C5802 and *S. hominis* C5835 isolates from environmental samples; two *S. sciuri* X3011 and X3041 isolates from food).

Genetic environment comparisons between the four MP1 producers included in this study and those previously described confirmed the high identity between the BGC structures of isolates from the genus *Staphylococcus* but with clear differences to the BGCs from other genera (Figure 4). The reference sequences used for the MP1 operon analysis were as follows: *Bacillus safensis* (SDG14_10_1), *B. cereus* (ATCC14579), *Listeria monocytogenes* (FDA802499), *Streptococcus pseudoporcinus* (NCTC5385), *M. caseolyticus* (115), *Staphylococcus agnetis* (4244), *Staphylococcus intermedius* (14503307), *Staphylococcus felis* (F30k1271111), *S. aureus* (358), *Staphylococcus hominis* (34) and *Staphylococcus sciuri* (IMDOS72p). Moreover, close to 100% identity was observed among *S. intermedius* (14503307) and *S. felis* (F30k1271111), between *S. aureus* (358) and *S. hominis* (34) and between the two independent *S. sciuri* isolates. In addition, the DNA sequence analysis of the MP1 genetic environment revealed the closest relationship between the operon identified in *M. caseolyticus* (115) with those found in *Staphylococcus*. The strongest differences were observed between *Staphylococcus* and *M. caseolyticus* (115) MP1 operons with those described in *Bacillus* spp. isolates (SDG14_10_1 or ATCC14579) (Figure 4).

For deeper research of the MP1 BGC detected among the isolates included in this study a global comparison with the NCBI database was performed. Individual BlastN analysis with each of the contigs carrying the MP1 BGC, revealed a close affinity between the MP1 operon of our four carrier genomes and a complete plasmid coding for MP1 BGC of *S. hominis*_34 (CP040733.1). Moreover, the tight relationship between the entire MP1 plasmid of *S. hominis*_34 and of *S. hominis* C5835, recovered from this study, is noteworthy. In this respect, the BlastN analysis revealed a high identity and

coverage percentage (>90%), differing in 5 genes coding for ATPases or genes with unknown function. This identity was lower when comparing the MP1 BGC of the *S. aureus* C5802 isolate with *S. hominis*_34. However, high identity was observed between the *S. aureus* C5802 and *S. hominis* C5835 MP1 operons, also when comparing the flanking areas so it can be suggested that these two isolates could share a common genetic element. Finally, the presence of identical additional genes upstream of the MP1 operon in the two *S. sciuri* from this study and the selected reference (MP1 coding plasmid of *S. sciuri* IMDOS72) should be mentioned. Further analysis should be carried out to confirm the relation of the four MP1 BGC detected in this study to mobile genetic elements such as plasmids, and their transmission capability.

Moreover, differences in the MP1 structural gene were studied at the protein level (Figure 5). Regarding to the amino acid sequence alignment, it is important to note that *Staphylococcus* isolates were all identical when considering the MP1 structural gene, except for *S. agnetis* from the database which presented two amino acid changes in the leader peptide. Moreover, the high identity between *Staphylococcus* and *M. caseolyticus* sequences was outstanding, which only differ in a few amino acids within the leader peptide. On the other hand, the comparison with the other isolates included in this study revealed huge differences in the leader peptide sequences and even punctual amino acid exchanges and insertions within the mature peptides of *L. monocytogenes* and *S. pseudoporcinus,* likely affecting the structure and/or antimicrobial activity of their bacteriocins.

Discussion

The new genome mining tools offer an important technological resource in the discovery of novel natural products based either on the detection of bacteriocin structural genes or other bacteriocin associated genes [59]. In this study, ~82% of the investigated bacteriocin-producing *Staphylococcus* isolates (18 out of 22 genomes) carried at least one bacteriocin coding gene among which a large diversity was observed independently of isolate origin (human, pet, wild animals, food and environmental). Here, five types of BGCs were identified among 18 bacteriocin-producing isolates, coding for bacteriocins of class I (lanthipepthides), class II (lactococcin972, bacSp222 and blp family bacteriocin), class IV (circular bacteriocins), NRP and thiopeptides. Interesting relationship between related *Staphylococcus* species and the type of bacteriocin has been observed.

Focusing on lanthipeptides, they are characterised by their small (<5 kDa) size, their post-translational modifications and they contain lanthionine or β-methyllanthionine residues in their structure [60,61]. In the present work, seven

lanthipeptide-like BGCs have been described among the 22 studied genomes and genomic comparisons with those bacteriocin clusters previously referred in the literature allowed us to classify these putative new bacteriocins into class I. Upon lanthipeptide BGC identification among bacteriocin-producing staphylococci, bioinformatic analyses indicated the highest identity of the bacteriocins predicted in this study to the reference ones: i) the BSA bacteriocin discovered from an MRSA strain involved in community-acquired infections [62]; ii) the bacCH91 bacteriocin reported by Wladyka and colleagues in 2013 [63], although they only reported the structural gene; iii) the epilancin15X antimicrobial peptide firstly detected by [64] in a clinical *S. epidermidis* isolate. These results showed a close relationship between the putative structural genes detected in the newly investigated isolates and those previously reported, which suggests the detection of new variants of both BSA and epilancin15X bacteriocins. In addition, a lanthipeptide-like BGC type (V) was detected in the *S. hyicus* C9585 genome showing identity with those recently reported elsewhere [65,66].

Interestingly, the BGCs detected in *S. warneri* and *S. epidermidis* bacteriocin-producing isolates seemed to be putative new lanthipeptide-like bacteriocin. Lanthipeptide BGC are characterized by the presence of a structural gene which is post-translationally modified by dehydratase, phosphatase, glycosidase, cyclase, oxidase enzymes and ABC transporters [18]. Moreover, the presence of a C-terminal core region in the structural gene of *S. simulans* C9832 suggests that this operon could code for a type I lanthipeptide while the one detected in *S. epidermidis* X3009 revealed higher identity to pep5 bacteriocin (Figures 2 and 3). Future work will focus on the optimization of the extraction conditions to obtain inhibition in the well diffusion assay and to finally confirm the proteinaceous nature of the inhibitory compound and to decipher the peptide structure. Moreover, the use of heterologous expression systems will be important for future validation of the *in-silico* screening studies [67,68,69].

Focusing on class II bacteriocins, lactococcin-like clusters were identified in six out of the 22 bacteriocin-producing isolates included. These bacteriocins are described as linear non-pediocin-like molecules. Among them, lactococcin972 produced by *Lactococcus lactis* subsp. *lactis* IPLA 972 is the most representative and frequent [70]. Figure S1 shows a lactococcin-like operon comparison, and all clusters contained the precursor of lactococcin972 domain.

In addition, the bacSp222 bacteriocin of class II has also been detected in this study. It was the first reported bacteriocin produced by *S. pseudintermedius* [54] and its genetic cluster organization revealed identity to aureocin A53. Moreover, bacsp222 is characterised by its bactericidal activity and

virulence capacity that acts in modulating the immune system of the host [54].

The recently proposed class IV bacteriocins are circular peptides, formed by the post-translational covalent linkage between their carboxy and amino termini [71]. Currently, aureocyclicin 4185 from *S. aureus* 4185 is the first circular bacteriocin of *Staphylococcus* included in this category [72]**.** In this respect, we identified here two putative novel BGCs encoding circular bacteriocins of the circularin A/uberolysin family without identity to the previously reported circular staphylococcins (Figure S2). Moreover, the *S. hyicus* C9581 isolate seems to carry the aureocyclicin 4185 bacteriocin coding genes.

Finally, cyclic lugdunin, which was the first NRP described among staphylococci and is considered as an independent class of antibacterials, was detected in two clinical *S. lugdunensis* isolates of the present work. Likewise, Zipperer and collaborators discovered lugdunin in a nasal *S. lugdunensis* isolate with antimicrobial activity against a wide range of Gram-positive bacteria including methicillin resistant *S. aureus* (MRSA) [57]. The NRPS operon is about 30 kbp and encodes four genes (*lugABCD*) for the biosynthesis of lugdunin. Not all *S. lugdunensis* produce antimicrobial peptides although the lugdunin BGC seems to be conserved in this species and interestingly, the GC-content indicates horizontal transfer to *S. lugdunensis* from another bacterial species [57]**.**

Regarding the bacteriocin classification proposed by de Freire Bastos in 2020, thiopeptides could be considered as a new class of staphylococcins (Class VI) [13]. Thiopeptides are sulfur-containing, ribosomally produced and highly post-translationally modified peptides with strong inhibitory and competitive potential [73,74]. Most likely, MP1 (originally designated micrococcin) was firstly discovered in a strain of *Micrococcus* by its activity against *Mycobacterium tuberculosis* and it was characterized as a hydrophobic and heat-stable molecule with high activity against a wide range of Gram-positive bacteria [75]**.** So far, MP1 has been isolated from different genera, including *Micrococcus*, *Staphylococcus, Streptococcus* and *Bacillus spp*., and origins (food, humans, and animals).

Focusing on *Staphylococcus*, MP1 production has been reported for isolates of *S. equorum* from cheese [76], *S. epidermidis* [77], *S. felis* from cats [78], *S. hominis* recovered from human skin [79], *S. sciuri* [29] and recently *S. aureus* [30]. In the present study, MP1 production has been verified at the genetic level among four bacteriocin-producing isolates (~18%) with high antimicrobial activity against both MSSA and MRSA: two *S. aureus* and *S. hominis* isolates from environmental samples (river water) and two *S. sciuri* isolates recovered from raw meat chicken.

Comparison of the MP1 BGCs (Figure 4) revealed major genetic differences between

the staphylococcal isolates and *Bacillus*. First, the number of structural genes can differ between both genera (one for *Staphylococcus* and up to four for *Bacillus cereus*). Next, the staphylococcal strains appear to produce only one product [77], while a mixture of similar thiopeptides with different posttranslational modifications [thiocillin I, II, III, MP1 and micrococcin P2 (MP2)] have been reported among *Bacillus* [80]. Only after horizontal gene transfer of the MP1 BGC into *S. aureus* RN4220 a yet uncharacterized by-product of MP1 could be detected [30]. In this study, the strong capacity of MP1 to force RN4220 to change its metabolic capacity via *citZ* mutation was highlighted. In addition, the comparison of the MP1 BGCs included in this study illustrates the high identity between the *Staphylococcus* isolates, especially when considering the same species and with *M. caseolyticus.*

Most staphylococcal BGCs appear to be associated with mobile genetic elements such as plasmids, transposons, IS-elements, or chromosomal islands [30,28,57]. Hence, BGCs can be transferred between strains and lineages and are important genetic determinants of competitive fitness within a given habitat [30]. Due to the great diversity of staphylococcal isolates and origins detected among our bacteriocin-producing isolates and the high frequency of MP1 carrier detection, the occurrence of certain mechanisms of BGC transfer could be assumed as mentioned above.

The analysis of the genomes included in this study allowed us to identify a wide diversity of BGCs and more concretely, the comparison of the genetic environment of MP1 revealed identity to the reference plasmids of *S. hominis* and *S. sciuri*, indicating that the BGCs are plasmid-encoded. However, although the detection of *rep* sequences by PlasmidFinder, third-generation sequencing technologies, such as PacBio [81] or Oxford Nanopore (ONT) [82] instruments are recommended to confirm the presence of transferrable BGC.

Conclusion

In conclusion, our findings revealed a great abundance and diversity of bacteriocin gene clusters including unique systems and unfrequently detected among staphylococcal genomes. In this respect, the genus *Staphylococcus* and specially CoNS isolates has been confirmed as a valuable source of new peptide structures with promising functionalities for treatment and prevention. Moreover, the OneHealth perspective should be accentuated as a good perspective for further research on the alternatives for the AMR crisis.

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Author Contributions

C.T., B.K., A.P., R.F.-F., and C.L. contributed to the design of the study. C.T., B.K., A.P., and C.L. contributed to the general supervision of the study. A.M.A.E. and M.T. carried out the processing of the sequences. R.F.-F., A.M.A.E and A.M. collaborated in the data analysis and R. F.-F. developed the first version of the manuscript. C.T., C.L., and B.K. made the first revision of the manuscript. C.T., M.Z. and A.P. contributed to project funding. All authors revised the different versions of the manuscript, read, and agreed to the submitted version of the manuscript.

Data Availability

All data generated or analysed during this study are available within this paper and its supplementary information files. References [75-79] corresponds to Supplementary material (Table S2).

Illumina reads for 21 staphylococcal isolates included in this study can be found at the BioProject PRJNA974190 and C5802 is registered with ERS659514 accession number (ENA). The respective BioSample numbers are indicated in Supplementary Table S2.

Ethics Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Competing Interests The authors declare no competing interests.

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Tables and Figures

Table 1. Details of the type of Bacteriocin Gene Clusters (BGC) predicted both by antiSMASH or BAGEL4 among the 22 genomes included in this study.

Isolate	Specie	Origin	BGC Type(s)[a,b,c]	BGC prediction[a,b,c]
X3041	*S. sciuri*	Food	Class II, thiopeptide	Lactococcin972, MP1
X3011	*S. sciuri*	Food	Class II, thiopeptide	Lactococcin972, MP1
C9179	*S. sciuri*	Wild-bird	NRPs	None[c]
C9213	*S. sciuri*	Wild-bird	NRPs	None[c]
C9529	*S. sciuri*	Wild-bird	NRPs	None[c]
C9585	*S. hyicus*	Wild-mammal	Lanthipeptide	Lanthipeptide V
C9581	*S. hyicus*	Wild-mammal	RiPP	Circular
C9838	*S. chromogenes*	Wild-mammal	RiPP	2 Circular
C9727	*S. chromogenes*	Wild-mammal	Lanthipeptide	Hominicin variant
C9832	*S. simulans*	Wild-mammal	Class II, lanthipeptide	Lactococcin972, new lanthipeptide
C5835	*S. hominis*	Environmental	Thiopeptide	MP1
C9255	*S. xylosus*	Wild-bird	Class II, RiPP	Lactococcin972, blp, circular
X2969	*S. warneri*	Food	Lanthipeptide	Epilancin15X variant
X3009	*S. epidermidis*	Food	Lanthipeptide	New lanthipeptide
C8478	*S. pseudintermedius*	Pet	Class II	Bacsp222
C8189	*S. pseudintermedius*	Human	Class II	Bacsp222
C4502	*S. pseudintermedius*	Pet	NRPs	None[c]
C5802	*S. aureus*	Environmental	Class II, thiopeptide	Lactococcin972, MP1
C8609	*S. aureus*	Wild-mammal	Class II, lanthipeptide	Lactotococcin972, BSA
X3410	*S. aureus*	Food	Lanthipeptide	BacCH91
C9954	*S. lugdunensis*	Human	NRPs	Lugdunin
C9161	*S. lugdunensis*	Human	NRPs	Lugdunin

[a]Abbreviation: MP1, micrococcin P1; NRPs, non-ribosomal-peptide-synthetase; RiPP, ribosomally synthesized and posttranslationally modified peptide.
[b]Bacteriocin names: bacsp222, BSA, bacCH91
[c]None: in these cases, the relation of the predicted secondary metabolites with antimicrobial substances could not be verified.

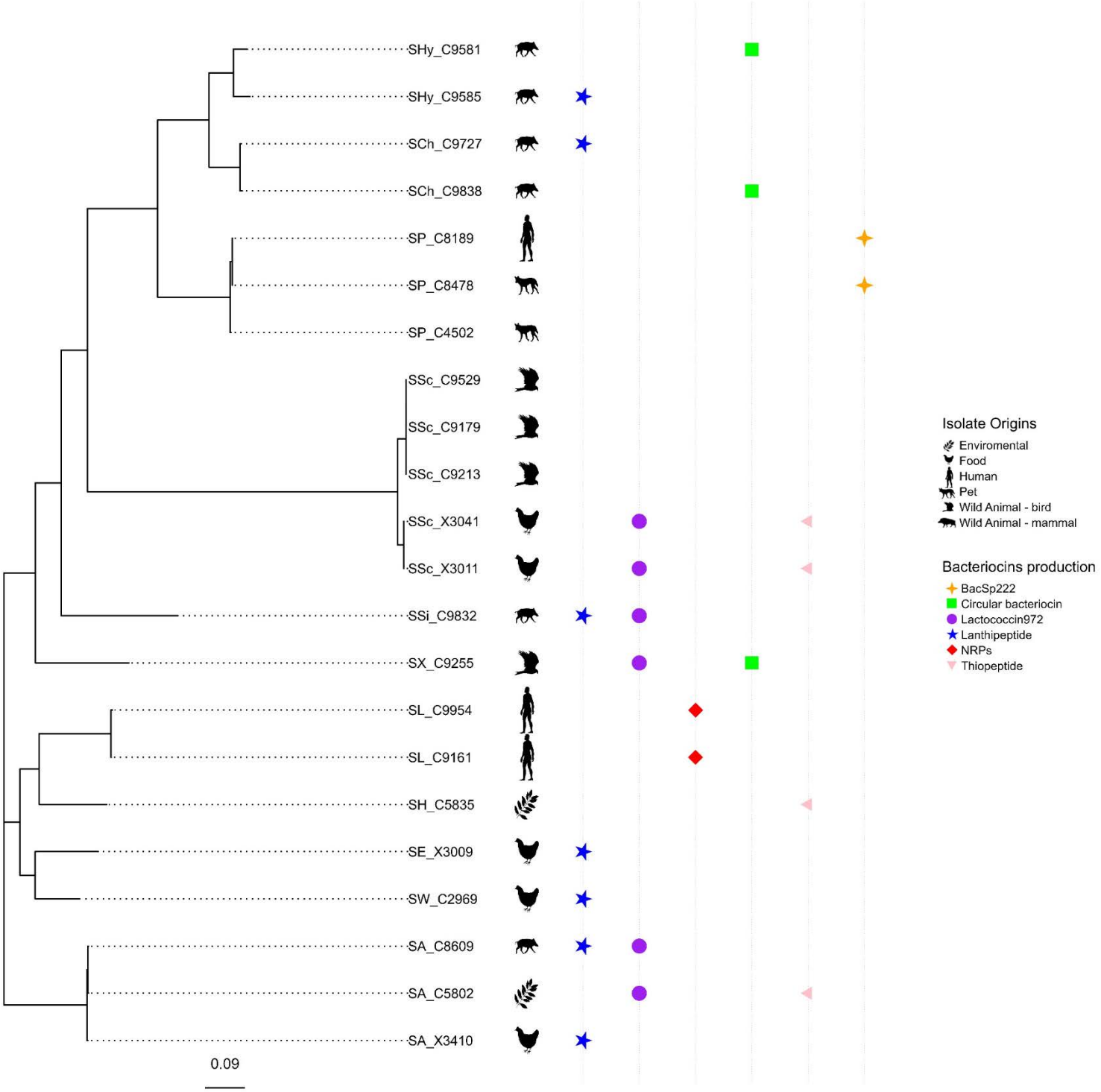


Figure 1. Maximum likelihood tree of protein markers of the 22 bacteriocin-producing isolates of different origins (environmental, food, human, pet, and wild animal both birds and mammals). The bacteriocin gene clusters (BGC) identified after *in silico* analysis are marked with stars: class I, lanthipeptides (blue star); class II, lactotoccin972 (purple circle) and bacsp222 (yellow cross); circular (green square), NRPS (red rhombus), thiopeptide (pink triangle). Abbreviation of specie: SA: *S. aureus*, SP: *S. pseudintermedius*, SSc: *S. sciuri*, SCh: *S. chromogenes*, SSi: *S. simulans*, SX: *S. xylosus*, SL: *S. lugdunensis*, SH: *S. hominis*, SE: *S. epidermidis*, SW: *S. warneri.*

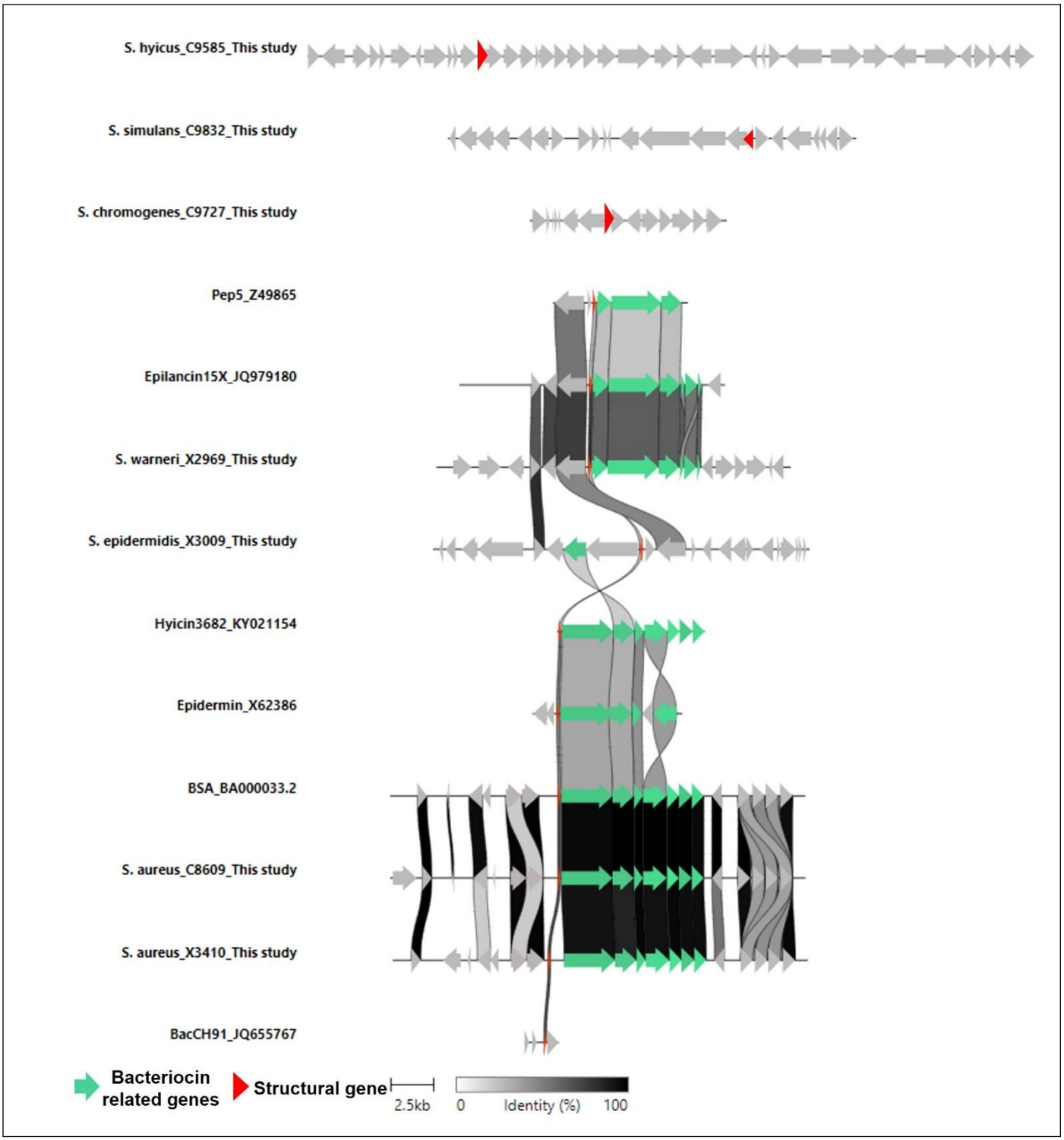


Figure 2. Genetic environment comparison between the bacteriocin gene clusters (BGCs) coding for lanthipeptides (class I) in the antimicrobial producing isolates detected in this study and those selected as references from the databases.

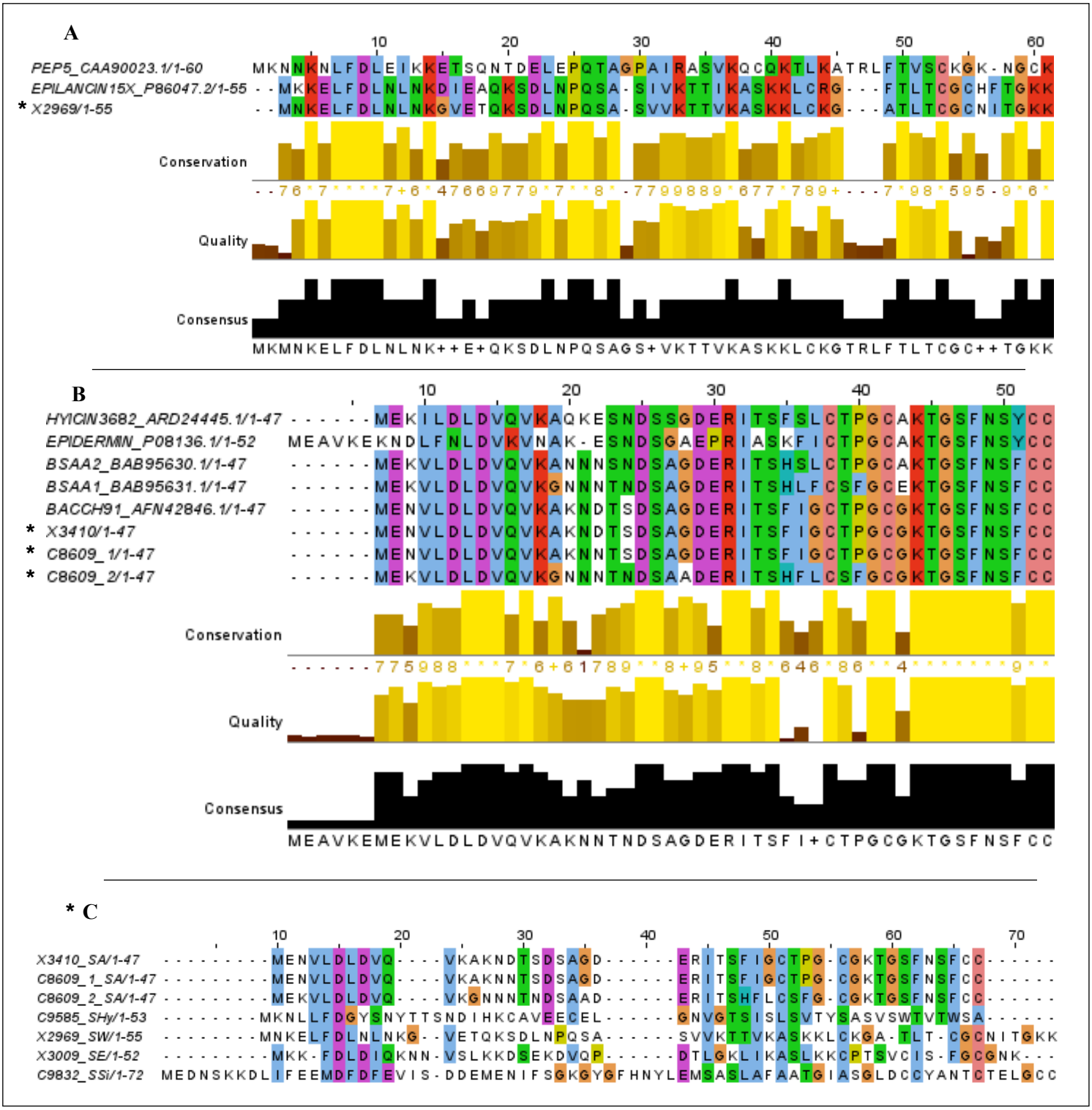


Figure 3. Sequence alignments of the structural peptides coding for lanthipeptide-like bacteriocins (detected in this study*) and those previously described and published in public databases used as reference: A) *S. warneri* X2969 and the prepeptide of epilancin15X and pep5 bacteriocins; B) Hyicin 3682, epidermin, BsaA1, BsaA2, bacCH91 peptides with those detected in *S. aureus* C8609 isolate and *S. aureus* X3410; C) Structural peptides detected among the genomes included in this study.

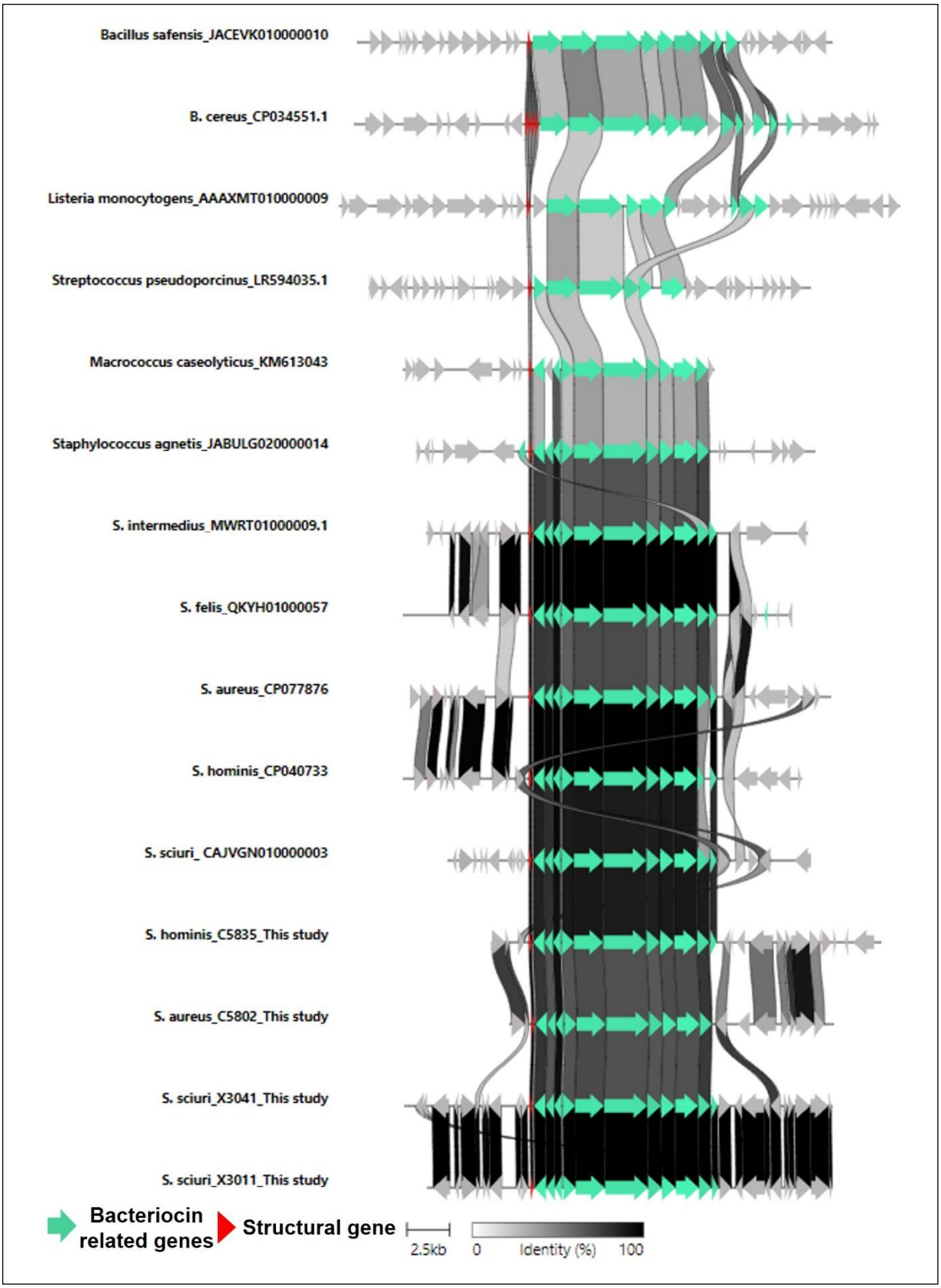


Figure 4. Genetic environment comparison of MP1 thiopeptide gene clusters of *B. safensis* (SDG14_10_1), *B. cereus* (ATCC14579), *L. monocytogenes* (FDA802499), *S. pseudoporcinus* (NCTC5385), *M. caseolyticus* (pBac115), *S. agnetis* (4244), *S. intermedius* (14S03307), *S. felis* (F30k1271111), *S. aureus* (358), *S. hominis* (34), *S. sciuri* (IMDOS72p), *S. hominis* (C5835, this study), *S. aureus* (C5802, this study), *S. sciuri* (X3041, this study), *S. sciuri* (X3011, this study).

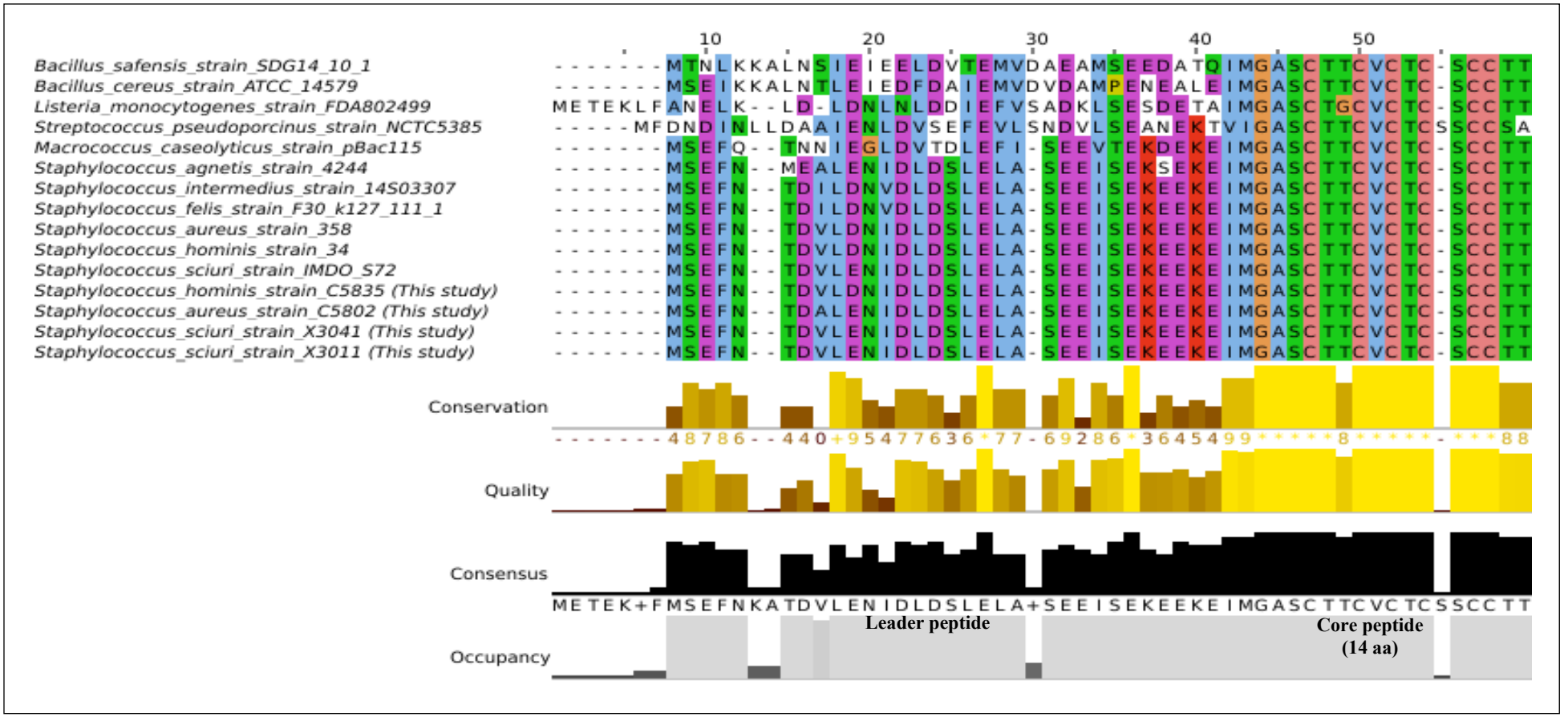


Figure 5. Differences at the protein level of the structural gene coding for the thiopeptide MP1: *B. safensis* (SDG14_10_1), *B. cereus* (ATCC14579), *L. monocytogenes* (FDA802499), *S. pseudoporcinus* (NCTC5385), *M. caseolyticus* (pBac115), *S. agnetis* (4244), *S. intermedius* (14S03307), *S. felis* (F30k1271111), *S. aureus* (358), *S. hominis* (34), *S. sciuri* (IMDOS72p), *S. hominis* (C5835, this study), *S. aureus* (C5802, this study), *S. sciuri* (X3041, this study), *S. sciuri* (X3011, this study).

Supplementary material

Table S1. Species, origins, and bacteriocin-producing isolates of the 1205 CoPS and CoNS isolates evaluated for antimicrobial activity in previous studies [31,32,33].

		Number of isolates tested/ Number of bacteriocin-producing isolates					
Type of staphylococci	**Species**	**Total**	**Human**	**Food**	**Wild animal**	**Pet**	**Environment**
CoPS	*S. aureus*	193/6	11/0	72/2	103/3	0/0	7/1
	S. pseudintermedius	60/16	9/1	1/0	0/0	50/15	0/0
	S. delphini	31/0	0/0	19/0	12/0	0/0	0/0
	Total CoPS*	**284/22**	**20/1**	**92/2**	**115/3**	**50/15**	**7/1**
CoNS	*S. sciuri*	352/15	0/0	23/2	320/13	0/0	9/0
	S. saprophyticus	75/0	0/0	39/0	11/0	0/0	25/0
	S. lentus	62/0	0/0	16/0	42/0	0/0	4/0
	S. xylosus	56/3	0/0	7/0	32/3	0/0	17/0
	S. epidermidis	54/5	4/0	17/4	21/1	0/0	12/0
	S. fleuretti	29/0	0/0	14/0	15/0	0/0	0/0
	S. chromogenes	37/10	0/0	7/3	28/7	0/0	2/0
	S. warneri	26/6	1/0	24/6	0/0	0/0	1/0
	S. vitulinus	24/0	0/0	5/0	19/0	0/0	0/0
	S. simulans	29/3	0/0	16/0	10/3	0/0	3/0
	S. arlettae	22/0	0/0	0/0	1/0	0/0	21/0
	S. cohnii	16/0	0/0	2/0	2/0	0/0	12/0
	S. equorum	16/0	0/0	2/0	13/0	0/0	1/0
	S. pasteuri	9/0	0/0	8/0	0/0	0/0	1/0
	S. hominis	15/2	1/0	3/0	8/1	0/0	3/1
	S. capitis	7/0	0/0	1/0	2/0	0/0	4/0
	S. hyicus	7/3	0/0	2/0	2/3	0/0	0/0
	S. succinus	6/0	0/0	0/0	5/0	0/0	1/0
	S. haemolyticus	8/0	0/0	0/0	5/0	0/0	3/0
	S. nepalensis	5/0	0/0	0/0	5/0	0/0	5/0
	S. kloosii	3/0	0/0	0/0	0/0	0/0	0/0
	S. schleiferi	3/0	0/0	0/0	3/0	0/0	0/0
	S. auricularis	1/0	0/0	0/0	0/0	0/0	1/0
	S. felis	1/0	0/0	0/0	1/0	0/0	0/0
	S. lugdunensis	57/23	57/23	0/0	0/0	0/0	0/0
	S. simiae	1/0	0/0	0/0	1/0	0/0	0/0
	Total CoNS*	**921/70**	**63/23**	**186/15**	**547/31**	**0/0**	**125/1**
Total CoPS+CoNS		**1205/92**	**83/24**	**278/17**	**662/34**	**50/15**	**132/2**

Table S2. Characteristics of the 22 bacteriocin-producing *Staphylococcus* isolates included in this study including the resistome and molecular characterization by WGS analysis (Bioproject accession number PRJNA974190).

[1]References: UR: University of La Rioja.
[2]Abbreviation: NI, not identified; NE; not studied.
[3]LSA: Lincosamides, Streptogramins and Aminoglycosides; *sal*A and *mec*A1 genes are intrinsically carried by *S. sciuri* species and it was not consider as an adquired resistance mechanism

					Antimicrobial resistance							
Isolate (ID)	**Origin**	**Reference of Phenotypical Characteristics[1]**	**Accession number**	**Molecular typing[2]**	**Beta-lactam**	**Macrolide**	**LSA[3]**	**Tetracycline**	**Phenicol**	**Fusidic-acid**	**Fosfomycin**	**Desinfectant**
S. pseudintermedius (C4502)	Pet-Dog	UR collection	SAMN35174351	NI	*blaZ*			*tet*(M)				
S. pseudintermedius (C8189)	Human	[83]	SAMN35174352	ST241	*blaZ*	*erm*(B)	*aph*(3')_III, *ant*(6)-Ia		cat_{pc221}			*qac*D
S. pseudintermedius (C8478)	Pet-Dog	[83]	SAMN35174353	ST241	*blaZ*	*erm*(B)	*aph*(3')_III, *ant*(6)-Ia		cat_{pc221}			*qac*D
S. aureus (C5802)	Environmental-Water	[84]	ERS659514	ST130								
S. aureus (C8609)	Wild animal-Mammal	[85]	SAMN35174354	ST11225								
S. aureus (X3410)	Food-Chicken	[31]	SAMN35174355	ST304								
S. sciuri (C9179)	Wild animal-Bird	[86]	SAMN35174356	NS	*mec*A1		*sal*A					*qac*D
S. sciuri (C9213)	Wild animal-Bird	[86]	SAMN35174357	NS	*mec*A1		*sal*A					*qac*D
S. sciuri (C9529)	Wild animal-Bird	[86]	SAMN35174358	NS	*mec*A1		*sal*A					*qac*D
S. sciuri (X3011)	Food-Chicken	[31]	SAMN35174359	NS	*BlaZ*, *mec*A1	*mph*C	*sal*A				*fos*D	*qac*J, *qac*D
S. sciuri (X3041)	Food-Chicken	[31]	SAMN35174360	NS	*BlaZ*, *mec*A1	*mph*C	*sal*A	*tet*(K)			*fos*D	*qac*J, *qac*D
S. chromogenes (C9838)	Wild animal-Mammal	[87]	SAMN35174361	NI			*sal*A	*tet*(K)				*qac*D
S. chromogenes (C9727)	Wild animal-Mammal	[87]	SAMN35174362	NI								
S. hyicus (C9581)	Wild animal-Mammal	[87]	SAMN35174363	NI	*mec*A1							*qac*D
S. hyicus (C9585)	Wild animal-Mammal	[87]	SAMN35174364	NI								*qac*D
S. warneri (X2969)	Food-Chicken	[31]	SAMN35174365	NS								*qac*D, *qac*J
S. epidermidis (X3009)	Food-Chicken	[31]	SAMN35174366	ST1025		*msr*A, *mph*C		*tet*(K)		*fus*B	*fos*B	*qac*D
S. xylosus (C9255)	Wild animal-Bird	[86]	SAMN35174367	NS								
S. hominis (C5835)	Environmental-Water	[84]	SAMN35174368	ST52/ST15	*blaZ*	*msr*A		*tet*(K)		*fus*B		
S. simulans (C9832)	Wild animal-Mammal	[87]	SAMN35174369	NS				*tet*(K)				*qac*C, *qac*D
S. lugdunensis (C9954)	Human	[32]	SAMN35174370	ST24								
S. lugdunensis (C9161)	Human	[32]	SAMN35174371	ST2								

Table S3. Details of the plasmidome detected in the genomes of the 22 bacteriocin-producing isolates.

