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Resistome, virulome and mobilome of nasal staphylococci from healthy humans and animals: A One Health approach with public health implications
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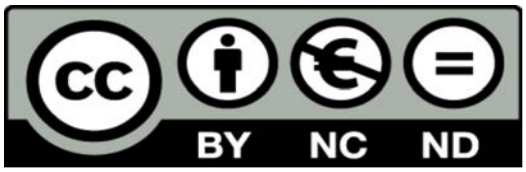

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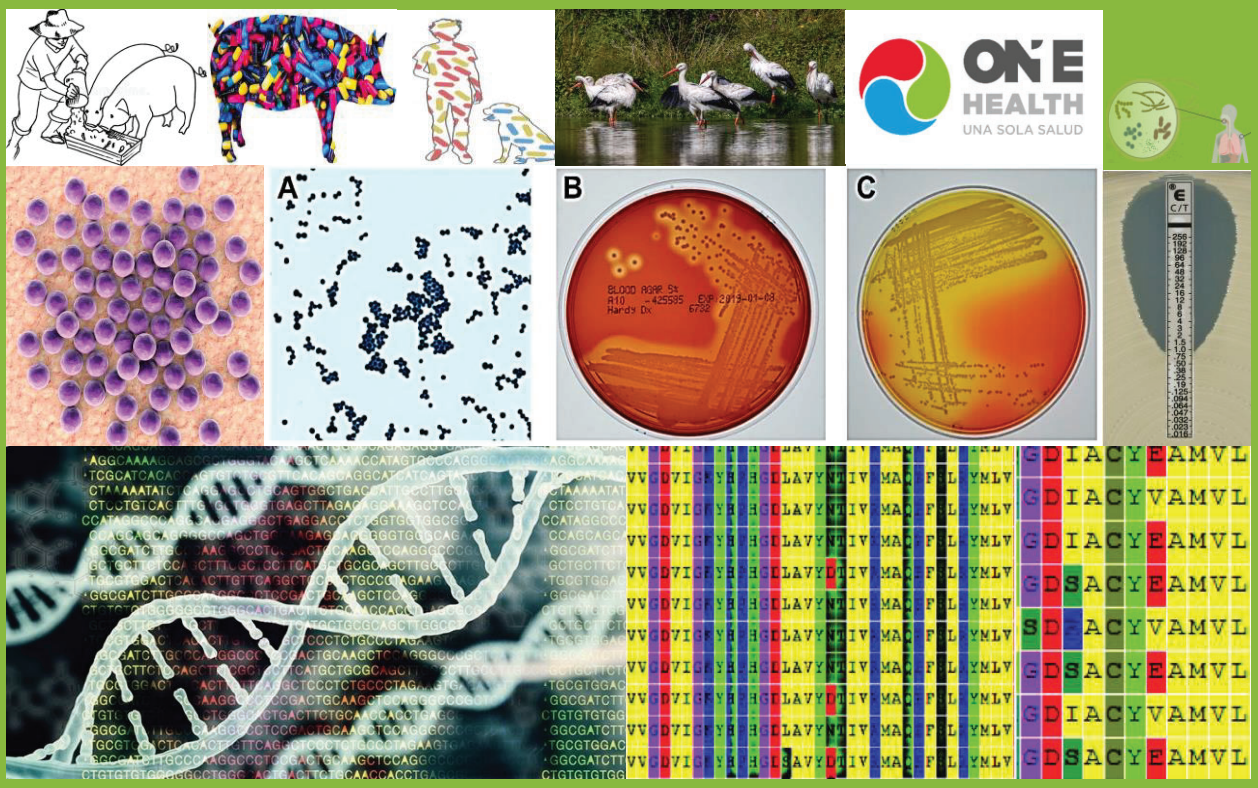
PhD thesis with International Mention

Idris Nasir Abdullahi

Logroño (Spain), 2023



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2023

Programa de Doctorado en Ciencias Biomédicas y Biotecnológicas

RESISTOMA, VIRULOMA Y MOBILOMA DE ESTAFILOCOCOS NASALES DE PERSONAS Y ANIMALES SANOS: UN ENFOQUE ONEHEALTH CON IMPLICACIONES EN SALUD PUBLICA

RESISTOME, VIRULOME AND MOBILOME OF NASAL STAPHYLOCOCCI FROM HEALTHY HUMANS AND ANIMALS: A ONE HEALTH APPROACH WITH PUBLIC HEALTH IMPLICATIONS

Idris Nasir Abdullahi

Directora: Carmen Torres Manrique

Directora: Carmen Lozano Fernández

Area of Biochemistry and Molecular Biology
Faculty of Science and Technology

DOCTORAL THESIS

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Thesis presented by **IDRIS NASIR ABDULLAHI** to qualify for the title of Philosophy of Doctor (PhD) with International Mention from the University of La Rioja

Logroño, October 2023

UNIVERSIDAD DE LA RIOJA

Area of Biochemistry and Molecular Biology
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The report titled "**Resistome, virulome and mobilome of nasal staphylococci from healthy humans and animals: A One Health approach with public health implications**", presented by **IDRIS NASIR ABDULLAHI,** an M.Sc. graduate in Medical Microbiology and Parasitology from the University of Ilorin (Nigeria), has been carried out in the Area of Biochemistry and Molecular Biology of the University of La Rioja, under our direction, and meets the conditions required to qualify for the degree of PhD in Biomedical and Biotechnological Sciences.

As recorded in Logroño, on 6th October 2023.

Signed by: Carmen Torres Manrique

Signed by: Carmen Lozano Fernández

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DEDICATION

For the true love, efforts, hurdles, sacrifice, unalloyed support and encouragement, the entirety of this doctorate thesis is dedicated to the loving and blessed memory of my beloved late mother, **Zainab Umar.** May the mercy and gladness of the almighty God be on her!

"No one leaves their house in search of knowledge but that angels will lower their wings in approval of what he is doing".

Source: Sunan Ibn Mājah 226, Grade: Sahih (authentic)

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LIST OF ABBREVIATIONS

AAI: average amino acid identity

agr: Accessory gene regulator

AMPs: Antimicrobial peptides

AMR: Antimicrobial Resistance

ANI: average nucleotide identity

APOBEC: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide.

ARG: antimicrobial resistance gene

BA: Blood agar

BHI: Brain Heart Infusion

BLAST: Basic Local Alignment Search Tool

bp: Base pairs

CA: Community-associated

Cas: CRISPR-associated proteins

CC: Clonal complex

CDS: Coding Sequence

CHL: Chloramphenicol

CHL^R: Chloramphenicol-resistant

chrDNA: chromosomal DNA

CI: Confidence interval

CIP: Ciprofloxacin

CLI: Clindamycin

CLSI: Clinical and Laboratory Standards Institute

CoNS: Coagulase-negative staphylococci

CoPS: Coagulase-positive staphylococci

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

CSPs: conserved signature proteins

d.f.: degree of freedom

dDDH: digital DNA–DNA hybridization

DNA: Deoxyribonucleic Acid

dNTPs: Deoxynucleotide Triphosphates

eDNA: extracellular DNA

EMBOSS: European Molecular Biology Open Software Suite

ERY: Erythromycin

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FOX: Cefoxitin

GDP: Gross Domestic Product

GEN: Gentamicin

HA: Hospital-associated

HCCA: α-Cyano-4-hydroxycinnamic acid

HCl: Hydrochloric acid

HGT: Horizontal Gene Transfer

HLA: human leukocyte antigen

HVR: Hypervariable region

IE: Infective endocarditis

IEC: Immune Evasion Cluster

IS: Insertion sequence

kb: kilo base

kb: Kilobase

LA: Livestock-associated

LPSN: List of Prokaryotic names with Standing in Nomenclature

LZD: Linezolid

LZD^R: Linezolid-resistant

MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

MGE: Mobile Genetic Element

MIC: Minimum Inhibitory Concentration

min: minute

MLS_A: Macrolides, Lincosamides and Streptogramins A

MLS_B: Macrolides, Lincosamides and Streptogramins B

MLST: Multilocus sequence typing

MRCoNS: Methicillin-resistant coagulase-negative staphylococci

MRSA: methicillin-resistant *Staphylococcus aureus*

MRSE: Methicillin-resistant *S. epidermidis*

MRSP: Methicillin-resistant *Staphylococcus pseudintermedius*

MSA: Mannitol Salt agar

MSCRAMM: Microbial surface components recognizing adhesive matrix molecules

MSSA: methicillin-sensitive *Staphylococcus aureus*

MSSP: Methicillin-susceptible *Staphylococcus pseudintermedius*

MUP: mupirocin

ND: Not detected

NHP: Non-human primates

Not applicable

NT: Not tested

NTO: nasal, tracheal and/or oral

OGRIs: overall genome-related indices

OR: Odd RatioNA:

ORSAB: Oxacillin screening agar base

OXA: Oxacillin

p: Probability

PBP Penicillin Binding Protein

PCR: Polymerase Chain Reaction

PEN: Penicillin

PEN^R: penicillin-resistant

PEN^S: penicillin-susceptible

pH: potential of Hydrogen

PNAg: Polysaccharide poly-N-acetylglucosamine

PSMs: Phenol-soluble modulins

PVL: Panton valentine Leucocidin

QRDR: Quinolone Resistance Determining Region

RAM: Resistencia antimicrobiana

rep: replicon

RNA ribonucleic acid

rpm: Revolutions per minute

rRNA: ribosomal Ribonucleic Acid

SAgs: Superantigens

SaPIs: *S. aureus* pathogenicity islands

SARM: *S. aureus* resistente a meticilina

SASM: *S. aureus* sensible a la meticilina

Sbp: Small basic protein

SCC*mec*: Staphylococcal Cassette Chromosome (SCC) *mec*

SCoN: *Staphylococcus* coagulasa-negativo

SCoP: *Staphylococcus* coagulasa-positivo

sec: seconds

SIG: *Staphylococcus intermedius* group

SNP: Single Nucleotide Polymorphism

SPSM: *Staphylococcus pseudintermedius* sensible a la meticilina

spa: staphylococcal protein A

spp: Species

ST: Sequence type

STR: Streptomycin

subsp. Subspecies

SXT: Sulfamethoxazole-trimethoprim

Tas: Teichoic acids

TEC: Teicoplanin

TET: Tetracycline

TET^R: Tetracycline resistant

Tn: Transposon

TOB: Tobramycin

TSST: Toxic Shock Syndrome Toxin

UNEP: United Nations Environment Programme

URT: Upper Respiratory Tract

VAN: Vancomycin

VBNC: Viable but-non culturable cells

WB: Wild birds

WGS: Whole Genome Sequencing

WM: wild mammals (excluding rodents and NHP)

WR: Wild rodents

φ: Prophage

χ^2: Chi square

ABSTRACT
RESUMEN

ABSTRACT

Antimicrobial resistance (AMR) and pathogenicity in *Staphylococcus* constitute one of the major global health challenges that need to be addressed using a holistic "One Health" approach. Nasal *Staphylococcus* microbiota in healthy hosts and their molecular characterizations could provide relevant information about the interconnection of the One Health ecosystems. The inclusion of wild animals has been considered necessary to obtain adequate epidemiological links of *Staphylococcus* across the "One Health" niches. This thesis elucidates and deepen the understanding of the diversity, molecular and genomic contents of *Staphylococcus* species in the nasal cavities of healthy humans (with or without animal contact), healthy pigs, healthy dogs, and healthy nestling storks, using culture-dependent strategies.

Of the 13 *Staphylococcus* species identified from the 87 nestling storks, S. *sciuri* (85.7%) and *S. aureus* (31%) were the predominant ones and significantly higher in nestlings of parent storks that fed in natural and landfill areas, respectively. All *S. aureus* strains were methicillin-susceptible (MSSA), and only one presented a multidrug resistance (MDR) phenotype. Regarding *S. aureus* lineages detected, CC398 (8.2%) was the most frequent one. Moreover, strains that carry *tst* (CC22 and CC30), *eta* (CC9) and *etb* (CC45) were detected in four storks. Of the 268 non-duplicated coagulase-negative staphylococci (CoNS), 10 *mecA*-positive strains (associated with SCC*mec* types III, IV and V) were identified. Remarkably, a multidrug-resistant-*S. lentus* strain harboured both *mecA* and *mecC* located in an SCC*mec* type VII hybrid.

Of the 57 healthy individuals who had no animal contact, (98.2%) carried staphylococci (seven species), of which the predominant species were *S. epidermidis* (87.7%) and *S. aureus* (36.8%). All the *S. aureus* were MSSA, but methicillin-resistant-(MR)CoNS was detected in 30.2% (carried in SCC*mec* types III, IV, and V). About 85.1% of the *S. aureus* strains carried one or more of *lukF/S-PV, tst, eta, etb, etd, sea, seb, sec, sed, see* and/or *sep* genes. Eight CCs of MSSA were identified, of which CC398 was the predominant (33.3%). About 77.8% of the CC398 strains harboured the *ermT* gene located on plasmid *rep*13 flanked by IS257. However, one of the MSSA-CC398 strains carried the *ermC* gene in *rep*10. Only the MSSA-CC398 (*ermT*-positive) strains were closely related (SNPs <50) and carried the *φ*Sa3 (IEC type-C). Diverse MDR-*S. epidermidis* strains were identified, which included ST2, ST59, ST173 and ST210 lineages.

Of the 34 dogs and 41 dog owners, *S. aureus* carriage was found in 34.1% of dog owners (including one methicillin-resistant *S. aureus* MRSA-CC5-t2220-SCC*mec* type-IV2B)

and 5.9% of dogs; *S. pseudintermedius* in 2.4% of humans and 32.4% of dogs, while *S. coagulans* was only detected in dogs (5.4%). Remarkably, one human co-carried *S. aureus*/*S. pseudintermedius*, while a dog co-carried the three CoPS species. Household density was significantly associated with *S. pseudintermedius* carriage in households (OR = 18.10, 95% CI: 1.24-260.93, $p = 0.034$). Closely related (<15 SNPs) *S. aureus* or *S. pseudintermedius* were found in humans or dogs in three households. About 56.3% of *S. aureus* carriers (dog or dog owner) harboured diverse intra-host *S. aureus* strains. MSSA-CC398 was the most frequent, but exclusive to humans. It is remarkable the detection of linezolid-resistant (LZD^R)-MRSA-CC5, mediated by novel point mutations at G2261A & T1584A in 23S rDNA. The *S. coagulans* strains were susceptible to all antimicrobials. Most of the *S. pseudintermedius* carried *lukS/F-I, siet,* and *sient* genes, and all *S. aureus* were negative for *lukF/S*-PV, *tst, eta* and *etb* genes. The predominant CoNS species from dogs and dog owners were *S. epidermidis* (26.5% and 80.4%, respectively). About 17.4% were methicillin-resistant (with SCC*mec* types II, III, IVc, V). One LZD^R-*S. epidermidis*-ST35 containing four mutations in L3 (I188V, G218V, N219I, L220D) and L4 (N158S) in a dog owner. Dogs and dog owners' carriers of *S. epidermidis* with similar AMR patterns and genetic lineages (ST59, ST61, ST166 and ST278) were identified in three households (14.8%).