Isolate (ID)	Rep Protein	Plasmid similarity NCBI	% Identity[1]	Coverage[1]
S. pseudintermedius (C4502)	-	-	-	-
S. pseudintermedius (C8189)	rep21_Plnu8	AM399080	82.21	579/1005
	repUS12_SAP014A	GQ900379	100	620/876
	rep7a_Pre25	X92945	99.79	939/939
S. pseudintermedius (C8478)	rep7a_Pre25	X92945	99.79	939/939
	rep21RC_Psk41	AF051917	89.58	240/950
	rep21_pLNU8	AM399080	82.49, 82.21, 81.28	337,579,358/1005
	rep21RC_pGO1	FM207042	89.58	240/927
	repUS12B_SAP014A	GQ900379	1000	620/876
S. aureus (C5802)	repA_repUS23	GQ900449	90.72	937/936
	rep2_repUS46	GQ900449	98.78	573/573
	rep3_rep5a	GQ900405	89.39	858/861
S. aureus (C8609)	-	-	-	-
S. aureus (X3410)	rep21_Pso385	AM990995	98.86	1050/1050
	rep7de_Pack6	AF093750	99.26	942/945
S. sciuri (C9179)	rep1_rep13	AM184099	92.57	619/924
	repA_N_rep19	GQ900399	94.42	556/984
	repA_N_rep23	EU366902, GU237136	77.94	408/1026
S. sciuri (C9213)	rep1_rep13	AM184099	92.41	619/924
	repA_N_rep23	EU366902, GU237136	77.89	407/1026
S. sciuri (C9529)	rep1_rep13	AM184099	92.57	619/924
	repA_N_rep19	GQ900399	94.42	556/984
	repA_N_rep23	EU366902, GU237136	77.94	408/1026
S. sciuri (X3011)	rep_trans_rep7a	U35036	88.85	942/945
	rep1_rep21	AM990995	96.66	1047/1050
	repA_N_rep19	FR821778	75.22	900/975
S. sciuri (X3041)	repA_N_rep15	AE017171	89.96	956/960
	repA_N_rep19	FR821778	75.22	900/975
	repA_N_rep21	AM990995	96.67	1050/1050
S. chromogenes (C9838)	rep_trans_rep7a	SAU38656	100	539/570
	repA_N_rep24a	GQ900389	83.87, 93.75	558/989, 448/989
S. chromogenes (C9727)	-	-	-	-
S. hyicus (C9581)	rep1_rep21	FR714928, GQ900464	80.91	503/1005
	rep_trans_rep7a	AB037671, NC007791, SAU38656, U36910	100	197/945
	repA_N_rep19	FR821778	78.22	450/975
S. hyicus (C9585)	-	-	-	-
S. warneri (X2969)	repA_N_rep19c	GQ900458	88.57	980/972
	repA_N_repUS23	GQ900449	93.6	906/936
	rep2_repUS46	GQ900449	88.27	571/573
S. epidermidis (X3009)	Rep1_rep21	AF051917, FM207042, FR714928, FR821780, GQ900464, GQ900485	Nind	Nind
	repA_N_rep39	GQ900381	88.52	897/957
	repA_N_repUS9	AF203376	89.18	924/921
S. xylosus (C9255)	repA_N_rep19c	CP003673	96.19	972/972
	rep1_rep21	AM990995	98.86	1050/1050
S. hominis (C5835)	rep1_rep13_pLNU9	AM399082	98.7	846/846
	rep1_rep21	FR714928, FR821780, GQ900461, GQ900464, GQ900485	Nind	Nind
	rep2_repUS46	GQ900449	90.56	551/573
	rep3_rep5c	AF447813	75	516/858
	rep3_rep5d	AF051916	89.91, 93.55	228/636, 636/636
	repA_N_rep19b	GQ900452	93.05	734/969
	repA_N_rep20	AP003367, GQ900453	Nind	Nind
	repA_N_rep39	GQ900465	98.83	511/954
	repA_N_rep40	AB125341	94.67	1257/1254
	repA_N_repUS70	GQ900405	92,08	808/1452
	reptrans_rep7a	AB037671, AM990993, GQ900417, NC007791, SAU83488, U36910	Nind	Nind
S. simulans (C9832)	rep1_rep13 (pLNU9)	AM399082	83.74	695/846
	rep1_rep21 (pKH12)	EU168704, GQ900461	81.32, 87.56	1001/996, 844/843
	rep_trans_rep7a	AB037671, AM990993, NC007791, SAU38656, U36910	100	Nind
S. lugdunensis (C9954)	-	-	-	-
S. lugdunensis (C9161)	-	-	-	-

[1]Abbreviations: Nind, not indicated; (-): rep proteins not identified.

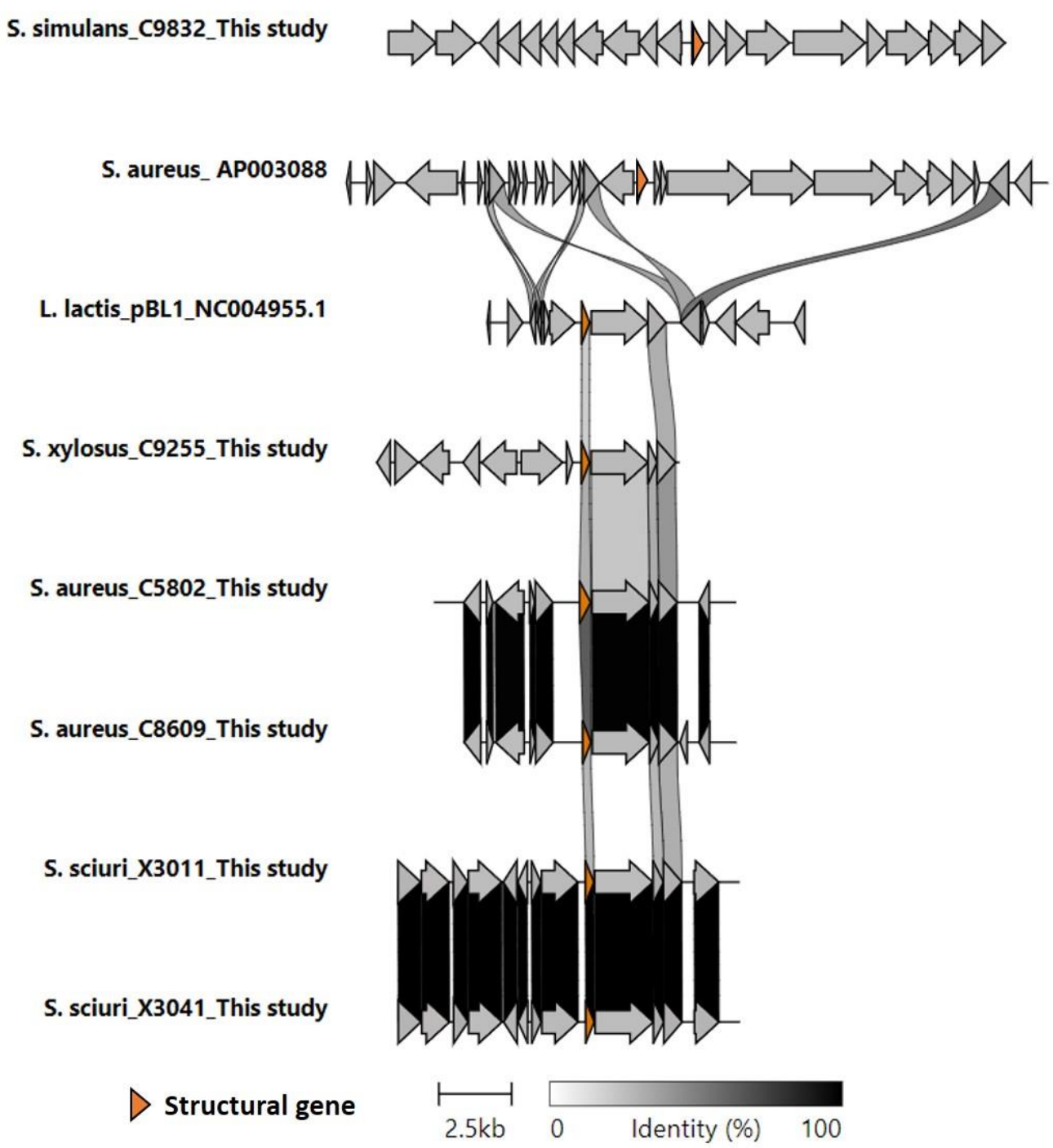


Figure S1. Genetic environment comparison between the bacteriocin gene clusters (BGCs) coding for the lactococcin972 class II bacteriocin.

*The accession numbers of the BGC coding for the bacteriocins used as reference are included in the figure.

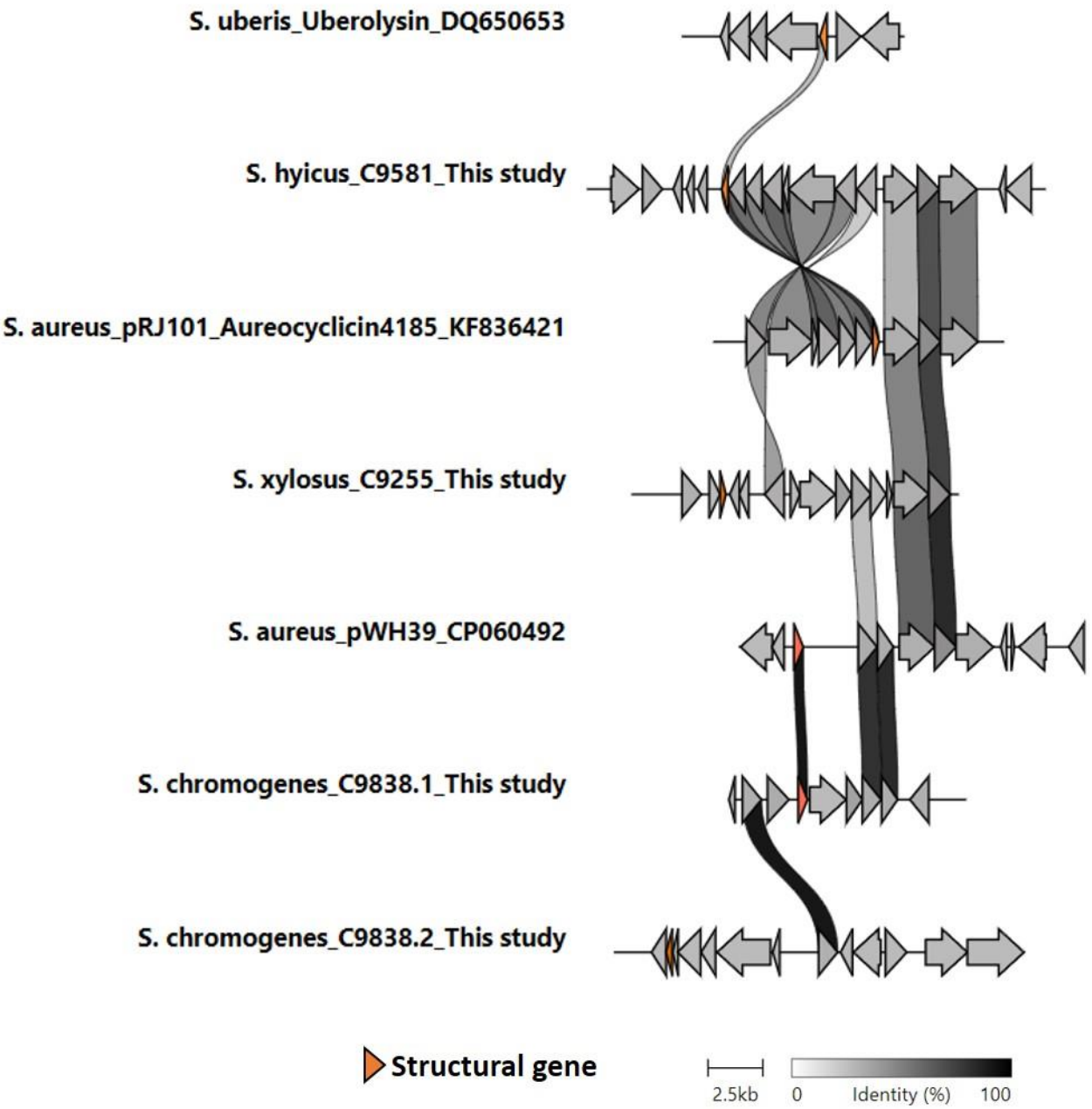


Figure S2. Genetic environment comparison between the bacteriocin gene clusters (BGCs) coding for the putative circular bacteriocins.

*The accession numbers of the BGC coding for the bacteriocins used as reference are included in the figure.

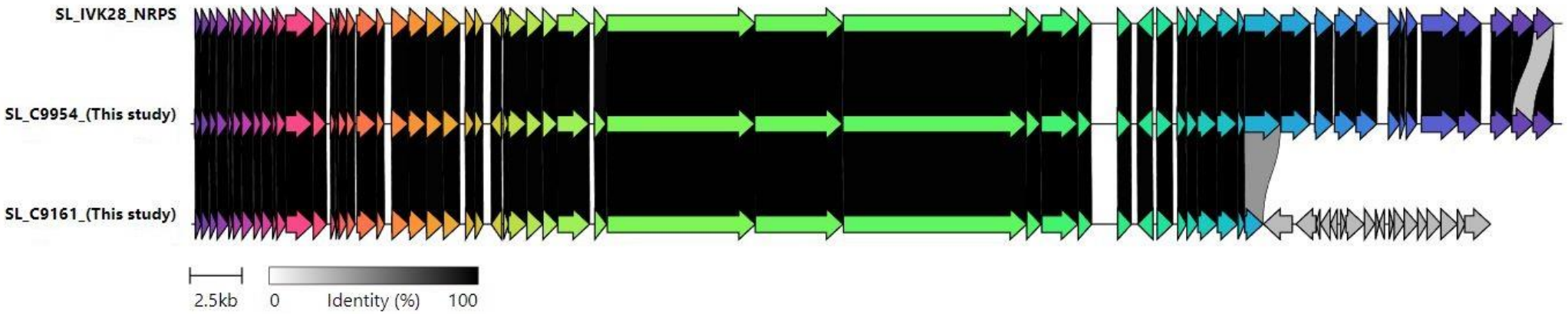


Figure S3. Genetic environment comparison between the gene clusters predicted to code for lugdunin Non-Ribosomal-Peptides (NRPs).

*The accession number for the BGC used as reference is included in the figure.

Capítulo 3

3. Capítulo 3: Estudio de actividad antimicrobiana y caracterización proteica

Las bacteriocinas son péptidos con actividad antimicrobiana que pueden inhibir el crecimiento de otras bacterias, incluidos patógenos de relevancia en clínica y en seguridad alimentaria como enterococos resistentes a vancomicina (ERV) y SARM.

Normalmente, las bacteriocinas presentan una fuerte actividad en bajas concentraciones (rango nanomolar), lo que permite pensar en ellas como alternativas potenciales a los antibióticos. Además, el uso de bacteriocinas solas o en combinación con otros compuestos antimicrobianos podría ser una estrategia para compensar la escasez de nuevas moléculas y la baja disponibilidad y eficacia de algunos antibióticos debido a la problemática de la resistencia.

Teniendo en cuenta el grave problema de salud mundial que supone la resistencia a los antibióticos, los aislados de *Staphylococcus* con actividad antimicrobiana presentan un alto potencial en el ámbito de la modulación de microbiomas o como fuente de compuestos antimicrobianos naturales.

En este capítulo se aborda el tercer objetivo que incluye: el análisis y caracterización de la naturaleza peptídica de las sustancias antimicrobianas producidas por las cepas Bac+, la pre-purificación de los péptidos antimicrobianos de las cepas de Bac+ seleccionadas y el estudio comparativo de su actividad antimicrobiana frente a patógenos de interés como SARM-CC398 en comparación con bacteriocinas comerciales (nisina), el análisis de la actividad combinada de bacteriocinas purificadas en este estudio y antibióticos de uso en clínica y los estudios de competencia entre cepas Bac+ y otras cepas de *Staphylococcus* de interés. Los resultados obtenidos han sido desarrollados en forma de un artículo científico y se presentan como resumen gráfico en la **Figura 19.**

3.1 Artículo 4..... Detection and evaluation of the antimicrobial activity of Micrococcin P1 isolated from commensal and environmental staphylococcal isolates against MRSA

3.1 Artículo 4 Detection and evaluation of the antimicrobial activity of Micrococcin P1 isolated from commensal and environmental staphylococcal isolates against MRSA

En este trabajo se incluyen 28 cepas de estafilococos productoras de bacteriocinas (Bac+) de 10 especies diferentes (*S. pseudintermedius*, *S. aureus*, *S. sciuri*, *S. chromogenes*, *S. warneri*, *S. epidermidis*, *S. xylosus*, *S. hominis*, *S. hyicus*, and *S. simulans*) y 5 orígenes (animals de vida libre, mascotas, humanos, alimentos y medio ambiente). Las 28 cepas Bac+ fueron seleccionadas a partir de estudios previos de esta tesis por su alto perfil de actividad antimicrobiana (AA) (capítulo 1) y por su contenido en operones codificantes de bacteriocinas de partir del estudio de sus genomas (capítulo 2).

Se llevó a cabo un estudio de la capacidad inhibitoria de las 28 cepas Bac+ frente a 6 microorganismos indicadores [*Listeria monocytogenes* CECT4032, *Enterococcus cecorum* X3809, *S. pseudintermedius* resistente a meticilina (SPRM) C3468, *S. sciuri* C9459; SARM C1570, y *S. aureus* sensible a meticilina (SASM) SA113]. Se evaluó la AA tanto por *spot-on-lawn* como mediante ensayos de difusión en agar de los sobrenadantes libres de células (SLC) y de los extractos obtenidos por métodos químicos (butanol, BT). Los extractos BT fueron analizados por espectrometría de masas para la detección de bacteriocinas.

Asimismo, se realizaron curvas de crecimiento para evaluar la AA de los SLC (del 100% al 0,5%) y BT (de 100 µg/mL a 0,2 µg/mL) de cuatro cepas de estafilococos seleccionadas (*S. aureus* C5802, *S. hominis* C5835, y 2 *S. sciuri* X3041 y X3011) frente a los aislados indicadores SARM-C1570 y *L. monocytogenes*-CECT4032 ($5x10^5$ UFC/mL). Se incluyó como control negativo el SLC de una cepa *S. hominis* no productora de bacteriocinas. Además, se realizó una comparativa con nisina (Sigma) evaluando su actividad antimicrobiana frente al aislado indicador SARM-C1570.

Por otro lado, se evaluó el efecto combinado por el método checkedboard del extracto BT de la cepa *S. sciuri* X3041 Bac+ y los siguientes antibióticos: oxacilina, eritromicina, clindamicina, gentamicina, tetraciclina y cloranfenicol y se utilizó como microorganismo indicador la cepa SARM-C1570. Finalmente, se llevaron a cabo estudios de competencia entre las cuatro cepas Bac+ seleccionadas y el indicador SARM-C1570 con el objetivo de evaluar su potencial inhibitorio a lo largo del tiempo. Se incluyó como control negativo tres cepas de estafilococos no productoras de bacteriocinas de las especies *S. aureus, S. hominis* y *S. sciuri*.

Los resultados obtenidos revelaron seis cepas con AA en sus SLC, mientras que todos los extractos BT inhibieron al menos una de las bacterias indicadoras ensayadas. La bacteriocina micrococcina P1 (MP1) se detectó por espectrometría de masas en 4 aislados productores (*S. aureus*-C5802; *S. hominis*-C5835, *S. sciuri*-X3041 y X3011). Las curvas de crecimiento realizadas con los SLC y los BT de los 4 productores de MP1 revelaron alta AA frente a SARM

y *L. monocytogenes*. Además, se observó sinergismo entre el extracto BT de *S. sciuri*-X3041 productor de MP1 y varios antibióticos frente al indicador SARM. Las combinaciones que mostraron sinergismo fueron: BT-clindamicina (>80% de las combinaciones) y BT-oxacilina (30%). En el caso de la combinación BT-cloranfenicol, se observaron valores de sinergismo y próximos a sinergismo en el 37% de las combinaciones evaluadas. Por último, los estudios de competencia revelaron potentes efectos inhibidores de los aislados productores de MP1 frente al indicador SARM. Estos resultados han permitido identificar 4 cepas de estafilococos productores de MP1 con gran eficacia de acción según los diferentes ensayos realizados en este estudio. En este sentido, se propone que *Staphylococcus* o sus bacteriocinas son candidatos interesantes con elevado potencial para posibles aplicaciones futuras.

3.2 Resumen gráfico del Capítulo 3

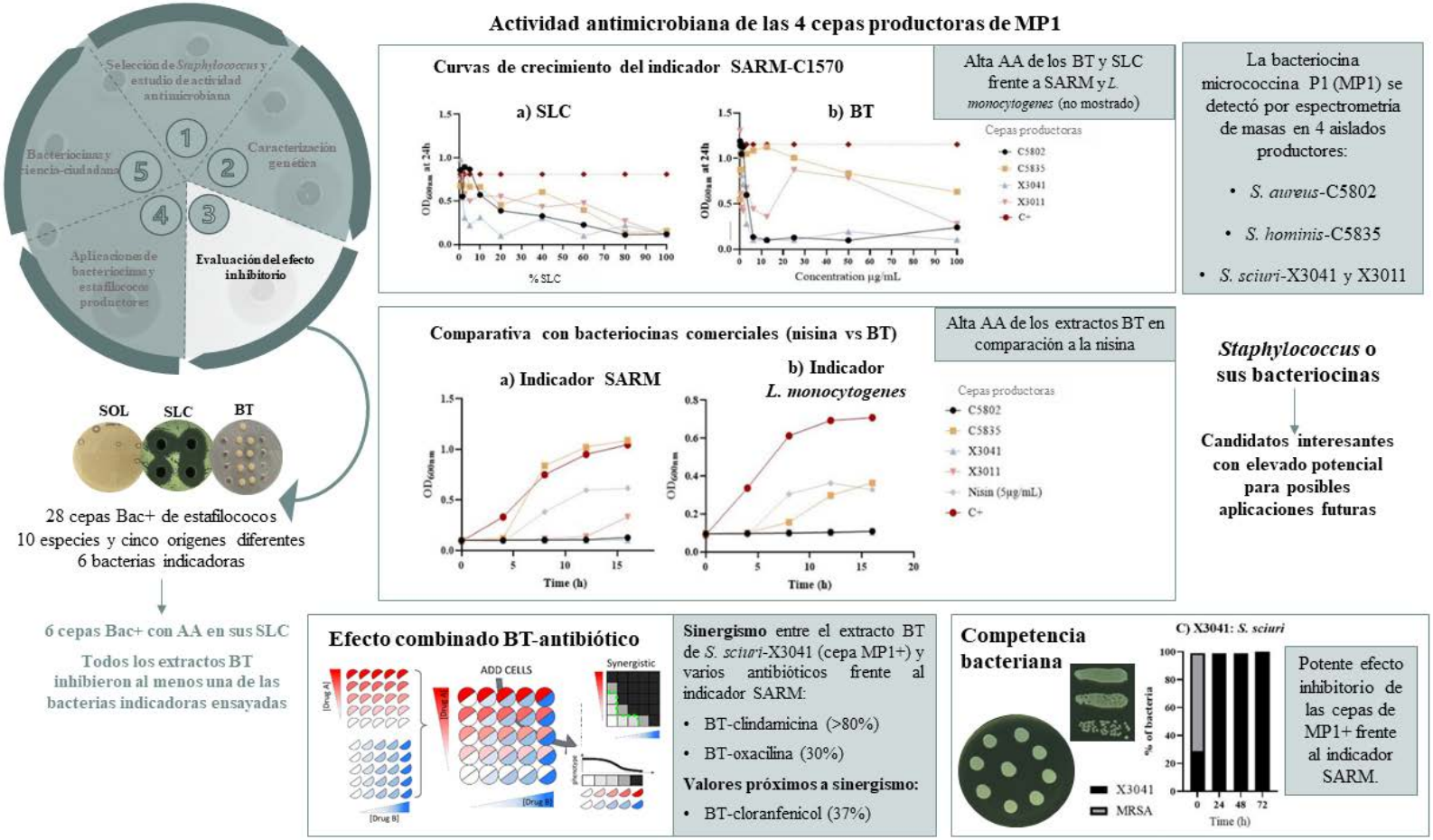
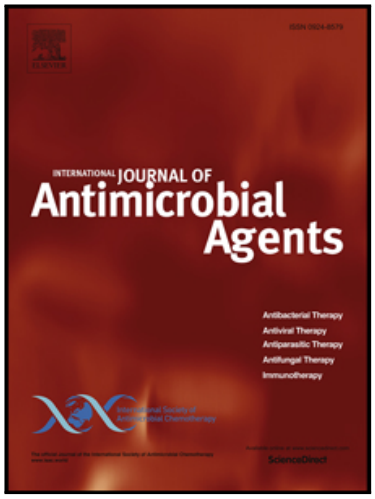


Figura 19 Resumen gráfico de la metodología y resultados obtenidos en el tercer capítulo referente al estudio detallado de actividad antimicrobiana y caracterización proteica de las bacteriocinas y cepas productoras detectadas en esta tesis (elaboración propia).

Artículo 4

Editor: Professor Geoffrey Coombs

Journal Pre-proof



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Detection and evaluation of the antimicrobial activity of Micrococcin P1 isolated from commensal and environmental staphylococcal isolates against MRSA

Rosa Fernández-Fernández[1], Carmen Lozano[1], Rocío Fernández-Pérez[2], Myriam Zarazaga[1], Andreas Peschel[3, 4, 5], Bernhard Krismer[3, 4, 5], Carmen Torres[1*]

[1]University of La Rioja, Area of Biochemistry and Molecular Biology, OneHealth-UR Research Group, 26006 Logroño, Spain

[2]University of La Rioja, Instituto de Ciencias de la Vid y del Vino (ICVV), Logroño, Spain

[3]University of Tübingen, Interfaculty Institute of Microbiology and Infection Medicine, Department of Infection Biology, 72076 Tübingen, Germany

[4]Cluster of Excellence EXC 2124 Controlling Microbes to Fight Infections, Tübingen, Germany

[5]German Center for Infection Research (DZIF), partner site Tübingen, Tübingen, Germany

[*]Corresponding author:

Prof. Carmen Torres

University of La Rioja, Area of Biochemistry and Molecular Biology,

OneHealth-UR Research Group, 26006 Logroño, Spain,

Phone. 34941299750; Email: carmen.torres@unirioja.es

ABSTRACT

Bacteriocins (of different origins) have been proposed as promising alternatives to face antimicrobial resistance-associated health problem. Isolates of the genus *Staphylococcus* are well-known bacteriocin producers, especially coagulase-negative species. Twenty-eight bacteriocin-producing staphylococcal isolates were selected from a previous study for an in-depth characterization. The antimicrobial activities (AA) of the producing isolates were studied by the *Spot-on-lawn* method and their crude cell-free-supernatants (CFS) and butanol extracts (BT) were evaluated by agar-diffusion assays against six indicator bacteria, including multidrug-resistant and zoonotic isolates (such as *Listeria monocytogenes* or methicillin-resistant *Staphylococcus aureus* [MRSA]). Six bacteriocin-producing isolates showed AA in their CFS whereas all staphylococcal BT extracts inhibited at least one of the indicator bacteria tested. Micrococcin P1 (MP1) bacteriocin was detected by mass-spectrometry in four producing isolates (*S. aureus*-C5802; *S. hominis*-C5835, *S. sciuri*-X3041 and *S. sciuri*-X3011). Growth curves performed with CFS and BT extracts of the four MP1-producers revealed a strong AA profile against MRSA and *L. monocytogenes*, even when considerably diluted. Moreover, synergism between the BT extract of MP1-producing *S. sciuri*-X3041 and several antibiotics against a MRSA indicator was observed: BT-clindamycin (>80%) and BT-oxacillin (30%) combinations. For the BT-chloramphenicol combination, synergism and near synergism values were observed in 37% of the combinations. Finally, competition studies revealed potent inhibitory effects of the MP1-producing isolates against the MRSA indicator. These results help us to identify *Staphylococcus* isolates or their bacteriocins as interesting candidates for potential future applications.

KEYWORDS: antimicrobial activity, bacteriocins, coagulase-negative staphylococci, micrococcin P1 (MP1), *Staphylococcus*.

1. INTRODUCTION

The genus *Staphylococcus* includes a huge number of species, which are present in the complex bacterial ecosystems of the human and animal microbiotas. It has been subdivided into coagulase-negative species (CoNS, most abundant and considered to be mostly commensals) and coagulase-positive species including *S. aureus,* which can also colonize mammals and occasionally causes severe infections **[1]**.

Bacteriocins are antimicrobial peptides of bacteria origin, mostly ribosomally synthesized, that are secreted into the natural environment **[2]**. Bacteriocins are broadly classified into class I (post-translationally modified) and class II (unmodified) groups. Focusing on *Staphylococcus*, lantibiotics are the most extensively studied subclass of bacteriocins **[3]**.

Considering the potential use of bacteriocins, previous reviews have highlighted their potential roles in food preservation **[4]**, their capacity to modulate the human microbiome **[5]** and their possible use as probiotic cultures in animal feed to promote growth, improve animal health and/or reduce infections **[6].**

Moreover, bacteriocins exhibit strong activity against susceptible target species, including numerous clinically significant pathogens **[7]**, which suggest them as potential alternatives to classical antibiotics for use in clinical applications **[8]**. In this respect, the use of bacteriocins alone or in combination with other antimicrobial compounds should be considered as a strategy to compensate for the decrease of effectiveness of the currently used antibiotics and the lack of development of novel molecules.

In a previous study performed by our research group, the production of bacteriocin-like inhibitory activities ($BLIS^+$) was analysed in a collection of 890 staphylococcal isolates of different species and origins **[9]**. From these isolates, sixty were identified as $BLIS^+$ and a subcollection of 28 *Staphylococcus* were selected because of their particularly strong inhibitory activity. These 28 isolates were included in the present work to analyse in depth the antimicrobial activity of the corresponding staphylococcal extracts, alone and in combination

with antibiotics, and to perform competition studies with the producing isolates to elucidate their potential applications.

2. MATERIAL AND METHODS

2.1. Isolates and growth conditions

Twenty-eight bacteriocin-producing *Staphylococcus* isolates of 10 different species (*S. pseudintermedius*, *S. aureus*, *S. sciuri*, *S. chromogenes*, *S. warneri*, *S. epidermidis*, *S. xylosus*, *S. hominis*, *S. hyicus*, and *S. simulans*) from different origins (wild animal, pet, human, food and environment) were selected from a previous study **[9],** due to their strong antimicrobial profile, for an in-depth evaluation of the inhibitory capacity of these isolates **(Table S1).** Six indicator bacteria were used in this study for detection of antimicrobial activity [*Listeria monocytogenes* CECT4032, *Enterococcus cecorum* X3809, methicillin-resistant *S. pseudintermedius* (MRSP) C3468, *S. sciuri* C9459; methicillin-resistant *S. aureus* (MRSA) C1570, and methicillin-susceptible *S. aureus* (MSSA) SA113].

2.2. Antimicrobial activity (AA) determination in agar media

The antimicrobial activity (AA) of the 28 bacteriocin-producing isolates was tested by the *Spot-on-lawn* method against the six indicator bacteria. Moreover, crude cell-free supernatant (CFS), and 1-butanol (BT) extracts of all bacteriocin-producing isolates were obtained as previously reported **[10],** and their AA were evaluated by a diffusion-assay. In case of BT extracts, the aqueous phase was discarded after centrifugation and the organic phase was evaporated at 44°C under reduced pressure. Finally, the extract was dissolved in acetonitrile:water (1:1) and the lyophilized product was resuspended at a concentration of 10 mg/mL in dimethyl sulfoxide to test the AA.

Basic medium agar plates (BM; 1% soy peptone 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, 0.1% K_2HPO_4, 1.5% agar, pH 7.2) were inoculated with an overnight culture of the indicator isolate (OD_{600} of 0.0025), and 50 µL of the CFS or BT extracts were filled in wells punched in the agar. Inhibition zones around the wells after incubation (24h, 37ºC) were recorded.

2.3. Bacteriocin identification

Lyophilized butanol extracts were resuspended in methanol and analysed by MALDI-TOF mass spectrometry (Microflex, Bruker) for bacteriocin detection. Moreover, a detailed analysis was performed and bacteriocin extracts were injected through an UPLC (Acquity, Waters) with a flux of 90% metanol-10% water (0.1mL/min) acidified with 0.1% of formic acid. The exact mass of the bacteriocin was analysed (<5ppm) by a LQT Orbitrap XL with an ESI ionizing source.

2.4. Antimicrobial activity (AA) in broth

Growth curves were performed in 96-well microtiter plates to evaluate the AA of crude CFS (from 100% to 0.5%) and BT extracts (from 100 µg/mL to 0.2 µg/mL) of four selected staphylococcal isolates in BM broth media (*S. aureus* C5802, *S. hominis* C5835, and 2 *S. sciuri* X3041 and X3011), against MRSA-C1570 and *L. monocytogenes*-CECT4032 indicator isolates ($5x10^5$ CFU/mL). In addition, CFS of a non-bacteriocin-producing isolate (*S. hominis* X3758) was included as negative control.

Plates were incubated at 37ºC for 24 h under shaking in a "Epoch2" microplate reader (BioTek), and the OD_{600} was measured every 10 minutes. Moreover, growth curves with nisin (Sigma) as a control were carried out against MRSA-C1570 indicator isolate **[11]**. The purity of the commercial product was taken into consideration (2.5%), for calculation of nisin concentration.

2.5. Combined effect of the antimicrobial extracts with antibiotics

Minimum Inhibitory Concentrations (MICs) of the MRSA-C1570 indicator isolate for oxacillin, erythromycin, clindamycin, gentamicin, tetracycline, and chloramphenicol, as well as for the selected BT extract of *S. sciuri*-X3041 isolate, were determined. The combined effect of the BT extract and commercial antibiotics was analysed by the checkerboard assay **[12]**. Briefly, twofold serial dilutions of both antibiotics (diluted along the abscissa) and BT extract (diluted along the ordinate) were performed on Mueller Hinton Broth (MHB; Roth, Karlsruhe, Germany) from at least double the MIC. The resulting microtiter plate was inoculated with the

indicator bacteria (MRSA-C1570) at a final concentration of $5 \cdot 10^5$ CFU/mL. Plates were incubated at 37°C for 24 h under continuous shaking. The fractional inhibitory concentration index (FIC) was calculated for each combination using the following formula: FICA + FICB = FIC, where FICA = MIC of drug A in combination/MIC of drug A alone, and FICB = MIC of drug B in combination/MIC of drug B alone. The FIC was interpreted as follow: synergism = FIC ≤ 0.5; indifference = 0.5 < FIC ≤ 4; antagonism = FIC > 4 **[13]**.

2.6. Competition studies

Four bacteriocin-producing and clindamycin-susceptible isolates (*S. aureus*-C5802, *S. hominis*-C5835, 2 *S. sciuri*-X3041 and *S. sciuri*-X3011) were selected for competition studies against the clindamycin-resistant MRSA-C1570 indicator isolate. Non-bacteriocin-producing isolates of the same species as the bacteriocin-producing isolates were used as negative controls (*S. aureus*-X4422, *S. hominis*-X3758 and *S. sciuri*-X3304). Thus, equal volumes adjusted to $1x10^8$ CFU/mL were mixed and 10 µL of bacteria co-culture were spotted in triplicate on BM agar and incubated at 37°C. Samples were taken at 0 h, 24 h, 48 h, and 72 h, serial diluted and plated on BM and BM-clindamycin (2.5µg/mL) agar for selection of the indicator MRSA isolate. Colony counts were determined after overnight incubation at 37°C, and the bacterial ratios of MRSA and the respective bacteriocin-producing isolate were calculated.

3. RESULTS AND DISCUSSION

3.1. Antimicrobial activity of staphylococcal extracts in agar media

Staphylococcus isolates with antimicrobial activity are gaining attention due to their potential use as probiotics **[14],** or as a resource to obtain new antimicrobial compounds as interesting alternatives to the use of conventional antibiotics **[15, 16].** In this study, the AA profile of the 28 *Staphylococcus* isolates obtained by the *Spot-on-lawn* method, as well as of their crude CFS and BT extracts against the 6 indicator bacteria, is summarized in **Table 1**.

The 28 tested isolates showed AA against at least one indicator strain in one of the conditions tested. It is to note that 64% of the antimicrobial producing isolates showed AA against the

MRSA indicator bacteria and 50% against *L. monocytogenes*. Moreover, the most susceptible indicator strains were *E. cecorum,* MRSP and MSSA (inhibited by >80% of bacteriocin-producing strains); followed by *S. sciuri* (inhibited by 75%), MRSA (64%) and *L. monocytogenes* (50%) **(Table 1)**.

On the other hand, six of the 28 staphylococcal isolates showed inhibitory activity in their CFS by agar-diffusion-assay against at least one of the six indicator isolates tested; these isolates were the following ones (n° of indicator bacteria inhibited): *S. sciuri*-X3011 and *S. sciuri*-X3041 (n=6), *S. simulans*-C9832 (n=6), *S. hominis*-C5835 (n=5), *S. aureus*-C5802 (n=2) and *S. pseudintermedius*-C8479 (n=1). Concerning the BT extracts, all 28 isolates showed antimicrobial activity against at least one of the six indicator isolates **(Table 1)**. It is to note that after extraction with 1-butanol a high number of indicator isolates were inhibited, indicating that many of the antimicrobial compounds were present only at low amounts in the culture supernatants, and their activities were only detectable after concentration by extraction as previously reported **[17].**

3.2. Bacteriocin identification

The thiopeptide type bacteriocin micrococcin P1 (MP1) was detected by mass spectrometry analysis in the four highly producing *Staphylococcus* isolates (14%) (*S. aureus*-C5802; *S. hominis*-C5835, *S. sciuri*-X3041 and *S. sciuri*-X3011). These four MP1-producing isolates carried the complete MP1 bacteriocin gene cluster as shown in a previous study **[18]**, which revealed high identity between them and with other operons previously reported. It is known that MP1 has antimicrobial activity mainly against Gram-positive bacteria and it was firstly discovered in *Micrococcus* although it has been also detected in other Gram-positive bacteria (*Staphylococcus, Streptococcus* and *Bacillus,* among others) **[18].** The mass spectrometry analysis did not render any other bacteriocin in the remaining 24 isolates **(Figure S1).**

3.3. Antimicrobial activity (AA) of staphylococcal extracts in broth media

A potent antimicrobial activity of the crude CFS and BT extracts of the four MP1-producing isolates (*S. aureus*-C5802, *S. hominis*-C5835, *S. sciuri*-X3041 and *S. sciuri*-X3011) against two indicator bacteria (MRSA-C1570 and *L. monocytogenes*-CECT4032) was revealed by growth curves **(Figure 1 and Figure 2)**.

The OD_{600} at 24 h of the two indicator bacteria incubated with different CFS (%) and BT extract (µg/mL) concentrations is shown in **Figure 1-I,** and the growth curves along the time are shown in **Figure 2,** including control growth (indicator bacteria without extracts)**.** In this experiment, the crude CFS of the four MP1-producing isolates, strongly reduced the growth of both indicator bacteria compared to the control, even when considerably diluted, up to 2.5% in some cases (X3041 CFS against the indicators *L. monocytogenes* and MRSA) **(Figure 1-I** and **Figure 2).** It should be noted that as the amount of CFS increased, the volumes of nutrient medium were lower, and this could have affected the detected results. For this reason, a CFS of a non-bacteriocin-producing isolate (*S. hominis*-X3758) was included as a negative control **(Figure S2)**. As expected, the lower amount of nutrient medium affected the growth of the indicator (*L. monocytogenes* and MRSA) isolates when increasing CFS volumes of X3758 isolate were used. However, this reduction in the growth of indicator isolates was much more substantial when CFS of MP1-producing isolates (C5802, C5835, X3041 and X3011) was used **(Figure S2).** With respect to the BT extract test, a decrease of the OD_{600} of the indicator control at 24 h and a logarithmic phase delay from 2 to 14 hours even at low concentrations of the extracts were identified in most of the cases and notably, the AA of the X3011 BT extract inhibited both indicators at concentrations of 3.1 µg/mL or even lower (0.8 to 2.5) **(Figure 1-II and Figure 2)**.

Moreover, nisin was selected as a reference because it is the only bacteriocin approved in food industry **[19]**. In this study, growth curves carried out with the non-purified BT extracts of the four selected MP1-producing staphylococcal isolates against two indicator bacteria (*L. monocytogenes* and MRSA) revealed a potent inhibitory effect in the case of *S. sciuri* (X3041 and X3011) and *S. aureus* (C5802) isolates even at low concentrations (6.3 µg/mL). These

results are shown in **Figure 1-III,** also including the antimicrobial effect of nisin (5 µg/mL) against the indicator bacteria MRSA and *L. monocytogenes*.

3.4. Analyses of synergistic effects between antibiotics and extracts

The association of bacteriocins with classic antibiotics and other compounds has been reported and it is expected to have important beneficial effects based on the promising synergistic effects although antagonistic effects should also be considered **[3]**.

It is plausible that synergistic combinations of bacteriocins and antibiotics may reduce the likelihood of resistance development, decreasing adverse effects and broadening the spectrum of activity **[12, 20, 21].** In this respect, checkerboard assay has revealed in our work synergistic effects against the MRSA-C1570 indicator strain for the combination of the BT extract of the MP1-producing *S. sciuri* X3041 isolate with clindamycin (> 80% of combinations tested) and oxacillin (30%), considering antibiotic concentrations of 2-625 µg/mL and 1-2 µg/mL, respectively. In addition, the combination BT-chloramphenicol showed synergism only at antibiotic concentrations of 15.6 µg/mL with near synergism values of 37% in both cases **(Table S2).** Recent studies reported synergism between MP1 and rifampicin showing that this antibiotic-bacteriocin combination was effective not only to eradicate but also to prevent recurrent infections **[22].** In this regard, although bacteriocins have potential applications on their own, the combination of bacteriocins with other existing antibiotics/antimicrobials could be a good option against antimicrobial resistance and therefore it should be considered as a viable alternative.

3.5. Competition among MP1-producing isolates and the MRSA-C1570 indicator strain.

Recent studies have revealed that coagulase-negative staphylococcal isolates compete with *S. aureus* for stable colonization of human skin reservoirs by production of antimicrobial factors **[23, 16].** Focusing on the competition assays, MRSA-C1570 was co-cultivated with the two MP1-producing *S. sciuri* isolates (X3041 and X3011), which showed strong inhibition against the MRSA-indicator isolate already after 24 h growth (>99% of viable counts were $MP1^{+}$) and

this effect was maintained after 48 h and 72 h. The MP1-producing methicillin-susceptible *S. aureus* isolate (C5802) also inhibited the MRSA indicator isolate at 24, 48 and 72 h ($MP1^+$ counts: 89%, 73%, 100%, respectively), although the killing effect was lower as for the MP1-producing *S. sciuri* isolates. For the *S. hominis* MP1-producing isolate a relevant inhibition effect was only found at 72 h (C5835, 52% of viable counts) **(Figure 3)**. Competition assays carried out with non-bacteriocin-producing isolates revealed a higher growth of the MRSA-indicator than when $MP1^+$ isolates were used **(Figure 3).** In conclusion, the out-competing effects of the MP1 producers against the MRSA-indicator could be confirmed in the present study, while the non-bacteriocin-producing isolates had no such properties. Further analysis will be performed to deeper characterize the production of MP1 and to identify the bacteriocins in the rest of the antimicrobial-producing isolates included in this study.

4. Conclusion

The emergence of antimicrobial resistance is an international health problem with highest priority that has raised the urgent need to explore novel sources of antimicrobial compounds. Among new possible alternatives to traditional antibiotics, we suggest the four MP1-producing isolates characterized in this study and especially the coagulase-negative staphylococci species as excellent candidates for future application studies.

DECLARATIONS

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Competing Interests: The authors declare no competing interests.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Sequence Information: Not applicable

AUTHOR CONTRIBUTIONS

C.T., B.K., A.P., and C.L. contributed to the design and the general supervision of the study. R.F.-F. developed the experimental work and the first version of the manuscript. C.T., C.L., and B.K. made the first revision of the manuscript. R.F.-P. contributed to some experimental laboratory work related to growth curves. C.T., M.Z. and A.P. contributed to project funding. All authors revised the different versions of the manuscript, read, and agreed to the submitted version of the manuscript.

Data availability All data generated or analysed during this study are available within this paper and its supplementary information files.