Of the 40 pigs, the highest carriage rate in pigs was *S. aureus* (65%) and *S. chromogenes* (22.5%), whereas in the 10 pig farmers, *S. aureus* (80%) and *S. epidermidis* (40%) were the most predominant ones. MRSA was detected in 60% of pigs and 70% of pig-farmers. Only six *S. aureus* strains were MSSA. All MRSA strains were CC398, but all the MSSA-CC9 strains were detected in only one farm (Farm C). All *S. aureus* strains were negative for *luk-S/F-PV, tst,* and *scn* genes. But one MSSA-CC45-t065 strain was *scn*-positive (IEC-type C) from a pig farmer. High repertoires of AMR genes were detected, including unusual ones. It was important the detection of a plasmid-bound (41.6kb) *cfr* in *S. saprophyticus* from a pig and a chromosomally located *cfr* in *S. epidermidis*-ST16 from a pig farmer, respectively. About 42.5% of the CoNS carriers presented similar AMR genes and or SCC*mec* types. It is also remarkable the detection of *ermT* located in plasmid *repUS18* in two MRSA strains from a pig and a pig farmer.

The whole genome sequencing data of 107 strains revealed the presence of multiple plasmids-bound AMR genes, which were predominant in *Staphylococcus* strains from pigs and pig farmers, but least in nestling storks. Moreover, transposons-linked AMR genes such as *ant9'*(Tn*554*), *ermA* (Tn*554*). *fexA* (Tn*554*, Tn*558*), *tet*(M) (Tn*916*, Tn*925*, Tn*6006*) and *dfrK* (Tn*559*) were identified. SNPs analyses identified spillover patterns of MRSA-CC398 between

pigs and pig farmers, whereas human-associated *S. aureus* (carrying *φ*Sa3) strains colonized the nesting storks. Remarkably, *sec*- and *sel*-carrying *S. epidermidis*-ST595 from a nestling stork was detected. Among the CoNS, *ermT* was in plasmid *repUS18* in one *S. borealis* strain. However, the *ermT* in *S. hyicus* was not associated with any plasmid. Complete CRISPR-Cas system was detected in 19.2% of the CoNS strains, of which cas-1, -2 and -9 predominated and especially in 75% of the *S. borealis* strains. All the *S. aureus* strains had no CRISPR-Cas. The phylogenetic analysis identified clusters of related *S. epidermidis* lineages with other countries (SNP <100).

This thesis showed the influence of ecological niches on AMR levels and the presence and/ or transmission of various epidemic *Staphylococcus* species and lineages across healthy humans and animals and their relatedness with international strains. Collectively, this report underscores the need to strengthen the genomic epidemiological approach and inclusion of all *Staphylococcus* species from all hosts (even the healthy ones) to adequately understand the global spread of antimicrobial-resistant strains and track pathogenic ones using the “One Health” model.

RESUMEN

La resistencia a los antimicrobianos (RAM) y la patogenicidad de estafilococos constituyen uno de los principales desafíos de salud mundial que deben abordarse mediante un enfoque holístico de "Una sola salud" (*One Health*). El estudio de este género bacteriano en la microbiota nasal de humanos y animales sanos y su caracterización molecular podría proporcionar información relevante sobre la interconexión de diferentes ecosistemas. La inclusión de animales salvajes es necesaria para obtener vínculos epidemiológicos adecuados de *Staphylococcus* en los nichos desde la perspectiva de “Una sola salud”. Esta tesis aclara y profundiza en la comprensión de la diversidad y de los contenidos moleculares y genómicos de cepas de *Staphylococcus* obtenidas de muestras nasales de humanos (con o sin contacto con animales), cerdos, perros y polluelos de cigüeña sanos, utilizando estrategias cultivo dependientes.

De las 13 especies de *Staphylococcus* identificadas entre los 87 pichones de cigüeña, *S. sciuri* (85,7%) y *S. aureus* (31%) fueron las predominantes, siendo la prevalencia de *S. sciuri* significativamente mayor en los pichones de cigüeñas que se alimentaban en áreas naturales y la de *S. aureus* en aquellos que se alimentaban en vertederos. Todas las cepas de *S. aureus* fueron sensibles a la meticilina (SASM) y sólo una presentaba un fenotipo de resistencia a múltiples fármacos (MDR). Respecto a las líneas genéticas detectadas en las cepas de *S. aureus*, CC398 (8,2%) fue la más frecuente. Además, en cuatro cigüeñas se detectaron cepas portadoras de *tst* (CC22 y CC30), *eta* (CC9) y *etb* (CC45). De los 268 estafilococos coagulasa negativos (SCoN) no duplicados, se identificaron 10 cepas positivas para *mecA* (con SCC*mec* tipos III, IV y V). Destacó la identificación de una cepa de *S. lentus* que fue multirresistente y albergaba tanto el gen *mecA* como *mecC* ubicados en un SCC*mec* híbrido tipo VII.

De los 57 individuos sanos que no tuvieron contacto con animales, 98,2% fueron portadores de estafilococos (siete especies), de las cuales las especies predominantes fueron *S. epidermidis* (87,7%) y *S. aureus* (36,8%). Todos los *S. aureus* fueron SASM, pero se detectaron SCoNS resistente a meticilina (RM) en el 30,2% (con SCC*mec* tipos III, IV y V). Aproximadamente el 85,1% de las cepas de *S. aureus* portaban uno o más de los genes *lukF/S-PV, tst, eta, etb, etd, sea, seb, sec, sed, see* y/o *sep*. En las cepas SASM se identificaron ocho CCs, de los cuales el CC398 fue el predominante (33,3%). Aproximadamente el 77,8% de las cepas CC398 albergaban el gen *ermT* ubicado en el plásmido *rep13* y flanqueado por IS*257*. Sin embargo, una de las cepas SASM-CC398 portaba el gen *ermC* junto al *rep10*. Solo las cepas SASM-CC398 (*ermT* positivas) estaban estrechamente relacionadas (SNP <50) y

portaban el *φ*Sa3 (IEC tipo C). Se identificaron cepas de *S. epidermidis* multiresistentes que pertenecieron a las líneas genéticas ST2, ST59, ST173 y ST210.

De los 34 perros y 41 dueños de perros, se encontraron cepas de *S. aureus* en el 34,1 % de los dueños de perros (incluido un *S. aureus* resistente a la meticilina SARM-CC5-t2220-SCC*mec* tipo-IV2B) y en el 5,9 % de los perros; de *S. pseudintermedius* en el 2,4% de los humanos y el 32,4% de los perros, y de *S. coagulans* sólo en perros (5,4%). Sorprendentemente, en uno de los humanos se detectó tanto *S. aureus* como *S. pseudintermedius*, mientras que un perro portaba las tres especies de CoPS detectadas. La densidad del hogar se asoció significativamente con colonización con *S. pseudintermedius* en los hogares (OR = 18,10, IC del 95 %: 1,24-260,93, p = 0,034). Se encontraron cepas de *S. aureus* o *S. pseudintermedius* estrechamente relacionadas (<15 SNP) en humanos o perros en tres hogares. Aproximadamente el 56,3% de los portadores de *S. aureus* (perro o dueño de perro) albergaban más de una cepa diferente de *S. aureus*. SASM-CC398 fue la líneas genética más frecuentemente detectada, pero exclusiva de humanos. Fue destacable la detección de cepas SARM-CC5 resistentes a linezolid (LZD^R), fenotipo mediado por nuevas mutaciones puntuales en G2261A y T1584A en el ADNr 23S. Las cepas de *S. coagulans* fueron sensibles a todos los antimicrobianos. La mayoría de *S. pseudintermedius* contenían los genes *lukS/F-I, siet* y *sient*, y todos los *S. aureus* fueron negativos para los genes *lukF/S-PV, tst, eta* y *etb*. Las especies de SCoN predominantes en perros y dueños de perros fueron *S. epidermidis* (26,5% y 80,4%, respectivamente). Alrededor del 17,4% fueron resistentes a la meticilina (con SCC*mec* tipos II, III, IVc, V). Se identificó una cepa de *S. epidermidis*-ST35 LZD^R que contenía cuatro mutaciones en L3 (I188V, G218V, N219I, L220D) y una en L4 (N158S) en el dueño de un perro. Se identificaron perros y dueños de perros portadores de *S. epidermidis* con patrones de RAM y líneas genéticas similares (ST59, ST61, ST166 y ST278) en tres hogares (14,8%).

En los 40 cerdos analizados, las especies más detectadas fueron *S. aureus* (65%) y *S. chromogenes* (22,5%), mientras que en los 10 trabajadores de granjas de cerdos, *S. aureus* (80%) y *S. epidermidis* (40%). fueron los más predominantes. SARM se detectó en el 60% de los cerdos y en el 70% de los trabajadores. Sólo seis cepas de *S. aureus* fueron SASM. Todas las cepas de SARM fueron CC398, mientras todas las cepas de SASM-CC9 se detectaron solo en una granja (Granja C). Todas las cepas de *S. aureus* fueron negativas para los genes *luk-S/F-PV, tst* y *scn*, excepto una cepa SASM-CC45-t065 procedente de un trabajador que fue *scn* positiva (IEC tipo C). Se detectaron gran cantidad de genes de resistencia, incluidos algunos inusuales. Fue importante la identificación del gen *cfr* unido a plásmido (41,6 kb) en una cepa de *S. saprophyticus* de un cerdo y localizado cromosómicamente en una cepa de *S. epidermidis*-

ST16 de un trabajador, respectivamente. Alrededor del 42,5% de los portadores de SCoN presentaron similares genes de resistencia a antimicrobianos y tipos de SCC*mec*. También fue destacable la detección del gen *ermT* localizado en el plásmido *repUS18* en dos cepas de SARM procedentes de un cerdo y de un trabajador.

Los datos completos de secuenciación del genoma de 107 cepas revelaron la presencia de múltiples genes de resistencia a antimicrobianos unidos a plásmidos, que fueron predominantes en las cepas de *Staphylococcus* de cerdos y trabajadores de granjas, pero menos en los pichones de cigüeña. Además, genes de resistencia unidos a transposones como *ant9'* (Tn*554*), *ermA* (Tn*554*). Se identificaron *fexA* (Tn*554*, Tn*558*), *tet*(M) (Tn*916*, Tn*925*, Tn*6006*) y *dfrK* (Tn*559*). Los análisis de SNP identificaron patrones de diseminación de cepas SARM-CC398 entre cerdos y trabajadores en contacto con esots animales, mientras que cepas de *S. aureus* asociadas a humanos (portadoras de φSa3) colonizaron las cigüeñas estudiadas. Sorprendentemente, se detectó una cepa de *S. epidermidis*-ST595 que contenía los genes *sec* y *sel* en una cigüeña. Entre los SCoN, el gen *ermT* se detectó en el plásmido *repUS18* en una cepa de *S. borealis*. Sin embargo, este gen (*ermT*) no se asoció con ningún plásmido en la cepa de *S. hyicus*. El sistema CRISPR-Cas completo se detectó en el 19,2% de las cepas SCoN, de las cuales predominó cas-1, -2 y -9 y especialmente en el 75% de las cepas de *S. borealis*. No se detectó CRISPR-Cas en ninguna de las cepas de *S. aureus*. El análisis filogenético identificó linajes de *S. epidermidis* relacionados con los encontrados en otros países (SNP <100).

Esta tesis ha mostrado la influencia de diferentes nichos ecológicos en los niveles de RAM y la presencia y/o transmisión de varias especies y líneas genéticas epidémicas de cepas de *Staphylococcus* entre humanos y animales sanos y su relación con cepas internacionales. En conjunto, este trabajo subraya la necesidad de fortalecer el enfoque epidemiológico genómico y la inclusión de todas las especies de *Staphylococcus* de todos los huéspedes (no solo cepas clínicas sino también individuos sanos) para comprender adecuadamente la propagación global de cepas resistentes a los antimicrobianos y rastrear aquellas que puedan resultar patogénicas utilizando el modelo "Una sola salud".

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1. **Abdullahi, I. N.,** Lozano, C., Zarazaga, M., Saidenberg, A. B. S., Stegger, M., & Torres, C. (2023). Clonal relatedness of coagulase-positive staphylococci among healthy dogs and dog-owners in Spain. Detection of multidrug-resistant-MSSA-CC398 and novel linezolid-resistant-MRSA-CC5. *Frontiers in microbiology* ***Q1***, 14, 1121564. https://doi.org/10.3389/fmicb.2023.1121564
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CHAPTER ONE

INTRODUCTION

1.1 Microbiome: Definition and factors that influence its composition

The human microbiome consists of a diverse community of microorganisms that reside in different anatomical sites within the bodies of humans and animals (Aggarwal *et al*., 2023). Consequently, the coevolution of the microbiome alongside the hosts has led to these microbial communities having a significant role in enhancing human and animal well-being (Groussin *et al.,* 2020). Therefore, disruptions in the human microbiome have the potential to induce or worsen several diseases (Aggarwal *et al*., 2023).

Microorganisms inhabit specific environments based on the availability of their optimal growth conditions (Berg *et al.,* 2020). Microorganisms can be located in several regions of the human body, both externally and internally, including entrance points (Aggarwal *et al*., 2023). Several external parts of human and animal bodies serve as habitats for bacteria, these include the skin, nasal passages, ocular regions, and even exposed areas beneath the fingernails (Aggarwal *et al*., 2023). Thus, the routes by which microorganisms gain access to the human and animal body include the respiratory tract (mainly through the nose and trachea), gastrointestinal tract (through the mouth, oral cavity, stomach, and intestines), urogenital tract, and breaches in the integrity of the skin. Based on the findings of Hou *et al*. (2022), various internal anatomical regions, such as the lungs, gut, bladder, kidneys, and vagina, are inhabited by several microbial communities.

Microorganisms have a propensity to flourish in an environment that is conducive to their growth and survival. Therefore, it is hypothesized that these microbes possess mechanisms they use to adjust the conditions found in the microbiome, which closely reflect their preferred natural habitat (Aggarwal *et al*., 2023). The variety and quantity of microorganisms in various regions of the body are influenced by environmental conditions, including temperature, pH, oxygen concentration, pressure, osmolarity, and nutrient source (Aggarwal *et al*., 2023). As an example, the human body maintains an ideal temperature that facilitates the habitation of many microbial species. Additional factors, such as the existence of nutrition sources such as sebum, can alter the pH of the skin and serve as a carbon source, hence promoting the proliferation of specific microbial communities (Kim *et al*., 2016).

The composition and diversity of the human microbiota are contingent upon both intrinsic and external factors. Intrinsic variables encompass the characteristics of bodily environments, as previously elucidated, wherein the physiological conditions of habitat locations create a conducive environment for the proliferation of certain microorganisms

(Aggarwal *et al*., 2023). Additional intrinsic factors that play a role in shaping the composition of the microbiome include genetic and ethnic background, sex, and age of the hosts. The stability of the human microbiome can often be observed once the microorganisms have successfully acclimated to their surrounding environment. In addition to endogenous factors that might potentially induce alterations in the microbiome over time, exogenous factors like dietary patterns, lifestyle choices, pharmaceutical interventions, geographical location, climatic conditions, and seasonal variations have the potential to influence the composition and dynamics of the microbial community (Rinninella *et al.*, 2019). The human microbiome exhibits robust growth in favourable conditions, which are contingent upon the inherent ecological characteristics of the body (Aggarwal *et al*., 2023). The perturbation of the body's natural environment leads to a consequential alteration in the microbial composition and diversity, as the microorganisms adjust to the modified conditions (Sanders *et al.*, 2021). This adaptive response has the potential to give rise to many diseases (Sanders *et al.*, 2021).