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FIGURE AND TABLES LEGEND

Table 1. Antimicrobial activity profile obtained by the *Spot-on-lawn* test (blue), crude cell free supernatant (orange) and 1-butanol extracts (green) with the 28 bacteriocin-producing isolates included in this study against the six indicator bacteria tested.

[a]Abbreviations: MRSP, methicillin resistant *S. pseudintermedius*; MRSA, methicillin resistant *S. aureus*; MSSA, methicillin susceptible *S. aureus;* AA, antimicrobial activity.

[b]Colour; detection of antimicrobial activity; White; non-antimicrobial activity for the different conditions.

Figure 1. Antimicrobial activity (AA) of the four MP1-producing isolates (*S. aureus* C5802, *S. hominis* C5835, and *S. sciuri* X3041 and X3011) against MRSA-C1570 (methicillin resistant *S. aureus*) and *L. monocytogenes*-CECT4032 indicator isolates in different conditions: I) OD_{600nm} values at 24 hours using different percentages of crude cell free supernatant (CFS, A and B) and different concentrations (in µg/mL) of 1-butanol extracts (BT, C and D) of MP1-producers; II) Minimum inhibitory concentration of the CFS (%) and BT extracts (µg/mL) of the MP1-producers; III) Comparison of the antimicrobial activity of nisin (5 µg/mL) and the non-purified 1-butanol extracts (6.3 µg/mL) of MP1-producing isolates. Positive controls (C+) of subfigures 1-I corresponds to the indicator strain without extracts and are represented with a red dotted line. Positive control of subfigures 1-III corresponds to the indicator strain without extracts or nisin.

Figure 2. Growth curves (OD_{600nm} values) of the two indicator strains (MRSA C1570 and *L. monocytogenes* CECT4032) in presence of different concentration of CFS (in percentage, subfigures A and B), or 1-butanol (BT in µg/mL, subfigures C and D) extracts of the four MP1-producing isolates (*S. aureus* C5802, *S. hominis* C5835, and *S. sciuri* X3041 and X3011). Positive controls (C+) correspond to the indicator strain growth without MP1 extracts.

Figure 3. I) Competition studies of the four MP1-producing staphylococcal isolates against the MRSA-C1570 indicator strain. II) Competition studies of the three non-bacteriocin-producing staphylococcal isolates used as control against the MRSA-C1570 indicator strain.

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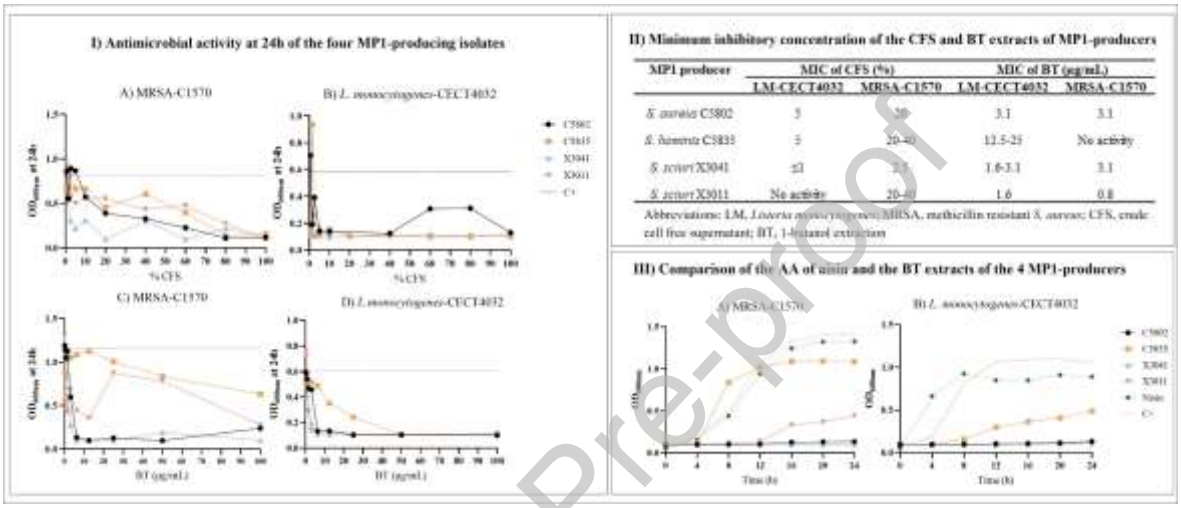

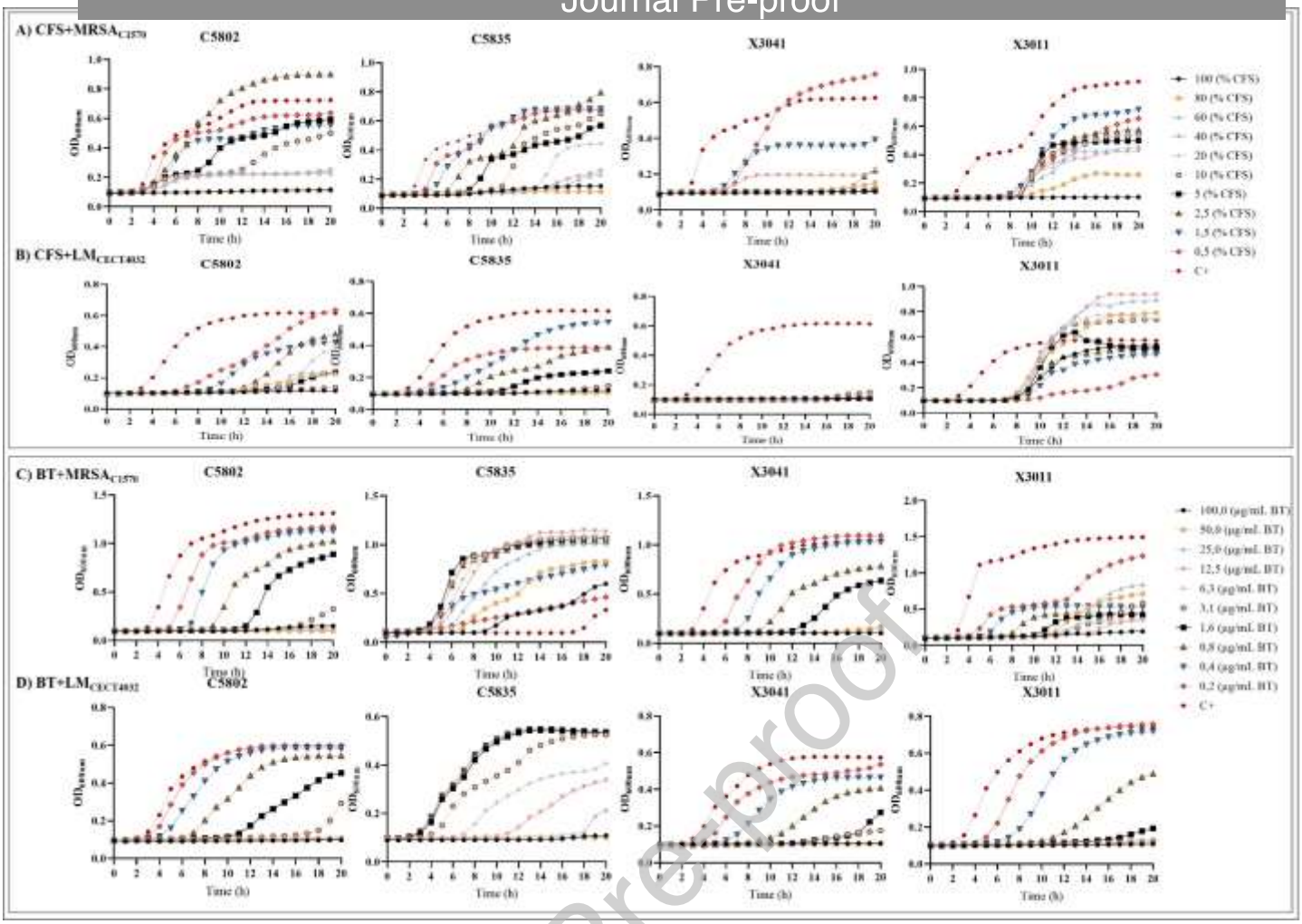

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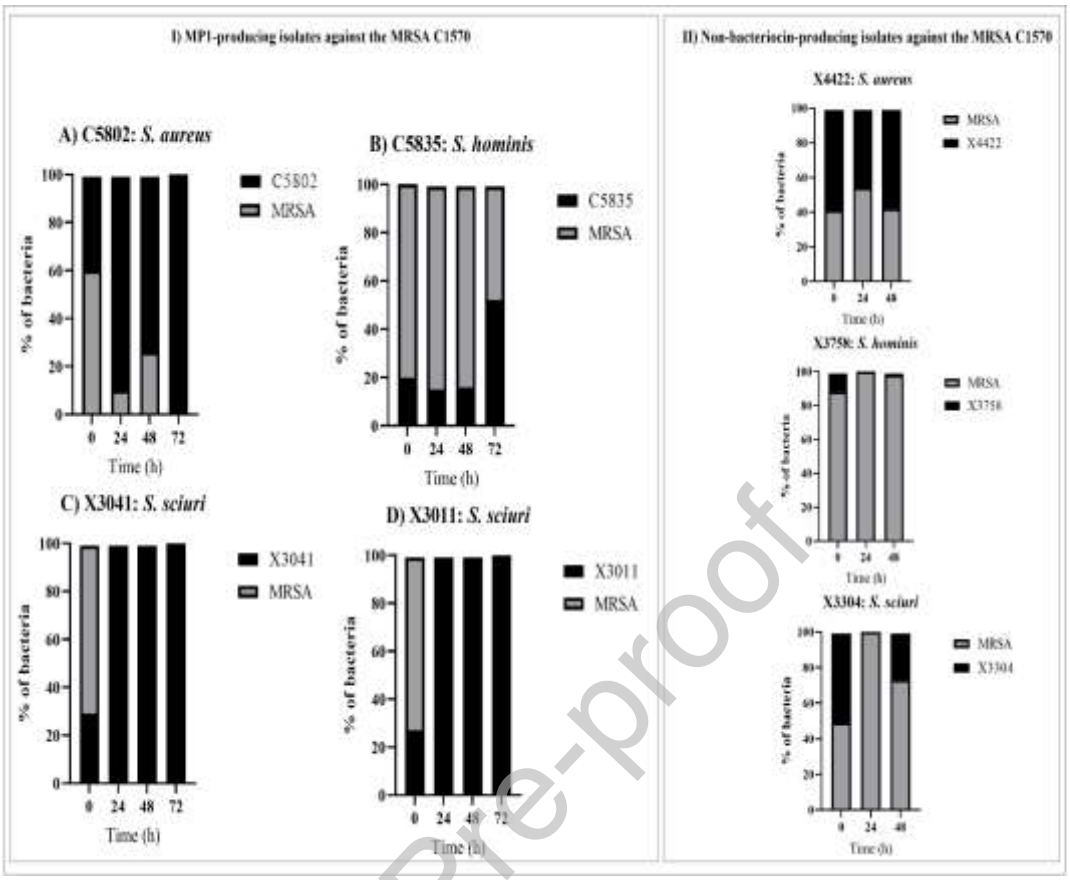

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Table 1. Antimicrobial activity profile obtained by the *Spot-on-lawn* test (blue), crude cell free supernatant (orange) and 1-butanol extracts (green) with the 28 bacteriocin-producing isolates included in this study against the six indicator bacteria tested.

Producer strain	*L. monocytogenes* CECT4032			*E. cecorum* X3809			MRSP C3468			*S. sciuri* C9459			MRSA C1570			MSSA SA113		
***S. aureus* C5802**																		
***S. aureus* C8609**																		
***S. aureus* C6770**																		
***S. aureus* X3410**																		
***S. pseudintermedius* C8189**																		
***S. pseudintermedius* C8478**																		
***S. pseudintermedius* C8479**																		
***S. pseudintermedius* C4502**																		
***S. pseudintermedius* C5357**																		
***S. hyicus* C9585**																		
***S. hominis* C5835**																		
***S. simulans* C9832**																		
***S. epidermidis* X2969**																		
***S. warneri* X3009**																		
***S. lugdunensis* C9954**																		
***S. lugdunensis* C9161**																		
***S. chromogenes* C9838**																		
***S. chromogenes* C9581**																		
***S. chromogenes* C9727**																		
***S. chromogenes* C9726**																		
***S. sciuri* X3041**																		
***S. sciuri* X3011**																		
***S. sciuri* C9175**																		
***S. sciuri* C9179**																		
***S. sciuri* C9213**																		
***S. sciuri* C9258**																		
***S. sciuri* C9529**																		
***S. sciuri* C9255**																		
% of isolates with AA	46	14	18	46	21	64	50	21	68	43	14	75	36	14	50	21	14	82
Total nº of inhibitions	14			26			25			21			18			24		

[a]Abbreviations: MRSP, methicillin resistant *S. pseudintermedius*; MRSA, methicillin resistant *S. aureus*; MSSA, methicillin susceptible *S. aureus;* AA, antimicrobial activity.
[b]Colour; detection of antimicrobial activity; White; non-antimicrobial activity for the different conditions.

Capítulo 4

4. Capítulo 4: Potenciales aplicaciones de las estafilococcinas y los estafilococos productores

La resistencia a los antibióticos ha supuesto importantes retos en salud pública y el sector agroalimentario, como son la lucha contra las bacterias multirresistentes, las infecciones en animales de producción, los problemas en la conservación y seguridad alimentaria, y también problemas derivados de la colonización por bacterias resistentes emergentes. En base a esta problemática, la comunidad científica aboga por la búsqueda y el uso de bacteriocinas entre otras sustancias antimicrobianas de origen natural como posibles alternativas al uso de antibióticos para controlar, prevenir o paliar los efectos negativos derivados de la infección bacteriana.

En el ámbito humano, las infecciones cutáneas son muy frecuentes a nivel global, especialmente las infecciones de piel y partes blandas. *S. aureus* es uno de los agentes infecciosos más relevantes en salud pública y además es un colonizador frecuente de la microbiota nasal. Su presencia está relacionada con una baja riqueza de especies y con el aumento de probabilidades a padecer procesos infecciosos. Además, la aparición de resistencias en los últimos años y las normativas cada vez más estrictas respecto al uso de antibióticos en producción animal han obligado a buscar y desarrollar alternativas, como las bacteriocinas, para el control y prevención de enfermedades infecciosas.

Recientemente se han descrito varios aislados de SCoN de piel y tejidos mucosos productores de bacteriocinas con interesantes actividades antimicrobianas, normalmente frente a microorganismos G+ potencialmente patógenos y, en unos pocos casos, también frente a Gram-negativos (G-). En concreto, se ha demostrado en humanos el papel protector de cepas de SCoN capaces de competir o limitar el crecimiento de *S. aureus*, incluido el SARM, garantizando el equilibrio del microbioma cutáneo en la dermatitis atópica. Asimismo, se ha observado como la presencia de cepas de *S. lugdunensis* (SCoN) productoras de bacteriocinas podría controlar la presencia de *S. aureus* en las fosas nasales por lo que podría tener un papel como agente modulador de la microbiota nasal y en consecuencia en la infección por dicho patógeno.

Asimismo, se deben tener en cuenta los retos no resueltos relacionados con la problemática de la RAM en la industria agroalimentaria, incluyendo el sector agrícola, la producción animal y la seguridad alimentaria. En este punto, hongos micoparásitos y patógenos bacterianos tales como *S. aureus, S. agalactie, E. coli, Clostridium, Enterococcus, S. suis, L. monocytogenes* adquieren gran relevancia.

Entre las medidas llevadas a cabo, cabe destacar el uso de bacteriocinas bien puras o semi-purificadas para combatir mastitis o enteritis aviar. Asimismo, se están llevando a cabo estudios centrados en el uso de bacterias productoras de sustancias antimicrobianas para diferentes usos:

a) probióticos para la acuacultura y/o producción animal aviar y porcina; b) cultivos protectores para garantizar la seguridad alimentaria; y c) agentes de biocontrol en agricultura.

Por todo ello, la identificación y caracterización de nuevas bacteriocinas y especialmente las producidas por aislados comensales es un objetivo claro de esta tesis. En este capítulo se presentan los resultados obtenidos tras abordar el cuarto objetivo en el cual se plantea: (a) Estudiar el papel modulador de cepas de estafilococos productoras de bacteriocinas en la microbiota nasal, usando un animal de vida libre (cigüeñas) como sistema modelo; (b) Analizar la actividad antimicrobiana de extractos prepurificados de cepas Bac+ seleccionadas en esta tesis frente a bacterias patógenas de interés en el sector agroalimentario y en salud pública **(Figura 20).**

4.1 Artículo 5 Detection of antimicrobial producing *Staphylococcus* from migratory birds: Potential role in nasotracheal microbiota modulation

4.2 Bacteriocin-producing *Staphylococcus* for agro-food applications: relevance of micrococcin P1

4.1 Artículo 5..... Detection of antimicrobial producing *Staphylococcus* from migratory birds: Potential role in nasotracheal microbiota modulation

En este trabajo se ha evaluado la actividad antimicrobiana y la capacidad de modulación de la microbiota en una colección de estafilococos procedentes de 136 muestras (84 traqueales y 52 nasales) de 87 cigüeñas blancas. Los animales incluidos pertenecían a cuatro colonias diferentes de cigüeñas situadas en el centro-sur de España y los estafilococos fueron aislados, identificados y caracterizados para evaluar su resistencia y virulencia en un estudio anterior del grupo OneHealth-UR **(Abdullahi et al. 2023a).**

Se incluyeron un total de 259 cepas de estafilococos de 13 especies diferentes [212 coagulasa-negativos (SCoN) y 47 coagulasa-positivos (SCoP)]. El estudio de actividad antimicrobiana (AA) por *spot-on-lawn* frente a 14 bacterias indicadoras permitió detectar 9 estafilococos Bac+ (3,5%) que inhibieron al menos uno de los indicadores evaluados, todos ellos G+. Los indicadores más inhibidos fueron estafilococos sensibles a meticilina seguidos de enterococos, *M. luteus, S. suis* y, por último, los estafilococos resistentes a meticilina.

Asimismo, se analizó la actividad antimicrobiana de los SLC y extractos BT en los 9 aislados Bac+. Destacaron tres cepas (*S. hominis*, *S. sciuri* y *S. chromogenes*) por presentar actividad inhibitoria en todas las condiciones probadas. De ellas, la cepa de *S. hominis* X3764 inhibió todos los indicadores G+ excepto a *L. monocytogenes.* Finalmente, se confirmó por PCR la presencia de genes codificantes de lantibióticos en las 9 cepas Bac+ y los ensayos con enzimas proteolíticas en las cepas Bac+ seleccionadas (*S. hominis* y *S. sciuri*) permitieron verificar la naturaleza proteica del compuesto con actividad antimicrobiana detectado en los SLC.

Por otro lado, se quiso conocer el posible papel modulador de los 9 estafilococos Bac+ y se analizó su actividad antimicrobiana por *spot-on-lawn* frente a las bacterias G+ siguiendo dos enfoques diferentes: (a) Actividad intra-muestra, para determinar si los aislados Bac+ inhiben a las bacterias G+ recuperadas en la misma muestra nasotraqueal de cigüeña que la cepa productora; (b) Actividad inter-muestra, para determinar si el aislado Bac+ inhibe a alguna de las 30 cepas de 29 especies G+ diferentes y nueve géneros elegidas como bacterias indicadoras representativas de la comunidad nasotraqueal de cigueñas. En este último caso, se seleccionó un aislado de cada especie G+ obtenido en las 87 cigueñas del estudio. Tres de las nueve cepas Bac+ revelaron potente actividad intra-muestra; asimismo destaca la actividad inter-muestra de una cepa de *S. hominis*, que inhibió el 73% de las 29 especies G+ representativas de la microbiota nasotraqueal de cigüeñas. Si bien es necesario profundizar en la caracterización y purificación de las potenciales bacteriocinas detectadas, este tipo de trabajos abren la puerta al interés de las cepas SCoN productoras de bacteriocinas o extractos de las mismas en distintas aplicaciones, especialmente en la modulación de la microbiota nasal como una novedosa aplicación en la

prevención de enfermedades causadas por agentes patógenos de interés, como es el caso de SARM.

4.2 Bacteriocin-producing Staphylococcus for agro-food applications: relevance of micrococcin P1

En este trabajo se ha evaluado la actividad inhibitoria de 15 cepas de estafilococos productoras de bacteriocinas detectadas en esta tesis, sus SLC y sus extractos BT prepurificados con el objetivo de verificar su potencial aplicación frente a 27 microorganismos patógenos o zoonóticos relevantes en salud pública y agroalimentación utilizados como indicadores. Se utilizaron 22 bacterias indicadoras G+, tres G- y dos hongos, seleccionados en función de la especie y/o a sus fenotipos de resistencia a antibióticos incluyendo cepas MDR y agentes zoonóticos de relevancia. Asimismo, los indicadores fueron agrupados en siete categorías en base a su potencial área de aplicación: a) mastitis e industria láctea; b) patógenos aviares y agentes zoonóticos; c) zoonosis porcinas; d) seguridad alimentaria; e) acuicultura; f) industria vitivinícola; y g) cultivo de hongos (*Agaricus bisporus*). La actividad antimicrobiana se evaluó por el método de *spot-on-lawn* (25 indicadores G+/-) mientras que la capacidad inhibitoria de los SLC y BT fueron analizados por difusión en agar frente a los 27 microorganismos indicadores (en el caso de los hongos sólo se estudió la actividad inhibitoria de los BT).

De este modo, se evidenció que todas las bacterias Bac+ inhibieron al menos a una bacteria indicadora G+ en una de las siete categorías evaluadas. Sin embargo, no se observó AA frente a bacterias G-. En cuanto a la actividad antimicrobiana detectada por *spot-on-lawn*, cabe destacar los altos porcentajes de inhibición detectados frente a tres cepas SARM, *E. faecium* C2321*, L. monocytogenes* CECT4032*, Lactococcus garviae, Pediococcus acidilactici* y *Leuconostoc mesenteroides*, que fueron inhibidos por ≥60% de las cepas Bac+. Asimismo, 6 aislados Bac+ de los 15 evaluados mostraron AA en sus SLC inhibiendo al 77% de los indicadores usados. Cabe destacar el incremento del potencial inhibitorio revelado en los extractos prepurificados con butanol, que inhibieron el crecimiento de todas las bacterias indicadoras G+ y los dos hongos evaluados. Por otro lado, se quiso probar la inmunidad cruzada entre las cepas Bac+ con el objetivo de identificar posibles consorcios bacterianos para su aplicación conjunta frente a un determinado patógeno. En este sentido, se evaluaron varios aspectos relacionados con la seguridad (hemólisis, actividad gelatinasa, resistencia a antibióticos y virulencia) que ayudaron a proponer algunas de las cepas SCoN incluidas en este trabajo como potenciales candidatas a estudio para aplicaciones futuras en consorcios conjuntos. Entre ellas, se encuentran tres cepas productoras de la bacteriocina Micrococcina P1, que tanto por *spot-on-lawn* como en sus SLC o extractos BT prepurificados tuvieron un gran potencial inhibitorio (inhibieron a más del 60% de los indicadores en todas técnicas estudiadas con los halos más intensos) por lo que se proponen en este estudio como las mejores candidatas para su uso en las categorías mencionadas.

4.3 Resumen gráfico del Capítulo 4

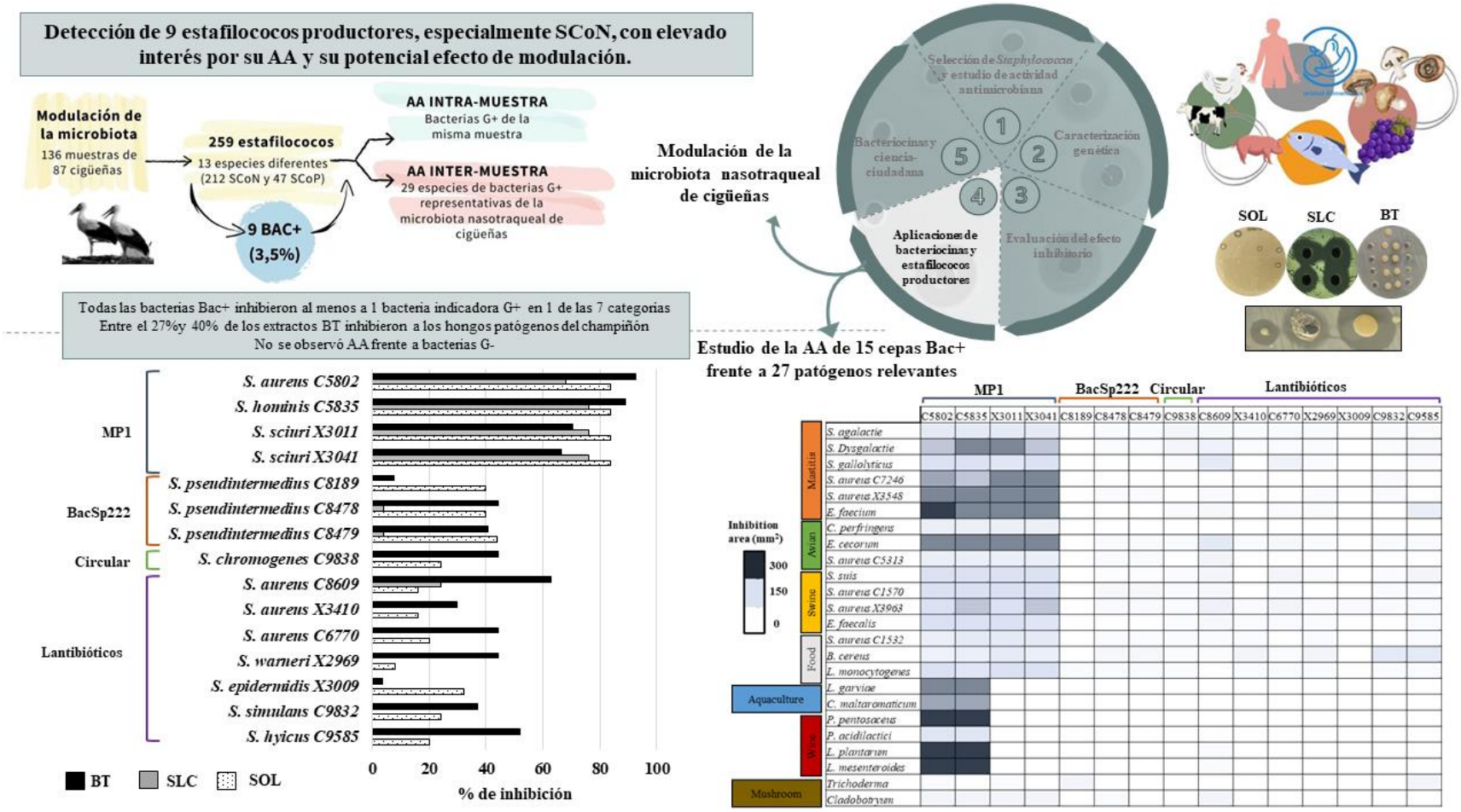

Figura 20 Resumen gráfico de la metodología y resultados obtenidos en el cuarto capítulo referente a las posibles aplicaciones de las estafilococcinas y los estafilococos productores (elaboración propia).

Artículo 5

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EDITED BY
Rustam Aminov,
University of Aberdeen, United Kingdom

REVIEWED BY
Katarzyna Garbacz,
Medical University of Gdańsk, Poland
Süleyman Yildirim,
İstanbul Medipol University, Türkiye

*CORRESPONDENCE
Carmen Torres
✉ carmen.torres@unirioja.es

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Detection of antimicrobial producing *Staphylococcus* from migratory birds: Potential role in nasotracheal microbiota modulation

Rosa Fernández-Fernández[1], Idris Nasir Abdullahi[1], Carmen González-Azcona[1], Adriana Ulloa[1], Agustí Martínez[1], Sara García-Vela[1,2], Ursula Höfle[3], Myriam Zarazaga[1], Carmen Lozano[1] and Carmen Torres[1]*

[1]Area of Biochemistry and Molecular Biology, One Health-UR Research Group, University of La Rioja, Logroño, Spain, [2]Department of Food Science, University of Laval, Québec City, QC, Canada, [3]SaBio (Health and Biotechnology) Research Group, Game and Wildlife Research Institute, Spanish National Research Council/University of Castilla–La Mancha, Ciudad Real, Spain

A collection of 259 staphylococci of 13 different species [212 coagulase-negative (CoNS) and 47 coagulase-positive (CoPS)] recovered from nasotracheal samples of 87 healthy nestling white storks was tested by the *spot-on-lawn* method for antimicrobial-activity (AA) against 14 indicator bacteria. Moreover, extracts of AP isolates were obtained [cell-free-supernatants (CFS) both crude and concentrated and butanol extracts] and tested against the 14 indicator bacteria. The microbiota modulation capacity of AP isolates was tested considering: (a) intra-sample AA, against all Gram-positive bacteria recovered in the same stork nasotracheal sample; (b) inter-sample AA against a selection of representative Gram-positive bacteria of the nasotracheal microbiota of all the storks (30 isolates of 29 different species and nine genera). In addition, enzymatic susceptibility test was carried out in selected AP isolates and bacteriocin encoding genes was studied by PCR/sequencing. In this respect, nine isolates (3.5%; seven CoNS and two CoPS) showed AA against at least one indicator bacteria and were considered antimicrobial-producing (AP) isolates. The AP isolates showed AA only for Gram-positive bacteria. Three of these AP isolates (*S. hominis* X3764, *S. sciuri* X4000, and *S. chromogenes* X4620) revealed AA on all extract conditions; other four AP isolates only showed activity in extracts after concentration; the remaining two AP isolates did not show AA in any of extract conditions. As for the microbiota modulation evaluation, three of the nine AP-isolates revealed intra-sample AA. It is to highlight the potent inter-sample AA of the X3764 isolate inhibiting 73% of the 29 representative Gram-positive species of the nasotracheal stork microbiota population. On the other hand, enzymatic analysis carried out in the two highest AP isolates (X3764 and X4000) verified

the proteinaceous nature of the antimicrobial compound and PCR analysis revealed the presence of lantibiotic-like encoding genes in the nine AP isolates. In conclusion, these results show that nasotracheal staphylococci of healthy storks, and especially CoNS, produce antimicrobial substances that could be important in the modulations of their nasal microbiota.

KEYWORDS

staphylococci, coagulase-negative staphylococci, bacteriocins, antimicrobial activities, storks, nasal microbiota

1. Introduction

Bacteria thrive in complex niches establishing inter-species and intra-environment relationships, including the ability to acquire and transfer several adaptation mechanisms (Krismer et al., 2017; Heilbronner et al., 2021). The nasal cavity is directly connected to the external media and in close contact with a wide diversity of microorganisms that can be acquired through inhalation (Biswas et al., 2015). Moreover, the microenvironment of the nasal cavity varies depending on the anatomical location.

The anterior nares (nostrils) are the most difficult area for the survival of microbes due to their acidic environment with high salinity (Geurkink, 1983). Therefore, microbes that live in the nasal cavity are subjected to a variety of stress conditions and they must counteract to survive and persist (Krismer et al., 2014). In this respect, species competition in the nasal cavity can be mediated by direct or indirect mechanisms such as the acquisition of nutrients, the production of antimicrobial substances and the activation of specific host defense mechanisms (Krismer et al., 2017).

Human nasal microbiota is mainly composed of *Staphylococcus, Cutibacterium, Corynebacterium,* and *Moraxella* (Zhou et al., 2014), and bacteria from other genera are less frequently found (Liu et al., 2015). Focusing on the *Staphylococcus* genus, human nasal isolates have been frequently described as producers of antimicrobial substances against bacterial competitors. For instance, *S. epidermidis* or *S. lugdunensis,* among others favorably ousted *S. aureus* (Janek et al., 2016; Zipperer et al., 2016). However, there could be limitations in the detection and/or production of antimicrobial activity due to some producing isolates require specific environmental stress conditions commonly present in the human nose, such as hydrogen peroxide release and iron limitation (Janek et al., 2016).

The nasal microbiota of animals has also been analysed revealing composition differences. For example, companion and farm animals or rodents have higher abundances of Proteobacteria as compared to humans (Weese et al., 2014; Chaves-Moreno et al., 2015; Misic et al., 2015), and some *S. aureus* lineages, including livestock-associated MRSA, are increasingly found in the noses of livestock (Bal et al., 2016). However, staphylococci and especially coagulase-negative staphylococci (CoNS) from wildlife remain largely understudied. In this respect, some studies carried out in Spain and Portugal revealed that wild animals (birds and mammals) are frequently colonized by CoNS and *S. sciuri* being one of the predominant species among this group of microorganisms (Sousa et al., 2016; Mama et al., 2019; Ruiz-Ripa et al., 2020).

Birds have been postulated as sentinels, reservoirs, and potential disseminators of antimicrobial resistance due to their interaction with the human interface, diverse ecological niches, and capacity to travel for long distances (Bonnedahl and Järhult, 2014). Consequently, the nasotracheal bacterial communities of white storks have recently been studied by our research group (Abdullahi et al., 2023). Moreover, storks can also be a source of antimicrobial substances thanks to the adaptation strategies of the isolates present in their bacterial communities. Here, the present study aims to detect and partially characterize the production profile of antimicrobial substances in *Staphylococcus* isolates from nasotracheal samples of nestling white storks obtained from a previous study (Abdullahi et al., 2023), and to evaluate their capacity as modulators of the nasotracheal microbiota of these animals.

2. Materials and methods

2.1. Staphylococcal isolates used for the detection of antimicrobial activity (AA)

A total of 259 Staphylococcal isolates of stork origin were included in this study and they were tested for the production of antimicrobial activity (AA). These isolates were of 13 different species (number of isolates): *S. aureus* (46), *S. sciuri* (138), *S. epidermidis* (16), *S. lentus* (14), S. *chromogenes* (11), *S. xylosus* (8), *S. hominis* (7), *S. simulans* (7), *S. saprophyticus* (6), *S. haemolyticus* (3), *S. hyicus* (1), *S. capitis* (1), and *S. arlettae* (1).

These 259 Staphylococcal isolates were obtained from 136 samples (84 tracheal and 52 nasal) of 87 nestling white storks in a previous study (Abdullahi et al., 2023). The animals included belonged to four different colonies of storks located in South-central Spain. For bacterial isolation, the nasal or tracheal samples were pre-enriched in brain heart infusion broth supplemented with 6.5% NaCl and after overnight incubation, four culture media were used for bacteria recovery [blood agar, mannitol salt agar, oxacillin screening agar base supplemented with oxacillin (ORSAB medium), and CHROMagar^{TM} LIN]. Finally, up to 12 different colonies were randomly selected per sample and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Bruker Daltonics, Bremen, Germany). Thus, 259 distinct staphylococci were included

TABLE 1 Staphylococcal isolates evaluated[a] for antimicrobial activity by the *spot-on-lawn* method against 14 indicator bacteria and antimicrobial-producing (AP) isolates detected.

Species	N° of isolates[a]	Origin (n° of isolates)		AP isolates	
		Nasal	Tracheal	N° of AP isolates	Stork sample ID code (origin)[b]
S. aureus	46	22	24	1	436 (T)
S. hyicus	1	0	1	1	538 (T)
S. sciuri	138	54	84	2	507 (T), 433 (T)
S. epidermidis	16	3	13	1	506 (T)
S. lentus	14	5	9	0	–
S. chromogenes	11	7	4	1	481 (N)
S. xylosus	8	6	2	0	–
S. hominis	7	0	7	1	507 (T)
S. saprophyticus	6	1	5	0	–
S. simulans	7	6	1	2	480 (N), 481 (N)
S. haemolyticus	3	0	3	0	–
S. capitis	1	0	1	0	–
S. arlettae	1	1	0	0	–
Total	259	105	154	9	–

[a]Isolates were obtained from a previous study (Abdullahi et al., 2023).
[b]T, tracheal; N, nasal.

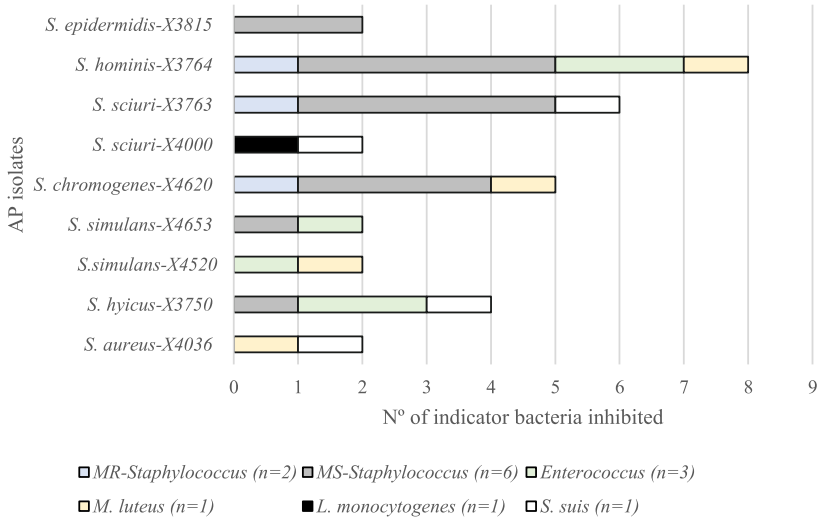


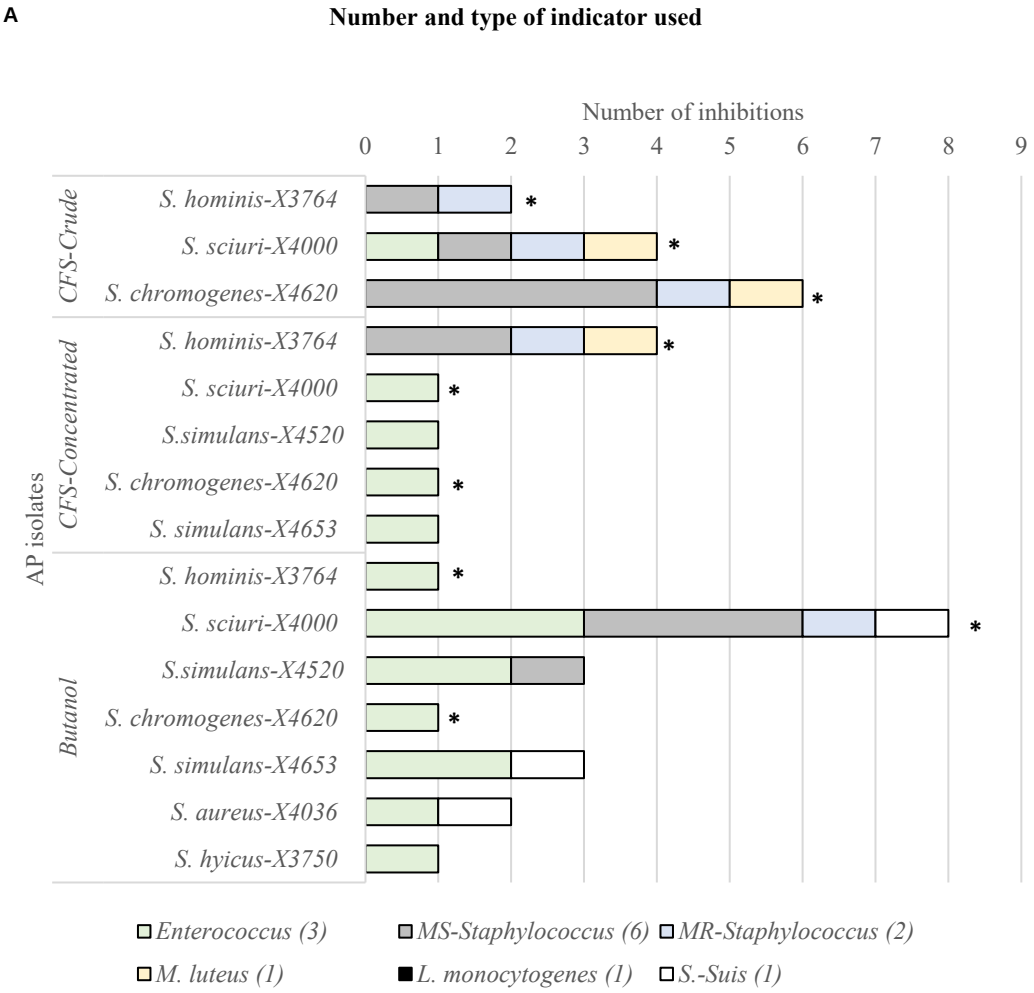
FIGURE 1
Antimicrobial activity of the nine antimicrobial producer (AP) isolates against 14 Gram-positive indicator bacteria grouped in six categories (*n*, number of isolates of each category), by the *spot-on-lawn* method.

in the present study (one isolate of each *Staphylococcal* species per animal), which corresponded to 2–5 staphylococci/animal.

2.2. Isolates used as indicator bacteria in the screening for detection of antimicrobial-producing (AP) staphylococci

Fourteen Gram-positive (G+) isolates of different genera and species were used as indicator bacteria to evaluate the AA of the collection of 259 *Staphylococcal* isolates of storks. The list of these 14 indicator bacteria is shown in **Supplementary Table 1** and includes relevant pathogenic, zoonotic, and multidrug-resistant (MDR) bacteria methicillin-resistant *S. aureus* (MRSA) (C1570), methicillin-susceptible *S. aureus* (MSSA) (ATCC29213), methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) (C2381), methicillin-susceptible *S. pseudintermedius* (MSSP) (C3468), *Staphylococcus lugdunensis* (C10107), *S. epidermidis* (C2663), *S. sciuri* (C9780), *Staphylococcus delphini* (C9459), *Enterococcus cecorum* (X3809), *Enterococcus faecalis* (ATCC29212), *Enterococcus faecium* (C2321), *Micrococcus luteus* (CECT241),

A



B

Summary of antimicrobial activity in extracts

	CFS Crude	CFS Concentrated	Butanol
Number of AP isolates	3	5	7
Total number of inhibitions	12	8	19

FIGURE 2
(A) Number of indicator bacteria (n = 14) inhibited by different preparations of the nine antimicrobial producer (AP) isolates of stork nasotracheal microbiota: crude CFS (boiled or filtrated), concentrated CFS, and after butanol extraction. **(B)** Number of AP isolates with antimicrobial activity in the three extract conditions tested and their respective total number of inhibitions. *Antimicrobial producing isolates with antimicrobial activity in the three conditions tested (*S. hominis* X3764, *S. sciuri* X4000, and *S. chromogenes* X4620).

Listeria monocytogenes (CECT4032), and *Streptococcus suis* (X2060). Two additional Gram-negative (G–) indicator bacteria were tested in the AP staphylococci detected: *Escherichia coli* (ATCC25922) and *P. aeruginosa* (PAO1).

2.3. Detection of antimicrobial activity (AA)

Four methods were used to determine antimicrobial activity:

1. *Spot-on-lawn* method: Indicator isolates were resuspended in Brain Heart Infusion broth (BHI; Condalab, Madrid, Spain) up to 0.5 MacFarland and 10 μL of the cultures were added to five-milliliter aliquots of Tryptic Soy Broth supplemented with 0.3% yeast extract (TSB; Condalab, Madrid, Spain) and 0.7% agar tempered at 45°C. Then, the mixture was seeded onto plates containing Tryptic Soy agar plus 0.3% yeast extract (TSA; Condalab, Madrid, Spain), and the putative antimicrobial-producing (AP) isolates were spotted on the surface and plates were incubated overnight at 37°C. When *Streptococcus suis* was the indicator bacteria, Columbia agar with 5% sheep blood (bioMérieux SA, France) was used instead of TSA. Growth inhibition was detected by a clearing zone with no bacterial growth around the AP isolate.
2. Crude cell-free supernatant (CFS): Antimicrobial-producing isolates were grown in 10 mL of BHI medium for 24 h

at 37°C, centrifuged (4.500 rpm, 10 min) and sterilized by boiling or filtration through a low-protein binding 0.45 μm Millipore filter.
3. Concentrated CFS: The resulting crude CFS was concentrated by speed vacuum and resuspended in dimethyl sulfoxide (DMSO).
4. Butanol extraction: 1-butanol was added to a fresh overnight BHI broth culture at a ratio of 1:2 and shacked for 1 h at 37°C. After phases differentiation, samples were centrifuged at 4.500 rpm for 15 min. The organic phase was tested for antimicrobial activity.