Microbial interactions exhibit a high degree of complexity, flexibility, and adaptability in response to physiological alterations. For instance, alterations in the availability of nutrients have the potential to induce changes in the relative proportions of community members and subsequently impact their functional capabilities. In addition to competition for nutrients, the resident bacterial community can engage in cross-feeding interactions by producing "waste" products that are subsequently metabolised by other species (Wagner, 2022). Thus, competition for nutrients and direct mortality through the release of antimicrobial substances are anticipated consequences (Friedman and Gore, 2017; Widder *et al*., 2016; Fredrickson, 2015). The interplay between cooperation and competition within microbial communities elicits both positive and negative feedback, hence exerting a significant influence on the overall functional activities of these communities (Leung and Poulin, 2008).

The One-Health concept acknowledges the interdependence between human health, animal health (including companion animals, cattle and their associated products, and wildlife), and the surrounding environment. The nasal commensal microbiota in both humans and animals exhibits a wide range of diversity and has a crucial and intricate function in safeguarding health and enhancing the immunological competence of the hosts (Di Stadio *et al*., 2020).

1.1.1 Methodology for studying microbiome composition

The assessment of alterations in the nasal microbial communities in both humans and animals has historically been conducted through the utilisation of conventional microbiological methodologies (Dorn *et al*., 2017). In recent years, there has been significant progress in the field of molecular-based techniques, particularly those focused on analyzing the 16S rRNA gene of bacteria. These approaches have facilitated the study of intricate microbial communities found in many locations within both human and animal hosts. Notably, previous studies have been conducted on the nasal microbiota of healthy humans, pigs, dogs and cats, as demonstrated by Chen *et al* (2022), Chrun *et al* (2021), Vientos-Plotts *et al*. (2017), Dorn *et al*. (2017) and Ericsson *et al*. (2016). However, available studies on wild animals were solely on gut microbiomes targeting faecal samples (Sun *et al*., 2023; Pannoni *et al*., 2022).

Respiratory tract microbiome research could encounter several methodological challenges, such as the selection of sampling tools (e.g., swabs, nasal rinses, and dry filter sheets) and sampling locations (Kumpitsch *et al*., 2019). Typically, the anterior nares, middle meatus, and nasopharynx are commonly selected as the ideal sampling locations due to their accessibility, as noted by Kumpitsch *et al*. (2019). Other regions are often less readily reachable for sampling purposes.

There exists a diverse array of platforms and technologies that can be employed to investigate microbiomes. These methodologies can broadly be classified into three categories: (a) marker gene analysis, (b) shotgun metagenomics, and (c) metatranscriptomics, metabolomics, and metaproteomics. Marker gene analysis is a widely employed approach in which targeted sequencing methods are predominantly utilized. One of these methods involves the sequencing of the 16S ribosomal RNA gene of bacteria (Jo *et al* (2016). The genes in question, although exhibiting a high degree of conservation, have undergone divergence over time (Galloway-Peña & Hanson, 2020). This divergence has resulted in the development of a distinctive barcode that can be utilised to assign certain taxonomies. Furthermore, this barcode can be quantified to determine the frequency of each member within the microbial community (Galloway-Peña & Hanson, 2020). A notable difficulty encountered in the examination of these marker genes involves the establishment and detection of a distinct sequence (Galloway-Peña and Hanson, 2020). Furthermore, it is worth noting that certain taxonomic groups can possess identical gene sequences (such as *Escherichia* and *Shigella*). Thus, this can complicate the determination of a distinct

sequence and the distinguishing of individual taxonomic groups (Vetrovsky and Baldrian, 2013). Furthermore, the amplification of each gene is conducted using polymerase chain reaction (PCR), and subsequently, all the PCR results are collectively sequenced (Xue *et al*., 2018). This approach could introduce several errors, potentially affecting the accurate identification of a distinct sequence (Xue *et al*., 2018).

At present, the predominant method for 16S sequencing often utilizes the sequencing capacity provided by the Illumina MiSeq platform, which allows for a sequencing length of 2×300. This sequencing length is commonly employed to target several variable regions and achieve optimal accuracy (Galloway-Peña & Hanson, 2020). The Illumina MiSeq platform involves the utilization of primers unique to certain regions, such as V1-V3 or V4. These primers are used to sequence both the forward and reverse strands, resulting in the generation of a complete amplicon for each taxon present in the microbial community (Callahan *et al*., 2019).

The utilisation of marker gene sequencing techniques has provided significant findings regarding the involvement of the microbiome in both healthy states and the development of diseases. However, it is important to note that this methodology primarily concentrates on a limited fraction of microbial genomes. Shotgun metagenomics approaches encompass a set of techniques that employ untargeted sequencing methods to comprehensively capture the complete repertoire of microbial genomes present inside a given sample (Quince *et al*., 2017). The comprehensive acquisition of genetic data from a microbiome specimen enables the examination of bacterial components but is contingent upon the availability of reference genomes and scientific understanding (Galloway-Peña and Hanson, 2020; Mukhopadhya *et al*., 2019).

Metatranscriptomic methodologies employ analogous analytical principles to shotgun metagenomics, with a specific focus on capturing the RNA transcripts originating from microbial cells. This enables the evaluation of bacterial expression activities (Bashiardes *et al*., 2016). Shotgun metagenomics and metatranscriptomic techniques predominantly utilise Illumina sequencing protocols, particularly the HiSeq or NovaSeq platforms, owing to their advantageous characteristics of high throughput and cost-effectiveness per base. Nevertheless, there has been a growing trend towards the utilisation of PacBio and Oxford Nanopore sequencing technologies. This shift is driven by the advantage of longer read lengths, which prove beneficial in facilitating gene calling and genetic mapping to reference genomes (Galloway-Peña and Hanson, 2020).

The primary objective of metabolomics analysis is to examine the metabolic profiles of the metabolites produced by bacteria and investigate the intricate interactions between these products, microbiota, and the metabolism of the hosts (Lamichhane *et al*., 2018). These methods frequently measure the concentrations of low molecular weight compounds, such as antibiotics, byproducts of antibiotics, and intermediates of host and/or bacterial metabolism (Lamichhane *et al*., 2018). Mass spectrometry is frequently employed in metabolomics to detect compounds that are already known (Zierer *et al*., 2018). Metaproteomics use mass spectrometry as a means to detect and quantify the proteins that are present inside a given microbiome (Blakeley-Ruiz *et al*., 2019). Metaproteomics and metabolomics are two rapidly developing technologies that are now significant in the advancement of microbiome research.

1.2 Nasal Microbiome: Anatomy and Composition

1.2.1 Anatomy of the nasal cavities of humans and animals

The upper respiratory tract (URT) in humans is composed of several anatomical structures, including the anterior nares, nasal cavity, sinuses, nasopharynx, eustachian tube, middle ear cavity, oral cavity, oropharynx, and larynx (Kumpitsch *et al*., 2019). The nasal cavity is divided into three distinct regions, namely the lower, middle, and superior meatus, which are demarcated by three nasal turbinates (Kumpitsch *et al*., 2019). The nasal cavity serves as a crucial interface between the human body and the external world. During the process of inhaling, the respiratory passages come into contact with many elements present in the surrounding environment, including germs, pollutants, and aeroallergens, among other substances (Kumpitsch *et al*., 2019).

The nasal cavity harbours a diverse range of bacteria, including both pathogenic and non-pathogenic strains. The presence of this wide array of bacteria can be ascribed to localised factors such as temperature and humidity, as described by Kumpitsch *et al* (2019). The spatial location inside the respiratory system may also play a role in shaping the variability observed in the nasal microbiome. For example, Kumpitsch *et al* (2019) found that the anterior nares exhibit lower levels of microbiome biodiversity compared to the middle meatus and sphenoethmoidal recesses. The lining of the anterior nares consists of keratinized squamous epithelium and sebaceous glands that secrete sebum, potentially influencing the diversity of bacteria present (Yan *et al*., 2013).

The turbinates in canines have a role in augmenting the surface area covered by the mucosa. Nevertheless, the overall surface area of the dog's mouth can be impacted by

factors such as the dimensions and configuration, as suggested by Taherali *et al* (2019). The nasal turbinates are anatomical structures that extend from the lateral walls of the nasal cavity and house a network of veins. As a result, a portion of the air inhaled by dogs, around 5-15%, is directed towards these turbinates (Galibert *et al*., 2016).

The respiratory system of the pig starts at the nostrils, which serve as the entry point for two nasal passages. The dorsal and ventral turbinate bones are present in these structures (Helke *et al*., 2015). The ventral turbinates are comprised of four slender primary bones, with two located on each side and separated by a cartilaginous septum. The respiratory tract is bordered by a mucous membrane, which is characterised by its smooth texture and is coated with viscous mucus (Helke *et al*., 2015). Additionally, the surface is adorned with little hair-like structures that possess the capability to sweep the mucous throughout its expanse through their undulating action (Helke *et al*., 2015). The process involves the transportation of mucus from the nasal passages, bronchial tree, and trachea into the pharynx, ultimately leading to its ingestion. The nasal cavity serves as the primary entry point for microorganisms into the respiratory tract, making it a crucial component of the body's initial defence mechanism against bacteria (Helke *et al*., 2015).

It has been observed that wild animals possess a nasal cavity that is characterised by a relatively brief connection pathway to the trachea. Similarly, the oral (buccal) cavity of these animals is found to be connected to the pharynx (Morand *et al*., 2014). Therefore, it is anticipated that microorganisms present in the nasal and oral cavities possess convenient pathways to reach the trachea and pharynx (Morand *et al*., 2014).

1.2.2 Microbiota composition in humans and animals

1.2.3 Microbiota in humans

According to Basis *et al*. (2015), the microbial composition of the anterior nares in individuals who are in good health is primarily characterised by the presence of three phyla, namely Actinobacteria, Firmicutes, and Proteobacteria. The anterior nares can be categorised into four unique genus profiles, which include *Staphylococcus, Propionibacterium, Corynebacterium,* and *Moraxella*. **Figure 1** illustrates that the middle meatus has a significant prevalence of the species *S. aureus, S. epidermidis,* and *Propionibacterium acnes*. The nasal microbiota of 178 adult participants was examined in a study, revealing that 88.2% of them were carriers of *Corynebacterium* spp, 83.7% were carriers of *Propionibacterium acnes,* and 90.4% were carriers of *S. epidermidis.* However,

the frequency of occurrence of these bacterial species could vary among individuals (Liu *et al*., 2015).

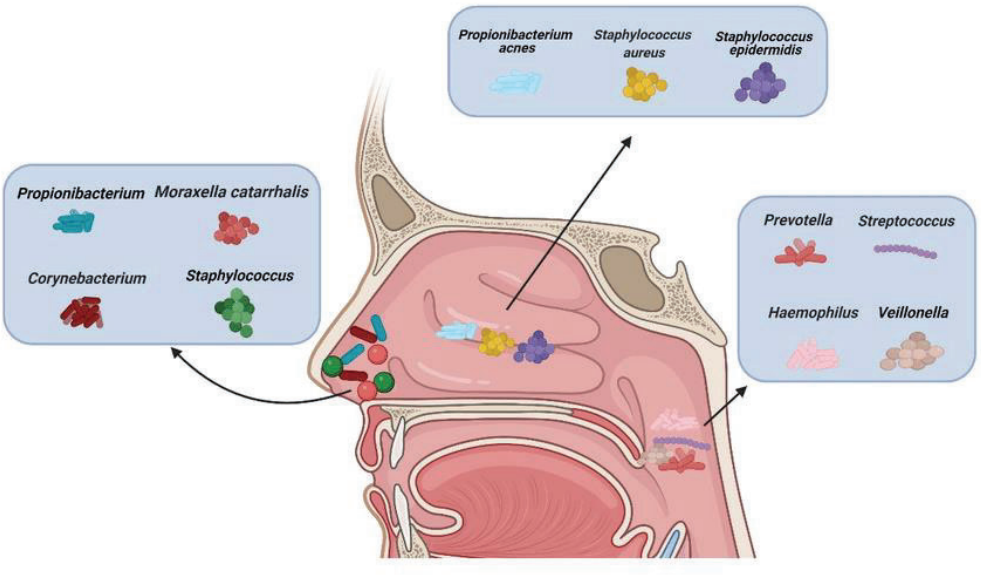


Figure 1. Types of microbiota in normal nasal mucosa and other parts of the upper airway (Tai *et al*., 2021)

A study conducted by Kaspar *et al*. (2016) revealed the presence of more than 150 distinct bacterial species in the nasal secretions of humans. *S. aureus*, an opportunistic pathogen, is highly prevalent among healthy humans, with around one-third of the human population harbouring this species in their noses (Brégeon *et al*., 2018). Asymptomatic nasal carriage of *S. aureus* is a significant contributing factor in the development of endogenous *S. aureus* infections, particularly following surgical procedures (Brégeon *et al*., 2018; Sakr *et al*., 2018).

Pig farmers are regularly exposed to a diverse and varied environment, which entails significant bacterial presence (Moor *et al*., 2021). Moreover, pigs serve as a possible source for several microorganisms that can be transmitted to humans and subsequently alter their microbiota (Kraemer *et al*., 2018). Moreover, the employment of a culture-independent approach in next-generation sequencing showed a significant correlation between household affiliation and microbial communities in both human and animal companions (Misic *et al*., 2015). Furthermore, Lax *et al*. (2014) presented evidence of significant interactions between the microbiota of humans, their households, and their pets in a separate longitudinal investigation. Nevertheless, despite its significance, the investigation into the transfer of microbiota between animals, people, and the environment has been conducted to a limited extent.

1.2.2.2 Migratory wild birds

A crucial area of inquiry in the study of migratory birds pertains to the relationship between bacterial microbiomes or colonisation and ecological characteristics, such as foraging behaviour and host habitat (Vittecoq *et al*., 2016). Hence, it is important to understand the microbial variations within the nasotracheal cavities of storks, considering their feeding patterns, habitat preferences, and movement ecology. A considerable number of white storks have developed the ability to depend on landfills as a food source throughout their migration and winter periods, as well as utilising rice and other cereal fields for foraging and resting purposes (Martín-Vélez *et al*., 2020).

According to Tortosa *et al*. (2002), certain storks have successfully formed colonies in proximity to garbage sites. During the breeding period, adult storks predominantly engage in foraging activities close to their nests, hence offering an opportunity to conduct a comparative analysis of the influence of foraging habitat on the composition of respiratory tract microbiota in nestlings (Pineda-Pampliega *et al*., 2021). These several elements have the potential to influence the composition and diversity of the nasal and gut microbiota in storks. To date, there has been a lack of research investigating the nasal microbiota of white storks.

1.2.2.3 Dogs

To date, there exist limited studies that compare the nasal microbiota between healthy canines and those afflicted with nasal pathologies, as this could provide credible data on the nasal microbiome of healthy dogs. In a study conducted by Tress *et al* (2017) on canines in good health, it was observed that *Moraxella* spp. was the predominant species, with *Phyllobacterium* spp., *Cardiobacteriaceae*, and *Staphylococcus* spp. following suit. The study indicates that the nasal cavity of canines harbours a diverse bacterial ecology. Consistent with prior studies that employed next-generation sequencing techniques to analyse bacterial composition, the examination of the nasal microbiome in canines deemed healthy unveiled the presence of nine distinct taxonomic groups. Notably, the dominant phyla observed were proteobacteria (82.8%), firmucutes (4.9%), Bacteroidetes (2.8%), and Cyanobacteria (2.1%).