For methods 2–4, 50 μL of the *Staphylococcal* extracts were filled on wells done on TSA agar plates previously inoculated with the indicator bacteria, and the plates were incubated for 24 h at 37°C. The AA was assessed by the analysis of the inhibition zones around the wells. Positive (a previously described AP-isolate) and negative (BHI medium, DMSO, butanol) controls under all conditions were included in the assays.

2.4. Analysis of microbiota modulation

In the Staphylococcal isolates that showed antimicrobial activity by the *spot-on-lawn* method (AP isolates), their antimicrobial activity was also analysed against G^+ bacteria obtained from the storks' samples (used in this case as indicator bacteria), using the same procedure. Two different approaches were used:

1. Intra-sample activity. The activity of the AP isolates against all the G+ bacteria recovered in the same stork nasotracheal sample of the selected AP isolate was analyzed.
2. Inter-sample activity. From the whole collection of bacteria obtained from the nasotracheal samples of the 87 storks included in this study (Abdullahi et al., 2023), one isolate of each G^+ species was selected (avoiding the selection as representants of the isolates recovered in the same samples in which AP bacteria were detected). Following these criteria, a collection of 30 isolates of 29 different G^+ species and nine genera was used as indicator bacteria (representative of the inter-sample community) (**Supplementary Table 2**).

2.5. Characterization of the antimicrobial compounds in AP staphylococci

1. Susceptibility to proteolytic enzymes. The following enzymes were assayed (treatment conditions): trypsin (pH = 7.6; 25°C), α-chymotrypsin (pH = 7.8; 25°C), proteinase-K (pH = 7.5; 37°C), papain (pH = 6.2; 25°C) and protease (pH = 7.5; 37°C) (Sigma). Boiled CFS of two selected AP isolates (*S. sciuri* X4000 and *S. hominis* X3764) were prepared as described above. After adjusting to optimal pH, aliquots were independently incubated for 1 h with 1 g/L of each enzyme. After treatment, the enzymes were inactivated by boiling and antimicrobial activity was assayed (Navarro et al., 2000). Hemoglobin was used as negative control in all assays under all conditions.
2. Bacteriocin gene detection. The presence of 22 bacteriocin structural genes was tested by PCR and sequencing in all the AP-isolates detected in this study (*aurA, aucA, epiA, sacaA/sacbA, gdmA, bacSp222, nsj, hyiA, hycS, bacCH91, bsaA2, acIA, ale-1, lss, nukA, nkqA, eciA, pepA, elxA, elkA, ecdA, orf4*), as well as three bacteriocin gene families described elsewhere (BS, GEST, and NUK) (Fernández-Fernández et al., 2022).

2.6. Antibiotic resistance phenotype of AP isolates

The susceptibility to 13 antibiotics was evaluated in the AP isolates by the disk diffusion method and they were interpreted using the European Committee on Antimicrobial Susceptibility Testing criteria (EUCAST, 2022). The antibiotics tested were as follows: penicillin, cefoxitin, oxacillin, erythromycin, clindamycin, gentamicin, tobramycin, tetracycline, ciprofloxacin, chloramphenicol, linezolid, trimethoprim–sulfamethoxazole, and mupirocin.

3. Results

3.1. Antimicrobial activity of the collection of staphylococci isolates of storks against Gram-positive bacteria

Antimicrobial activity was detected by the *spot-on-lawn* method in 9 of the 259 staphylococci (3.5%) of stork origin tested, and they were considered as AP-isolates: *S. aureus* X4036, *S. hyicus* X3750, *S. sciuri* X3763 and X4000, *S. epidermidis* X3815, *S. chromogenes* X4620, *S. hominis* X3764, and *S. simulans* X4520 and X4653 (**Table 1**). These AP isolates were obtained from seven different storks and revealed AA against at least one of the 14 G^+ indicator bacteria tested. **Figure 1** represents the number of G^+ bacteria inhibited by each of the nine AP-bacteria, grouping the indicators in six categories: methicillin-resistant and -susceptible (MR and MS, respectively) *Staphylococcus, Enterococcus, M. luteus, L. monocytogenes*, and *S. suis*. Nevertheless, the AP staphylococci did not show AA against the two G– isolates tested (*E. coli* and *P. aeruginosa*). For this reason, all the next steps were performed only with G^+ indicator bacteria.

In this respect, the isolate with broader AA was *S. hominis* X3764, that inhibited all but one G^+ indicator (*L. monocytogenes*). The most susceptible indicator category was MS-staphylococci (inhibited by six AP-isolates, 66.7%), followed by enterococci, *M. luteus* and *S. suis* (44.4%) and MR-staphylococci (33.3%). Interestingly, one out of the nine AP-isolates (*S. sciuri* X4000) revealed AA against *L. monocytogenes*. Moreover, most of the AP-isolates showed AA against more than one G^+ indicator category tested. However, *S. epidermidis* X3815 isolate revealed a narrow inhibition profile, inhibiting only the MS-staphylococci indicators (**Figure 1**).

TABLE 2 Activity of the antimicrobial-producing (AP) isolates against Gram-positive (G^+) bacteria cohabiting in the same stork (intra-activity) or representative of the nasotracheal microbiota of the storks (inter-activity).

Stork sample/origin	AP-isolate	Intra-activity[a]	Inter-activity[b]
538/Tracheal	X3750-S. *hyicus*	*S. sciuri, S. lentus, S. saprophyticus*	*S. aureus, E. durans, E. hirae, M. caseolyticus, M. luteus, Glutamicibacter sp., C. falsenii, C. aurimucosum, Corynebacterium sp.*
507/Tracheal	X3763-S. *sciuri*	*E. faecalis, S. sciuri*	None
507/Tracheal	X3764-S. *hominis*	***S. sciuri,*** *E. faecalis*	*S. sciuri, S. aureus, S. chromogenes, S. epidermidis, S. xylosus, S. lentus, S. saprophyticus, S. hyicus, S. arlettae, S. capitis, E. faecium, E. gallinarum, E. durans, E. hirae, M. caseolyticus, L. garvieae, M. luteus, V. lutrae, Glutamicibacter sp., Corynebacterium sp., C. falsenii, C. aurimucosum*
506/Tracheal	X3815-S. *epidermidis*	*Corynebacterium sp., E. faecium.*	*S. aureus, S. capitis*
433/Tracheal	X4000-S. *sciuri*	*E. faecalis, S. sciuri*	*E. durans, Glutamibacter sp.*
436/Tracheal	X4036-S. *aureus*	*S. sciuri, E. hirae, S. aureus, S. sciuri*	None
480/Nasal	X4520-*S. simulans*	***M. caseolyticus,*** *S. xylosus, S. sciuri*	*M. caseolyticus, E. durans, Glutamicibacter sp. C. falsenii, Corynebacterium sp., C. aurimucosum*
481/Nasal	X4620-*S. chromogenes*	***M. caseolyticus,*** *S. chromogenes*	*S. lentus, S. simulans*
481/Nasal	X4653-S. *simulans*	*M. caseolyticus, S. chromogenes*	*E. durans, M. caseolyticus, Glutamicibacter sp., C. falsenii, Corynebacterium sp., C. aurimucosum*

[a]For intra-activity, the G^+ species cohabitant with AP-isolate in the same stork sample, that showed inhibition activity, are marked in bold.
[b]For inter-activity, 30 isolates of 29 different G^+ species representative of the G^+ nasotracheal diversity of storks were tested as indicator bacteria (see Table 3 and Supplementary Table 2).

Regarding extracts obtained in different conditions, only three AP-isolates (*S. hominis* X3764, *S. sciuri* X4000, and *S. chromogenes* X4620) revealed inhibitory capacity in all the conditions tested: crude CFS (filtered or boiled), CFS concentrated and butanol extraction. Moreover, other four isolates showed AA in the concentrated extracts: 55.6% revealed bioactivity in the concentrated CFS and this percentage increased to 77.8% after butanol treatment. Two additional isolates considered as AP by the *spot-on-lawn* method (*S. epidermidis* X3815 and *S. sciuri* X3763) were negative for all the extracts conditions against all the 14 G^+ indicators tested (Figure 2).

Going deeper into the AA of the extracts obtained under different conditions, Figure 2 also represents the G^+ indicator bacteria category inhibited in each of the extract conditions. This shows that extracts of all conditions tested (CFS crude and concentrated and butanol) inhibited MS-*Staphylococcus*, MR-*Staphylococcus*, and *Enterococcus* while none of them was active against *L. monocytogenes*. Interestingly, only the crude or concentrated CFS of some AP isolates showed AA against *M. luteus*, and *S. suis* was inhibited exclusively after butanol extraction of some AP isolates. Moreover, the three types of extracts were active against at least three indicator categories and as expected, concentrated CFS and butanol extracts showed a higher diversity of AP isolates and indicators.

3.2. Microbiota modulation

To analyse the ability for managing bacterial communities, the intra-sample AA of the nine AP isolates were firstly studied against the previously isolated G^+ bacteria of the same sample as the producer isolate (Table 2). Only three of the AP-isolates (*S. hominis* X3764, *S. simulans* X4520, and *S. chromogenes* X4620) inhibited one of the G^+ bacteria isolated in their respective samples (one *S. sciuri* and four *M. caseolyticus* isolates). Focusing on *S. hominis* X3764, it revealed AA against one of the two *S. isolates* isolates recovered in the same stork tracheal sample. However, *S. hominis* X3764 did not show antimicrobial activity against co-habitant *E. faecalis* isolates. In the same way, the AP isolates *S. simulans* X4520 and *S. chromogenes* X4620 inhibited the *M. caseolyticus* isolates co-habitant in the same nasotracheal sample when used as indicator bacteria (Table 2).

In addition, the inter-sample AA of the nine AP isolates was tested against a collection of 30 G^+ bacteria of 29 different species and nine genera representative of the G^+ bacterial diversity of the stork nasotracheal microbiota (Table 3). The high AA of *S. hominis* X3764, inhibiting 73% of the isolates selected as a representative G^+ microbial stork community is noteworthy. On the other hand, six AP isolates revealed AA against 7–33% of the G^+ indicators tested. However, *S. sciuri* X3763 and *S. aureus* X4036 isolates lacked inhibitory capacity against any of those indicator bacteria (Table 3). Focusing on the AA of these AP-isolates, *Glutamicibacter sp.*, (66.7%), *M. caseolyticus*, and *E. durans* (55.6%, respectively) were the most susceptible indicator bacteria inhibited by of AP-isolates (Table 3).

However, considering each AP-isolate independently, the isolates of some species as *S. hominis, S. haemolyticus*, *S. pasteuri*, *E. faecalis, E. canis*, and *S. gallolyticus* were completely resistants to the antimicrobial activity of all the AP-isolates tested. Moreover, it is to note that some of those species (*E. faecalis*) were isolated in the same sample as the AP strains X3764 and X4000 (Table 3).

3.3. Characterization of the antimicrobial compound

It is of interest the detection of two AP-isolates (*S. hominis* X3764 and *S. sciuri* X4000) with the widest AA profile and

TABLE 3 Antimicrobial activity of the nine antimicrobial-producing (AP) isolates against a representative Gram-positive stork microbial community (30 isolates of 29 species used as indicator bacteria).

		Antimicrobial producing isolates									
Species of indicator bacteria	Isolate ID code	*S. hyicus* X3750	*S. sciuri* X3763	*S. hominis* X3764	*S. epidermidis* X3815	*S. sciuri* X4000	*S. aureus* X4036	*S. simulans* X4520	*S. chromogenes* X4620	*S. simulans* X4653	N° of AP isolates that inhibited the indicator bacteria (%)
S. sciuri	X4121	–	–	+	–	–	–	–	–	–	1 (11%)
S. aureus	X4013	+	–	+	+	–	–	–	–	–	3 (33%)
S. aureus	X4409	+	–		–	–	–	–	–	–	1 (11%)
S. chromogenes	X4697	–	–	+	–	–	–	–	–	–	1 (11%)
S. epidermidis	X4146	–	–	+	–	–	–	–	–	–	1 (11%)
S. xylosus	X4413	–	–	+	–	–	–	–	–	–	1 (11%)
S. lentus	X4149	–	–	+	–	–	–	–	+	–	2 (22%)
S. simulans	X4525	–	–		–	–	–	–	+	–	1 (11%)
S. hominis	X3726	–	–		–	–	–	–	–	–	0
S. saprophyticus	X4145	–	–	+	–	–	–	–	–	–	1 (11%)
S. hyicus	X3750	–	–	+	–	–	–	–	–	–	1 (11%)
S. haemolyticus	X3784	–	–		–	–	–	–	–	–	0
S. arlettae	X4721	–	–	+	–	–	–	–	–	–	1 (11%)
S. capitis	X3968	–	–	+	+	–	–	–	–	–	2 (22%)
S. pasteuri	X4093	–	–		–	–	–	–	–	–	0
E. faecalis	X4126	–	–		–	–	–	–	–	–	0
E. faecium	X4688	–	–	+	–	–	–	–	–	–	1 (11%)
E. gallinarum	X4634	–	–	+	–	–	–	–	–	–	1 (11%)
E. durans	X4532	+	–	+	–	+	–	+	–	+	5 (56%)
E. canis	X3928	–	–		–	–	–	–	–	–	0
E. hirae	X4037	+	–	+	–	–	–	–	–	–	2 (22%)
M. caseolyticus	X4488	+	–	+	–	–	–	+	+	+	5 (56%)
Lactococcus garvieae	X4417	–	–	+	–	–	–	–	–	–	1 (11%)
Streptococcus gallolyticus	X4698	–	–		–	–	–	–	–	–	0
M. luteus	X4481	+	–	+	–	–	–	–	+	–	3 (33%)
Vagococcus lutrae	X4122	–	–	+	–	–	–	–	–	–	1 (11%)
Glutamicibacter sp.	X4102	+		+		+		+	+	+	6 (67%)
C. falsenii	X4270	+	–	+	–	–	–	+	–	+	4 (44%)
Corynebacterium sp.	X4486	+	–	+	–	–	–	+	–	+	4 (44%)
C. aurimucosum	X4660	+	–	+	–	–	–	+	–	+	4 (44%)
n=30 (%)*		10 (33%)	0	22 (73%)	2 (7%)	2 (7%)	0	6 (20%)	5 (17%)	6 (20%)	–

*Number of indicator bacteria (%) inhibited by each antimicrobial-producing (AP) isolate. Color: in dark green are marked the AP isolate with the strongest inhibition capacity against the major number of indicators and the indicator isolate that was inhibited by a higher number of AP isolates. In soft gray is shaded all the positive interactions of the AP isolates against the indicators.

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TABLE 4 Characteristics of the nine antimicrobial producer isolates of the nasotracheal microbiota of storks.

Isolate	Stork sample ID code (origin)[a]	AMR phenotype[b]	Bacteriocin genes
S. aureus X4036	436 (T)	Susceptible	Lantibiotic-like
S. hyicus X3750	538 (T)	Susceptible	Lantibiotic-like
S. sciuri X3763	507 (T)	PEN	Lantibiotic-like
S. sciuri X4000	433 (T)	PEN, CIP	Lantibiotic-like
S. epidermidis X3815	506 (T)	ERY	Lantibiotic-like
S. chromogenes X4620	481 (N)	Susceptible	Lantibiotic-like
S. hominis X3764	507 (T)	PEN, FOX, ERY	Lantibiotic-like
S. simulans X4520	480 (N)	PEN	Lantibiotic-like
S. simulans X4653	481 (N)	Susceptible	Lantibiotic-like

[a]T, tracheal; N, nasal.
[b]Antibiotics tested were the following ones: PEN, penicillin; FOX, cefoxitin; ERY, erythromycin; CIP, ciprofloxacin; oxacillin, clindamycin, tetracycline, gentamicin, tobramycin, linezolid, chloramphenicol, trimethoprim-sulfamethoxazole, and mupirocin; AMR: antimicrobial resistance.

strong inhibitory capacity in their respective extracts obtained in all conditions tested: crude (boiled and filtered), concentrated CFS and butanol extraction. These isolates were selected, and the susceptibility of their extracts to the enzymatic activity of trypsin, α-chymotrypsin, proteinase-K, papain, and protease was tested to verify the peptidic nature of the antimicrobial substances of AP isolates. The absence of AA against MRSA and MRSP indicators in the CFS of AP isolates after enzymatic treatment allowed to confirm the peptide nature of the antimicrobial substance present on these isolates.

Moreover, the presence of 22 bacteriocin structural genes were analysed by PCR and sequencing in the nine AP isolates. Genes encoding lantibiotic-like antimicrobial peptides were detected in all the nine AP isolates (**Table 4**).

3.4. Antibiotic resistance profile of the AP isolates

Antibiotic susceptibility testing was performed with the collection of nine AP isolates. In this sense, 44.4% of the AP isolates showed susceptibility to the antimicrobial agents tested and the remaining isolates presented low resistance rates, highlighting the absence of MDR isolates. In this respect, 44.4% of the isolates showed resistance to penicillin, 22.2% to erythromycin and only 11.1% revealed resistance to cefoxitin or ciprofloxacin (**Table 4**).

4. Discussion

Antimicrobial resistance is a dynamic and multifaceted One-Health problem involving humans, animals, and the environment (Prestinaci et al., 2015). In this respect, the urgency to find new alternatives to antibiotics has induced the scientific community to focus on the antimicrobial substances produced by bacteria isolated from natural sources among which bacteriocins are of particular interest.

However, studies reporting the frequency of antimicrobial activity in bacteria of wildlife and livestock animals are limitated (Poeta et al., 2007; Almeida et al., 2011), and those focused on *Staphylococcus* are even scarcer (Fernández-Fernández et al., 2022), and storks have never been studied in this respect. The current work represents to the best of our knowledge, the largest study of antimicrobial activity, as well as the presence of bacteriocin structural genes in nasotracheal *Staphylococcus* isolates recovered from healthy wild storks.

Considering the inhibitory capacity of a collection of 259 *Staphylococcal* isolates recovered from nasotracheal samples of healthy storks in this study, nine of the isolates (3.5%) revealed antimicrobial activity against at least one of the 14 G^+ indicators tested including methicillin-resistant and susceptible *Staphylococcus, Enterococcus*, and *L. monocytogenes,* among others. The AP isolates did not show antimicrobial activity against Gram-negative bacteria, such as *E. coli* and *P. aeruginosa*. Recent studies have detected *Staphylococcus* with antimicrobial activity among staphylococci of wild mammals and birds (excluding storks) (Fernández-Fernández et al., 2022), human nares (Janek et al., 2016), and those recovered from food (Van der Veken et al., 2020).

Among the *Staphylococcus* genus, several bacteriocins have been isolated from commensal CoNS species. Many of them display inhibitory activity against *S. aureus*, a CoPS widely considered an important pathogen of both humans and animals and implicated in a wide range of infections (Laux et al., 2019; Newstead et al., 2020).

To better characterize the inhibitory effect of the identified AP isolates, the antimicrobial activity of CFS was studied. However, bacteriocin production is costly and it is often regulated depending on cell density and environmental factors (Heilbronner et al., 2021), so we decided to extract the antimicrobial compounds from the inner cell. Thus, three of the nine AP isolates showed inhibition capacity in all the extract conditions tested (CFS crude or concentrated as well as butanol extract). However, when concentrating the extracts, most of the AP-isolates revealed bioactivity mainly against *Enterococcus* indicator bacteria. Nevertheless, two AP isolates (detected by the *spot-on-lawn* method) were negative for all the extract conditions and against all the indicators tested.

Regarding genetics, the nine AP isolates detected were positive for a lantibiotic-like structural gene although we can not discard that other structural bacteriocin genes, not tested in this study or not already described, could be responsible for the antimicrobial activity. A high variety of lantibiotics have been reported on

Staphylococcus isolates such as epidermin, epilancins, and various nukacins, among others, showing antimicrobial activity exclusively against G^+ bacteria (Laux et al., 2019).

According to microbiota composition, each community reflects specific niche conditions, and associations among species and concrete locations have been described (Wei et al., 2022). On the other hand, it is widely accepted that antibacterial molecules that inhibit major microbial competitors have a particularly important role in shaping the microbiome (Krismer et al., 2017; Lewis and Pamer, 2017; García-Bayona and Comstock, 2018).

Based on bacteriocin effects on the microbial community, the present study demonstrates the strong inhibitory capacity of the recovered AP-isolates. Especially, *S. hominis* X3764 could act as a microbiota modulator due to its high inter-sample AA against 73% of the G^+ representative stork community and the intra-sample activity, inhibiting the *S. sciuri* isolate recovered from stork 507. In this respect, potent antimicrobial activity has been previously reported in nasal staphylococci of human origin (80%) against bacteria of the nasal ecosystem (Janek et al., 2016).

As for the nasal microbiome, *S. aureus* is also regarded as a human commensal that colonizes asymptomatically about 30% of human nares (Laux et al., 2019). However, nasal carriage of *S. aureus* predisposes to invasive infection (Zipperer et al., 2016). For example, it has been described that nasal *S. lugdunensis* can prevent *S. aureus* colonization by producing an unusual antimicrobial compound termed lugdunin (Zipperer et al., 2016). Moreover, *S. epidermidis* has been reported as an *S. aureus* inhibitor although there is no clear correlation between the absence of *S. aureus* with the presence of *S. epidermidis* (Bierbaum and Sahl, 2009; Iwase et al., 2010; Yan et al., 2013).

On the other hand, recent studies with antimicrobial-producing isolates indicate that a specific bacteriocin may not affect all microbiome members equally and may only affect those with closer physical proximity. Therefore, bacteriocin-producing isolates have an important role in niche competition and colonization conferring to the producer an ecological superiority against other microbiome constituents without natural bioactive compounds that allows maintaining of stable communities and can lead to the redistribution of microbiome members into sub-niches (Heilbronner et al., 2021).

Although migratory and resident wild birds are not implicated directly in the development of antimicrobial resistance, they are considered important reservoirs and vectors of zoonotic and antimicrobial-resistant bacteria (Gómez et al., 2016; Wang et al., 2017; Abdullahi et al., 2021). In combating the global antimicrobial resistance problem, they could be be considered due to their potential ability to carry *Staphylococcus* spp. which produce effective antimicrobial compounds of relevance in biomedical and food production sciences. Fortunately, half of the AP isolates were susceptible to all antibiotics tested and none of them was MDR.

5. Conclusion

Exploring the mechanisms of how bacteriocins affect microbiota dynamics requires an improved understanding of the functions of these diverse molecules. Many studies have been carried out to elucidate the exclusion mechanisms of *S. aureus* from the human nose. However, more studies should be undertaken to clarify which antimicrobial substances are produced by nasal commensals among other strategies used in their competition with nasal microbiota for nutrients and adhesion sites.

In this respect, the two highly AP isolates identified in this study (*S. hominis* X3764 and *S. sciuri* X4000) could be excellent candidates for further studies as an alternative to the alarming situation of antibiotic resistance. This highlights the relevant role of the nasotracheal microbiota of storks as a model for the control of bacterial communities by bacteriocin-producing isolates and their transmission to humans, other animals, and the environment.

Moreover, the evaluation of bacteriocins production by staphylococci from wild animal can contribute to their potential application on other hosts and ecosystems.

Data availability statement

The original data presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

The sampling procedures performed in the previous study in which the staphylococci isolates were obtained (Abdullahi et al., 2023, in press), and were approved by the ethical committee for animal experimentation of the University of Castilla-La Mancha and authorized by the regional government of Castilla-La Mancha (permit no. VS/MLCE/avp_21_198); moreover, all applicable international, national, and/or institutional guidelines for the care and ethical use of animals, specifically directive 2010/63/EU and Spanish laws 9/2003 and 32/2007, and RD 178/2004 and RD 1201/2005 were followed.

Author contributions

CT, CL, and RF-F designed the study, made the first analysis of the data, and prepared the draft of the manuscript. RF-F, UH, INA, AU, and CG-A performed sampling, recovery of isolates, and experimental work. RF-F, SG-V, and AM participated in the graphical design of data and some methodological issues. CT and CL supervised the study. All authors read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2023.1144975/full#supplementary-material

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Supplementary material

Table S1. Characteristics of the 14 Gram-positive (G^+) indicator bacteria used in this study for the screening of antimicrobial activity production in the 259 staphylococci of storks and the two Gram-negative indicator bacteria tested for the 9 antimicrobial-producing (AP) isolates.

Specie (nº of isolates)	**Isolate ID code**	**Origin**	**Relevant Antimicrobial Resistance Phenotype/Genotype**	**Reference**
Staphylococcus aureus (2)	C1570	Human-skin	Methicillin/*mec*A (MDR)	UR collection
	ATCC29213	Reference strain		ATCC collection
Staphylococcus pseudintermedius (2)	C2381	Pet-nasal	Methicillin/*mec*A	UR collection
	C3468	Pet-nasal		UR collection
Staphylococcus lugdunensis (1)	C10107	Human-blood		UR collection
Staphylococcus epidermidis (1)	C2663	Human-blood	Methicillin/*mec*A and Linezolid	UR collection
Staphylococcus sciuri (1)	C9780	Wild boar-nasal		UR collection
Staphylococcus delphini (1)	C9459	Vulture-nasal		UR collection
Enterococcus faecalis (1)	ATCC29212	Reference strain		ATCC collection
Enterococcus faecium (1)	C2321	Human-wound	Vancomycin/*van*A	UR collection
Enterococcus cecorum (1)	X3809	Storks-nasal		UR collection
Listeria monocytogenes (1)	CECT4032	Reference strain		CECT collection
Micrococcus luteus (1)	CECT241	Reference strain		CECT collection
Streptococcus suis (1)	X2060	Pig		UR collection
Escherichia coli (1)	ATCC25922	Reference strain		ATCC Collection
Pseudomonas aeruginosa (1)	PAO1	Reference strain		

Table S2. Gram-positive bacteria (30 isolates of 29 different species and 9 genera) selected as representative of the nasotracheal diversity of storks used as indicator bacteria in inter-sample antimicrobial production assays (strains selected of Abdullahi et al., 2023, in press)

Species of indicator bacteria	Isolate ID code
Staphylococcus sciuri	X4121
S. aureus	X4013
S. aureus	X4409
S. chromogenes	X4697
S. epidermidis	X4146
S. xylosus	X4413
S. lentus	X4149
S. simulans	X4525
S. hominis	X3726
S. saprophyticus	X4145
S. hyicus	X3750
S. haemolyticus	X3784
S. arlettae	X4721
S. capitis	X3968
S. pasteuri	X4093
Enterococcus faecalis	X4126
E. faecium	X4688
E. gallinarum	X4634
E. durans	X4532
E. canis	X3928
E. hirae	X4037
Macrococcus caseolyticus	X4488
Lactococcus garvieae	X4417
Streptococcus gallolyticus	X4698
Micrococcus luteus	X4481
Vagococcus lutrae	X4122
Glutamicibacter sp.*	X4102
Corynebacterium sp.	X4486
C. falsenii	X4270
C. aurimucosum	X4660

**Glutamicibacter* has been recently recognised into a new genus and it was considered previously as *Arthrobacter*.

Bacteriocin-producing staphylococci for agro-food applications: relevance of micrococcin P1

Bacteriocin-producing staphylococci for agro-food applications: relevance of micrococcin P1

Fernández-Fernández Rosa[1], Lozano Carmen[1], Campaña Allelen[1], Fernández-Pérez Rocío[1], Peña Raquel[2], Zarazaga Myriam[1], Carrasco Jaime[2], Torres Carmen[1*]

Abstract

Antimicrobial producing isolates and their bacteriocins hold a great promise for the treatment and prevention of microbial diseases being an attractive alternative to antibiotics. Here, it was evaluated the inhibitory activity of 15 bacteriocin-producing (BP) staphylococci and their prepurified extracts (butanol, BT) against a collection of 27 pathogenic or zoonotic microorganisms (including Gram-positive and -negative bacteria and molds) of relevance in public health and agro-food fields. The indicators were grouped into seven categories based on their potential area of application: a) mastitis and dairy industry; b) poultry pathogens and zoonotic agents; c) swine zoonoses; d) food safety; e) aquaculture; f) wine industry; and g) mushroom cultivation. Moreover, cross-immunity assays between BP staphylococci were carried out to identify potential bacteria combinations to enhance their activity against pathogens. Finally, the hemolytic and gelatinase activities were tested in BP staphylococci. A strong inhibitory capacity of BP staphylococci was verified against relevant Gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus, Listeria monocytogenes* or *Clostridium perfringens* among others, while no activity was detected against Gram-negative bacteria. Interestingly, several BT extracts inhibited the two mold indicators included in this study as representants of mushroom pathogens. All these assays added to the antibiotic resistance phenotype and virulence characteristics previously described in this BP staphylococci allowed us to propose the micrococcin P1 producer *S. hominis* strain C5835 (>60% of indicators inhibited with intense halos by all methods) as potential candidate for future applications in the afore mentioned categories alone or in combination with other BP isolates (*S. warneri* X2969).

Keywords: Bacteriocin, bacteriocin-producing staphylococci, bacterial consortia, agro-food, public health.

Article recently submitted

*Corresponding author:
Carmen Torres: carmen.torres@unirioja.es
Author information:
[1]University of La Rioja, Area of Biochemistry and Molecular Biology, OneHealth-UR Research Group, 26006 Logroño, Spain
[2] Regional institute for agri-food and forest research and development (IRIAF), Department ecology of cultivated mushrooms, Castilla La Mancha, Spain
The authors declare no conflict of interest

Introduction

The emergence of antibiotic-resistant pathogenic microorganisms resulting of the long-term overuse of antibiotics in humans and animals has become an unresolved old challenge to public health worldwide that need to be addressed. In this respect, natural antimicrobial agents, including bacteriocins, have attracted extensive attention as a new microbial barrier in both the food and veterinary sectors **(Soltani et al. 2022).** Moreover, under the One-Health perspective it is important to deal bacterial pathogens that cause severe diseases in animals and that affects the production of livestock industries (bovine, avian and swine) with important economic activities worldwide **(Fairbrother et al., 2005; Gottschalk, 2012)**.

Among the most alarming human pathogens are *Escherichia coli* and *Staphylococcus aureus* **(Lagha et al., 2017),** especially methicillin-resistant isolates (MRSA) that has been reported as an important pathogen in both community and hospital settings, causing skin, wound, bloodstream, and other types of infection **(WHO, 2014; ECDC, 2009; ECDC, 2015).** Moreover, these bacteria and other relevant pathogens are also involved in livestock production highlighting *Streptococcus suis* in swine, *C. perfringens* in poultry and other gram-positive/negative pathogens engaged in bovine infections such as *S. aureus, Mycoplasma spp., S. uberis, S. dysgalactiae, S. agalactiae, E. coli* and *Klebsiella pneumoniae*. Concretely, *S. aureus* is responsible for about 50% of the mastitis cases, and only 10–30% of the cases are curable upon using antibiotics **(Gomes and Henriques 2016, Mella et al., 2017).**

Potential alternatives to antibiotics, including antimicrobial peptides, bacteriophages, nanomedicines, probiotics, phytochemicals, or photodynamic light therapy among others have been proposed **(Wang et al., 2020; Anyaegbunam et al., 2022; Mba et al., 2022a,b)**. Bacteriocins are ribosomal synthesized antimicrobial peptides produced by bacteria that hold great promise (both purified or partially purified) as pharmabiotics in prophylaxis and treatment of target pathogenic bacteria **(Darbandi et al., 2022; Huang et al., 2021; Telhig et al., 2022; Gillor, 2007).** Besides, such peptide toxins are a viable strategy to replace conventional antibiotics or to potentiate their effects against pathogens **(Drider et al., 2016).**

Some *Staphylococcus* and especially coagulase-negative *Staphylococcus* (CoNS) species are frequently found as commensals of humans and animals being infrequently associated with infections, showing less pathogenicity that coagulase-positive isolates. Moreover, staphylococci with antimicrobial activity have been recently reported highlighting the possible interest of these *Staphylococcus* producing isolates or their antimicrobial compounds as antimicrobial, antitubercular, antifungal,

antiviral and anticancer strategies **(Jo et al., 2022; Khusro et al., 2018; Khusro et al., 2020; Cebrián et al., 2020; Mangrolia and Osborne, 2020)**.

A collection of fifteen bacteriocin producer (BP) staphylococci with activity against Gram-positive bacteria (including different species of *Enterococcus*, *Staphylococcus* and *Listeria*) were previously characterized by our group at genetic and protein level **(Fernández-Fernández et al., 2022, and 2023a,b)**. In order to maximise the potential applications of these isolates, the present study aims to evaluate the antimicrobial activity of these BP staphylococci and their pre-purified bacteriocins against a wide collection of microorganisms of interest as indicators for agro-food and public health applications.

Material and methods

Isolates and growth conditions

Fifteen BP *Staphylococcus* isolates were selected to evaluate their inhibitory capacity against 27 pathogens used as indicator microorganisms, including multidrug-resistant (MDR) and zoonotic isolates of relevance. The BP staphylococci included in this work were obtained and characterized in previous studies **(Fernández-Fernández et al., 2022 and 2023a,b)** and their main characteristics are shown in **Supplementary Table S1.** These BP staphylococci contained the bacteriocin gene clusters encoding: thiopeptide micrococcin P1 (MP1, *S. aureus* C5802, *S. hominis* C5835, *S. sciuri* X3041 and X3011); lanthipeptides (*S. aureus* C8609, *S. aureus* X3410, *S. aureus* C6770*, S. epidermidis* X3009*, S. warneri* X2969*, S. simulans* C9832 and *S. hyicus* C9585); circular bacteriocins (*S. chromogenes* C9838) and bacSp222 (*S. pseudintermedius* C8189, C8478 and C8479). The microorganisms were selected as indicators due to their species and/or their antimicrobial resistance phenotypes and were classified in seven categories based on their main field of potential applications **(Table 1)**: dairy livestock mastitis, avian pathogen zoonoses, swine zoonoses, food safety, aquaculture, wine industry and mushroom cultivation. Although these indicator isolates were categorized in only one potential application field, it is important to remark that they could be included for the study of several ones, but for simplicity they were only included in the main field of potential application. Despite most of the indicator microorganisms were Gram-positive bacteria, three Gram-negative bacteria were also included as indicators (*Escherichia coli, Salmonella* and *Pseudomonas aeruginosa*) to test the activity of potential staphylococcins in this group of organisms. These indicators were considered in a separate group. The antimicrobial activity of these BP staphylococci against six indicator bacteria of the genera *Staphylococcus*, *Enterococcus* and *Listeria* was tested in

previous studies. **(Fernández-Fernández et al. 2023b)**

Brain Heart Infusion (BHI, Condalab Spain) was used as the standard growth medium both for liquid and solid cultures (1.5% of agar). Antimicrobial Activity (AA) determinations were performed in Triptic Soy Agar supplemented with 0.3% of yeast extract (TSA, Condalab Spain). Anaerobic conditions were used for *Clostridium perfringens, Pediococcus spp, Lactobacillus plantarum* and *Leuconostoc mesenteroides* indicators. Columbia agar with 5% sheep blood (bioMérieux, France) was used when the indicator isolate was *Streptococcus suis* and potato dextrose agar media (PDA) was used in case of molds. Microorganism indicators were incubated overnight at 37ºC during 24h except those selected as representants for agriculture (molds and lactic acid bacteria) and aquaculture applications that were grown at 25ºC during 24h to 96h.

Detection of antimicrobial activity (AA)

The AA of the BP staphylococci was evaluated by the s*pot-on-lawn* method, and the inhibitory capacity of two different bacterial extracts [cell-free-supernatants (CFS) and butanol extracts (BT)] was tested by agar diffusion assays. Extracts were obtained as indicated elsewhere **(Fernández-Fernández et al., 2023b)** with slight modifications: the CFS was sterilized by boiling (100ºC, 5 min); and the BT extracts were resuspended in the minimum needed volume of dimethyl sulfoxide (DMSO, 0.7-0.9 mL). Indicator isolates were resuspended in 0.9% sterile saline solution at a bacteria concentration equivalent to 0.5 MacFarland turbidity and were seeded on TSA plates. Wells of 6 mm of diameter were punched in the inoculated agar plates and filled with 50 µL of the CFS staphylococcal samples whereas 10 µL of the BT extract was added to a blank disc. Plates were incubated overnight at the corresponding temperatures, and diameters of inhibition zones were measured (mm) and recorded.

Detection of antifungal activity (AF)

The inhibitory capacity of the BT extracts was tested by disc diffusion assay against two mold mycopathogens responsible of cobweb and green mould in mushroom farms (*Cladobotryum mycophilum* 13900 and *Trichoderma atroviride* TAV1 isolated from commercial farms in Spain). The spores were collected in sterile water and rubbing onto the PDA surface with a glass rod (100 µl per plate). The spore suspensions were quantified by using a counting chamber, adjusted to 10^4 spores/mL and used as inoculum.

Intensity of the inhibitory capacity

The inhibition intensity of the 15 BP isolates against the 27 indicator stains was determined considering the inhibition diameter measuring the halloes in millimeters (mm) after s*pot-on-lawn* and

BT assays as follows: area of zone πr^2−area of well πr^2 **(O'Sullivan et al., 2019)**.

Cross-immunity among the BP staphylococci

The cross-immunity to the bacteriocins produced by the 15 BP isolates included in this study was evaluated. For that, all BP isolates were tested against each other by the s*pot-on-lawn* method described above using each BP stahylococci both as a producer and as a target indicator isolate **(O'Sullivan et al., 2019).**

Hemolysis and gelatinase activity

Overnight cultures of BP *Staphylococcus* isolates in BHI broth were spotted onto Columbia agar with 5% sheep blood (bioMérieux, France) and then incubated at 37°C overnight. Hemolytic activities of the isolates were recorded by the presence of Beta (β) hemolysis when detecting a clear and complete hemolysis of blood cells around the BP inoculated isolate.

Moreover, gelatinase production was evaluated in BP staphylococci. Isolates were spotted onto TSA plates containing 1.5% of skim milk and incubated overnight at 37°C. Then, plates were cooled to ambient temperature for 2h and evaluated for the appearance of a transparent halo around the colonies (positive for gelatinase test) **(Reuben et al., 2019)**.

Results

Inhibitory capacity of bacteriocin-producing (BP) staphylococci

The antimicrobial and antifungal activities of the 15 BP *Staphylococcus* isolates were analysed by the *spot-on-lawn* and agar diffusion assays against 27 microorganisms selected as indicators of agronomic and public health interest distributed in the following categories: dairy livestock mastitis, avian pathogen zoonoses, swine zoonoses, food safety, aquaculture, wine industry and mushroom cultivation **(Table 1)**. Three Gram-negative (G^-) bacteria were also included as indicators in a separate group. All the 22 Gram-positive (G^+) bacteria and the two molds were inhibited by at least one of the BP staphylococci in one of the three conditions evaluated. Nevertheless, none of the BP isolates showed AA against the three G- bacteria used as indicators in any of the assays evaluated.

The percentage of inhibition of the BP staphylococci against each indicator microorganism included in this study is presented in **Table 1,** differentiating by the three different methods. It is shown in dark colour those indicators that were inhibited by ≥60% of BP staphylococci. According to the *spot-on-lawn* method, 9 indicator isolates were the most inhibited: *S. aureus* X3548, *E. faecium* C2321, *S. aureus* C5313*, S. aureus* C1570, *S. aureus* C1532*, L. monocytogenes* CECT4032*, Lactococcus garviae, Pediococcus acidilactici* and *Leuconostoc mesenteroides*. In the case of CFS method, inhibition rates of each indicator, when

positive, oscillate between 27% and 40% (this last, in the case of *S. agalactie*). In this respect, none of CFS of the 15 BP isolates were active against *C. perfringens* X9740 or *S. aureus* C5313 and C1532 isolates. Finally, the inhibitory capacity of BT extracts against the G^+ and mold indicators included in this study was notably increased and it is to note the high percentages of inhibition (up to 87%) detected in the BT extracts against some indicator bacteria classified into dairy livestock mastitis, swine zoonoses and food safety field of applications. Nevertheless, both CFS and BT extracts revealed limited inhibitory capacity (percentages from 13% to 33%) against acid lactic bacteria included in aquaculture and wine industry fields. Finally, it highlights the antifungal activities of BT extracts against pathogens involved in mushroom agriculture *Trichoderma atroviride* TAV1 (n=4, 27%) and *Cladobotryum mycophilum* 13900 (n=6, 40%) **(Table 1)**. Interestingly, the BT extracts of 6 BP isolates (MP1-producers: X3011, X3041, C5802, C5835; lanthipeptide-like: C8609, C6770) inhibited the growth in the cohabitant-symbiotic microbiota of the *Cladobotryum mycophilum* 13900 mycelium.

Figure 1 shows the percentage of the indicator microorganisms inhibited by each of the 15 BP staphylococci (grouped by the type of bacteriocin produced), according to the three methods used. High percentages of inhibition were detected by the s*pot-on-lawn* method (8%-88%), CFS (4%-72% only showed in 7 BP isolates) or BT extraction (up to 89%). Moreover, the **Figure 2** represents the percentages of indicator isolates inhibited by each BP staphylococci (by the three methods), clustering the indicators by the field of potential application. Thus, MP1 staphylococcal producers were active against almost all indicator pathogens and what is more interesting in all the methods analysed **(Figures 1 and 2).** In relation to bacSp222 producers, two profiles of antimicrobial activity were observed (**Figures 1 and 2**): a) *S. pseudintermedius* C8478 and C8479 isolates revealed the same antimicrobial activity by the three methods, highlighting the inhibitory capacity of their CFS against dairy livestock mastitis pathogens (**Figure 2.B**); b) *S. pseudintermedius* C8189 showed antimicrobial activity by *spot-on-lawn* and after BT extraction (lower percentages of inhibition than C8478 and C8479 isolates), although no inhibitory activity was detected in the CFS of *S. pseudintermedius* C8189. Finally, the BT extract of the BP isolate C8189 showed activity against pathogens involved in food safety and agriculture while the *S. pseudintermedius* C8478 and C8479 inhibited indicator microorganisms enclosed on 4 or 5 fields of interest, respectively **(Figure 2.C).**

In relation to the inhibitory activity spectrum of *S. chromogenes* C9838 isolate, **Figure 1** reveals a raise in the percentage

of inhibition of BT extract in comparison with *spot-on-lawn* assay or CFS (no activity); the BT extract was active against pathogens of 3 categories **(Figure 2.C)**. Finally, the lanthipeptide producing isolates revealed different patterns of activities in all the conditions evaluated **(Figure 1)**. The three *S. aureus* isolates (C8609, C6770 and X3410) showed similar activities by s*pot-on-lawn* and BT extracts but only the CFS of C8609 isolate revealed antimicrobial activity. Interestingly, *S. warneri* X2969 and *S. epidermidis* X3009 revealed specific inhibitory capacity by *spot-on-lawn* against indicator microorganisms included in wine industry category and by BT extraction against swine zoonoses **(Figure 2.A and 1.C, respectively).**

Potential applications for bacteriocin or BP staphylococci

The intensity of inhibition of the 15 BP staphylococci or their pre-purified extracts was evaluated against seven categories of indicator microorganisms (related to the main field of interest for potential application) and it has been represented in **Figure 3** (measure of their inhibition halloes in mm^2). Most of BP staphylococci were active either by the *spot-on-lawn* or by BT extraction against all the indicator categories. In general, BP isolates presented a wide profile of action showing inhibitory activity against microorganisms of all the seven categories analyzed. Concretely, BT extracts showed special activity against microorganisms of interest on dairy livestock mastitis, avian pathogen zoonosis, aquaculture and wine industry (**Figure 3.B**). Interestingly, the BT extracts of 8 BP isolates were active against two major mushroom pathogens**.** On the other hand, it should be highlighted the effect of the MP1 producers which inhibits at least one indicator of each of categories evaluated and revealed the strongest antimicrobial activity both by *spot-on-lawn* and BT extracts **(Figure 3.A and B),** especially the CoNS *S. hominis* C5835 and the CoPS *S. aureus* C5802. Finally, *S. aureus* X3410, *S. epidermidis* X3009 and *S. pseudintermedius* C8189 showed lower inhibitory capacity acting only against 1 to 3 indicator categories with reduced halloes.

Going deeper on the characterization of the 15 BP staphylococci, a cross-immunity assay was performed using all these isolates both as bacteriocin producers and also as indicator bacteria (**Table 2**); a white box indicates absence of antimicrobial activity in the pair producer-indicator bacteria, a grey box represents presence of antimicrobial activity and a green box indicates absence of antimicrobial activity between two BP staphylococci used both of them as producers and indicator bacteria. The four MP1-producing isolates inhibited almost all BP staphylococci but did not reveal AA against themselves, as expected. Interestingly, all MP1-producing bacteria were immune to the inhibitory activity of C8609, C6770 and X2969 as BP isolates

(carriers of genes encoding lanthipeptides) **(Table 2)**. Interestingly, the MP1-producing isolate *S. hominis* C5835 was resistant to the inhibitory activity of 12 of the BP isolates (excepting for X3009 and C9832 isolates, carriers of genes of lanthipeptides) and what is more, it did not show AA when testing against *S. warneri* X2969. Moreover, the three *S. pseudintermedius* isolates producing bacSp222 bacteriocin, were resistant to the inhibitory activity of *S. aureus* C8609 isolate (lanthipeptide). In this respect, considering the mutual interactions among the BP staphylococci, it can be proposed the following BP staphylococci potential combinations: lanthipeptide producing bacteria i) C6770+C8609, ii) C6770+C9585, iii) C8609+C9585, iv) X3410+X2969; v) The lanthipeptide producing isolate X2969 with the MP1 producing isolate C5835. In all these combinations the two BP staphylococci did not show antimicrobial activity against the partner (marked in green box in **Table 2).**

Preliminar characterization of pathogenesis

Six out of the 15 BP isolates were β-hemolytic, and all were CoPS (3 *S. aureus* and 3 *S. pseudintermedius*). Moreover, five isolates revealed gelatinase activity including both CoPS (3 *S. pseudintermedius*) and CoNS isolates (one *S. hyicus* and one *S. chromogenes*). Therefore, subsequent screening for the possible uses as probiotic of these isolates should be performed in the future.

Discussion

Currently, the use of healthy products with an environmentally respectful background is demanded. Bacteriocins have enormous research and commercialization potential and hold great promise for the prevention and/or treatment of bacterial diseases being an attractive alternative to antibiotics or chemical preservatives that can be used also as semi-purified compounds or bioactive powders **(Soltani et al., 2021).** Several studies have indicated the economical and health benefits of livestock production of bacteriocins or bacteriocin-producing isolates as probiotics, as preventive treatments against diseases or in combination with antibiotics for livestock production **(Soltani et al. 2022; Al Atya et al., 2016; LeBel et al., 2013; Diez-Gonzalez, 2007; Joerger, 2013; Vandeplas et al., 2010; Malinowski et al. 2019; Fernández et al., 2008; Godoy-Santos et al. 2019; Pieterse and Todorov 2010).** However, its application is still limited at least with concerns to *Staphylococcus*. The study of the pathogenic characteristics of both bacteriocins and bacteriocin-producing isolates is required in order to verify the lack of toxicity for the host as well as confirm their effect and sustainability as potential probiotics.

Therefore, the discovery and characterization of new alternative antimicrobials such as bacteriocins will likely become crucial to combat antimicrobial resistance. In this respect, the present study aims to contribute to the knowledge of potential applications of staphylococcins and also the BP isolates. This study presents interesting antimicrobial activities verified by several methods against pathogens, including MDR, involved in relevant infections and important problems of livestock industry such as dairy livestock mastitis, poultry pathogen zoonoses, swine zoonoses, food safety, aquaculture, mushroom cultivation, and wine production. According to the pathogens considered in this study for the livestock industry, all G^+ bacteria were inhibited by at least 4 BP staphylococci, whereas none of the producers revealed antimicrobial activity against G^- indicators. As expected, higher inhibitory activities were detected when BT extracts were used. It is to note 8 BP staphylococci with antimicrobial activity against more than the 30% of the indicators (*S. aureus* C5802, *S. hominis* C5835, *S. sciuri* X3041 and X3011, *S. pseudintermedius* C8478, C8479 and C818, *S. epidermidis* X3009). These BP isolates produced micrococcin P1, bacSp222 and lanthipeptide-like bacteriocins, previously reported to inhibit critical G^+ pathogenic bacteria such as *Mycobacterium tuberculosis*, MRSA, *Listeria monocytogenes*, vancomycin-resistant enterococci, or *Clostridium difficile* among others **(Huang et al., 2021; Wladyka et al., 2015; Ovchinnikov et al., 2021; Degiacomi et al., 2016).**

Considering the veterinary field, we highlight that all the BP staphylococci evaluated in this study inhibited at least one indicator microorganism included in bovine mastitis and swine zoonoses categories, being these among the main sources of livestock production. The use of bacteriocins as natural antimicrobial substances for mastitis control and prevention has been considered **(Pieterse and Todorov, 2010; Bennett et al., 2021 and 2022).** A recent *ex vivo* study proposes the use of staphylococcins against pathogens isolated from bovine mastitis evidencing that, after 24 h of incubation, aureocin A53 caused a strong reduction in staphylococcal or streptococcal populations, respectively, eliminating all detectable viable cells **(Marques-Bastos et al. 2022).** In addition, it is of great interest to solve the problem of nosocomial transmission of *S. aureus* and especially MRSA-CC398 between pigs and humans, a new livestock-associated lineage, considered an emerging public health problem. On this basis, a recent *ex vivo* and *in vivo* murine model study has confirmed the protective role of the porcine skin microbiota, including bacteria producing antimicrobial substances, against MRSA colonization model (**Wei et al., 2023)**. Here, we detected five bacterial combinations of lanthipeptide producing

isolates and the MP1 producer *S. hominis* C5835 isolate of interest in future studies by enhancing their antimicrobial activity against MRSA, among other pathogens. Moreover, in a previous study the producing isolate C5835 and its pre-purified extract revealed intense inhibitory capacity against an MRSA isolate **(Fernández-Fernández et al., 2023b)** which allow us to consider this producing isolate as good candidate for potential applications. Similar results were presented by Eveno and colleagues in 2021, suggesting the use of BP isolates with synergistic interactions *in vivo* as consortium probiotic supplement to promote the health of piglets **(Eveno et al. 2021).**

In addition to bovine and swine livestock, the poultry industry has progress through improvements in genetic makeup, proper management, and advancements in nutritional science **(Gado et al., 2019).** Among these measures, it is to highlight the potential use of bacteriocin producing isolates as suitable alternative to antibiotics to combat or prevent infections of relevant pathogens in poultry farming such as MRSA and *C. perfringens*, as well as for improving the performance and productivity of birds **(García-Vela et al., 2023; Reuben et al., 2019)**. In this respect, the results presented in this study revealed a potent inhibitory capacity against avian pathogen zoonoses including *C. perfringens*, MRSA and *E. cecorum* indicators by different methods. Concretely, it is to note the four MP1 producing staphylococci and the lanthipeptide-like producing isolates C8609, C6770 and X3410 which inhibited 100% of avian pathogens (n=3) by *spot-on-lawn*.

In respect of fish pathogens, *Lactococcus garviae* is a well-known bacterium considered as an emerging zoonotic pathogen **(Meyburgh et al., 2017).** Focusing on our results, *Lactococcus garviae* was one of the most susceptible indicators and it was inhibited by the *spot-on-lawn* assay, by CFS and by semi-purified compounds (BT). On the other hand, *Carnobacterium maltaromaticum* can also act as a pathogen although representants of the genus *Carnobacteria* have been suggested as probiotic cultures for aquaculture **(Leisner et al., 2007).**

Lactic acid bacteria (LAB) have been widely used in the food industry and concretely represents a key factor for wine production **(Petruzzi et al., 2017).** In this respect, synergism between commercialised bacteriocins and conservants such as sulfur dioxide have been reported controlling the growth of spoilage lactic acid bacteria involved in wine production: *Oenococcus, Lactobacillus, Leuconostoc* and *Pediococcus* **(Rojo-Bezares et al., 2017).** In this study, it has been detected non-purified extracts (BT) with activity against *Pediococcus pentosaceus, Pediococcus*

acidilactici, Lactobacillus plantarum and *Leuconostoc mesenteroides* revealing their potential uses in the wine-industry.

In respect to mushroom cultivation, fungal diseases are among the most serious disorders damaging and affecting the quality of the yield of relevant crops such as mushroom **(Fletcher and Gaze, 2008; Largeteau et al., 2010)**. However, the scarce range of available products added to the occurrence resistance derived to the continuous exposure to the same active substances evidence the need of alternative for effective products to mitigate resistance occurrence **(Carrasco et al., 2017).** Concretely, we focus on two of the most harmful fungal diseases in cultivated mushrooms, the causatives agent of cobweb disease (*Cladobotryum* spp.) and green mould disease (*Thrichoderma* spp.). Interestingly, antifungal activity was observed in 6 out the 15 BP isolates tested in this study towards these mycoparasites inhibiting also the mycelial growth of *Cladobotryum mycophilum* by the antagonism towards its respective cohabitant-symbiotic microbiota, suggesting that the growth of this pathogenic fungi could be repressed by the noted detrimental effect on its mutualistic microbiota. Thus, bacteriocins could be of interest as a biological control agent of some mycoparasites by its detrimental action on essential bacteria of the pathogen microbiome. Nevertheless, more research should be performed in this field to deepen in these activities and the potential mechanisms of action. This is a singular observation in our approach since commonly mycoparasites are commercially treated by chemical fungicides but the barely unknown fungal-bacterial interactions could play a role to facilitate pathogen control through active bacteriocin producers. Its configuration may have a direct impact on yield performance and disease occurrence **(Carrasco et al., 2019; Vieira et al., 2019; Carrasco et al., 2020),** contributing to enhance the production of protein alternative to meat-based western diets.

Conclusion

Focusing on the present study, it has been shown the inhibitory capacity of the 15 BP staphylococci or their pre-purified BT extracts against pathogens for all the categories, especially after extraction. In addition, cross-immunity assay helped us to propose five potential combinations of BP isolates to enhance their activity against a specific pathogen. Finally, the CoNS lack of hemolytic and gelatinase activity so further studies should be performed to verify the potential use of the isolate or their bacteriocin as probiotic or antimicrobial agent, respectively. Besides all the above, the three CoNS producers of MP1 bacteriocin and especially the *S. hominis* C5835 isolate or their bacteriocin declare themselves as good candidates for agro-food applications, especially dairy livestock mastitis, avian pathogen

zoonosis, aquaculture and wine industry, alone or in combination with other BP staphylococci (*S. warneri* X2969).

Author contributions

R. F.-F., and R.P. performed most of the experiments and R.F.-P. and A.C. contributed to some experimental laboratory work related to butanol extracts and the growth indicator microorganism. and made the first version of the manuscript and R.F.-F., C.L., J.C., M.Z., C.T. analyse the data and R.F.-F., made the first revision of the manuscript. C.T., and M.Z. contributed to project funding. All authors reviewed the results and approved the final version of the manuscript.

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Data Availability

All data generated and reported during this study are included or indicated in the published article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors agreed for publication.

Conflict of interest

The authors declare no competing interests.

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Tables and Figures

Table 1. Percentage of inhibition exhibited by the 15 bacteriocin-producing (BP) bacteria against the 27 indicator bacteria classified in seven categories and Gram-negative isolates, using three methods (*Spot-on-lawn*; cell-free-supernatant, CFS; butanol-extraction, BT).

[a]It has been coloured in dark blue the indicator bacteria inhibited by ≥60% of BP staphylococci, in slight blue those inhibited by 20% to 60% of BP bacteria and in white those inhibited by less of 20% of BP staphylococci.
[b]Gram-negative bacteria were considered separately due to the fact they were not inhibited by any of the BP staphylococci included in this study or their bacteriocin extracts.
Abbreviations: UR, University of La Rioja; CECT, Spanish Type Culture Collection; ATCC, American Type Culture Collection; MRSA, methicillin resistant *Staphylococcus aureus;* VanR, vancomycin resistant; LinR, linezolid resistant; CC, clonal complex; NT, not tested.

Table 2. Cross immunity assay between the 15 BP staphylococci classified according to the bacteriocins produced.
*All BP staphylococci isolates were used as producer and as indicator bacteria in this experiment.

Figure 1. Percentage of indicators inhibited by each of the 15 bacteriocin producing (BP) staphylococci isolates (number of isolates tested) considering three methods: *spot-on-lawn* (n=25), cell-free-supernatant (CFS, n=25) and butanol extraction (BT, n=27) according to the type of bacteriocin produced.

*The antifungal activity of the 15 BP staphylococci against the two moulds included in this study was only evaluated with the BT extracts by disc diffusion assay.

Figure 2. Percentage of indicators inhibited by each of the 15 bacteriocin producing (BP) staphylococci isolates considering categories of application. It was used three methods (number of isolates tested): Figure 2.A) spot-on-lawn (n=25); Figure 2. B) cell-free-supernatant (CFS, n=25); Figure 2.C) butanol extraction (BT, n=27). Moreover, BP isolates are organised according to the type of bacteriocin produced.

Figure 3. Intensity of inhibition detected in the 15 BP staphylococci against 22 or 24 indicator microorganisms (excluding Gram-negative) classified in categories based on their potential applications. Two methods were used (number of indicators): A) *spot-on-lawn* (n=22), B) BT extracts (n=24).

*The intensity of the inhibitory activity was calculated by the inhibition zones as indicated in material and methods.

Supplementary material

Table S1. Characteristics of the 15 bacteriocin-producer (BP) staphylococci isolates included in this study[a].

[a]Data of the isolates were obtained in previous studies (Fernández-Fernández et al., 2023a,b)
[b]Abbreviations: ND, not detected; WGS, whole genome sequencing; NE, not studied.
[c]Antibiotics tested were the following ones: PEN, penicillin; cefoxitin; oxacillin; ERY, erythromycin; CLI, clindamycin; CIP, ciprofloxacin; TET, tetracycline; gentamicin, tobramycin; linezolid; chloramphenicol; SXT, trimethoprim-sulfamethoxazole; FA, fusidic acid and mupirocin.
[d]*sal*(A): intrinsic resistance gene in *S. sciuri.*

Figure S1. Antimicrobial activity of five bacteriocin staphylococci against different indicator bacteria represented from left to right 1-3: 1.-Butanol extracts, 2.-Cell-free-supernatant (diffusion assay) and 3.-Bacteria (*spot-on-lawn*). A) *S. aureus* C5802 against MRSA X3963; B) *S. hominis* C5835 against *E. faecalis* C9951; C) *S. sciuri* X3011 against *L. monocytogenes*; D) *S. aureus* C8609 against *S. gallolyticus*; E) *S. sciuri* X3041 against *B. cereus.*

Table 1. Percentage of inhibition exhibited by the 15 bacteriocin-producing (BP) bacteria against the 27 indicator bacteria classified in seven categories and Gram-negative isolates, using three methods (*Spot-on-lawn*; cell-free-supernatant, CFS; butanol-extraction, BT).

	Indicator microorganism		Percentage of BP staphylococci showing inhibitory activity against the indicator by the method[a]:		
Field of interest	**Indicator ID**	**Relevant information: antibiotic resistance, genetic lineage (CC) or virulence**	***Spot-on-lawn***	**CFS**	**BT**
Dairy livestock mastitis	*Streptococcus agalactie* X9738		27%	40%	87%
	S. dysgalactie X9739		33%	27%	87%
	S. gallolyticus X9742		40%	33%	60%
	Staphylococcus aureus C7246	MRSA-*mecC*-CC130	33%	27%	33%
	S. aureus X3548	MRSA-*mecA*-CC121-virulent	60%	27%	60%
	Enterococcus faecium C2321	VanR-*vanA*	60%	27%	60%
Avian pathogen zoonoses	*Clostridium perfringens* X9740		53%	0%	27%
	E. cecorum X3809		47%	33%	87%
	S. aureus C5313	MRSA-*mecA*-CC5	60%	0%	47%
Swine zoonoses	*S. suis* X2060		47%	27%	80%
	S. aureus C1570	MRSA-*mecA*-CC398	67%	27%	73%
	S. aureus X3963	MRSA-*mecA*-CC9	53%	33%	93%
	E. faecalis C9951	LinR-*optrA*,*fexA*	33%	27%	67%
Food safety	*S. aureus* C1532	MRSA-*mecA*-CC5-enterotoxigenic	60%	0%	73%
	Bacillus cereus X10062		47%	27%	87%
	Listeria monocytogenes CECT4032		60%	27%	87%
Aquaculture	*Lactococcus garviae* UR		67%	27%	13%
	Carnobacterium maltaromaticum UR		47%	27%	13%
Wine industry	*Pediococcus pentosaceus* UR		47%	27%	13%
	P. acidilactici UR		60%	27%	13%
	Lactobacillus plantarum UR		33%	33%	20%
	Leuconostoc mesenteroides UR		67%	33%	20%
Mushroom cultivation	*Trichoderma atroviride* TAV1		NT	NT	27%
	Cladobotryum mycophilum CM13900		NT	NT	40%
[b]Gram-negative	*Salmonella* spp. X10061		0%	0%	0%
	Escherichia coli ATCC25922		0%	0%	0%
	Pseudomonas aeruginosa PAO1		0%	0%	0%

[a]It has been coloured in dark blue the indicator bacteria inhibited by ≥60% of BP staphylococci, in slight blue those inhibited by 20% to 60% of BP bacteria and in white those inhibited by less of 20% of BP staphylococci.
[b]Gram-negative bacteria were considered separately due to the fact they were not inhibited by any of the BP staphylococci included in this study or their bacteriocin extracts.
Abbreviations: UR, University of La Rioja; CECT, Spanish Type Culture Collection; ATCC, American Type Culture Collection; MRSA, methicillin resistant *Staphylococcus aureus;* VanR, vancomycin resistant; LinR, linezolid resistant; CC, clonal complex; NT, not tested.

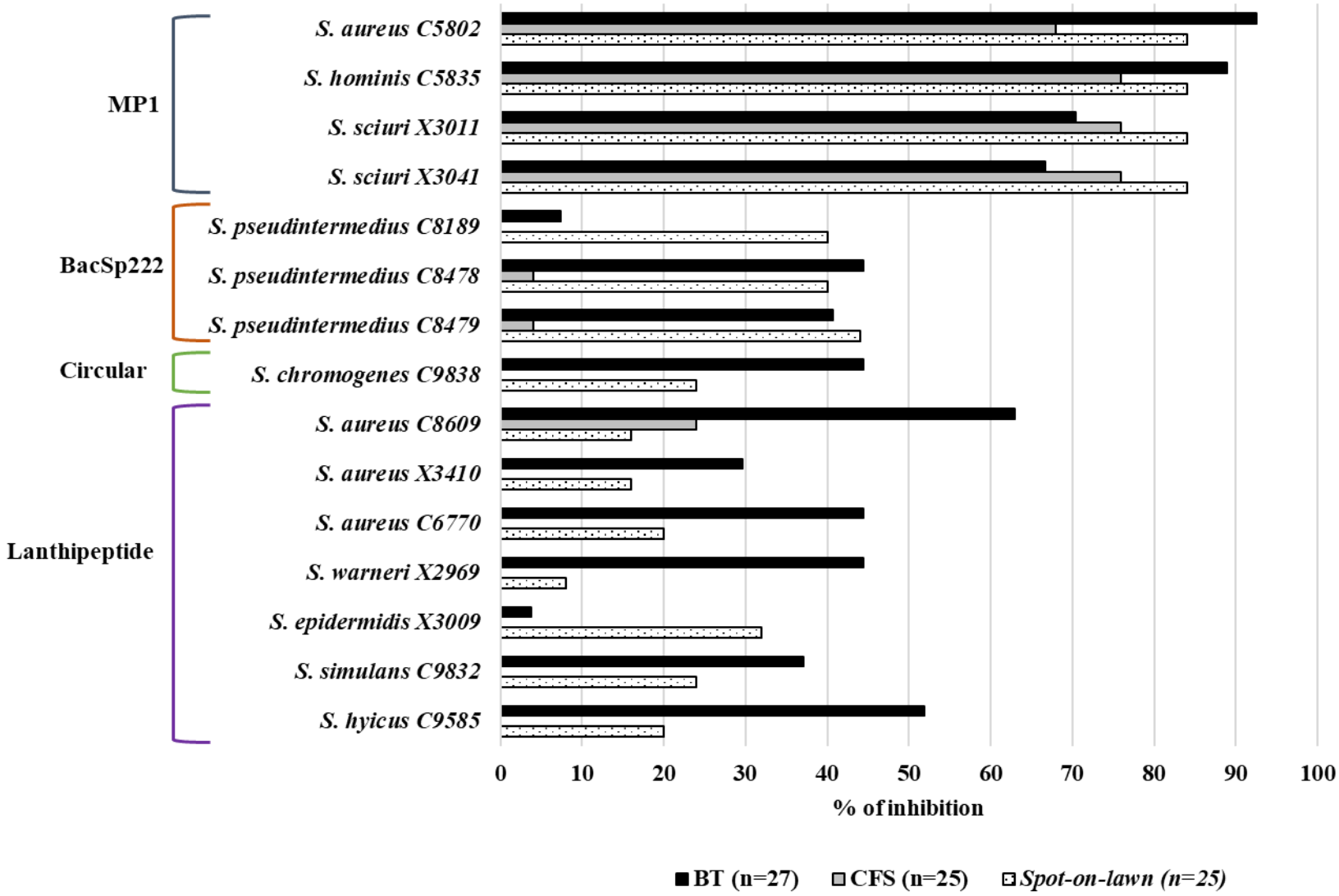

Figure 1. Percentage of indicators inhibited by each of the 15 bacteriocin producing (BP) staphylococci isolates (number of isolates tested) considering three methods: *spot-on-lawn* (n=25), cell-free-supernatant (CFS, n=25) and butanol extraction (BT, n=27) according to the type of bacteriocin produced.

*The antifungal activity of the 15 BP staphylococci against the two moulds included in this study was only evaluated with the BT extracts by disc diffusion assay.

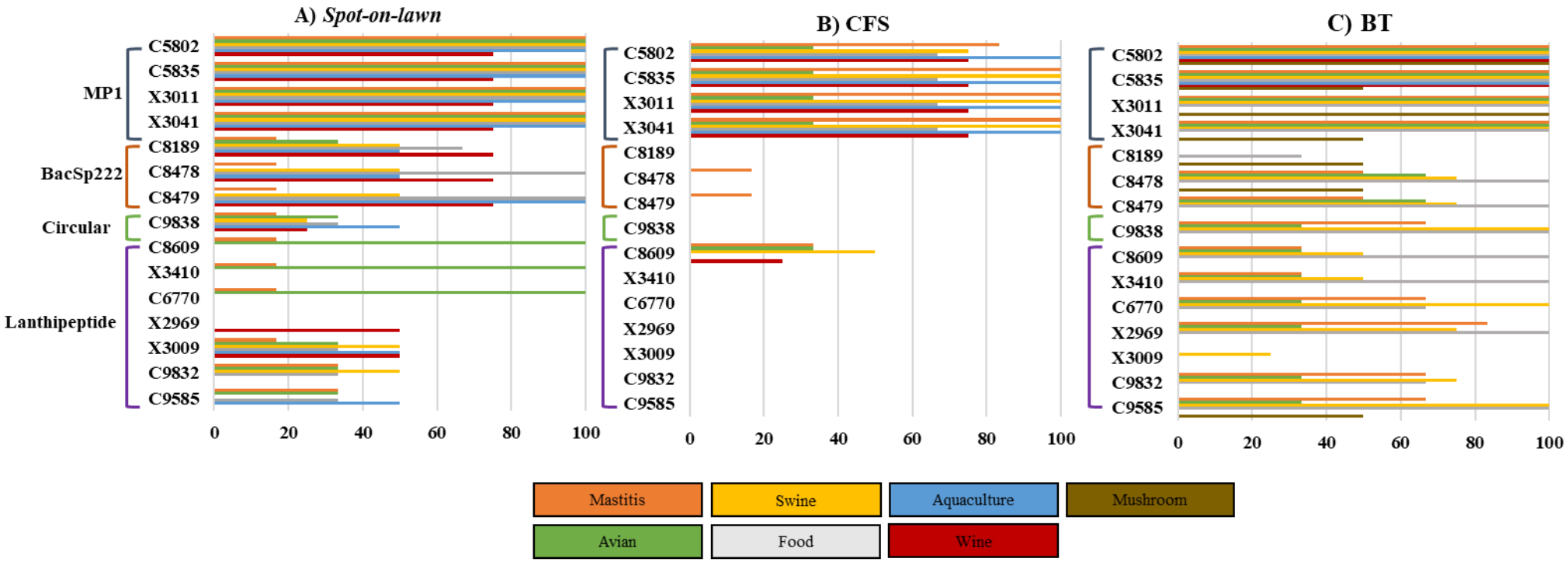

Figure 2. Percentage of indicators inhibited by each of the 15 bacteriocin producing (BP) staphylococci isolates considering categories of application. It was used three methods (number of isolates tested): Figure 2.A) *spot-on-lawn* (n=25); Figure 2. B) cell-free-supernatant (CFS, n=25); Figure 2.C) butanol extraction (BT, n=27). Moreover, BP isolates are organised according to the type of bacteriocin produced.

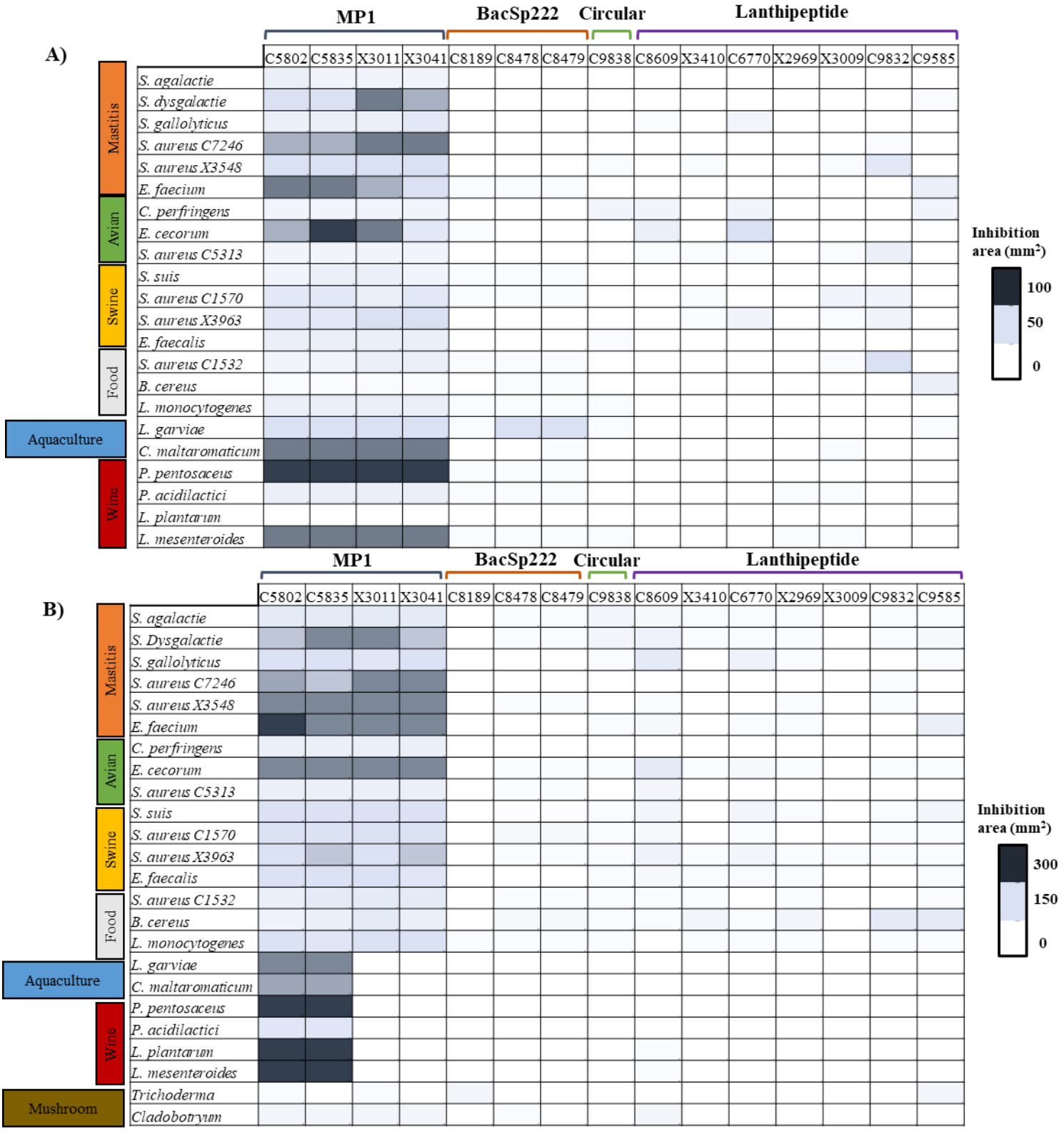

Figure 3. Intensity of inhibition detected in the 15 BP staphylococci against 22 or 24 indicator microorganisms (excluding Gram-negative) classified in categories based on their potential applications. Two methods were used (number of indicators): A) *spot-on-lawn* (n=22), B) BT extracts (n=24).

*The intensity of the inhibitory activity was calculated by the inhibition zones as indicated in material and methods.

Table 2. Cross immunity assay between the 15 BP staphylococci classified according to the bacteriocins produced.

			PRODUCER														
			Micrococcin P1				BacSp222			Circular	Lanthipeptide						
	Bacteriocin	ID code	C5802	C5835	X3011	X3041	C8189	C8478	C8479	C9838	C8609	X3410	C6770	X2969	X3009	C9832	C9585
INDICATOR	Micrococcin P1	C5802	AI														
		C5835		AI													
		X3011			AI												
		X3041				AI											
	BacSp222	C8189					AI										
		C8478						AI									
		C8479							AI								
	Cyclic	C9838								AI							
	Lanthipeptide	C8609									AI						
		X3410										AI					
		C6770											AI				
		X2969												AI			
		X3009													AI		
		C9832														AI	
		C9585															AI

AI — Absence of inhibitory activity of BP isolate against itself

Absence of inhibitory activity of BP isolate against indicator

Antimicrobial activity of BP isolate against indicator

No antimicrobial activity between BP isolates both used as indicator or producer

*All BP staphylococci isolates were used as producer and as indicator bacteria in this experiment.

Supplementary material

Table S1. Characteristics of the 15 bacteriocin-producer (BP) staphylococci isolates included in this study[a].

			Bacteriocin detected by[b]		Antibiotic Resistance (AR)			
ID Code	**Specie**	**Origin**	**WGS**	**Mass spectrometry**	**AR Phenotype[c]**	**AR Genotype**	**Virulence Content**	**Molecular Typing**
C5802	*S. aureus*	Environmental-water	Micrococcin P1, Lactococcin972	Micrococcin P1	PEN	*blaZ*	*luk*MF′, *luk*ED, *etD2*	t843-ST130-CC130/III
C8609	*S. aureus*	Wild animal	BSA, Lactococcin972	ND	Susceptible	-	-	t11225-CC425/II
X3410	*S. aureus*	Food-Chicken	BacCH91	ND	Susceptible	-	-	t10234/I
C6770	*S. aureus*	Wild animal	NS	ND	Susceptible	-	-	t1125-CC5/II
C8189	*S. pseudintermedius*	Human-Dog	BacSp222	ND	ERY-CLI	*erm*(B)	*luk*S/F-I, *siet*	ST241/III
C8478	*S. pseudintermedius*	Human-Dog	BacSp222	ND	ERY-CLI	*erm*(B)	*luk*S/F-I, *siet*	ST241/III
C8479	*S. pseudintermedius*	Human-Dog	BacSp222	ND	ERY-CLI	*erm*(B)	*luk*S/F-I, *siet*	ST241/III
C9585	*S. hyicus*	Wild animal-mammal	LanthipeptideV-like	ND	Susceptible	-	-	-
C9838	*S. chromogenes*	Wild animal-mammal	Cyclic	ND	Susceptible	-	-	-
C5835	*S. hominis*	Environmental-water	Micrococcin P1	Micrococcin P1	Susceptible	-	-	-
X3011	*S. sciuri*	Food-Chicken	Micrococcin P1, Lactococcin972	Micrococcin P1	ERY-CLI-CIP-SXT	*sal*(A)[d], *dfr*A, *erm*(B)	-	-
X3041	*S. sciuri*	Food-Chicken	Micrococcin P1, Lactococcin972	Micrococcin P1	CLI-FA	*sal*(A)[d], *erm*(B), *Inu*(A)	-	-
C9832	*S. simulans*	Wild animal-mammal	Lanthipeptide-New	ND	TET-FA	*tet*(K)	-	-
X2969	*S. warneri*	Food-Chicken	Epilancin15X-Like	ND	Susceptible	-	-	-
X3009	*S. epidermidis*	Food-Chicken	Lanthipeptide -New	ND	ERY-FA	*msr*(A), *mph*(C)	-	ST1025

[a]Data of the isolates were obtained in previous studies (Fernández-Fernández et al., 2023a,b)
[b]Abbreviations: ND, not detected; WGS, whole genome sequencing; NE, not studied.
[c]Antibiotics tested were the following ones: PEN, penicillin; cefoxitin; oxacillin; ERY, erythromycin; CLI, clindamycin; CIP, ciprofloxacin; TET, tetracycline; gentamicin, tobramycin; linezolid; chloramphenicol; SXT, trimethoprim-sulfamethoxazole; FA, fusidic acid and mupirocin.
[d]*sal*(A): intrinsic resistance gene in *S. sciuri.*

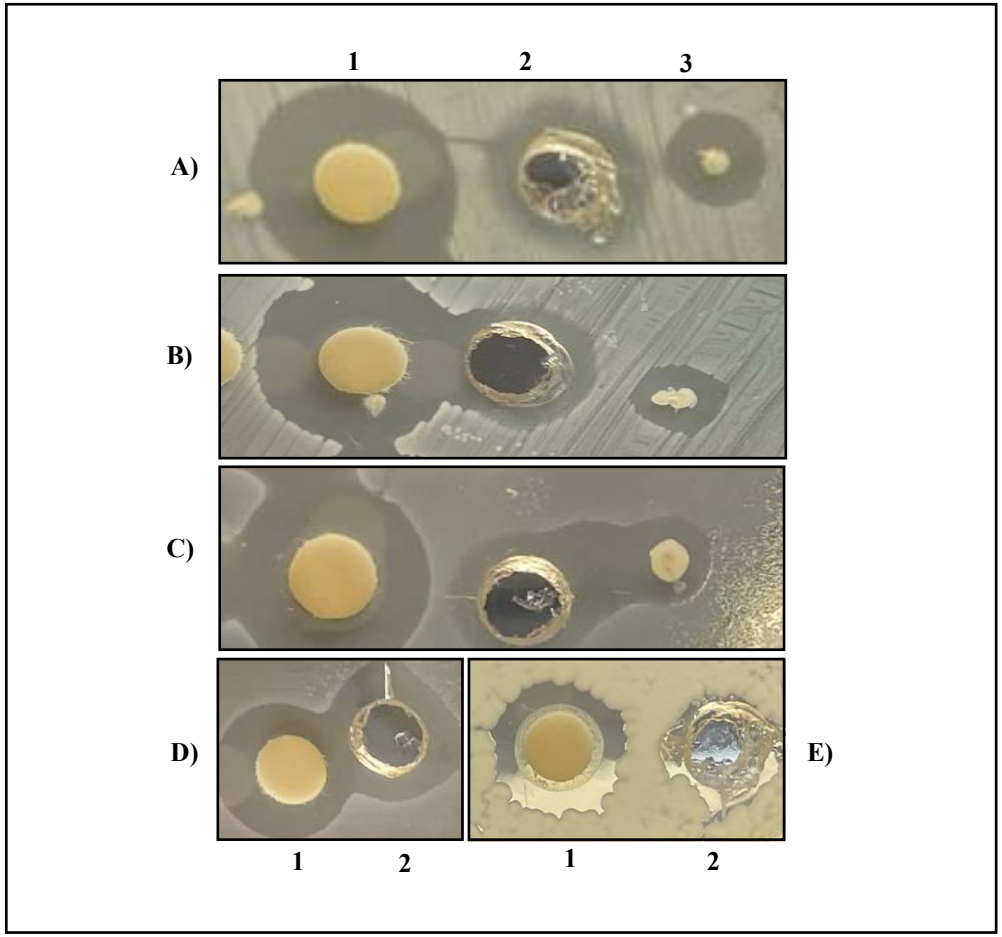

Figure S1. Antimicrobial activity of five bacteriocin staphylococci against different indicator bacteria represented from left to right 1-3: 1.-Butanol extracts, 2.-Cell-free-supernatant (diffusion assay) and 3.-Bacteria (*spot-on-lawn*). A) *S. aureus* C5802 against MRSA X3963; B) *S. hominis* C5835 against *E. faecalis* C9951; C) *S. sciuri* X3011 against *L. monocytogenes*; D) *S. aureus* C8609 against *S. gallolyticus*; E) *S. sciuri* X3041 against *B. cereus.*

Capítulo 5

5. Capítulo 5: Detección de bacteriocinas en microorganismos del suelo: ciencia ciudadana y transferencia del conocimiento

El suelo alberga una gran biodiversidad y biomasa de microorganismos. Las bacterias del suelo viven en un entorno superpoblado y muy competitivo, con recursos limitados y condiciones en constante cambio. Existen numerosos antibióticos producidos por microorganismos aislados del suelo, desde la penicilina, hasta algunos nuevos, como la teixobactina; por lo que el suelo es un inmenso reservorio de microorganismos con gran potencial para encontrar nuevos compuestos antimicrobianos.

La RAM se ha convertido en un grave problema de salud mundial y para solventar la falta de conocimiento en este tema organizaciones mundiales como las Naciones Unidas (2015) plantean en la Agenda 2030 para el Desarrollo Sostenible la urgente necesidad de educación y capacitación en diferentes sectores de la población. En este sentido, se han desarrollado estrategias pedagógicas novedosas como MicroMundo, proyecto creativo de aprendizaje-servicio implementado originalmente en 2012, en Estados Unidos con designación "Small World Initiative" (SWI) recientemente instaurado en España.

MicroMundo combina la recogida de muestras de suelo y el laboratorio para descubrir nuevas moléculas antimicrobianas naturales como agentes terapéuticos. Este proyecto ha sido implementado en la Universidad de La Rioja, como una iniciativa de escuela abierta para promover vocaciones científicas entre estudiantes de niveles preuniversitarios e incentivar su interés por la Ciencia mediante el contacto directo con el trabajo de toma de muestras y de laboratorio. De este modo, se ha conseguido acercar la investigación microbiológica y molecular por medio de la búsqueda de microorganismos con actividad antimicrobiana como posibles alternativas a la RAM y mejorar los conocimientos en materia de resistencia a antimicrobianos y su uso responsable, uno de los desafíos de salud pública mundial más importantes del siglo XXI. Esta iniciativa pone de manifiesto la importancia de la transferencia del conocimiento desde la universidad hasta los niveles educativos básicos. Además, es de gran relevancia elevar esta problemática a la sociedad con actividades divulgativas que permitan comprender la situación y concienciar sobre la necesidad de actuación.

En base a estas premisas, este capítulo aborda el último objetivo de la tesis donde se describen los resultados de la búsqueda de sustancias antimicrobianas en bacterias de diversos géneros obtenidos a partir de muestras de suelo recogidas en actividades divulgativas llevadas a cabo bajo proyectos de ciencia abierta y colaborativa.

5.1 Artículo 6..... Citizen contribution for searching for alternative antimicrobial activity substances in soil

Artículo 6..... Citizen contribution for searching for alternative antimicrobial activity substances in soil

En este trabajo se evaluó la actividad antimicrobiana de bacterias obtenidas en muestras de suelo a partir del proyecto de aprendizaje-servicio MicroMundo-UR **(Figuras 21 y 22)**, la biodiversidad de los aislados seleccionados como posibles productores de antimicrobianos, y el perfil de resistencia a los antibióticos, incluyendo tanto *Staphylococcus* como otros géneros bacterianos.

En total, 132 aislados (5% del total analizado) fueron seleccionadas a partir de 130 muestras de suelo como potenciales productores de antimicrobianos al presentar actividad inhibitoria frente al menos una de las bacterias indicadoras (*Escherichia coli* y *Staphylococcus epidermidis*). Estas bacterias fueron identificadas a nivel de especie por medio de espectrometría de masas MALDI-TOF. En cuanto a la diversidad observada entre los aislados productores, el género más frecuente fue *Bacillus*, seguido de *Pseudomonas, Paenibacillus* y *Serratia*. Posteriormente, la actividad antimicrobiana (AA) de los 132 aislados se estudió frente a una colección más amplia de 15 bacterias indicadoras (seis géneros y trece especies, incluidos patógenos relevantes). De las 132 bacterias potencialmente productoras identificadas en la primera fase se verificaron como productores 32 de ellos para su futura caracterización. En este sentido, 18 de los 32 aislados mostraron una baja actividad antimicrobiana (AA) (<20% de indicadores inhibidos), 12 aislados tuvieron actividad media (20%-70% de indicadores inhibidos), y se encontraron dos aislados con elevada capacidad inhibitoria (*Brevibacillus laterosporus* X7262 y *Staphylococcus hominis* X7276), que mostraron AA frente al 80% de los 15 indicadores probados. Este resultado despertó gran interés puesto que permitió detectar SCoN con gran potencial en muestras ambientales lo que abre los horizontes a la búsqueda y aplicación de nuevas sustancias antimicrobianas de origen natural. Este aislado *S. hominis* Bac+ será caracterizado en detalle en estudios futuros comparando con el resto de *S. hominis* reportados en la bibliografía y los detectados en otros capítulos de esta tesis doctoral. Finalmente, se estudió la seguridad de estas cepas productoras evaluando su fenotipo de resistencia a antibióticos por el método de difusión disco-placa. Se observó que el 48% de las bacterias productoras de antimicrobianos fueron sensibles a todos los antibióticos probados.

Gracias a la ciencia ciudadana se han aislado bacterias ambientales productoras de antimicrobianos de gran interés que pueden tener interés como agentes de biocontrol entre otras alternativas al problema de la RAM. Por otro lado, este proyecto ha permitido evidenciar los beneficios derivados del vínculo establecido entre ciencia y educación por medio de proyectos de innovación educativa para sensibilizar sobre el problema de la resistencia a los antibióticos y contribuir a la búsqueda de nuevas soluciones. Además, se ha conseguido concienciar sobre la importancia de los antibióticos desde el enfoque “ciencia de todos para todos”.

5.2 Anexo al Capítulo 5

Figura 21 Relación entre la universidad y la educación secundaria que conecta la investigación y la educación, buscando la actividad antimicrobiana del suelo, promoviendo vocaciones científicas y difundiendo la cultura científica de la resistencia a los antimicrobianos.