The study conducted by Tress *et al*. (2017) revealed a significant presence of Moraxellaceae, specifically *Moraxella* spp and *Pasteurella multocida*, in healthy canines

($p < 0.05$). It is worth mentioning that differences in dogs' breed and environmental hygiene could affect the nasal microbiota (Tress *et al.*, 2017).

According to Isaiah *et al*. (2017), the bacterial phyla that were most found in nasal samples were Proteobacteria, followed by Bacteroidetes. Furthermore, there was a significant ($p<0.001$) in the abundance of *Moraxella, Leucobacter, Helcococcus*, and *Cardiobacterium* at the genus level in nasal samples compared to oral samples.

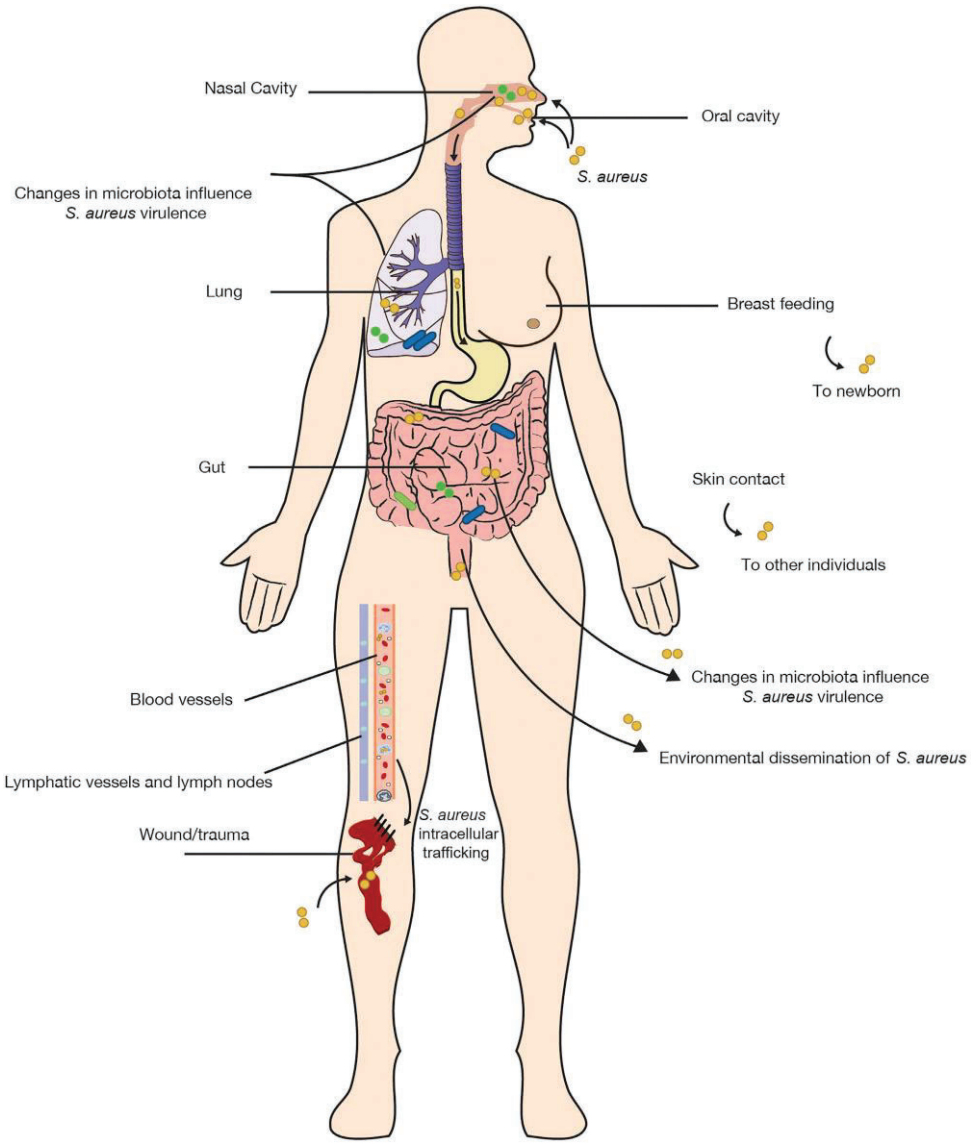


Figure 2. Routes of *S. aureus* acquisition, dissemination in the human body and transmission. *Staphylococcus aureus* can enter the human body via direct or indirect interpersonal contacts, contaminated food products, trauma and surgery (Raineri *et al.*, 2022).

1.2.2.4 Pigs

In their study, Chrun *et al*. (2021) provided an analysis of the predominant phyla found within the nasal cavity of healthy pigs. The study identified five primary phyla in

healthy pigs, namely Proteobacteria (48.3%), Bacteroidetes (21.7%), Firmicutes (16.2%), Cyanobacteria (5.2%), and Ternicutes (1.5%). At the family level, the most abundant taxonomic group was Moraxellaceae (38.9%), followed by Prevotellaceae (9.2%), Weeksellaceae (7.3%), Streptophyla (4.5%), Ruminococcaceae (3.6%), Lactobacillaceae (3.3%), Neisseriaceae (2.4%), Aerococcaceae (2.4%), Enterobacteriaceae (2.3%), Rickettsiales, mitochondria (2.2%), Lachnospiraceae (2.2%), Streptococcaceae (2.0%), Staphylococcaceae (1.6%), Veillonellaceae (1.4%), Paraprevotellaceae (1.0%), and Mycoplasmataceae (0.8%) (Chrun *et al*., 2021). The potential roles of antibiotic-induced dysbiosis should be emphasised, given the utilisation of several prophylactic antimicrobial drugs across different stages of pig production (Payen *et al*., 2023).

1.3 Nasal microbiota and interactions between bacteria

The nasal microbiota and health conditions can mutually influence each other. The study conducted by Frank *et al*. (2010) found a negative correlation between the colonisation of *S. aureus* and the presence of other bacteria, such as *S. epidermidis*. The antagonizing effect observed between bacteria may be attributed to interdependent inhibition processes by active metabolites of the bacteria (Krismer *et al*., 2017).

Certain bacterial species could release biochemicals that have an inhibitory effect on the growth of *S. aureus,* hence regulating its growth and population **(Figure 3).** For example, the formation of hydrogen peroxide (H_2O_2) by *Streptococcus pneumoniae* has been found to have bactericidal effects on *S. aureus,* as demonstrated in studies conducted by Regev-Yochay *et al*. (2006) and Selva *et al*. (2009). Another study conducted on both *in vitro* and on human subjects has provided evidence that lugdunin, a bioactive molecule synthesised by *S. lugdunensis* through a non-ribosomal process, exhibits the ability to inhibit nasal colonisation of *S. aureus* through its bactericidal properties (Zipperer *et al*., 2016).