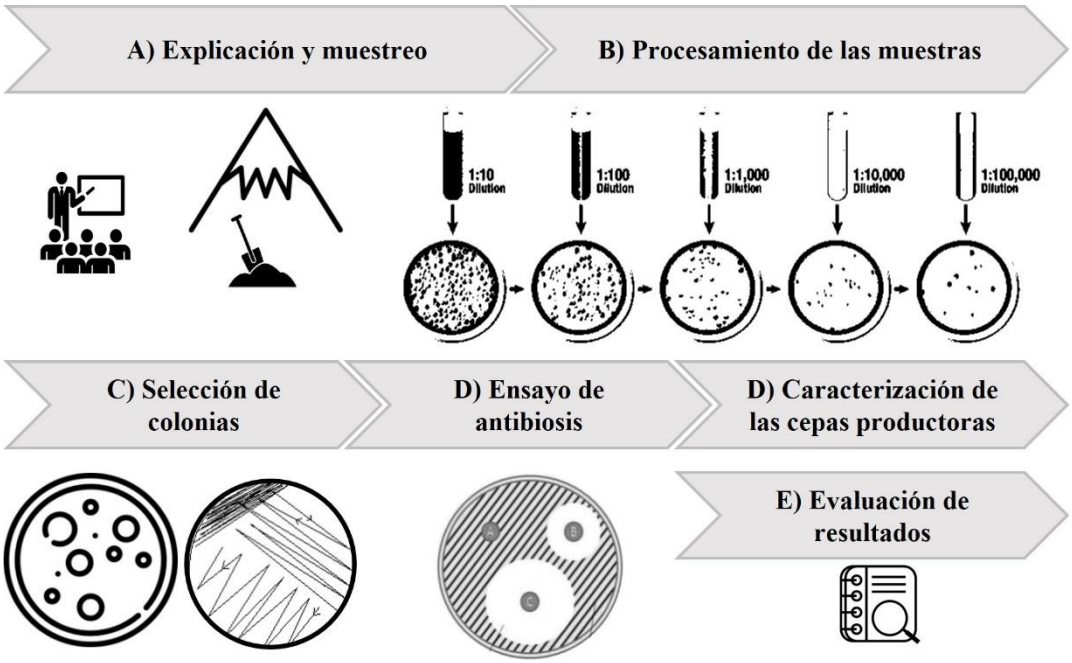

Figura 22 Resumen gráfico de la metodología llevada a cabo por los estudiantes de secundaria durante el proyecto MicroMundo.

Artículo 6

antibiotics

MDPI

Article

Citizen Contribution for Searching for Alternative Antimicrobial Activity Substances in Soil

Rosa Fernández-Fernández [1,†], Beatriz Robredo [2,*,†], Enrique Navajas [2] and Carmen Torres [1,*]

[1] Area of Biochemistry and Molecular Biology, Faculty of Science and Technology, University of La Rioja, 26006 Logroño, Spain
[2] Area of Didactic of Experimental Sciences, Faculty of Science and Technology, University of La Rioja, 26006 Logroño, Spain
* Correspondence: beatriz.robredo@unirioja.es (B.R.); carmen.torres@unirioja.es (C.T.)
† These authors contributed equally to this study.

Abstract: Antimicrobial resistance (AMR) is problematic worldwide, and due to the loss of efficiency of many antibiotics, the pressure to discover alternative antimicrobial molecules has increased. Soil harbors a great biodiversity and biomass of microorganisms, and many antibiotics are produced by soil microbiota. Therefore, soil is a promising reservoir to find new antimicrobial agents. In this respect, novel pedagogical strategies regarding the AMR global crisis have recently been developed in different countries worldwide. Highlighted is the service-learning project "MicroMundo" integrated in a global Citizen Science project called "Tiny Earth". Hence, the present work aimed at determining the antimicrobial activity of soil bacteria, the biodiversity of the selected isolates as putative antimicrobial producers, and their antibiotic resistance profile. Moreover, through the MicroMundo project, we tried to illustrate the relevant link between science and education and the benefits of implementing service-learning methodologies to raise awareness of the AMR problem and to contribute to the search for new alternatives. A total of 16 teachers, 25 university students and 300 secondary school students participated in the search for antimicrobial activity on a collection of 2600 isolates obtained from a total of 130 soil samples analysed. In total, 132 isolates (5% of total tested) were selected as potential antimicrobial producers when two indicator bacteria were used (Escherichia coli and Staphylococcus epidermidis); the most frequent genus among these isolates was Bacillus, followed by Pseudomonas, Paenibacillus and Serratia. The antimicrobial activity (AA) of the 132 potential antimicrobial producers was studied in a second step against 15 indicator bacteria (of six genera and thirteen species, including relevant pathogens). Of the 132 potentially producing bacteria, 32 were selected for further characterization. In this respect, 18 isolates showed low AA, 12 isolates were considered as medium producers, and 2 highly antimicrobial-producing isolates were found (Brevibacillus laterosporus X7262 and Staphylococcus hominis X7276) showing AA against 80% of the 15 indicators tested. Moreover, 48% of the antimicrobial-producing bacteria were susceptible to all antibiotics tested. Due to citizen science, antimicrobial-producing bacteria of great interest have been isolated, managing to raise awareness about the problem of AMR.

Keywords: antimicrobial-producing bacteria; soil; antimicrobial resistance; MicroMundo

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1. Introduction

Antimicrobial resistance (AMR) has risen an awareness alarm, being one of the most urgent challenges for current medicine and society due to the emergence of multi-drug resistant (MDR) pathogens [1,2]. Concretely, the science community is especially concerned about the antimicrobial resistance associated with the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) [3].

The loss of efficacy of many antibiotics increases the pressure to identify new effective approaches [4]. In the last decade, intensive studies have looked at the potential of natural

antibacterial molecules as next-generation therapeutics against pathogens [4,5]. Concretely, the ribosomally synthesized peptides of bacterial origin, also named as bacteriocins, are one of the most promising bioactive compounds with antimicrobial properties against other bacteria [5]. The ability to synthetize bioactive peptides is one of the oldest defensive mechanisms of microorganisms, and many microorganisms produce at least one bacteriocin [6]. These not essential secondary metabolites increase the bacterial chances of adaptation in a hostile environment, and they have been proposed as a good alternative to combat pathogens and MDR bacteria [5,7].

Soil harbors a great biodiversity and biomass of microorganisms [8,9]. Soil bacteria live in a crowded and highly competitive environment with limited resources and constantly changing conditions. There are many antibiotics produced by microorganisms isolated from soil, from penicillin, the first reported, to some new ones such as malacidins and teixobactin [10]. Actinomycetes, which are the most common bacteria in soil, produce 60% of antibiotics in clinical use [11]. Thus, the potential to find new antimicrobial compounds in this immense reservoir of microorganisms is enormous, and scientists are beginning to realize how little is known regarding soil microorganisms.

To address these problems, novel pedagogical strategies on the AMR global crisis have recently been developed in different countries worldwide. Among such strategies is "MicroMundo" [12], integrated in a global Citizen Science project on AMR called "Tiny Earth" (TE; https://tinyearth.wisc.edu/, accessed on 1 December 2022) originally implemented in 2012 in the United States, with "Small World Initiative" designation (SWI; http://www.smallworldinitiative.org/, accessed on 1 December 2022).

MicroMundo was developed in a service-learning environment, in which different educational levels are integrated [12]. The program seeks to raise awareness of the problem of AMR among students by participating in a creative research project that combines soil sample collection and laboratory work to discover new antimicrobial agents [13].

Hence, the present work aimed at determining the antimicrobial activity of bacteria of the soil, the biodiversity of the selected isolates as putative producers, and their antimicrobial resistance profile. Moreover, we tried to illustrate the relevant link between science and education and the benefits of implementing service-learning methodologies to raise awareness of AMR and to contribute to the search for new alternatives.

2. Material and Methods

2.1. MicroMundo: Service-Learning Methodology

During the 2020–2022 school years, the service-learning project called MicroMundo was developed in La Rioja region (Spain) through two scales of practical training, involving university and secondary education.

The initial step of the MicroMundo project was carried out at the university (University of La Rioja), taught by a qualified professor (SWIPI: Partner Instructor) to master students (n = 25) or biology secondary school teachers (n = 12) (SWITAs: technical assistants). The SWIPI trained the teaching abilities of SWITAs through periodic sessions, and at the end, they discussed the logistics of SWI actions to analyze the experimental results and to design data-recording sheets.

The second step was performed in secondary schools (La Rioja region) under the general supervision of SWIPI, using expendable material and equipment from the university. In this process, the 37 SWITAs (master students and secondary school teachers) and 299 students (SWISs) of 12 secondary schools were involved. The SWITAs trained the program to the SWISs through periodic sessions. They made up a total of 130 groups, each of whom analyzed a soil sample.

Practical work was divided into five 2 h sessions following the methodology previously explained [14]. Firstly, soil samples were diluted and grown on Tryptic Soy Agar (TSA) (Condalab, Madrid, Spain) plates for colony selection (20 isolates per sample). An antimicrobial activity test was performed with all 20 isolates obtained on each soil sample, using *Staphylococcus epidermidis* C2663 (Gram-positive bacteria) and *Escherichia coli* C408

(Gram-negative bacteria) as indicator microorganisms (Table S1). Indicator bacteria were inoculated in saline solution and spread as a lawn onto TSA plates. Then, bacteria to be tested for antimicrobial activity production were transferred with a sterile toothpick. Plates were evaluated by students after 24 h, and isolates with putative inhibition halloes were selected in this initial screening and transferred to the university for verification and further characterization (second screening).

2.2. Second Screening of Antimicrobial Activity by the Spot-on-Lawn Method

The selected strains with potential antimicrobial activity obtained in the first screening at school level were further analyzed and characterized in a second screening process at the university. For that purpose, these strains were tested by the *spot-on-lawn* method against 15 indicator strains of 6 different genera and 13 species, including relevant pathogens (Supplementary Table S1). Bacteria were grown in brain heart infusion (BHI) agar (Condalab, Madrid, Spain) for 24 h at 37 °C. To prepare test plates, a suspension of the indicator strain was prepared in a tube of 3 mL of sterile saline solution to obtain a turbidity of 0.5 MacFarland, and it was spread with a sterile swab in 0.3% yeast extract-supplemented solid Tryptic Soy Agar (TSA) plate (Condalab, Madrid, Spain). Then, each fresh solid culture of bacterium to be tested for antimicrobial activity was transferred with a sterile toothpick to the agar plates seeded with each of the 15 indicators tested. Plates were incubated at 37 °C for 24 h to evaluate the halos of inhibition. Isolates were considered antimicrobial producers (AP) when they showed a clear and sharp inhibition zone against at least one of the 15 indicator isolates. Depending on the antimicrobial activity (hallo of inhibition), it was expressed as + (<3 mm), ++ (3 < x < 10 mm), or +++ (>10 mm).

2.3. Bacterial Identification

Isolates with potential antimicrobial capacity obtained in the first screening (n = 132) were identified by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, using the standard protein extraction protocol recommended by the commercial device of Bruker Daltonics, Bremen, Germany. The antimicrobial-producing isolates of interest that could not be identified by MALDI-TOF were identified by amplification and sequencing of the 16S rDNA gene, using the following primer sequences (F-GTGCCAGCAGCCGCGGTAA, R-AGACCCGGGAACGTATTCAC) and PCR conditions: 94 °C, 2 min; 28 cycles (94 °C, 30 s; 45 °C, 1 min; 72 °C, 1 min) and final 72 °C, 7 min [15].

2.4. Antibiotic Susceptibility Testing of Antimicrobial-Producing Strains

The antibiotic susceptibility profile was determined in the antimicrobial-producing isolates verified in the second screening, by the disk diffusion test in Mueller Hinton (MH) agar (Condalab, Madrid, Spain). The antibiotics used for that purpose differed depending on the bacterial genera and are indicated in Supplementary Table S2. The EUCAST guidelines [16] were used for the antibiotic susceptibility testing, and *Staphylococcus* spp. breakpoints were used for Gram-positive bacteria, except for *Bacillus* spp., for which EUCAST indicates a specific breakpoint for some antimicrobial agents [16]. In the case of Gram-negative bacteria, the breakpoints of enterobacterales and *Pseudomonas* were used, depending on the genera.

2.5. Diversity of Antimicrobial-Producing Bacteria and Statistics

The 130 soil samples were grouped based on the geographic coordinates of the collection site. In this way, 5 geographical zones were distinguished: La Rioja East, La Rioja Central, La Rioja West, Logroño and Outside (Supplementary Figure S1). Statistics comparison between the antimicrobial-producing bacteria detected by the first screening and the non-antimicrobial-producing isolates included in the 5 established clusters was performed using the Fisher test, and significant differences were considered for $p < 0.05$. Moreover, diversity and statistical analyses were carried out considering each antimicrobial-producing

isolate as an independent item. Isolates that could not be identified were excluded for diversity analysis; thus, a collection of 104 isolates was included. Renyi profile was performed to compare alpha diversity between the 5 clusters. The Renyi profile was used to compare the species diversity among zones with vegan 2.6–2 package from R (4.2.1). The averages of the Renyi profile values were calculated using rarefied samples. Significant differences in these values were assessed with an ANOVA test. Tukey's post hoc test was used to identify differences between zones. All analyses were carried out with R, version 4.1.2.

3. Results

A total of 130 soil samples were analyzed at school level, and 2600 isolates were obtained (20 isolates/sample) and tested in a first screening for antimicrobial activity; 132 isolates showed potential inhibitory capacity in the first screening test performed in the school and using only two indicator bacteria (*S. epidermidis* and *E. coli*). Statistical differences were not observed between antimicrobial-producing and non-antimicrobial-producing isolates based on their geographical location.

Identification by MALDI-TOF at genus level of these 132 putative antimicrobial-producing isolates detected in soil samples revealed 19 genera and 48 species with the following microbial diversity (number of isolates): *Acinetobacter* (1), *Arthrobacter* (2), *Bacillus* (40), *Bradybacterium* (1), *Brevibacillus* (1), *Enterobacter* (3), *Escherichia* (2), *Klebsiella* (1), *Microbacterium* (2), *Micrococcus* (1), *Paenibacillus* (12), *Pseudomonas* (27), *Staphylococcus* (2), *Serratia* (6), *Stenotrophomonas* (1), *Streptomyces* (1), *Olivibacter* (1), *Variovarax* (1) and *Viridibacillus* (1). Moreover, 26 out of the 132 isolates (19.7%) could not be identified by MALDI-TOF mass spectrometry (Table 1). Diversity at genera level is represented in Figure 1, where the number of isolates of *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Serratia* as well as those unidentified are shown. Genera with low numbers of representants were considered together in a group called "Others" (n = 19 isolates). A high prevalence of the genus *Bacillus* followed by *Pseudomonas*, *Paenibacillus* and *Serratia* (all isolates belong to the species *S. plymuthica*) is of note.

Table 1. Genus and species identification of the 132 potential antimicrobial-producing isolates obtained in the first screening and those 32 isolates verified in the second screening.

Genus	Species	Number of Isolates First Screening	Number of Isolates Second Screening
Acinetobacter	*Acinetobacter radioresistens*	1	
Arthrobacter		2	
	Arthrobacter citreus	1	1
	Arthrobacter ilicis	1	
Bacillus		40	
	Bacillus marisflavi	1	
	Bacillus atrophaeus	2	2
	Bacillus cereus	6	1
	Bacillus cibi	1	
	Bacillus megaterium	4	1
	Bacillus mycoides	4	1
	Bacillus pumilus	7	5
	Bacillus safensis	2	2
	Bacillus simplex	3	
	Bacillus thuringiensis	2	
	Bacillus weihenstefanensis	3	
	Bacillus spp.	5	2

Table 1. *Cont.*

Genus	Species	Number of Isolates First Screening	Number of Isolates Second Screening
Bradybacterium	*Bradybacterium* spp.	1	1
Brevibacillus	*Brevibacillus laterosoporus*	1	1
Enterobacter	*Enterobacter cloacae*	3	
Escherichia	*Escherichia coli*	2	
Klebsiella	*Klebsiella aerogenes*	1	1
Microbacterium	*Microbacterium arborescensens*	2	1
Micrococcus	*Micrococcus luteus*	1	
Paenibacillus		12	
	Paenibacillus amylolyticus	6	
	Paenibacillus apiarus	2	2
	Paenibacillus gluconolyticus	1	
	Paenibacillus lautus	1	
	Paenibacillus polymyxa	1	1
	Paenibacillus xylanilyticus	1	
Pseudomonas		27	
	Pseudomonas brasicacearum	2	
	Pseudomonas brenneri	1	
	Pseudomonas caricapapayae	1	1
	Pseudomonas chlororaphis	3	1
	Pseudomonas kilonensis	5	3
	Pseudomonas koreensis	2	
	Pseudomonas mandelii	1	
	Pseudomonas mosselii	1	
	Pseudomonas putida	1	
	Pseudomonas savastanoi	1	
	Pseudomonas thivervalensis	2	
	Pseudomonas umsongensis	1	
	Pseudomonas spp.	6	2
Staphylococcus	*Staphylococcus hominis*	2	1
Serratia	*Serratia plymuthica*	6	
Stenotrophomonas	*Stenotrophomonas rhizophila*	1	
Streptomyces	*Streptomyces avidinii*	1	1
Olivibacter	*Olivibacter soli*	1	1
Variovorax	*Variovorax paradoxus*	1	
Viridibacillus	*Viridibacillus arenosi*	1	
Not identified		26	
Total		132	32

Serratia	*Serratia plymuthica*	6	
Stenotrophomonas	*Stenotrophomonas rhizophila*	1	
Streptomyces	*Streptomyces avidinii*	1	1
Olivibacter	*Olivibacter soli*	1	1
Variovorax	*Variovorax paradoxus*	1	
Viridibacillus	*Viridibacillus arenosi*	1	
Not identified		26	
Total		132	32

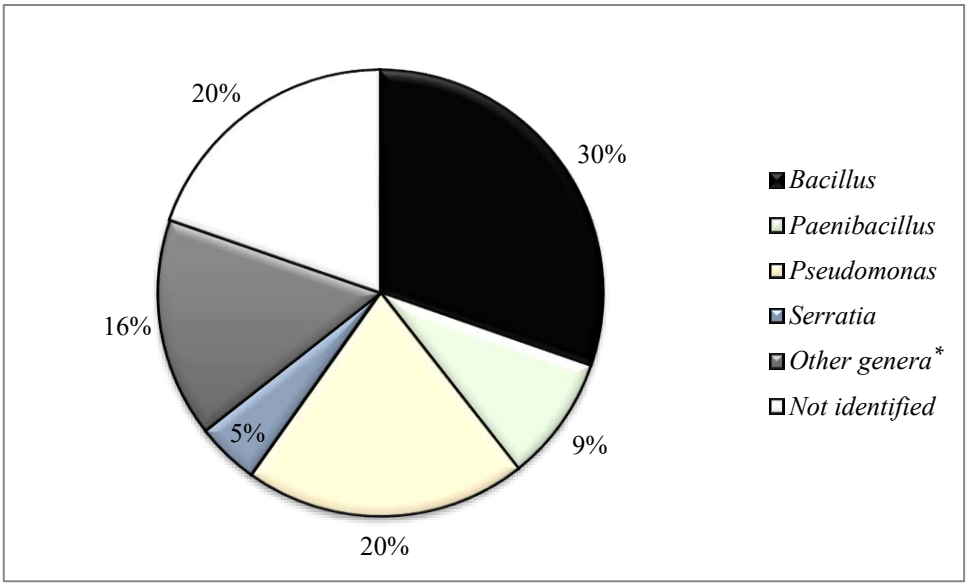

Figure 1. Diversity at genus level of the antimicrobial-producing bacteria isolated from soil samples in the first initial screening. *Other genera: include isolates of genera with less than six representants; see Table 1.

Renyi profiles allowed us to differentiate clusters according to the diversity of potential producers (Figure S1). The community with an overlapping diversity profile was considered the most diverse. Thus, the decreasing ranking of clusters was as follows: La Rioja Central, La Rioja West and Logroño (same alpha values), La Rioja East and Outside (Figure S2). This revealed that antimicrobial-producing isolates of La Rioja Central had the best distribution of richness (number of different genera/species) and abundance (number of isolates of each genus/species detected) (Table S3 and Figure S2).

3.1. Verification of Antimicrobial Activity of Antimicrobial-Producing Bacteria in a Second Screening Process against 15 Indicator Bacteria

The 132 isolates obtained in the first screening with potential antimicrobial activity production were tested at the university in a second screening by the *spot-on-lawn* method against 15 indicator bacteria. Then, 32 out of the 132 tested isolates were finally selected for presenting clear antimicrobial activity after several repetitions against at least one indicator bacteria (Table 2). Identification of 29 out of 32 isolates was obtained by MALDITOF, and the remaining 3 (X7264, X7265 and X7266) were identified by amplification and sequencing of the 16S rDNA gene. Twenty-three of the 32 antimicrobial-producing isolates were Gram-positive (15 species and 6 genera), and nine were Gram-negative (6 species and 3 genera), and they were further characterized. Most of antimicrobial antimicrobial-producing bacteria were of the genera *Bacillus* (43.8%) and *Pseudomonas* (21.9%) (Table 2).

The most susceptible indicators detected in this study with antimicrobial-producing isolates were the following: *S. epidermidis*, *M. luteus*, and methicillin-resistant and -susceptible *S. aureus* (MRSA and MSSA, respectively) (Tables 2 and 3). Seven Gram-positive producing isolates showed antimicrobial activity against the Gram-negative indicators used (*E. coli* and *P. aeruginosa*). It is to note the high inhibition produced by both Gram-positive and Gram-negative antimicrobial-producing bacteria against the indicators of the genus *Staphylococcus*, including methicillin-resistant staphylococci. In addition, three Gram-positive antimicrobial-producing isolates (*B. mycoides* X7258; *B. laterosporus* X7262 and *S. hominis* X7276) inhibited the indicator *L. monocytogenes*, a relevant pathogen in the food industry. Finally, excluding the species *E. cecorum* (with 48% of inhibition), *Enterococcus* was the most resistant indicator genus, only inhibited by Gram-positive antimicrobial-producing isolates, as expected (Tables 2 and 3).

Table 2. Activity profile of the 32 antimicrobial-producing isolates against the 15 indicator bacteria tested.

	Antimicrobial Activity [a] on the Indicator Bacteria [b]															
Producing Isolate	***E. coli***	***P. aeruginosa***	**MRSA**	**MSSA**	**MRSP**	**MSSP**	***S. delphini***	***S. sciuri***	***S. epidermidis***	***E. faecalis***	***E. faecium*** [c]	***E. cecorum***	***E. gallinarum***	***L. monocytogenes***	***M. luteus***	**No (%)**
A. citreus X7246				+					+						+	3 (20)
B. cereus X7247			+			+			+			+			+	5 (33)
B. safensis X7248			+			+			+			+			+	5 (33)
B. safensis X7249	+		+			+			++			+			+	6 (40)
B. pumilus X7250	+		+	+		+	+		+			+			+	8 (53)
B. atrophaeus X7251			+			+			+			+			+	5 (33)
B. atrophaeus X7252	+		+	+		+			+			+			+	7 (47)
Bacillus spp. X7253				+					+						+	3 (20)
Bacillus spp. X7256.			+	+		+	+	+	+			+			+	8 (53)
B. pumilus X7254			+			+	+		+			+			+	6 (40)
B. megaterium X7255				+					+						+	3 (20)
B. pumilus X7257		+							+						+	3 (20)
B. mycoides X7258			+	+		+	+	+	+					+	+	8 (53)
B. pumilus X7259			+	+	+	+	+		+			+			+	8 (53)
B. pumilus X7260			+	+	+	+	+		+						+	7 (47)
Bradybacterium spp. X7261				+					+							2 (13)
B. laterosoporus X7262	+		+	+	+	+	+	+	+	+		+		+	+	12 (80)
M. arborescensens X7263			+	+	+	+	+		+						+	7 (47)
P. apiarus X7264	+		+				+		+						+	5 (33)
P. apiarus X7267			+						+						+	3 (20)
P. polymyxa X7268	+		+	+		+	+		+			+			+	8 (53)
S. hominis X7276			+	+		+	+	+	+	+	+	+	+	+	+	12 (80)
S. avidinii X7277			+	+					+						+	4 (27)
Number of inhibitions (%)	6 (26)	1 (4)	18 (78)	15 (65)	4 (17)	15 (65)	11 (48)	4 (17)	23 (100)	2 (9)	1 (4)	12 (52)	1 (4)	3 (13)	22 (92)	
O. soli X7265				+					+						+	3 (20)
K. aerogenes X7266	+			+		+										3 (20)
P. kilonensis X7269			+	+	+	+	+	+	+++						+	8 (53)
P. kilonensis X7270			+				+		+++						+	4 (27)
P. kilonensis X7271			+	+					+						+	4 (27)
Pseudomonas spp. X7272			+	+					+							3 (20)
Pseudomonas spp. X7273			+	+					+							3 (20)
P. chlororaphis X7274	+	+	+	+	+		+		+						+	8 (53)
P. caricapapayae X7275	+	+		+					+						+	5 (33)
Number of inhibitions (%)	3 (33)	2 (22)	6 (67)	8 (89)	2 (22)	2 (22)	3 (33)	1 (11)	8 (89)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	6 (67)	

[a] Depending on the antimicrobial activity, it was expressed as + (<3 mm), ++ (3 < x < 10 mm), and +++ (>10 mm). Only positive results are indicated. [b] Abbreviations: MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; MRSP; methicillin-resistant *S. pseudintermedius*; MSSP, methicillin-susceptible *S. pseudintermedius*. [c] Vancomycin resistant strain.

Table 3. Antimicrobial activity profile of the 32 antimicrobial-producing isolates obtained in the second screening against 15 indicator bacteria (number of isolates).

Producing Isolates	Number of Positive Results of the Antimicrobial-Producing Isolates against the Following Indicator Bacteria:						
	E. coli (1)	*P. aeruginosa* (1)	MR-*Staphylococcus* [a] (2)	MS-*Staphylococcus* [a] (5)	*Enterococcus* (4)	*L. monocytogenes* (1)	*M. luteus* (1)
A. citreus (1)				2			1
Bacillus spp. (14)	3	1	13	41	9	1	14
Bradybacterium spp. (1)				2			
B. laterosoporus (1)	1		2	5	2	1	1
M. arborescensens (1)			2	4			1
Paenibacillus spp. (3)	2		3	7	1		3
S. hominis (1)			1	5	4	1	1
S. avidinii (1)			1	2			1
O. soli (1)				2			1
K. aerogenes (1)	1			2			
Pseudomonas spp. (7)	2	2	8	18			5

[a] Abbreviations: MR, methicillin resistant; MS, methicillin susceptible.

Three levels were differentiated regarding production based on the percentages of indicator bacteria inhibited by the antimicrobial-producing isolates: low (<35%), medium (from 35 to 70%) and high (>70%). In this respect, 18 isolates showed low antimicrobial activity, 12 isolates were considered as medium producers, and 2 isolates were found to be high producers with antimicrobial activity against 80% of the indicators tested (*Brevibacillus laterosporus* X7262 and *Staphylococcus hominis* X7276) (Table 2). Neither of the two highly producing isolates were active against *P. aeruginosa*, but *B. laterosporus* X7262 inhibited *E. coli*, the other Gram-negative indicator strain. On the other hand, both highly producing strains showed antimicrobial activity against methicillin-resistant and -susceptible (MR and MS) staphylococci (from 90% to 100% of inhibition), *L. monocytogenes* and *M. luteus*. The *S. hominis* X7276 strain revealed antimicrobial activity against all *Enterococcus* isolates used as indicators (100%), and *B. laterosporus* X7262 inhibited 50% of them (Figure 2).

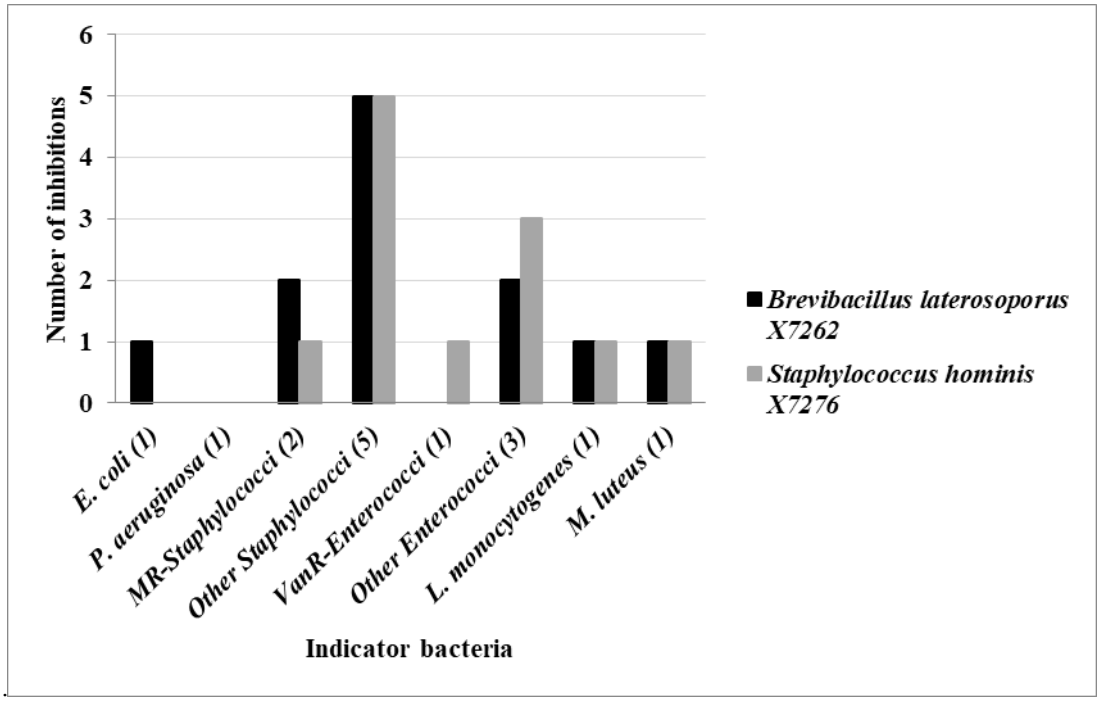

Figure 2. Number of indicator bacteria inhibited by the two highly producing isolates (*Brevibacillus laterosporus* X7262 and *Staphylococcus hominis* X7276).

3.2. Antibiotic Resistance Phenotype of the Antimicrobial-Producing Isolates

In total, 48% of the 32 antimicrobial-producing isolates showed susceptibility to all the antibiotics tested. Considering Gram-positive isolates, resistance was mostly detected for cefoxitin (22%) and penicillin and tobramycin (17%). Meropenem/imipenem and ciprofloxacin resistance were also found among *Bacillus* isolates. Four Gram-positive producing isolates (17%) were multidrug resistant (MDR). With respect to Gram-negative, 33% of them were susceptible to all the antibiotics tested (all belonging to *Pseudomonas* genus). Four *Pseudomonas* spp. showed resistance to ticarcillin, and two of them were also

for cefoxitin (22%) and penicillin and tobramycin (17%). Meropenem/imipenem and ciprofloxacin resistance were also found among *Bacillus* isolates. Four Gram-positive producing isolates (17%) were multidrug resistant (MDR). With respect to Gram-negative, 33% of them were susceptible to all the antibiotics tested (all belonging to *Pseudomonas* genus). Four *Pseudomonas* spp. showed resistance to ticarcillin, and two of them were also resistant to aztreonam. Moreover, two Gram-negative isolates were resistant to ampicillin and cefoxitin, one of them being MDR (*Olivibacter* X7265) (Table 4).

Table 4. Antimicrobial resistance phenotype of the selected 32 antimicrobial-producing isolates obtained in the second screening.

Type of Bacteria	Number of Isolates	Genus	Species [a]	Antimicrobial Resistance Phenotype [b]
Gram-positive	1	*Arthrobacter*	*A. citreus*	Susceptible
	7	*Bacillus* spp.	*B. pumilus* [2], *B. safensis*, *B. megaterium*, *B. mycoides*, *Bacillus* spp. [2]	PEN [3]-FOX [4]-MER [3]-IMI [2]-S [2]-TOB [3]-CLI -GEN-SXT-CIP [3]
	7	*Bacillus* spp.	*B. pumilus* [3], *B. cereus*, *B. artrophaeus* [2], *B. safensis*	Susceptible [7]
	1	*Bradybacterium*	*Bradybacterium* spp.	Susceptible
	1	*Brevibacillus*	*B. laterosporus*	Susceptible
	1	*Microbacterium*	*M. arborescensis*	Susceptible
	2	*Paenibacillus*	*P. apiarus* [2]	PEN-FOX-TOB
	1	*Paenibacillus*	*P. polymyxa*	Susceptible
	1	*Staphylococcus*	*S. hominis*	Susceptible
	1	*Streptomyces*	*S. avidinii*	Susceptible
Gram-negative	1	*Klebsiella*	*K. aerogenes*	AMP-AMC-FOX
	1	*Olivibacter*	*O. soli*	AMP-FOX-CTX-CAZ-C-TOB
	4	*Pseudomonas*	*P. chlororaphis*, *P. caricapapayae*, *P. kilonensis* [2]	TIC [4]-ATM [2]
	3	*Pseudomonas*	*Pseudomonas* spp. [2], *P. kilonensis*	Susceptible

[a] Superscript 2, 3 indicates the number of isolates of each species. [b] Superscript 2, 3, 4, 7 indicates the number of isolates with a specific characteristic, when more than one.

4. Discussion

Soil contains a highly diverse collection of bacteria, making it a very attractive starting point for efforts to discover molecules with antimicrobial activity [17]. In this sense, the present work carried out a massive soil sampling due to the citizen collaboration of professors, teachers, university students and secondary education students, under the MicroMundo project.

Therefore, from a collection of 2600 bacteria, 132 putative antimicrobial producers were obtained in the first screening, which represent 5% of the total isolates recovered. When processing these samples in the laboratory during the second screening, 100 producers were lost, probably due to the stricter criteria of antibacterial effect verification at the university, considering only clear zones of inhibition as putative markers of bacteriocins. However, many other antimicrobial substances have been described apart from antimicrobial peptides with different phenotypes of inhibition halos not considered for this study. On the other hand, bacteriocins are known to be produced in response to signals received from a potential competitor, which then elicits an antagonistic response [18]. Therefore, in this study, the 32 isolates, which showed constant antimicrobial activity throughout the second screenings carried out, were selected for their subsequent characterization.

This work provides information on the soil biodiversity of bacteria with potential inhibitory capacity. Renyi profiles of La Rioja zones reveal a higher diversity in La Rioja Central, although a higher number of antimicrobial-producing isolates among the 132 firstly identified were detected among Logroño samples. In this regard, *Bacillus* (30%) and *Pseudomonas* (20%) were the most predominant genera, in accordance with what was observed by Huang et al., 2021 [19]. However, other genera were found in this study, such

as *Paenibacillus* or *Serratia*. These results highlight the potential of soil as a reservoir of bacteria that produce antimicrobial agents; thus, further characterization of isolates could be of interest.

In recent years, bacteria such as *Pseudomonas* spp. and *Bacillus* spp. have been studied and used as biological control agents for plant diseases [20,21], including the antibiosis mechanism for competition for nutrients and niches [22]. *Bacillus* is a genus well known as a producer of antibacterial substances such as lipopeptides, phenols, proteases, and bacteriocins [23]. Species of the genus *Pseudomonas* produce several secondary metabolites that affect other bacteria, fungi, or predators of nematodes and protozoa, such as bacteriocins, ranging from small microcin to large tailocin [24].

Thus, as expected, 14 *Bacillus* spp. and 7 *Pseudomonas* spp. out of the 32 bacteria finally selected as clear producers of antimicrobial substances were identified in this work. According to the *spot-on-lawn* results, higher activity was found against Gram-positive indicator bacteria than against Gram-negative indicator bacteria, being the *Staphylococcus* genera, (including MR-*Staphylococci*), the most susceptible indicator bacteria. It is widely known that most microbial metabolites have specific antimicrobial potential, and they act at the target sites [2]. Seven Gram-positive isolates showed antimicrobial activity against the Gram-negative indicators used. In addition, *Brevibacillus laterosporus* X7262 and *Staphylococcus hominis* X7276 stand out as high producers, which show antimicrobial activity against MS-staphylococci, *L. monocytogenes* and *M. luteus*.

Brevibacillus laterosporus is an aerobic, spore-forming, entomopathogenic microorganism commonly isolated from soil. Some strains have potential activity as biological control agents [25]. In addition, several applications of this bacterium as a biological control agent have been described, highlighting the high toxicity against mosquito larvae among other insects and the activity that promotes growth and improves productivity in bee colonies [26–28].

As for *S. hominis*, it is a normal skin commensal coagulase negative staphylococci (CoNS) described as a bacteriocin producer such as hominicin [29] and nukacin KQU-131 [30], among others. Moreover, recent studies have detected bacteriocin-like-producing staphylococci of environmental origin, including *S. hominis* [31]. Due to their high tolerance to an acidic environment, the resistance to bile, and the capacity to adhering to an epithelial cell line, *S. hominis* has been proposed as a good candidate for probiotic treatments against *S. aureus* [32]. In this sense, Nakatsuji et al., 2017 [33], reported that human *Staphylococcus* commensal species produce antimicrobial peptides that protect us against pathogens that control skin microbiota imbalances, and they demonstrated that a personalized probiotic CoNS cream could alleviate the symptoms of skin dysbiosis such as atopic dermatitis.

In short, advanced and combinatorial therapies that include antibiotics or new molecules with antimicrobial activity could be used as an alternative solution to combat AMR from a biotechnological and biomedical perspective and to solve problems in the agriculture and food industries, among others [34]. The one-health perspective makes clear the need for an ecosystem union to achieve improved objectives in the problem of AMR. Citizens must be integrated into this system, knowing the problem of the urgent need to find antimicrobial molecules, becoming aware of it, and contributing to research through this type of citizen science and service-learning initiatives such as MicroMundo.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics12010057/s1, Figure S1: Coordinates of all soil samples analyzed in this study. Each cluster is differentiated by colors; Figure S2: Renyi diversity profile of the antimicrobial-producing microbial community included in each of the following zones: La Rioja Central, La Rioja East, La Rioja West, Logroño and Outside; Table S1: Characteristics of the 15 indicator bacteria used in this study for the screening of antimicrobial activity production; Table S2: Antibiotics used for disk-diffusion test in Gram-positive and -negative antimicrobial-producer isolates (EUCAST); Table S3: Number of isolates included in each of the established regions based on their sampling location.

Author Contributions: C.T., B.R. and R.F.-F. designed the study; R.F.-F., B.R. and E.N. performed the experiments; R.F.-F. and B.R. made the first analysis of the data and prepared the draft of the paper. All authors have read and agreed to the published version of the manuscript.

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Supplemental material

Table S1. Characteristics of the 15 indicator bacteria used in this study for the screening of antimicrobial activity production.

Specie (no of strains)	Strain	Origin	Relevant Antimicrobial Resistance Phenotype/Genotype
***Staphylococcus aureus* (2)**	C1570 ATCC29213	Human	Methicillin/*mecA*
***Staphylococcus delphini* (1)**	C9459	Wild animal	
***Staphylococcus pseudintermedius* (2)**	C2381 C3468	Pet Pet	Methicillin/*mecA*
***Staphylococcus epidermidis* (1)**	C2663	Human	Methicillin/*mecA* and Linezolid
***Staphylococcus sciuri* (1)**	C9780	Wild animal	
***Enterococcus faecalis* (1)**	ATCC29212		
***Enterococcus faecium* (1)**	C2321		Vancomycin/*vanA*
***Enterococcus gallinarum* (1)**	C2310		
***Enterococcus. cecorum* (1)**	X3809		
***Listeria monocytogenes* (1)**	CECT4032		
***Micrococcus luteus* (1)**	C157		
***Escherichia coli* (1)**	ATCC25922		
***Pseudomonas aeruginosa* (1)**	C3282		

Table S2. Antibiotics used for disk-diffusion test in Gram-positive and Gram-negative antimicrobial producer isolates (EUCAST).

Type of bacteria	Genera	Antibiotic	Abbreviation
Gram-positive	*Arthrobacter*	Penicillin	PEN
	Bacillus	Cefoxitin	FOX
	Bradybacterium	Imipenem	IMI
	Brevibacillus	Meropenem	MER
	Microbacterium	Vancomycin	VAN
	Paenibacillus	Erythromycin	ERY
	Staphylococcus	Clindamycin	CLI
	Streptomyces	Tetracycline	TET
		Gentamicin	GEN
		Tobramycin	TOB
		Streptomycin	S
		Trimethoprim-sulfamethoxazole	SXT
		Ciprofloxacin	CIP
		Chloramphenicol	C
		Linezolid	LZD
Gram-negative	*Klebsiella*	Ampicillin	AMP
	Olivibacter	Amoxicillin-clavulanic acid	AMC
		Cefoxitin	FOX
		Cefotaxime	CTX
		Ceftazidime	CAZ
		Imipenem	IMI
		Tetracycline	TET
		Ciprofloxacin	CIP
		Trimethopim-sulfamethoxazole	SXT
		Gentamicin	GEN
		Tobramycin	TOB
		Chloramphenicol	C
	Pseudomonas	Ticarcillin	TIC
		Levofloxacin	LEV
		Meropenem	MER
		Aztreonam	AZT
		Ceftazidime	CAZ
		Ciprofloxacin	CIP

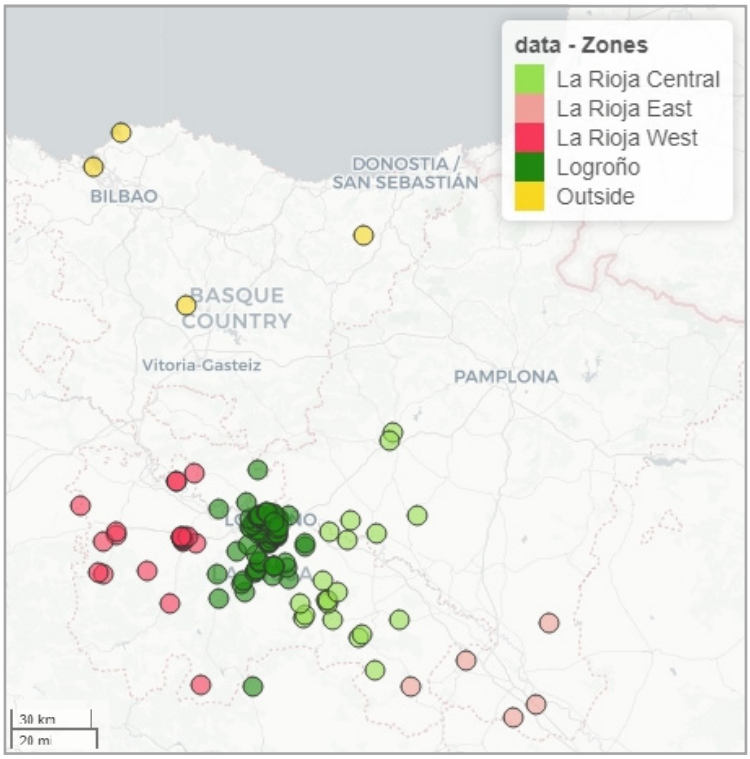


Figure S1. Coordinates of all soil samples analysed in this study. Each cluster is differentiated by colours.

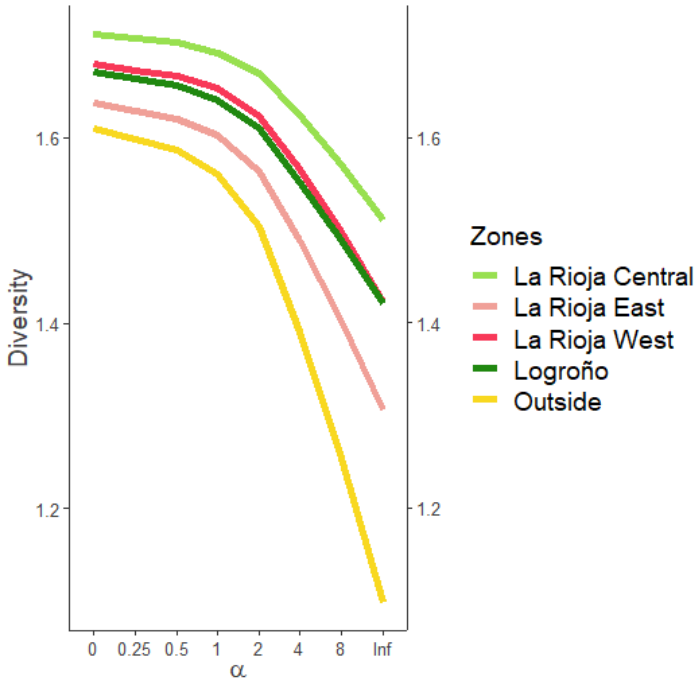
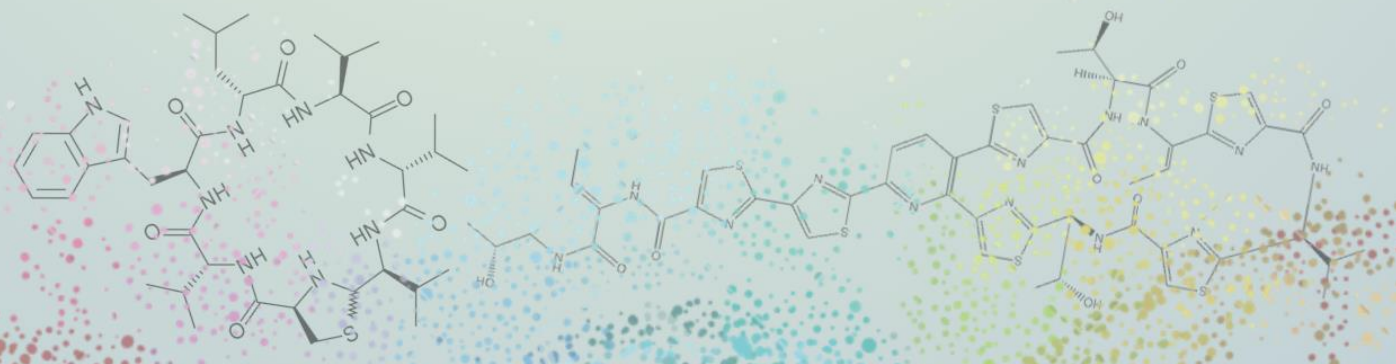


Figure S2. Renyi diversity profile of the antimicrobial-producing microbial community included in each of the following zones: La Rioja Central, La Rioja East, La Rioja West, Logroño and Outside.

Table S3. Number of isolates included in each of the established regions based on their sampling location.

	Total		Non-producing isolates		Producing isolates			
Cluster	Total isolates	No of samples	Non-producing isolates	No of samples	Producing isolates	No of samples	No of different Genera	No of different Species
La Rioja Central	400	20	378	12	22	8	7	12
La Rioja East	100	5	93	4	7	1	3	7
La Rioja West	420	21	400	12	20	9	4	11
Logroño	1580	79	1506	58	74	21	12	26
Outside	80	4	71	2	9	2	2	5
Total	2580	129	2448	88	132	41		

DISCUSIÓN



Discusión

1. Capítulo 1: Actividad antimicrobiana en el género *Staphylococcus*

¿En qué nichos se encuentran cepas de estafilococos productores de bacteriocinas y con qué frecuencia?

Los estafilococos son conocidos por su capacidad de producción de bacteriocinas **(Bastos et al. 2009; Janek et al. 2016)** y numerosos estudios han evaluado la actividad antimicrobiana en cepas de estafilococos de distintos orígenes detectando entre un 6% a 51% de cepas productoras **(Giambiagi-Marval et al., 1990; dos Santos Nascimento et al., 2005; Ceotto et al., 2009; Brito et al., 2011; Fagundes et al., 2011; de Souza Duarte et al., 2013; Braem et al., 2014).** Según nuestro conocimiento, los aislados de estafilococos procedentes de humanos y alimentos han sido los más estudiados y la reciente revisión sobre estafilococcinas incluida en esta tesis evidencia la escasez de trabajos centrados en la detección de estafilococcinas procedentes de animales de vida libre.

En base a ello, en esta tesis doctoral se ha llevado a cabo el mayor estudio, por nosotros conocido, de detección y caracterización de producción de actividad antimicrobiana en cepas de *Staphylococcus* (1205 aislados de 29 especies) aisladas a partir de orígenes muy diversos incluyendo animales de vida libre (mamíferos y aves silvestres), animales de compañía, alimentos, humanos y medio ambiente. Cabe destacar el estudio de actividad antimicrobiana en estafilococos procedentes de cigüeñas salvajes sanas nunca estudiadas previamente para la detección de bacteriocinas. De este modo, se detectaron 92 cepas productoras observándose los siguientes porcentajes de cepas Bac+ con respecto al número de aislados totales analizados en cada origen: animales de compañía (30%), humanos (29%), alimentos (6,1%), animales de vida libre (5,1%) y medio ambiente (1,5%). Asimismo, estos aislados productores representaron el 16,3%, 26%, 18,5%, 37% y el 2,2% de los 92 aislados Bac+, respectivamente.

Destaca la elevada tasa de producción de cepas Bac+ detectada entre los aislados de animales de compañía, que podría explicarse por el hecho de que todos los aislados analizados de este origen pertenecían a la especie *S. pseudintermedius*. Estudios previos describieron una cepa de *S. pseudintermedius* productora de bacteriocinas procedente de un caso de infección cutánea en animal de compañía (perro) **(Wladyka et al. 2015).** Otros estudios recientes han identificado cepas de estafilococos productoras de bacteriocinas en muestras procedentes de mascotas (gato), como es el caso de la micrococcina P1 descubierta por primera vez en *S. felis* **(O'Neill et al. 2021)**.

Asimismo, y como cabía esperar, la detección de actividad antimicrobiana en estafilococos de origen humano fue elevada en esta tesis (29%), identificándose un total de 24 cepas Bac+ de

origen humano, una cepa *S. pseudintermedius* que curiosamente se obtuvo de una muestra clínica de una persona que convivía con mascotas y 23 *S. lugdunensis*. Estudios previos llevados a cabo en saliva humana mostraron que la frecuencia de detección de estafilococos con actividad antimicrobiana fue de un 5,2% (**Nakamura et al. 1983**). Sin embargo, si se han notificado altas frecuencias de producción de bacteriocinas en estafilococos nasales de origen humano (80%) con actividad inhibitoria de interés frente a bacterias del ecosistema nasal como bacterias indicadoras **(Janek et al. 2016).**

¿Qué especies de estafilococos son más comúnmente productoras de bacteriocinas?

La producción de bacteriocinas ha sido reportada en el género *Staphylococcus* destacando *S. aureus* y *S. epidermidis* como especies altamente productoras de bacteriocinas generalmente pertenecientes a la clase II y clase I, respectivamente **(Bastos et al. 2009)**. Asimismo, se han encontrado otras cepas de estafilococos con actividad antimicrobiana de especies de SCoP (*S. hyicus* y *S. pseudintermedius*) y SCoN, destacando las siguientes especies: *S. capitis, S. cohnii, S. chromogenes, S. equorum, S. hominis, S. gallinarum, S. lugdunensis. S. simulans y S. warneri* **(Fernández-Fernández et al. 2023a).**

Atendiendo a las especies de SCoP, se han identificado cepas de *S. aureus* y *S. pseudintermedius* con actividad antimicrobiana de interés representando el 6,5% y 17,3%, respectivamente del total de las 92 cepas de estafilococos Bac+ detectadas. Tras la caracterización de 5 cepas de *S. aureus*, se ha observado la presencia de operones codificantes de bacteriocinas muy diversos incluyendo lantibióticos, la lactococcin972 y la micrococcina P1. Asimismo, se han detectado 3 cepas de *S. pseudintermedius* aisladas de una infección en humano por zoonosis con su mascota (perro) y portadoras de la bacSp222, bacteriocina descrita por Wladyka y colaboradores en 2015.

Por otro lado, esta tesis pone de manifiesto la capacidad de los SCoN, como productoras de bacteriocinas donde destacan las especies *S. chromogenes* (27%) y *S. warneri* (23,1%) con mayores porcentajes de aislados Bac+. Los porcentajes de aislados Bac+ en otras especies de estafilococos fueron menores: *S. hominis* (13,3%), *S. simulans* (10,3%), *S. epidermidis* (9,6%), *S. xylosus* (5,4%) y *S. sciuri* (4,3%). Algunas de estas especies han sido reportadas como productoras de bacteriocinas en muestras de leche, donde cabe destacar *S. chromogenes* (39%) y *S. epidermidis* (25%) como especies de mayor interés **(Rahmdel et al. 2019).**

Asimismo, *S. epidermidis* fue la quinta especie de CoNS con mayores tasas de actividad antimicrobiana en nuestro estudio, siendo los aislados Bac+ recuperados de alimentos (n=4) y de animales de vida libre (cigüeñas, n=1), aunque ninguno de los aislados de humanos analizados presentó actividad antimicrobiana. Por otra parte, *S. lugdunensis* es una especie de estafilococo comensal de la piel humana y estudios recientes han mostrado que puede producir un nuevo péptido antimicrobiano cíclico denominado lugdunina, que se incluye en una nueva clase de

antibacterianos debido a su síntesis no ribosomal (NRP) **(Zipperer et al. 2016)**. En este estudio se identificaron 23 aislados de *S. lugdunensis* Bac+ y uno de ellos (C9954) mostró una elevada actividad inhibidora frente a más del 70% de las bacterias indicadoras, incluidas cepas MDR, siendo un candidato interesante para una caracterización más profunda. Asimismo, cabe destacar que todas las cepas Bac+ portaron los genes que codifican la producción de lugdunina. Sin embargo, la frecuencia de producción no fue considerada en este caso, si bien se sabe que todas las cepas de *S. lugdunensis* incluidas en la base de datos portan los genes codificantes para la bacteriocina lugdunina.

En este sentido, el género *Staphylococcus* posee un gran potencial como reservorio de bacteriocinas siendo de especial interés analizar cepas de SCoN de diversas especies y aisladas en nichos diferentes. Habitualmente, las cepas SCoN son consideradas bacterias no patógenas y colonizadoras comunes, con alta capacidad para competir contra otras bacterias, incluyendo bacterias patógenas **(Janek et al. 2016; O'Sullivan et al. 2019).**

¿Cúal es el perfil de actividad antimicrobiana más frecuente en estafilococos?

En su gran mayoría las estafilococcinas muestran actividad inhibitoria frente a microorganismos patógenos muy diversos tales como *Streptococcus, Enterococcus, Corynebacterium, Bacillus,* y *Clostridioides,* entre otros **(de Freire Bastos et al. 2020).** Asimismo, se han descrito estafilococcinas con actividad frente a otras especies de estafilococos consideradas patógenas o patógenas oportunistas tanto de humanos como de animales, incluidas cepas de SASM y SARM e implicadas en una amplia gama de infecciones **(Kranjec et al. 2020; Van der Veken et al. 2020; Laux et al. 2019).**

El 7,6% de las cepas evaluadas en esta tesis presentaron actividad antimicrobiana frente a al menos uno de los indicadores G+ evaluados, incluyendo bacterias multirresistentes, y también bacterias con mecanismos relevantes de RAM o con interés zoonótico como *Staphylococcus* resistentes y sensibles a la meticilina, *Enterococcus* y *L. monocytogenes*, entre otros. En particular, fue especialmente relevante la actividad antimicrobiana detectada frente a *C. perfringens*, bacteria anaerobia y patógeno importante en la producción avícola **(Nhung et al. 2017)**. Finalmente, los aislados Bac+ no mostraron actividad antimicrobiana frente a bacterias G-, como *E. coli* y *P. aeruginosa,* resultado esperado teniendo en cuenta otros estudios previos sobre la actividad inhibitoria de las estafilococcinas **(Bastos et al. 2009; O´Sullivan et al. 2020; Lynch et al. 2019; Marques-Bastos et al. 2020).**

Aspectos por considerar en la producción de bacteriocinas

Existe gran diversidad de variables que influyen en las tasas de producción de bacteriocinas detectadas in vitro como son los métodos y condiciones de detección, los distintos orígenes y especies de los estafilococos evaluados, así como el tipo de bacteria indicadora empleada. Por ello, es necesario en muchos casos optimizar las condiciones de cultivo (tiempos de incubación y temperaturas) y los requisitos nutricionales. Ejemplo de ello lo encontramos en un estudio en el que se detectó una cepa de la especie *S. lugdunensis* que sólo producía lugdunina bajo condiciones limitantes de hierro y en medio sólido, por lo que no pudo ser detectada en el sobrenadante **(Zipperer et al. 2016).** En esta tesis también tuvimos problemas para detectar actividad antimicrobiana en el SLC de cepas de esta especie microbiana, siendo interesante estudiar en un futuro si los requerimientos nutricionales fueron la causa de ello.

La técnica empleada para la detección de actividad antimicrobiana es de gran relevancia. Con frecuencia, se emplean ensayos de difusión en agar mediante el uso de *spot-on-lawn* **(del Campo et al. 2001; Twomey et al. 2021).** Este análisis nos permite evaluar la capacidad de producción de sustancias antimicrobianas en multitud de posibles cepas productoras frente a una o varias cepas indicadoras de manera rápida y sencilla. Además, también se pueden emplear ensayos de difusión para evaluar la actividad de sobrenadantes libres de células (SLC) o extractos químicos obtenidos a partir de aislados productores. En este sentido nos podemos encontrar con que cepas que muestran actividad por uno de los métodos, por ejemplo, por "*spot-on-lawn*", no lo muestran empleando SLC o viceversa.

En esta tesis se presentan varias limitaciones que tienen que ver con la falta de equidad en el número de estafilococos de cada una las especies evaluadas y en sus orígenes. Asimismo, todos los ensayos para la detección de actividad antimicrobiana han sido realizados bajo condiciones estándar de cultivo en todas las técnicas empleadas. Todo ello puede influir en la tasa de detección de cepas productoras que por otro lado es difícilmente comparable entre sí por el tipo de selección de la muestra. No obstante, el abordaje llevado a cabo ha permitido identificar un gran número de cepas de estafilococos productoras procedentes de especies y orígenes de gran interés con potentes actividades inhibitorias frente a patógenos relevantes en salud pública y agentes zoonóticos.

2. Capítulo 2: Estudio genético de las cepas con actividad antimicrobiana

¿Qué herramientas tenemos para la detección genética de estafilococcinas?

La identificación de genes codificantes de bacteriocinas por medio de PCR permite la detección de genes codificantes de compuestos antimicrobianos de forma rápida, fácil y económica. Mediante PCR es posible detectar genes estructurales de bacteriocinas, entre otros genes presentes en el operón, siempre que sean conocidos y su secuencia esté disponible. Hasta la fecha, este

método se utiliza activamente para el cribado de bacterias productoras de lantibióticos **(Zimina et al. 2020)**, sobre todo centrado en la detección de bacteriocinas descritas en bacterias lácticas. Sin embargo, aunque el género *Staphylococcus* ha sido descrito como productor de bacteriocinas, existe una falta de información acerca de los protocolos y secuencias de cebadores necesarios para el estudio de estafilococcinas.

Con el objetivo de facilitar a la comunidad científica la detección y caracterización de bacteriocinas producidas por cepas de estafilococos, esta tesis doctoral presenta una recopilación de las estafilococcinas descritas hasta la fecha, y los genes que codifican dichas sustancias antimicrobianas (incluyendo acceso directo a GenBank y ProteinBank) **(Fernández-Fernández et al. 2023a).** Además, se ha llevado a cabo un análisis filogénetico de las secuencias genéticas y aminoacídicas de los genes estructurales estableciendo familias, así como cebadores y condiciones de reacción para poder identificarlas. Asimismo, se ha puesto de manifiesto la falta de uniformidad en cuanto a la clasificación de las bacteriocinas, por lo que esta tesis propone un sistema de clasificación de bacteriocinas basado en la relación filogenética de los genes estructurales de bacteriocinas tanto a nivel de nucleótidos como de aminoácidos.

Por otro lado, la secuenciación del genoma completo mediante las nuevas herramientas *in silico* se ha convertido en una tecnología importante para el descubrimiento de nuevos productos naturales con actividad antimicrobiana **(Morton et al. 2015)**. Recientemente se han descrito numerosas bases de datos basadas en la detección de genes estructurales o de otros genes asociados de bacteriocinas **(Egan et al. 2018),** entre las que se encuentran BACTIBASE, antiSMASH, BAGEL, APD3, ANTIMIC, DRAMP, o URMITE **(Blin et al. 2019; de Jong et al. 2006; Drissi et al. 2015; Wang et al. 2016; Kang et al. 2019).** En esta tesis se ha empleado antiSMASH y BAGEL por ser las bases de datos más usadas.

¿Es frecuente la detección de Operones Codificantes de Bacteriocinas (OCB) en estafilococos?

Algunos autores consideran que la producción de bacteriocinas es una característica común de las bacterias, ya que la mayoría de ellas pueden producir estos péptidos antimicrobianos **(Cotter et al. 2005)**. Además, se sabe que las cepas estafilocócicas pueden ser portadoras de uno o más grupos de genes responsables de la producción de bacteriocinas **(de Freire Bastos et al. 2020)**.

Los análisis de detección de genes codificantes de estafilococcinas llevados a cabo en esta tesis por PCR y/o secuenciación de los amplicones obtenidos, nos permitieron identificar 48 cepas portadoras de algún gen codificante de bacteriocinas. En concreto, se detectaron genes estructurales que codifican bacteriocinas de tipo lantibióticos en 20 aislados, tanto SCoN (n=12) como SCoP (n=8).

Además, el gen de la bacteriocina recientemente descrita BacSp222 **(Wladyka et al. 2015)** se detectó en tres de los aislados Bac+ *S. pseudintermedius*; sin embargo, este gen no se encontró en las otras especies de estafilococos analizadas en esta tesis ni en otros trabajos reportados en la bibliografía. Cabe destacar la detección del gen que codifica una bacteriocina circular similar a la uberolisina en dos de nuestros aislados Bac+ de *S. chromogenes* analizados. Las bacteriocinas circulares se han descrito comúnmente en *Bacillus* y rara vez se han identificado en especies de *Staphylococcus*, a excepción de la Aureocyclina 4185 descrita en *S. aureus* **(Potter et al. 2014)**. La detección de esta posible bacteriocina circular en dos aislados de *S. chromogenes* sería el primer reporte en esta especie de SCoN, lo que indica la posible transferencia de material genético entre especies estafilocócicas o la posible detección de una nueva bacteriocina. Cabe destacar que cuatro de nuestros aislados con actividad antimicrobiana alta (X3041, X3011 y C5835) o media (X2969) carecían de los 23 genes codificantes de bacteriocinas tras el análisis por PCR. Finalmente, los análisis de PCR y secuenciación confirmaron la presencia de *lugD* en el 100% de los aislados Bac+ de *S. lugdunensis* estudiados. Este gen es el precursor de la bacteriocina lugdunina y conforma el operón NRP con los otros cuatro genes denominados *lugA, B, C* y *D* **(Zipperer et al. 2016)**.

En esta tesis, se ha llevado a cabo, además, el estudio del genoma completo de 22 aislados de estafilococos de interés, seleccionados en base al criterio OneHealth tratando así de incluir cepas SCoP y SCoN, de distintas especies y orígenes. Se observó que en torno al 82% de los aislados de *Staphylococcus* productores de sustancias antimicrobianas (18 de 22 genomas) portaban al menos un gen codificante de bacteriocina entre los cuales se observó una gran diversidad. Asimismo, se ha puesto de manifiesto una interesante relación entre las especies de *Staphylococcus* relacionadas y el tipo de bacteriocina con independencia del origen del aislado (humano, mascota, animales salvajes, alimentos y ambiental). Dicha relación está en consonancia con los resultados obtenidos en otros estudios donde algunas bacteriocinas muestran claramente una estrecha relación con ciertas especies como la lugdunina-*S. lugdunensis* o la bacSp222-*S. pseudintermedius* **(Zipperer et al. 2016 y Wladyka et al. 2015)**. En este trabajo se identificaron cinco tipos de OCB entre 18 aislados productores de antimicrobianos, que codificaban bacteriocinas de clase I (lantipéptidos), clase II (lactococcina972, BacSp222 y bacteriocina de la familia blp), clase IV (bacteriocinas circulares), NRP (lugdunina) y tiopéptidos (micrococcina P1, MP1).

Cabe destacar la detección de lantibióticos y de la MP1 por su alta frecuencia en la colección y su elevado interés en la comunidad científica. Previamente se había descrito una gran variedad de lantibióticos en aislados de *Staphylococcus* que mostraban actividad antimicrobiana exclusivamente frente a bacterias G+ **(Laux et al. 2019)**, como por ejemplo en el genoma de *S. capitis* **(Kumar et al. 2017)**. Entre los lantibióticos destacan la epidermina, epilancinas y diversas

nukacinas, entre otros **(Laux et al. 2019)**. Sin embargo, aunque la producción de MP1 en aislados de estafilococos ha sido descrita en *S. equorum* procedente de queso **(Carnio et al. 2000)**, *S. epidermidis* **(Bennallack et al. 2014)**, *S. felis* procedente de gatos **(O'Neill et al. 2021)**, *S. hominis* recuperado de piel humana **(Liu et al. 2020)**, *S. sciuri* **(Van der Veken et al. 2022)** y recientemente *S. aureus* **(Krauss et al. 2023),** su detección en este género no es tan frecuente. Por ello, resulta de especial interés que en esta tesis se haya detectado por WGS del tiopéptido MP1 en cuatro cepas Bac+ (~18%), dos aislados de muestras ambientales (*S. aureus* y *S. hominis*) y dos aislados de *S. sciuri* recuperados de carne cruda de pollo.

¿La presencia de genes u operones codificantes de bacteriocinas (OCB) implica la producción del compuesto?

Gracias a las nuevas técnicas de secuenciación masiva podemos detectar la presencia de operones con potencialidad para codificar bacteriocinas (OCB). Sin embargo, es posible que: (i) una cepa posea más de un OCB; (ii) no exprese ningún OCB; (iii) porte varios OCB pero sólo exprese uno de ellos; (iv) tenga expresión condicionada por el fenómeno de quorum sensing.

Atendiendo a los resultados de esta tesis, los ensayos a nivel genético han permitido detectar en muchos casos genes estructurales de bacteriocinas, aunque no podemos descartar que otros genes bien no detectados en este estudio o no descritos, pudieran ser responsables de la actividad antimicrobiana. Para ello, es importante complementar estos estudios con ensayos que nos permitan obtener extractos puros o prepurificados del compuesto antimicrobiano, así como analizar la posible presencia de bacteriocinas conocidas gracias a técnicas como la espectrometría de masas.

Asimismo, es posible que aislados sin actividad antimicrobiana posean genes codificantes de bacteriocinas. Este fenómeno ha sido evidenciado en esta tesis en los aislados de *S. lugdunensis*. En este sentido, el gen *lugD* se detectó en los 23 aislados Bac+, pero 24 de los 33 aislados Bac- fueron positivos para el gen *lugD.* Se sabe que este operon está presente en todos los genomas de *S. lugdunensis* disponibles en las bases de datos **(Zipperer et al. 2016)**. Sería interesante analizar en el futuro la relación entre la presencia/expresión de este operón y la actividad antimicrobiana de los aislados de *S. lugdunensis* **(Fernández-Fernández et al. 2022b)**.

Por otro lado, la no detección de genes codificantes de bacteriocinas puede deberse a pequeñas mutaciones en la región del cebador (en el caso de emplear la técnica PCR) o a la presencia de nuevas bacteriocinas no descritas ni recogidas en las bases de datos genéticas (si se emplea la secuenciación masiva). Por todo ello, la secuencia de estudio tradicional, basada en el cribado de aislados con actividad antimicrobiana ha de ser considerada **(Carson et al. 2017)**. Según esto, se debe llevar a cabo una caracterización bioquímica y genética en detalle para comparar con los

datos existentes sobre las bacteriocinas ya reportadas en la bibliografía y confirmar si se trata de la detección de variantes o nuevas moléculas con capacidad antimicrobiana.

¿Son idénticos todos los OCB que codifican la misma bacteriocina? Implicaciones de la detección de variantes de bacteriocinas o bacteriocinas nuevas

El estudio de genes codificantes de estafilococinas por PCR y en algunos casos gracias a la secuenciación masiva, , permitió confirmar el tipo de bacteriocina producida en muchas de las cepas Bac+ y en otras se observó la posible presencia de variantes que pueden resultar de gran interés para estudios futuros. En cuanto a las bacteriocinas de clase I predichas en esta tesis (lantibióticos), se sugiere la detección de nuevas variantes de las bacteriocinas BSA y epilancina15X. Se consideró posible variante a aquellos OCB cuyo gen estructural presentó menos del 90% de identidad con las bacteriocinas disponibles en las bases de datos, pero mantuvo la estructura y secuencia de genes. Además, en el genoma de *S. hyicus* C9585 se detectó un nuevo OCB de clase I que mostraba similitud con los lantibióticos de tipo (V) descritos muy recientemente **(Pei et al. 2022; Xu et al. 2020)**. Curiosamente, dos nuevos OCB fueron detectados en los aislados Bac+ *S. warneri* y *S. epidermidis* los cuales parecen codificar bacteriocinas de tipo lantibiótico que han de ser estudiados en trabajos futuros optimizando las condiciones de extracción para descifrar la estructura del péptido. En este sentido, el uso de sistemas de expresión heterólogos por medio de la transferencia del OCB en estudio a una cepa no productora es importante para la futura validación de los estudios de cribado *in silico* **(Piper et al. 2011; van Heel et al. 2016; Mesa-Pereira et al. 2017)**. Frecuentemente, este proceso se lleva a cabo mediante la generación de clones que adquieren por transducción un plásmido portador de OCB.

Se identificaron por WGS la bacteriocina de clase II bacsp222 y el NRPs lugdunina en tres cepas de *S. pseudintermedius* y dos *S. lugdunensis*, respectivamente. En ambos casos, las secuencias fueron idénticas a las previamente descritas en la literatura **(Wladyka et al. 2015; Zipperer et al. 2016)**. No se encontró ninguna cepa productora de bacteriocinas de clase III, pero sí se identificaron tres nuevos OCB que codifican bacteriocinas circulares de la familia circularina A/uberolisina y aureocyclicina 4185 potencialmente nuevas ya que presentaron baja similitud con las estafilococcinas circulares descritas anteriormente **(Fernández-Fernández et al. 2023c).**

Finalmente, cabe destacar la detección de MP1 en cuatro cepas que presentaron muy alta actividad antimicrobiana (*S. aureus*-C5802; *S. hominis*-C5835, *S. sciuri*-X3041 y *S. sciuri*-X3011). Estas cepas inhibieron a más del 80% de indicadores G+ analizados y presentaron grandes halos de inhibición. Curiosamente, fueron negativas para los ensayos de detección de bacteriocinas por PCR, lo que evidencia que el cribado previo por métodos fenotípicos es de gran importancia. Los operones de las cuatro cepas fueron muy semejantes entre sí y con los descritos para otros

estafilococos y también con *Macrococcus caseolyticus*, aunque mostraron claras diferencias con el OCB previamente descrito en *Bacillus* **(Fernández-Fernández et al. 2023c).**

En definitiva, esta tesis hace patente la importancia de conocer los OCB pudiendo identificarse nuevas bacteriocinas o variantes de las ya conocidas. Se deben realizar comparativas entre los operones encontrados y las secuencias disponibles en las bases de datos con el objetivo de dilucidar el grado de similitud. No obstante, debemos tener siempre presente que pequeños cambios en la secuencia pueden afectar a la estructura del compuesto dificultando en ese caso su detección por espectrometría de masas. En cualquier caso, se necesitan criterios únicos que ayuden a delimitar cuando un compuesto se considera idéntico, variante o nueva bacteriocina.

3. **Capítulo 3: Caracterización de la actividad antimicrobiana de bacteriocinas pre-purificadas y de las cepas productoras**

   ¿Es frecuente detectar compuestos con actividad antimicrobiana en el sobrenadante? La importancia de recurrir a varias técnicas para evaluar la actividad inhibitoria

Los SLC obtenidos a partir de cultivos microbianos han sido conocidos durante décadas por su efecto inhibitorio frente al crecimiento de otras bacterias **(Frickmann et al. 2018).** Se ha observado que los estafilococos secretan sustancias con actividad antimicrobiana muy diversas que pueden detectarse en el SLC, pero su producción y excreción son altamente dependientes de la especie, la cepa, así como de las condiciones de incubación **(Frickmann et al. 2018)**. Algunos estudios llevados a cabo con aislados de SCoN pusieron de manifiesto que la mayoría de las proteínas con actividad antimicrobiana (en torno al 80%) se encuentran en gran cantidad en el interior del citoplasma celular y no son apenas detectadas en el SLC. Por ello, normalmente se requiere de la extracción de bacteriocinas mediante métodos físico-químicos **(Jang et al. 2020)**.

En esta tesis, se ha evaluado la capacidad de inhibición tanto de los SLC como de los extractos obtenidos mediante extracción química con butanol (BT) de 37 cepas Bac+. De este modo, se han detectado 9 aislados de estafilococos con actividad inhibitoria en sus SLC frente al menos uno de los indicadores probados. Además, >90% de los extractos de BT mostraron actividad antimicrobiana frente al menos uno de los indicadores evaluados. Esto indica que muchos de los compuestos antimicrobianos estaban presentes sólo en cantidades bajas en los sobrenadantes de cultivo, y sus actividades sólo eran detectables después de la extracción química (**Frickmann et al. 2018; Zipperer et al. 2016**). Este fenómeno es ampliamente conocido por la comunidad científica sabiendo que, en muchos casos, la cantidad de la sustancia antimicrobiana obtenida en el SLC puede ser muy pequeña e indetectable **(Twomey et al. 2021).**

Los extractos de butanol además se sometieron a espectrometría de masas con el fin de intentar detectar e identificar la bacteriocina responsable de la actividad antimicrobiana. Se identificó solo una bacteriocina en 4 aislados de *Staphylococcus* que eran altamente productores (14%) y resultó ser la bacteriocina MP1.

¿Cómo se comportan las bacterias patógenas en presencia de estafilococcinas o de cepas Bac+?

Para evaluar como se comportan las bacterias patógenas en presencia de estafilococcinas, se recurre con frecuencia a analizar cómo evolucionan a lo largo del tiempo en presencia de SLC o de extractos químicos procedentes de la cepa Bac+ de interés (curvas de crecimiento y muerte). Sin embargo, para analizar el comportamiento de los patógenos en presencia de bacterias Bac+ se deben llevar a cabo estudios de competencia **(Concepción-Acevedo et al. 2015, Gallet et al. 2012).**

En esta tesis, la actividad antimicrobiana de los extractos BT de las cepas productoras de MP1 fue ensayada en medio líquido frente a dos cepas indicadores, SARM-CC398 y *L. monocytogenes.* Tras 24 horas de incubación, se observó como ambos indicadores presentaron claramente un menor crecimiento en presencia de diferentes concentraciones del extracto cuando se comparaban con el control (cepa indicadora sin extracto). Además, en ambas cepas indicadores las curvas de crecimiento sufrieron un retraso cuando estaban en presencia del extracto. Por otro lado, las curvas de muerte evidenciaron el efecto bacteriostático de la MP1. Entre las cepas productoras de MP1 nos encontramos con aislados pertenecientes a la especie *S. sciuri*. Aunque sólo unos pocos estudios han demostrado capacidad inhibitoria en aislados de *S. sciuri* **(Fernández-Fernández et al. 2022a; Van der Veken et al. 2022),** las relevantes actividades antimicrobianas proporcionadas en este trabajo destacan a esta especie de SCoN como altamente productora de compuestos antimicrobianos con aplicaciones prometedoras.

Se realizaron además, estudios comparativos de la actividad antimicrobiana de los extractos de BT no purificados de cepas productoras de MP1 con la bacteriocina comercial nisina. Cada vez más, la nisina es utilizada en la industria alimentaria **(Ross et al. 2002)** ya que presenta gran capacidad inhibitoria frente a bacterias G+, como *L. monocytogenes* y *Clostridium botulinum* formador de esporas **(Gharsallaoui et al. 2016)**. Dado que la nisina es la única bacteriocina aprobada como conservante alimentario a una concentración no superior a 12,5 mg de nisina pura por kilogramo, se seleccionó esta bacteriocina para llevar a cabo este trabajo lo que permitió evidenciar la intensa actividad antimicrobiana de los extractos BT obtenidos en el trabajo incluido en esta tesis.

Por otro lado, en esta tesis, se quiso estudiar el potencial inhibitorio a lo largo del tiempo de cepas de estafilococos productoras de bacteriocinas frente a un patógeno de relevancia en salud pública.

En el trabajo presentado en el capítulo 3 se escogieron los cuatro aislados productores de MP1 (2 *S. sciuri*, 1 *S. hominis* y 1 *S. aureus*) por ser una de las bacteriocinas más frecuentes y mejor caracterizadas a lo largo de esta tesis y por despertar el mayor potencial como agente antimicrobiano. Por otro lado, se seleccionó como indicador al agente zoonótico SARM-CC398 con fenotipo de multirresistencia. Además, para considerar las diferencias metabólicas y de adaptación dependientes de la especie, se estudió la competencia entre el indicador SARM y aislados no productores de bacteriocina (1 *S. sciuri*, 1 *S. hominis* y 1 *S. aureus*). De este modo, los estudios de competencia célula-célula permitieron demostrar el fuerte efecto inhibitorio de las cepas productoras de bacteriocinas frente a SARM **(Fernández-Fernández et al. 2023d).**

En este sentido, se ha confirmado que algunos aislados de SCoN parecen competir con *S. aureus* por la colonización estable de los reservorios de piel humana mediante la producción de factores antimicrobianos **(Nakatsuji et al. 2017; Iwamoto et al. 2019; Lee et al. 2019).** Asimismo, este gran efecto inhibitorio fue observado por Zipperer y colaboradores en 2016 en donde revelaron que una cepa de *S. lugdunensis* con actividad antimicrobiana inhibía el crecimiento de *S. aureus* de forma eficiente, tanto que, tras 72 horas, dicho indicador no pudo ser recuperado en placa incluso partiendo de inóculos bacterianos superiores.

¿Y si combinamos agentes antimicrobianos? La importancia del efecto sinérgico

Si bien las bacteriocinas tienen aplicaciones potenciales por sí solas, su combinación con antibióticos clásicos ha sido propuesta como una alternativa viable y eficaz a la resistencia y se espera que tenga importantes efectos beneficiosos **(Mathur et al. 2017).**

Es posible que las combinaciones sinérgicas de bacteriocinas y antibióticos puedan reducir la probabilidad de desarrollo de resistencias, disminuyendo los efectos adversos y ampliando el espectro de actividad **(Orhan et al 2005; Soltani et al. 2012; Sopirala et al. 2010).** En este sentido, el ensayo checkerboard ha revelado efectos sinérgicos para la combinación del extracto BT del aislado *S. sciuri* X3041 productor de MP1 frente a la cepa indicadora SARM-C1570 con los siguientes antibióticos: clindamicina (> 80% de las combinaciones ensayadas) y oxacilina (30%), considerando concentraciones antibióticas de 2-625 μg/mL y 1-2 μg/mL, respectivamente. Además, la combinación BT-cloranfenicol mostró sinergismo sólo a concentraciones antibióticas de 15,6 μg/mL y valores de casi sinergismo en las concentraciones 7,8 y 31,3 μg/mL. Estudios recientes reportaron sinergismo entre MP1 y rifampicina mostrando que esta combinación antibiótico-bacteriocina era efectiva no sólo para erradicar sino también para prevenir infecciones recurrentes **(Ovchinnikov et al. 2021).**

4. **Capítulo 4: Bacteriocinas y cepas productoras como alternativa eficaz en el sector agroalimentario y en salud pública**

¿Qué posibles aplicaciones tienen las cepas Bac+ detectadas?

Debido al creciente problema de la resistencia a antibióticos se ha propuesto el uso tanto de bacteriocinas como de los cultivos protectores como alternativas eficaces para combatir patógenos en diferentes aplicaciones agroalimentarias y en salud pública. *Staphylococcus* es un género de interés por ser ubicuo y metabólicamente versátil, además de ser conocido como productor de estafilococcinas.

Atendiendo a las aplicaciones veterinarias, las bacteriocinas o las cepas productoras han sido propuestas para tratar o prevenir infecciones en el ganado. Entre ellas, cabe destacar la mastitis bovina, considerada una de las enfermedades más prevalentes e investigadas **(Narayama et al. 2018; Ruegg 2017).** En este sentido, un reciente estudio *ex vivo* propone el uso de la bacteriocina A53 frente a patógenos aislados de mastitis bovina evidenciando que, tras 24 h de incubación, la aureocina A53 provocó una fuerte reducción en las poblaciones de estafilococos o estreptococos, respectivamente, eliminando todas las células viables detectables **(Marques-Bastos et al. 2023).** Por otro lado, es de gran interés solventar la problemática derivada de la transmisión nosocomial de *S. aureus* y especialmente de cepas SARM pertenecientes al linaje CC398 entre el ganado porcino y el ser humano. En base a ello, **Wei y colaboradores (2023)** han confirmado que la microbiota de la piel porcina, incluyendo bacterias productoras de sustancias antimicrobianas, actua como barrera protectora frente a la colonización de SARM.

En cuanto a los estafilococos, en esta tesis se ha confirmado el potencial de 15 cepas de *Staphylococcus* productores de bacteriocinas o sus estafilococcinas como agentes antimicrobianos o antifúngicos que pueden ser considerados como buenos candidatos en áreas de interés entre las que destacamos la industria láctica, los animales de producción, la industria alimentaria y la agricultura. Dentro de ellos, son de especial interés los SCoN Bac+ sin mecanismos de resistencia a antibióticos ni factores de virulencia. Además, se ha detectado cinco consorcios bacterianos formados por cepas productoras de lantibióticos (epilancin15X, BSA/BacCh91, lantipeptido de tipo V) y la cepa de *S. hominis* productora de MP1, que pueden ser de interés en estudios futuros potenciando su actividad antimicrobiana frente a patógenos relevantes como SARM o *L. monocytogenes.*

El uso de comunidades bacterianas para prevenir la colonización bacteriana se ha propuesto como alternativa reciente para evitar la infección por SARM y se están observando resultados prometedores en modelos murinos **(Wei et al. 2023).** En este sentido, las comunidades bacterianas con papel protector pueden ser prometedoras en otras áreas de aplicación (producción animal, humanos o agricultura). Además, sería interesante estudiar el efecto combinado de bacterias Bac+ o de bacterias y bacteriocinas frente a un patógeno, con el fin de conocer los posibles efectos potenciadores de sus respectivas capacidades inhibitorias, así como sus cambios metabólicos.

¿Por qué evaluar el contenido de mecanismos genéticos asociados a la resistencia, virulencia y EGM en cepas productoras?

Actualmente, se plantea el uso de bacterias con actividad antimicrobiana por su amplio potencial en aplicaciones biotecnológicas o como moduladores de la microbiota original. Para ello, se ha de garantizar la seguridad y ausencia de citotoxicidad en aquellas cepas candidatas a un posible uso profiláctico o terapéutico. En este contexto, se debe asegurar la ausencia de mecanismos de resistencia a antibióticos adquiridos o la falta de contenido en factores de virulencia **(Eveno et al. 2021)**. Asimismo, es importante evaluar el contenido en elementos genéticos móviles de las cepas de interés. Esto, permite conocer la presencia de plásmidos, islas de patogenicidad o transposones, evaluando el contenido genético con potencialidad a ser transferido entre bacterias de forma horizontal.

En esta tesis se ha evidenciado la presencia de genes de resistencia a antibióticos, en muchos casos intrínsecos de especie y, por tanto, sin capacidad de transferencia horizontal. En cuanto al fenotipo y genotipo de resistencia de las cepas Bac+ identificadas en esta tesis, se detectó un 25,7% de cepas SCoN Bac+ sensibles a todos los antibióticos estudiados y un 27,3% para los aislados SCoP Bac+. Además, es de especial interés que ninguno de los aislados SCoN portó genes asociados a mecanismos de virulencia frecuentes en *S. aureus*, si bien fueron detectados en cepas SCoP productoras. Sin embargo, aunque las cepas de SCoN carecen de muchos de los factores de virulencia comunes de *S. aureus*, conviene puntualizar que se han identificado otros mecanismos de virulencia en este grupo estafilocócico **(França et al. 2021).** Asimismo, queda pendiente estudiar la posible vehiculización de estos operones en plásmidos u otros elementos genéticos móviles para lo cual se recurrirá en un futuro a tecnologías de secuenciación de tercera generación como PacBio **(Rhoads y Au, 2015)** o Oxford Nanopore (ONT) **(Lu et al. 2016).**

Importancia de los estafilococos como moduladores de la microbiota

El uso de bacterias como probióticos o cultivos protectores es un área muy prometedora como alternativa al uso de antibióticos de gran interés en salud pública **(Khusro et al. 2019).** Para su posible aplicación resulta de gran importancia evaluar la composición de las diferentes comunidades bacterianas y cómo se comportan las cepas objeto de estudio tanto de forma individual como en cultivos mixtos **(Chevin et al. 2011).**

Según la composición de la microbiota, cada comunidad refleja unas condiciones de nicho específicas, y se han descrito asociaciones entre especies y lugares concretos **(Wei et al. 2022).** Por otro lado, está ampliamente aceptado que las moléculas antibacterianas que inhiben a los principales competidores microbianos tienen un papel especialmente importante en la conformación del microbioma **(Krismer et al. 2017; Lewis y Pamer, 2017; García-Bayona y Comstock, 2018).** En este sentido, se ha informado de la potente actividad antimicrobiana en

estafilococos nasales de origen humano (80%) frente a bacterias del ecosistema nasal **(Janek et al. 2016).**

Por ejemplo, se ha descrito que *S. lugdunensis* nasal puede prevenir la colonización por *S. aureus* mediante la producción de un compuesto antimicrobiano inusual, anteriormente indicado, la lugdunina **(Zipperer et al. 2016).** Además, se ha informado de que *S. epidermidis* es un inhibidor de *S. aureus*, aunque no existe una correlación clara entre la ausencia de *S. aureus* y la presencia de *S. epidermidis* **(Bierbaum y Sahl, 2009; Iwase et al. 2010; Yan et al. 2013).** Estos estudios se han centrado en las interacciones de las bacterias cohabitantes de la microbiota nasal de humanos y en los efectos de las bacterias productoras de bacteriocinas sobre la microbiota. Un estudio reciente en animales ha evidenciado el potencial de la microbiota de la piel porcina como reservorio poco explorado de especies comensales cutáneas con potencial para prevenir la colonización y la infección por SARM, patógeno responsable de la causa más común de infecciones de piel y tejidos blandos **(Wei et al. 2023).**

En esta tesis, se propone de manera novedosa el modelo cigüeña para conocer los mecanismos que rigen la diversidad bacteriana en medios naturales y animales de vida libre, entre los cuales cabe destacar la producción de bacteriocinas. Los resultados han revelado la fuerte capacidad inhibidora inter-muestra de los estafilococos productores. Especialmente la cepa Bac+ de *S. hominis* X3764 podría actuar como modulador de la microbiota, ya que inhibió a más del 73% de la comunidad G+ representativa de la microbiota nasotraqueal de cigüeñas (30 cepas y 29 especies diferentes).

Por otra parte, estudios recientes con aislados productores de antimicrobianos indican que una bacteriocina específica puede no afectar por igual a todos los miembros de la microbiota y afectar solo a los que se encuentran más próximos físicamente. Por lo tanto, los aislados productores de bacteriocinas desempeñan un papel importante en la competencia y colonización de nichos, lo que confiere al productor una superioridad ecológica frente a otros componentes del microbioma sin compuestos bioactivos naturales que permite el mantenimiento de comunidades estables y puede conducir a la redistribución de los miembros del microbioma en sub-nichos **(Heilbronner et al. 2021).**

En base a esto, se evaluó también la actividad inhibitoria intra-muestra determinando si las muestras con presencia de cepas Bac+ eran más o menos diversas y la actividad de las bacterias Bac+ frente a sus cohabitantes G+. Sólo tres de los 9 estafilococos Bac+ detectados en esta tesis procedentes de muestras nasotraqueales de cigüeñas (*S. hominis* X3764, *S. simulans* X4520 y *S. chromogenes* X4620) inhibieron una de las bacterias G+ aisladas en sus respectivas muestras (una *S. sciuri* y cuatro aislados de *Macrococcus caseolyticus*). Centrándonos en *S. hominis* X3764,

reveló actividad intra-muestra frente a una de las dos cepas de *S. sciuri* recuperadas en la misma muestra nasotraqueal y no inhibió a *E. faecalis*.

5. Capítulo 5: El suelo como fuente de bacteriocinas y medio de divulgación

La perspectiva OneHealth deja clara la necesidad de la unión entre diferentes ecosistemas para abordar el problema de la resistencia a antibióticos. Entre estos ambientes, el suelo posee un enorme potencial para encontrar nuevos compuestos antimicrobianos. En este sentido, muchos de los antibióticos conocidos son producidos por microorganismos aislados del suelo, desde la penicilina, el primero del que se tiene constancia, hasta algunos nuevos como las malacidinas y la teixobactina **(Hover et al. 2018).**

Por otro lado, diferentes trabajos han evidenciado la falta de conocimiento o confusión en la población general sobre la situación actual de la problemática de resistencia a antibióticos y el uso correcto de antibióticos **(Haltiwanger et al. 2001, Keselman et al. 2004, Romine et al. 2013, Robredo y Torres, 2021).** Por ello, los ciudadanos deben integrarse en este sistema, para conocer la urgente necesidad de encontrar moléculas antimicrobianas y contribuir a su solución a través iniciativas de ciencia ciudadana y aprendizaje-servicio.

¿Es frecuente detectar bacterias con actividad antimicrobiana en el suelo? ¿cuáles son los géneros más productores?

Las bacterias del suelo viven en un entorno altamente competitivo, con recursos limitados y condiciones en constante cambio. No obstante, el suelo alberga una gran biodiversidad y biomasa de microorganismos **(Curtis et al. 2002; Schloss y Handelsman, 2006)**. Para asegurar su supervivencia, los microorganismos presentes en el suelo se han adaptado desarrollando capacidades diversas destacando la producción de metabolitos secundarios entre las que se incluyen las bacteriocinas.

En esta tesis se ha evaluado la actividad antimicrobiana de las bacterias del suelo aisladas en el proyecto MicroMundo, y se ha estudiado también la biodiversidad de los aislados seleccionados y su perfil de resistencia a los antimicrobianos. Además, se trató de ilustrar el relevante vínculo entre ciencia y educación y los beneficios de aplicar metodologías de aprendizaje-servicio para concienciar sobre la RAM y contribuir a la búsqueda de nuevas alternativas. Los resultados obtenidos en el trabajo incluido en esta tesis ponen de manifiesto la gran biodiversidad de bacterias edáficas con potencial capacidad inhibitoria y el potencial del suelo como reservorio de bacterias productoras de agentes antimicrobianos, por lo que una mayor caracterización de los aislados podría ser de interés **(Fernández-Fernández et al. 2022c)**. Los géneros más predominantes fueron *Bacillus* (30%) y *Pseudomonas* (20%) de acuerdo con lo observado por

Huang y colaboradores en 2021 si bien se encontraron otros géneros en menor abundancia, como *Paenibacillus* o *Serratia*.

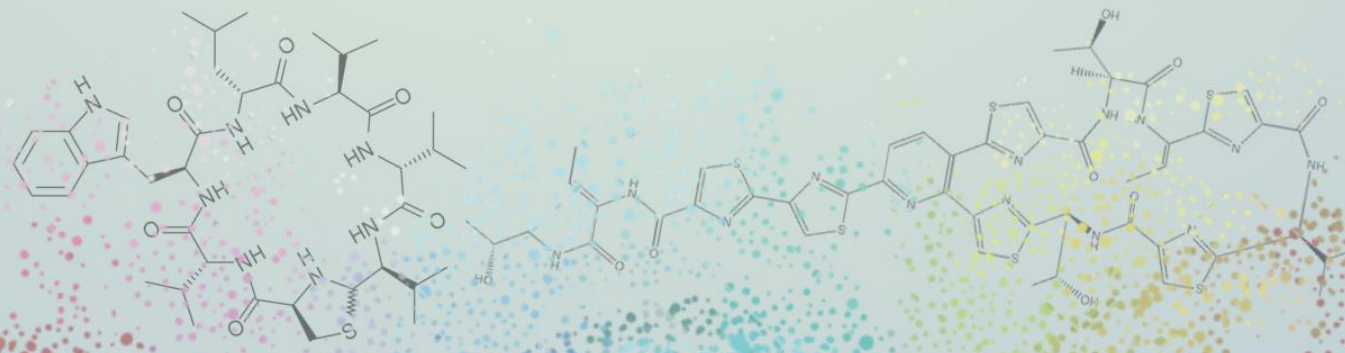
Así, se identificaron 14 *Bacillus* spp. y 7 *Pseudomonas* spp. productoras de sustancias antimicrobianas. En los últimos años, bacterias pertenecientes a ambos géneros han sido estudiadas y utilizadas como agentes de control biológico de enfermedades de plantas **(Chowdhury et al. 2015; Balthazar et al. 2022),** incluyendo la actividad antimicrobiana como mecanismo de competencia por nutrientes y nichos **(Dimkić et al. 2022).** *Bacillus* es un género bien conocido como productor de sustancias antibacterianas como lipopéptidos, fenoles, proteasas y bacteriocinas **(Zhao et al. 2022; Sumi et al. 2015).** Las especies del género *Pseudomonas* producen varios metabolitos secundarios que afectan a otras bacterias, hongos o depredadores de nematodos y protozoos, como las bacteriocinas, que van desde la pequeña microcina hasta la gran tailocina **(Ghequire y De Mot, 2014).**

Se observó actividad antimicrobiana frente a bacterias indicadoras G+ (mayoritariamente) y frente G-, siendo el género *Staphylococcus*, (incluyendo resistentes a meticilina), las bacterias indicadoras más sensibles. Además, las cepas *Brevibacillus laterosporus* X7262 y *Staphylococcus hominis* X7276 destacaron como altos productores, mostrando actividad antimicrobiana frente a estafilococos sensibles a meticilina, *L. monocytogenes* y *M. luteus*. En este sentido es de especial interés la detección de una cepa SCoN Bac+ procedente de suelo que ha de ser caracterizada en un futuro por su gran potencial en aplicaciones biotecnológicas y en salud pública.

6. Bacterias ¿problema o solución?

Por todos es sabido que la resistencia a los antimicrobianos se ha convertido en un problema global de alta prioridad. Además, numerosos estudios han evidenciado que la diseminación de bacterias resistentes es cada vez más frecuente en ambientes naturales como el suelo, aguas superficiales y residuales, y animales de vida libre lo que evidencia el estado crítico de salud al que se enfrenta la comunidad científica. En este sentido, la comunidad científica considera como una de las necesidades prioritarias encontrar alternativas eficaces a los antibióticos. Entre las propuestas, destacan las bacteriocinas y concretamente aquellas producidas por bacterias no patogénicas. Esta tesis ha permitido evidenciar la presencia de estafilococos productores en orígenes muy diversos escogidos en base a la perspectiva OneHealth, permitiendo proponer tanto a las cuatro cepas productoras de MP1 como a la bacteriociona como alternativas prometedoras a los antibióticos. Por otro lado, se han detectado actividades antimicrobianas de interés en aislados SCoN productores de bacteriocinas muy diversas, incluyendo variantes y nuevas moléculas. Asimismo, las cepas propuestas carecen de mecanismos de resistencia y virulencia lo que sugiere un uso potencial en el área agroalimentaria y en salud pública, si bien estos datos son preliminares y requieren de una verificación exhaustiva en el futuro.

CONCLUSIONES
CONCLUSIONS



Conclusiones/Conclusions

1. El 7,6 % de las 1205 cepas de estafilococos estudiadas por el método de *spot-on-lawn* mostraron actividad inhibitoria frente a bacterias indicadoras G+, incluyendo *Staphylococcus aureus* sensible y resistente a meticilina (SASM y SARM, respectivamente).
2. Las cepas de estafilococos productoras de sustancias antimicrobianas (Bac+) pertenecieron a 11 especies diferentes, mayoritariamente SCoN, y fueron aisladas de diferentes ecosistemas (animales de vida libre, de humanos, alimentos, mascotas y medio ambiente).
3. 8 de las 92 cepas Bac+ mostraron alta actividad antimicrobiana (AA) inhibiendo más del 70% de los indicadores evaluados.
4. Más del 90% de las cepas Bac+ mostraron actividad en sus extractos prepurificados (BT), siendo mucho menor la AA detectada en los sobrenadantes libre de células (SLC).
5. Un tercio de los estafilococos Bac+ fue sensible a todos los antibióticos analizados (principalmente SCoN), y además carecían de los genes de virulencia estudiados.
6. El estudio genético de cepas Bac+ permitió la identificación de diversos genes codificantes de bacteriocinas tales como lantipéptidos, lactococcin972, bacsp222, blp, uberolisina, aureocyclicina 4185, lugdunina y micrococcina P1 (MP1). Destaca la alta frecuencia de operones de lantipéptidos, algunos de ellos potencialmente nuevos
7. La bacteriocina micrococcina P1 (MP1) se detectó a nivel genómico y por espectrometría de masas en 4 aislados Bac+ [*S. aureus*-C5802 (agua superficial); *S. hominis*-C5835 (agua superficial), *S. sciuri*-X3041 y X3011 (carne cruda de pollo] y las curvas de crecimiento (con SLC y BT), estudio de actividad antimicrobiana combinada con antibióticos y los estudios de competencia frente a SARM confirmaron la gran eficacia de acción de estas 4 cepas de estafilococos productoras de MP1 o de sus extractos prepurificados.
8. El uso del modelo de cigüeña ha permitido analizar potenciales interacciones entre cepas de estafilococos Bac+ y el resto de la microbiota nasotraqueal de estos animales.
9. Se han detectado cepas Bac+ con potencial de aplicación en distintas áreas de agroalimentación y salud pública (mastitis e industria láctea, patógenos aviares y agentes zoonóticos, zoonosis porcinas, seguridad alimentaria, acuicultura, industria vitivinícola y cultivo de hongos) destacando la potente actividad de las cepas productoras de MP1.
10. Los estudios de inmunidad cruzada entre cepas Bac+ han permitido proponer cinco potenciales combinaciones de estafilococos con actividad antimicrobiana (cepas productoras de lantibióticos y de MP1); si bien es necesario la realización de estudios futuros.
11. El proyecto de aprendizaje-servicio MicroMundo evidenció la alta diversidad de cepas productoras de sustancias antimicrobianas en las muestras de suelo, donde cabe destacar la cepa de *Staphylococcus hominis* X7276, que inhibió al 80% de los indicadores evaluados.

12. El género *Staphylococcus* y especialmente los aislados de SCoN se han confirmado como una valiosa fuente de péptidos antimicrobianos con funcionalidades prometedoras en agroalimentación y salud pública.

Conclusiones/Conclusions

1. 7.6 % of the 1205 staphylococcal isolates tested by the *spot-on-lawn* method showed inhibitory activity against G+ indicator bacteria, including methicillin-susceptible and -resistant *Staphylococcus aureus* (MSSA and MRSA, respectively).
2. The bacteriocin-producing staphylococcal isolates (Bac+) belonged to 11 different species, mostly CoNS, and were isolated from different ecosystems (wildlife animals, humans, food, pets and environment).
3. 8 out of the 92 Bac+ isolates showed high antimicrobial activity (AA) inhibiting more than 70% of the indicators evaluated.
4. More than 90% of Bac+ isolates showed AA in their pre-purified extracts (BT), considerably higher than the AA detected in cell-free supernatants (CFS).
5. One third of the Bac+ staphylococci were susceptible to all antibiotics tested (mainly CoNS), and also lacked the virulence genes studied.
6. The genetic study of Bac+ isolates allowed the identification of several bacteriocin encoding genes such as lanthipeptides, lactococcin972, bacsp222, blp, uberolysin, aureocyclicin 4185, lugdunin and micrococcin P1 (MP1). Of note is the high frequency of lanthipeptides operons, some of them potentially novel.
7. The MP1 bacteriocin was detected at the genomic level and by mass spectrometry in 4 Bac+ isolates [*S. aureus*-C5802 (surface water); *S. hominis*-C5835 (surface water), *S. sciuri*-X3041 and X3011 (raw chicken meat) and growth curves (with SLC and BT), antimicrobial activity study combined with antibiotics and MRSA competence studies confirmed the high efficacy of action of these 4 MP1-producing staphylococcal isolates or their prepurified extracts.
8. The use of the stork model has allowed the analysis of potential interactions between Bac+ staphylococcal isolates and the rest of the nasotracheal microbiota of these animals.
9. Bac+ isolates with potential application in different areas of agro-food and public health (mastitis and dairy industry, poultry pathogens and zoonotic agents, swine zoonosis, food safety, aquaculture, wine industry and mushroom cultivation) have been detected, highlighting the potent activity of MP1-producing isolates.
10. Cross-immunity studies between Bac+ isolates have allowed to propose five potential combinations of staphylococci with antimicrobial activity (lanthipeptide and MP1-producing isolates); although future studies are needed.
11. The MicroMundo service-learning project demonstrated the high diversity of antimicrobial-producing isolates in the soil samples, with the *Staphylococcus hominis* X7276 isolate standing out, which inhibited 80% of the indicators evaluated.

12. The genus *Staphylococcus* and especially CoNS isolates have been confirmed as a valuable source of antimicrobial peptides with promising functionalities in agro-food and public health.

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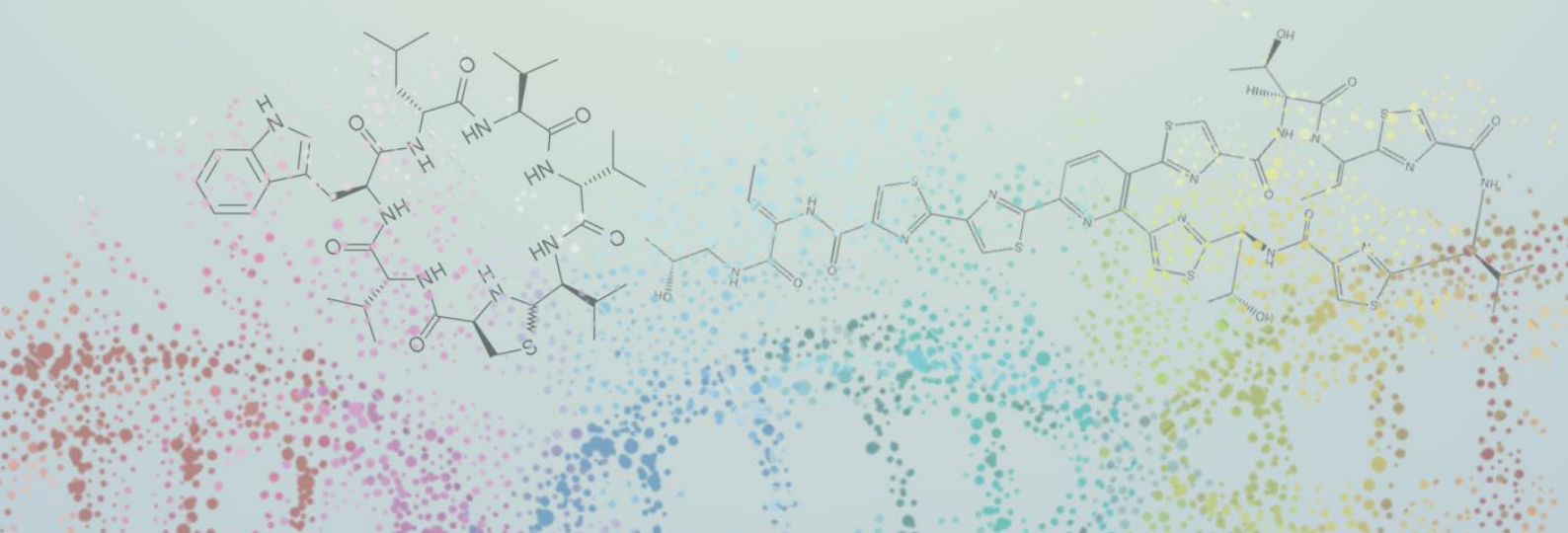

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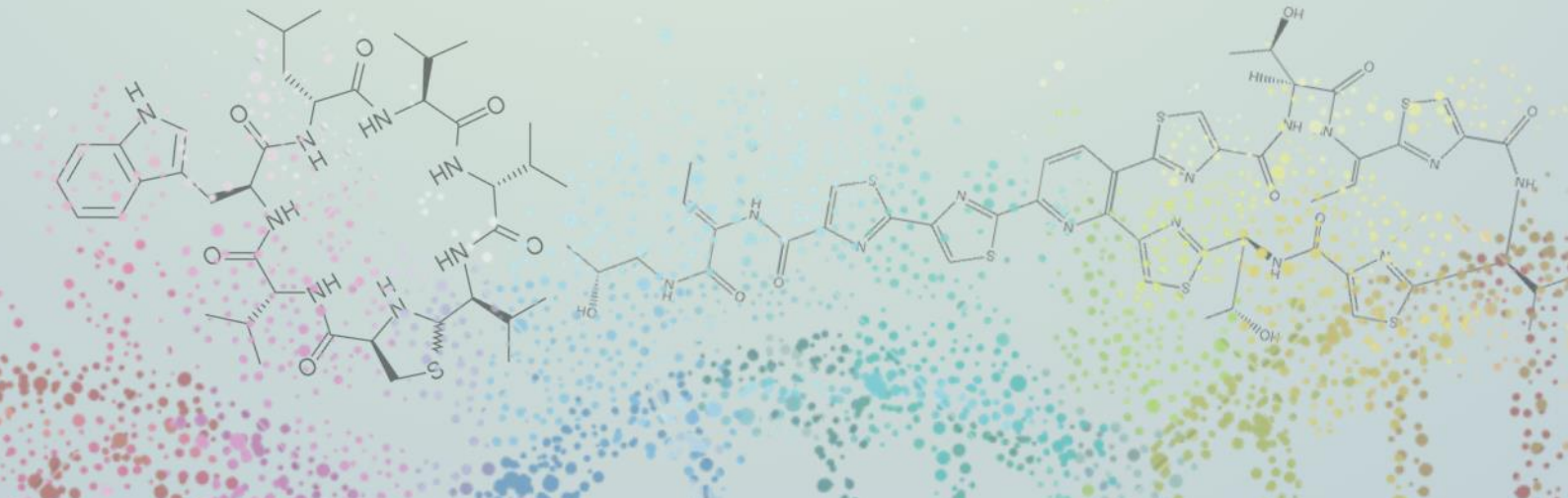
ANEXOS



Anexos

Tabla S1 Especies, orígenes y aislados productores de bacteriocinas del total de 1205 estafilococos SCoN y SCoN incluidos en esta tesis para evaluar su actividad antimicrobiana.

		Número de aislados estudiados/ Número de cepas productoras de bacteriocinas					
	Especies	Total	Humanos	Alimentos	Animales de vida libre	Mascotas	Medio ambiente
SCoP	*S. aureus*	193/6	11/0	72/2	103/3	0/0	7/1
	S. pseudintermedius	60/16	9/1	1/0	0/0	50/15	0/0
	S. delphini	31/0	0/0	19/0	12/0	0/0	0/0
	*Total SCoP**	284/22	20/1	92/2	115/3	50/15	7/1
SCoN	*S. sciuri*	352/15	0/0	23/2	320/13	0/0	9/0
	S. saprophyticus	75/0	0/0	39/0	11/0	0/0	25/0
	S. lentus	62/0	0/0	16/0	42/0	0/0	4/0
	S. xylosus	56/3	0/0	7/0	32/3	0/0	17/0
	S. epidermidis	54/5	4/0	17/4	21/1	0/0	12/0
	S. fleuretti	29/0	0/0	14/0	15/0	0/0	0/0
	S. chromogenes	37/10	0/0	7/3	28/7	0/0	2/0
	S. warneri	26/6	1/0	24/6	0/0	0/0	1/0
	S. vitulinus	24/0	0/0	5/0	19/0	0/0	0/0
	S. simulans	29/3	0/0	16/0	10/3	0/0	3/0
	S. arlettae	22/0	0/0	0/0	1/0	0/0	21/0
	S. cohnii	16/0	0/0	2/0	2/0	0/0	12/0
	S. equorum	16/0	0/0	2/0	13/0	0/0	1/0
	S. pasteuri	9/0	0/0	8/0	0/0	0/0	1/0
	S. hominis	15/2	1/0	3/0	8/1	0/0	3/1
	S. capitis	7/0	0/0	1/0	2/0	0/0	4/0
	S. hyicus	7/3	0/0	2/0	2/3	0/0	0/0
	S. succinus	6/0	0/0	0/0	5/0	0/0	1/0
	S. haemolyticus	8/0	0/0	0/0	5/0	0/0	3/0
	S. nepalensis	5/0	0/0	0/0	5/0	0/0	5/0
	S. kloosii	3/0	0/0	0/0	0/0	0/0	0/0
	S. schleiferi	3/0	0/0	0/0	3/0	0/0	0/0
	S. auricularis	1/0	0/0	0/0	0/0	0/0	1/0
	S. felis	1/0	0/0	0/0	1/0	0/0	0/0
	S. lugdunensis	57/23	57/23	0/0	0/0	0/0	0/0
	S. simiae	1/0	0/0	0/0	1/0	0/0	0/0
	Total SCoN*	921/70	63/23	186/15	547/31	0/0	125/1
Total SCoP+SCoN		1205/92	83/24	278/17	662/34	50/15	132/2

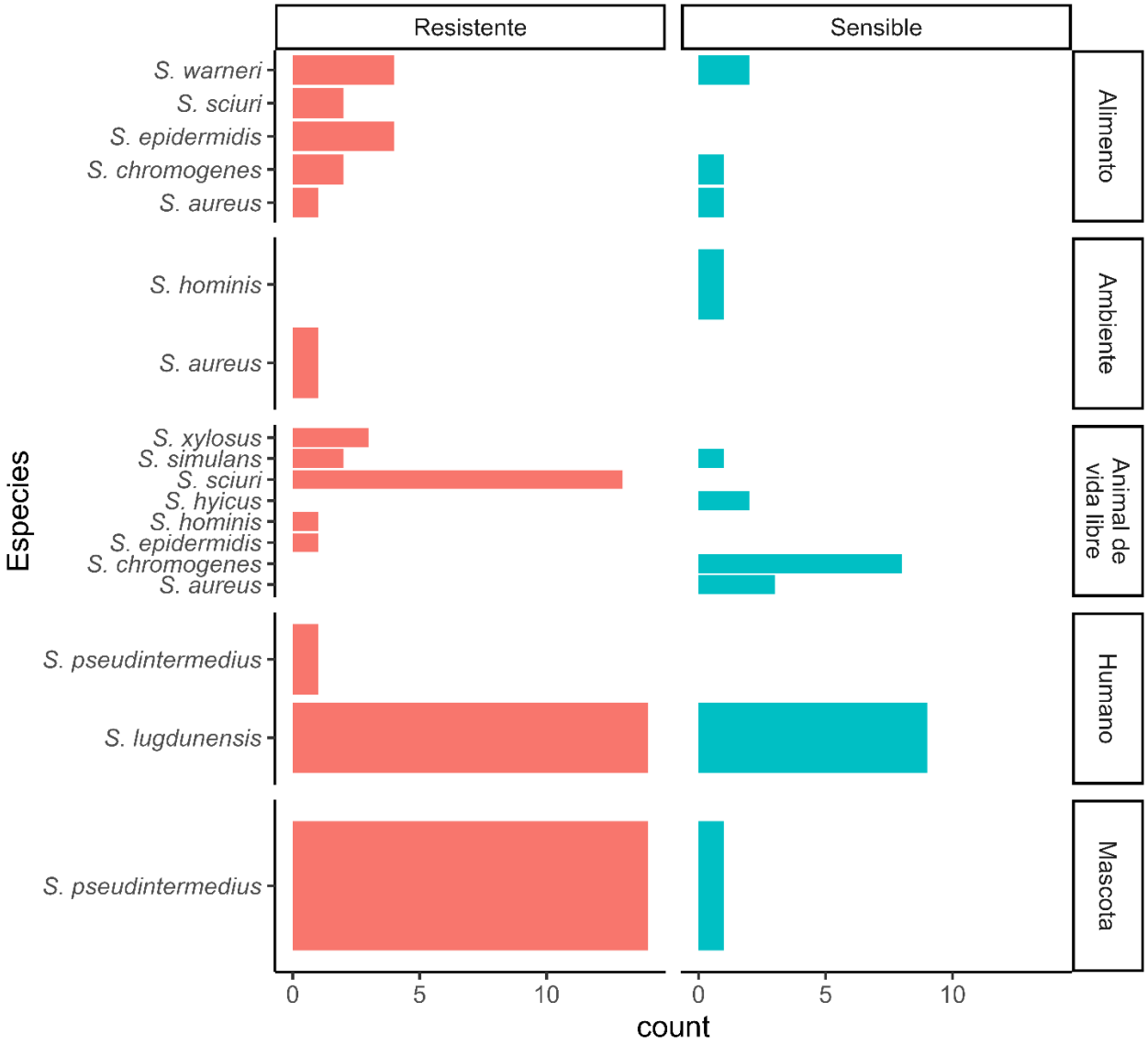


Figura S1 Número de cepas sensibles o resistentes a antibióticos en función de su especie y origen del total de las 92 cepas Bac+ identificadas en esta tesis.

Tabla S2 Fenotipo y genotipo de resistencia, tipado molecular y contenido de genes de virulencia y codificantes de bacteriocinas en las 60 cepas con actividad antimicrobiana detectadas de las 890 estudiadas en el Artículo 1 incluido en el primer capítulo de esta tesis **(Fernández-Fernández et al. 2022a).**

Cepa	Especies	Origen	Fenotipo de resistencia[a]	Genotipo de resistencia	Genes de virulencia	*spa*-MLST-CC/ *agr*	Gen estructural de bacteriocina	Referencia
C3869	*S. pseudintermedius*	Mascota	PEN-OXA-ERY-CLI-STR-TET-SXT	*bla*Z, *mec*A, *erm*(B), *tet*(M), *dfr*G	*luk*S/F-I, *siet, se-int*	ST258/II/ (SCC*mec*IV)	-	(Ruiz-Ripa et al. 2021)
C5344	*S. pseudintermedius*	Mascota	PEN-ERY-CLI-STR-TET-CHL-SXT	*bla*Z, *erm*(B), *tet*(M), cat_{pC221}, *dfr*G	*luk*S/F-I, *siet, se-int*	ST2171/III	-	(Ruiz-Ripa et al. 2021)
C5345	*S. pseudintermedius*	Mascota	PEN-ERY-CLI-STR-TET-CHL-SXT	*bla*Z, *erm*(B), *tet*(M), cat_{pC221}, *dfr*G	*luk*S/F-I, *siet, se-int*	ST2171/III	-	(Ruiz-Ripa et al. 2021)
C3877	*S. pseudintermedius*	Mascota	PEN-STR-TET	*bla*Z, *aadE*, *tet*(M)	*luk*S/F-I, *siet, se-int, expB*	ST2172/III	-	(Ruiz-Ripa et al. 2021)
C3873	*S. pseudintermedius*	Mascota	PEN	*bla*Z	*luk*S/F-I, *siet, se-int*	ST2173/III	-	(Ruiz-Ripa et al. 2021)
C3874	*S. pseudintermedius*	Mascota	Sensible	-	*luk*S/F-I, *siet, se-int*	ST2170/III	-	(Ruiz-Ripa et al. 2021)
C5357	*S. pseudintermedius*	Mascota	PEN	*bla*Z	*luk*S/F-I, *siet, se-int*	ST356/III	-	(Ruiz-Ripa et al. 2021)
C4502	*S. pseudintermedius*	Mascota	SXT	*dfr*G	*luk*S/F-I, *siet, se-int*	ST160/III	Lantibiótico	UR[c]
C4506	*S. pseudintermedius*	Mascota	FA	-	*luk*S/F-I, *siet, se-int,*	ST161/III	-	UR[c]
C4525	*S. pseudintermedius*	Mascota	PEN-ERY-CLI-STR- CHL-SXT	*bla*Z, *ant*(6)-Ia, *erm*(B), *dfr*G, *Inu*(A), cat_{pc221}	*luk*S/F-I, *siet, se-int*	ST162/III	-	UR[c]
C4486	*S. pseudintermedius*	Mascota	PEN-TET	*bla*Z, *tet*(M)	*luk*S/F-I, *siet, se-int, sec1,*	ST20/III	-	UR[c]
C4476	*S. pseudintermedius*	Mascota	PEN-TET-SXT	*bla*Z, *tet*(M), *dfr*G	*luk*S/F-I, *siet, se-int,*	ST160/III	-	UR[c]
C2912	*S. pseudintermedius*	Mascota	PEN	*bla*Z	*luk*S/F-I, *siet*	ST29/III	-	(Lozano et al., 2017)
C8189	*S. pseudintermedius*	Humano	ERY-CLI	*erm*(B)	*luk*S/F-I, *siet*	ST241/III	Bacsp222	(Lozano et al., 2017)
C8478	*S. pseudintermedius*	Mascota	ERY-CLI	*erm*(B)	*luk*S/F-I, *siet*	ST241/III	Bacsp222	(Lozano et al., 2017)
C8479	*S. pseudintermedius*	Mascota	ERY-CLI	*erm*(B)	*luk*S/F-I, *siet*	ST241/III	Bacsp222	(Lozano et al., 2017)
C5802	*S. aureus*	Ambiente	PEN	*bla*Z	*luk*MF′, *luk*ED, *etD2*	t843-ST130-CC130/III	Lantibiótico	(Gómez et al., 2017)
C6770	*S. aureus*	Animal de vida libre	Sensible	-	-	t1125-CC5/II	Lantibiótico	(Ruiz-Ripa et al., 2019)
C8609	*S. aureus*	Animal de vida libre	Sensible	-	-	t11225-CC425/II	Lantibiótico	(Ruiz-Ripa et al., 2019)
X3410	*S. aureus*	Alimento	Sensible	-	-	t10234/I	Lantibiótico	Este estudio
X3417	*S. aureus*	Alimento	PEN-ERY-CLI[I]	*bla*Z, *mrs*(A), *erm*(C), *ermT, Inu*(A)	-	t1451-CC398/I (IEC+C)	-	Este estudio

Tabla S2 Continuación.

Cepa	Especies	Origen	Fenotipo de resistencia[a]	Genotipo de resistencia	Genes de virulencia	*spa*-MLST-CC/ *agr*	Gen estructural de bacteriocina	Referencia
C9175	*S. sciuri*	Animal de vida libre	CLI^{I}	*Inu*(A), *sal*(A)	-	-	-	(Ruiz-Ripa et al., 2020)
C9179	*S. sciuri*	Animal de vida libre	CLI^{I}	*sal*(A)	-	-	-	(Ruiz-Ripa et al., 2020)
C9185	*S. sciuri*	Animal de vida libre	CLI^{I}-FA	*sal*(A)	-	-	-	(Ruiz-Ripa et al., 2020)
C9188	*S. sciuri*	Animal de vida libre	CLI^{I}	*Inu*(A), *sal*(A)	-	-	-	(Ruiz-Ripa et al., 2020)
C9191	*S. sciuri*	Animal de vida libre	CLI^{I}	*sal*(A)	-	-	-	(Ruiz-Ripa et al., 2020)
C9193	*S. sciuri*	Animal de vida libre	CLI-FA	*Inu*(A), *sal*(A)	-	-	-	(Ruiz-Ripa et al., 2020)
C9203	*S. sciuri*	Animal de vida libre	CLI^{I}	*sal*(A)	-	-	-	(Ruiz-Ripa et al., 2020)
C9213	*S. sciuri*	Animal de vida libre	CLI^{I}-FA	*sal*(A)	-	-	-	(Ruiz-Ripa et al., 2020)
C9231	*S. sciuri*	Animal de vida libre	CLI^{I}-FA	*sal*(A)	-	-	-	(Ruiz-Ripa et al., 2020)
C9258	*S. sciuri*	Animal de vida libre	ERY CLI -TOB-CIP-FA	*erm*(B), *msr*(A), *sal*(A), *ant*(4')-Ia	-	-	-	(Ruiz-Ripa et al., 2020)
C9529	*S. sciuri*	Animal de vida libre	CLI	*sal*(A)	-	-	-	(Ruiz-Ripa et al., 2020)
X3011	*S. sciuri*	Alimentos	ERY-CLI-CIP-SXT	*sal*(A), *dfr*A, *erm*(B)	-	-	-	Este estudio
X3041	*S. sciuri*	Alimentos	CLI-FA	*sal*(A), *erm*(B), *Inu*(A)	-	-	-	Este estudio
C9838	*S. chromogenes*	Animal de vida libre	Sensible	-	-	-	Uberolysina	(Mama et al., 2018)
C9853	*S. chromogenes*	Animal de vida libre	Sensible	-	-	-	-	(Mama et al., 2018)
C9726	*S. chromogenes*	Animal de vida libre	Sensible	-	-	-	Lantibiótico	(Mama et al., 2018)
C9727	*S. chromogenes*	Animal de vida libre	Sensible	-	-	-	Lantibiótico	(Mama et al., 2018)
C9567	*S. chromogenes*	Animal de vida libre	Sensible	-	-	-	-	(Mama et al., 2018)
C9581	*S. chromogenes*	Animal de vida libre	Sensible	-	-	-	Uberolysina	(Mama et al., 2018)

Tabla S2 Continuación.

Cepa	Especies	Origen	Fenotipo de resistencia[a]	Genotipo de resistencia	Genes de virulencia	*spa*-MLST-CC/ *agr*	Gen estructural de bacteriocina	Referencia
C9826	*S. chromogenes*	Animal de vida libre	Sensible	-	-	-	-	(Mama et al., 2018)
X3283	*S. chromogenes*	Alimento	Sensible	-	-	-	-	Este estudio
X3300	*S. chromogenes*	Alimento	TET	*tet(M), tet(K)*	-	-	-	Este estudio
X3390	*S. chromogenes*	Alimento	TET-CIP	*tet(L), tet(M)*	-	-	-	Este estudio
X3007	*S. warneri*	Alimento	PEN-ERY	*blaZ, erm(B)*	-	-	-	Este estudio
X3015	*S. warneri*	Alimento	PEN-ERY	*blaZ, erm(B)*	-	-	-	Este estudio
X3023	*S. warneri*	Alimento	Sensible	-	-	-	-	Este estudio
X2969	*S. warneri*	Alimento	Sensible	-	-	-	-	Este estudio
X3027	*S. warneri*	Alimento	TET	*tet(K)*	-	-	-	Este estudio
X3044	*S. warneri*	Alimento	PEN-TET-CIP	*blaZ, tet(K)*	-	-	-	Este estudio
X3009*	*S. epidermidis*	Alimento	ERY-FA	*msr(A), mph(C)*	-	ST1025b	Lantibiótico	Este estudio
X3026	*S. epidermidis*	Alimento	ERY	*erm(B)*	-	-	-	Este estudio
X3047	*S. epidermidis*	Alimento	PEN-FOX-ERY-SXT	*msr(A), mecA*	-	-	-	Este estudio
X3353	*S. epidermidis*	Alimento	PEN-FOX-TET-FA	*mecA*	-	-	-	Este estudio
C9255	*S. xylosus*	Animal de vida libre	PEN	*blaZ*	-	-	Lantibiótico	(Ruiz-Ripa et al., 2020)
C9576	*S. xylosus*	Animal de vida libre	FA	-	-	-	-	(Mama et al., 2018)
C9793	*S. xylosus*	Animal de vida libre	FA	-	-	-	-	(Mama et al., 2018)
C5835	*S. hominis*	Ambiente	Sensible	-	-	-	-	(Gómez et al., 2017)
C9585	*S. hyicus*	Animal de vida libre	Sensible	-	-	-	Lantibiótico	(Mama et al., 2018)
C9832	*S. simulans*	Animal de vida libre	TET-FA	*tet(K)*	-	-	Lantibiótico	(Mama et al., 2018)

[a]PEN, penicilina; OXA; oxacilina; FOX, cefoxitina; ERY, eritromicina; CLI, clindamicina; CLI[I], clindamicina inducicle; TOB, tobramicina; STR, estreptomicina; TET, tetraciclina; CIP, ciprofloxacino; CHL, cloranfenicol; SXT, trimetoprim–sulfametoxazol; FA, ácido-fusídico.

[b]Cepa tipada como ST1025. La caracterización del MLST se llevó a cabo úinicamente en esta cepa debido al interesante espectro de actividad antimicrobiana y la ausencia de genes codificantes de bacteriocinas conocidos.

[c]UR: Universidad de La Rioja

Tabla S3 Origen, fenotipo y genotipo de resistencia a antibióticos y presencia de *lug*D (gen codificante de la bacteriocina lugdunina) en las 23 cepas con actividad antimicrobiana de las 56 evaluadas en el Artículo 2 incluido en el primer capítulo **(Fernández-Fernández et al. 2022b).**

			Resistencia a antibióticos		
Cepa	**Origen[a]**	**Muestra**	**Fenotipo[b]**	**Genotipo**	*lugD*
C10107	Sangre	Infección	PEN	*bla*Z	+
C9142	Sangre	Infección	PEN	*bla*Z	+
C9151	Catéter	Colonización	Sensible	-	+
C9148	Catéter	Colonización	Sensible	-	+
C9147	Catéter	Colonización	Sensible	-	+
C9161	Catéter	Colonización	Sensible	-	+
C9954	Catéter	Colonización	Sensible	-	+
C9145	Catéter	Colonización	PEN	*bla*Z	+
C10341	Catéter	Colonización	FOS	-	+
C9146	Catéter	Colonización	Sensible	-	+
C10052	IPPB	Infección	PEN	*bla*Z	+
C10320	IPPB	Infección	PEN	*bla*Z	+
C10511	IPPB	Infección	Sensible	-	+
C9894	IPPB	Infección	ERY-CLI^{I}	*msr*(A)	+
C10343	IPPB	Infección	Sensible	-	+
C9342	IPPB	Infección	FOS	-	+
C9890	IPPB	Infección	PEN-FOS	*bla*Z	+
C9892	IPPB	Infección	PEN	*bla*Z	+
C9893	IPPB	Infección	PEN	*bla*Z	+
C9897	IPPB	Infección	PEN-OXA-TOB	*bla*Z, *mec*A, *ant*(4')-Ia, *acc*(6')-*aph*(2'')	+
C9980	Orina	Infección	PEN	*bla*Z	+
C9159	Orina	Infección	PEN-FOS	*bla*Z	+
C9911	Orina	Infección	Sensible	-	+

[a]IPPB: infección de piel y partes blandas.
[b]Abreviaturas de los casos positivos en la evaluación del fenotipo de resistencia a antibióticos: PEN, penicilina; OXA, oxacilina; FOS, fosfomicina; ERY, eritromicina; CLI^{I}, clindamicina inducible; TOB, tobramicina.

Tabla S4 Caracterización de las 9 cepas con actividad antimicrobiana de las 259 evaluadas a partir de la microbiota nasotraqueal de cigüeñas. Estos datos han sido incluidos en el Artículo 6 del cuarto capítulo de esta tesis **(Fernández-Fernández et al. 2023b).**

Especie	Código de muestra (Origen)[a]	Fenotipo de resistencia a antibióticos[b]	Genes de bacteriocinas
S. aureus X4036	436(T)	Sensible	Lantibiótico
S. hyicus X3750	538(T)	Sensible	Lantibiótico
S. sciuri X3763	507(T)	PEN	Lantibiótico
S. sciuri X4000	433(T)	PEN, CIP	Lantibiótico
S. epidermidis X3815	506(T)	ERY	Lantibiótico
S. chromogenes X4620	481(N)	Sensible	Lantibiótico
S. hominis X3764	507(T)	PEN, FOX, ERY	Lantibiótico
S. simulans X4520	480(N)	PEN	Lantibiótico
S. simulans X4653	481(N)	Sensible	Lantibiótico

[a]Abreviatura: T, traqueal; N, nasal

[b]Antibióticos evaluados (abreviatura mostrada en los casos positivos): penicilina (PEN), cefoxitina (FOX), oxacilina, eritromicina (ERY), ciprofloxacino (CIP), tetraciclina, gentamicina, tobramicina, linezolid, cloranfenicol, trimetropim-sulfametoxazol, mupirocina.

Tabla S5 Microorganismos indicadores utilizados en los ensayos para la detección de actividad antimicrobiana.

Especie (nº cepas)	Código de Identificación	Código colección	Origen	Fenotipo/Genotipo Resistencia a antibióticos
S. aureus (7)	C1570		Humano	Meticilina/*mecA* (MDR)
	C7246		Humano	Meticilina/*mec*C
	C5313		Humano	Meticilina/*mecA*
	X3548			Meticilina/*mecA/Virulenta*
	X3963			Meticilina/*mecA*
	C1532			Meticilina/*mecA*/enterotoxigénica
	C411	ATCC29213		
S. delphini (1)	C9459		Animal vida libre	
S. pseudintermedius (12)	C2381		Mascota	Meticilina/*mecA*
	C3930		Mascota	Meticilina/*mecA*
	C2382		Mascota	Meticilina/*mecA*
	C8187		Humano	
	C2915		Humano	
	C2912		Mascota	
	C8189		Humano	
	C3917		Humano	
	C8188		Humano	MDR
	C8368		Humano	
	C3007		Mascota	
	C3468		Mascota	
S. epidermidis (1)	C2663		Humano	Meticilina/*mecA* y Linezolid
S. haemolyticus (1)	C2709		Humano	Linezolid
S. lugdunensis (6)	C9927		Humano	
	C9981		Humano	
	C9159		Humano	
	C10107		Humano	
	C9954		Humano	
	C9980		Humano	
S. sciuri (1)	C9780		Animal vida libre	
Enterococcus casseliflavus (1)	C1232			
E. durans (1)	C1433			Vancomicina*/vanA*
E. faecalis (3)	C3735			Vancomicina */vanB2*
	C410	ATCC29212		
	C9951			Linezolid/*optrA, fexA*
E. faecium (1)	C2321			Vancomicina */vanA*
E. gallinarum (1)	C2310			
E. hirae (1)	C1436			Vancomicina */vanA*
E. cecorum (1)	X3809		Animal de vida libre	
Streptococcus suis (4)	X2057		Humano	
	X2058		Humano	
	X2060		Animal de producción	
	X2061		Animal de producción	
S. agalactie (1)	X9738			
S. dysgalactie (1)	X9739			
S. gallolyticus (1)	X9742			

Tabla S5 Continuación.

Especie (nº cepas)	Código de Identificación	Código colección	Origen	Fenotipo/Genotipo Resistencia a antibióticos
Clostridium perfringens (1)	X9740			
Bacilllus cereus (1)	X10062		Suelo	
Lactococcus garviae (1)	UR[a]		Peces	
Carnobacterium maltaromaticum (1)	UR[a]		Peces	
Listeria monocytogenes (1)	C137	CECT4032		
Micrococcus luteus (1)	C157	CECT241		
Pediococcus pentosaceus (1)	UR[a]		Vino	
P. acidilactici (1)	UR[a]		Vino	
Lactobacillus plantarum (1)	UR[a]		Vino	
Leuconostoc mesenteroides (1)	UR[a]		Vino	
Trichoderma atroviride (1)	TAV1		Champiñón	
Cladobotryum mycophilum (1)	CM13900		Champiñón	
Escherichia coli (1)	C408	ATCC25922		
Pseudomonas aeruginosa (1)	C3282	PAO1		
Salmonella spp.	X10061			

[a]UR: Universidad de La Rioja.

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