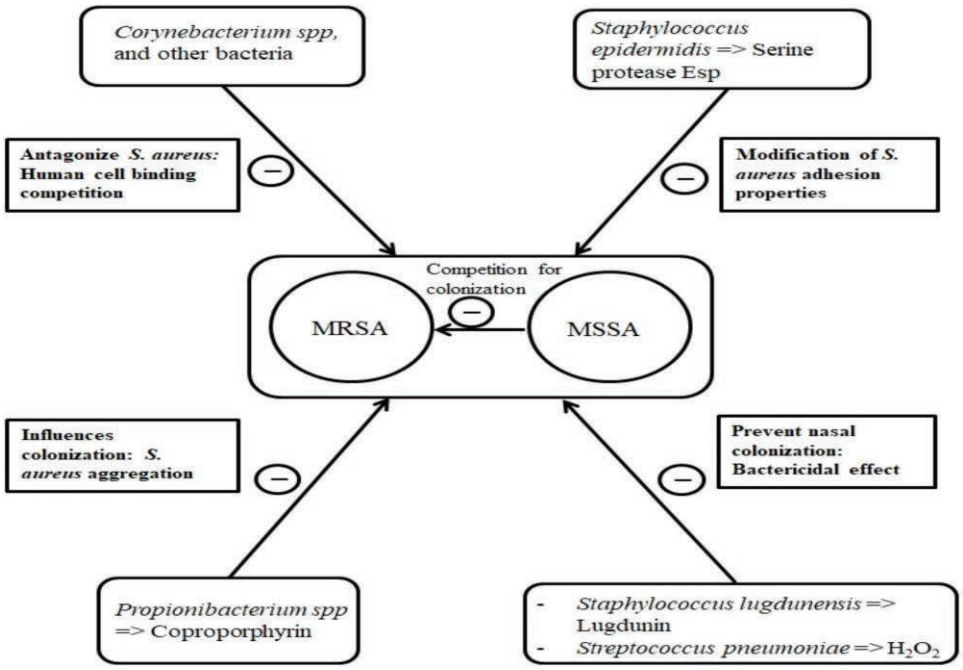


Figure 3. Bacterial biochemicals are used to antagonize *S. aureus* in the nasal cavity (Zipperer *et al*., 2016).

In certain instances, the chemicals released by bacteria can alter the adhesion properties of *S. aureus.* Certain strains of *S. epidermidis* have demonstrated the ability to produce the serine protease Esp, which has been observed to eradicate nasal *S. aureus* in humans without any consequences to their health (Iwase *et al*., 2010). This elimination is likely achieved through the degradation of staphylococcal surface proteins and human receptors that play a crucial role in the interaction between the host and the pathogen (Sugimoto *et al*., 2013).

Additionally, it has been observed that *Propionibacterium* species have the ability to generate coproporphyrin III, a metabolite of porphyrin, which has been found to stimulate the aggregation of *S. aureus* (Wollenberg *et al*., 2014). This aggregation process has been shown to have an impact on *S. aureus* nasal colonisation, as demonstrated in a study conducted by Wollenberg *et al* (2014). The antagonistic effect of *Corynebacterium* species on *S. aureus* is reported to occur through several mechanisms including competition for binding to human cells (Lina *et al*., 2003), as demonstrated in **Figure 3**. In the earlier study by Uehara *et al*. (2000) on 156 healthy people individuals. It was discovered that after administering up to 15 inoculations of *Corynebacterium* spp into the nasal cavity of *S. aureus* carriers, there was a total eradication rate of 71% for the nasal *S. aureus* (Uehara *et al*., 2000). In recent experiments, it has consistently been shown that *Dolosigranulum pigrum* antagonistically limits the growth of *S. aureus* in the nose of healthy humans and

could modulate the nasal microbiome (Mostolizadeh *et al*., 2022; De Boeck *et al*., 2021; Brugger *et al*., 2020).

Furthermore, there have been descriptions of antagonisms occurring within the same *Staphylococcus* species. The study conducted by Dall'Antonia *et al* (2005) suggested that there is a competitive colonisation between methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). The findings of the study indicate that MSSA may have a protective effect against MRSA carriage.

1.3.1 Antimicrobial resistance problem: One Health and Global Health context

Historically, bacterial infections have consistently emerged as significant causative agents of infectious diseases in both human and animal populations. The discovery and subsequent utilisation of antibiotics have facilitated the efficient treatment of bacterial infections while concurrently leading to a rise in antimicrobial resistance (AMR) due to multiple reasons (Uddin *et al*., 2021). The primary determinants of AMR encompass the inappropriate and excessive utilisation of antimicrobial agents, inadequate provision of clean water, sanitation, and hygiene for both humans and animals, substandard infection prevention and control practises in healthcare facilities, limited availability of medicines and vaccines, insufficient awareness and knowledge, and deficiencies in legislative frameworks (Velazquez-Meza *et al*., 2022; WHO, 2015).

The global health issues posed by the emergence and dissemination of AMR and resistant microorganisms are significant. Based on the figures provided in 2019, it was determined that there were 1.27 million fatalities worldwide that might be directly ascribed to illnesses due to antimicrobial-resistant pathogens **(Figure 4)**. If appropriate actions are not implemented to address the present rates of dissemination of AMR, projections indicate that it will impose a global economic burden of 3.4 trillion dollars by the year 2030 and lead to more than 10 million annual fatalities by 2050 (Murray *et al*., 2022; O'Neill 2016). According to the United Nations Environment Programme (UNEP, 2019), if left unaddressed, AMR has the potential to cause an additional 24 million people to fall into the category of severe poverty during the coming decade.

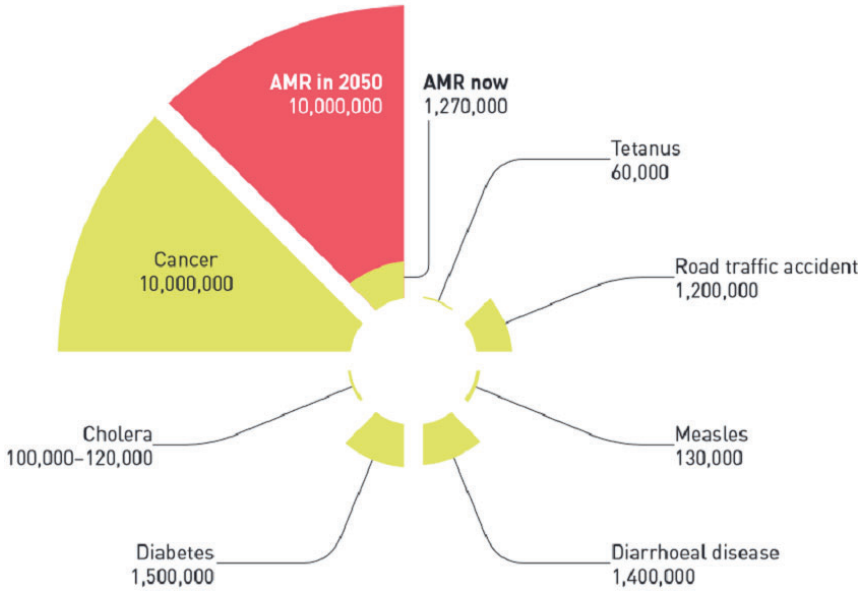


Figure 4. Predicted global mortality from AMR compared to common causes of death today (O'Neill 2016; Murray *et al*., 2022).

Understanding the pathways through which AMR is disseminated is crucial in addressing its consequences (Despotovic *et al*., 2023). A wide range of ecosystems within the biosphere can contribute to the emergence, development, and spread of AMR to varying extents (Despotovic *et al*., 2023). Comprehending the issue of AMR, particularly its transmission, necessitates the adoption of two interconnected approaches: One Health and Global Health (Laborda *et al*., 2022; Hernando-Amado *et al*., 2020). The field of One Health investigates the influence of various interconnected ecosystems, such as hospitals, water bodies, food systems, pets, wild animals, farms and the environment on the emergence and spread of AMR (Hernando-Amado *et al*., 2019). On the other hand, Global Health examines the repercussions of AMR emergence in a specific geographical location on other regions across the globe (Berndtson, 2020).

Numerous critical analyses and scholarly publications have been conducted about the impact of farming, hospital environments, communities, domestic animals, the food chain, and water on the emergence and dissemination of AMR concerning human health. Nevertheless, it is widely acknowledged that wildlife can potentially influence AMR by serving as a potential reservoir and sentinel for antibiotic-resistant bacteria **(Figure 5).**

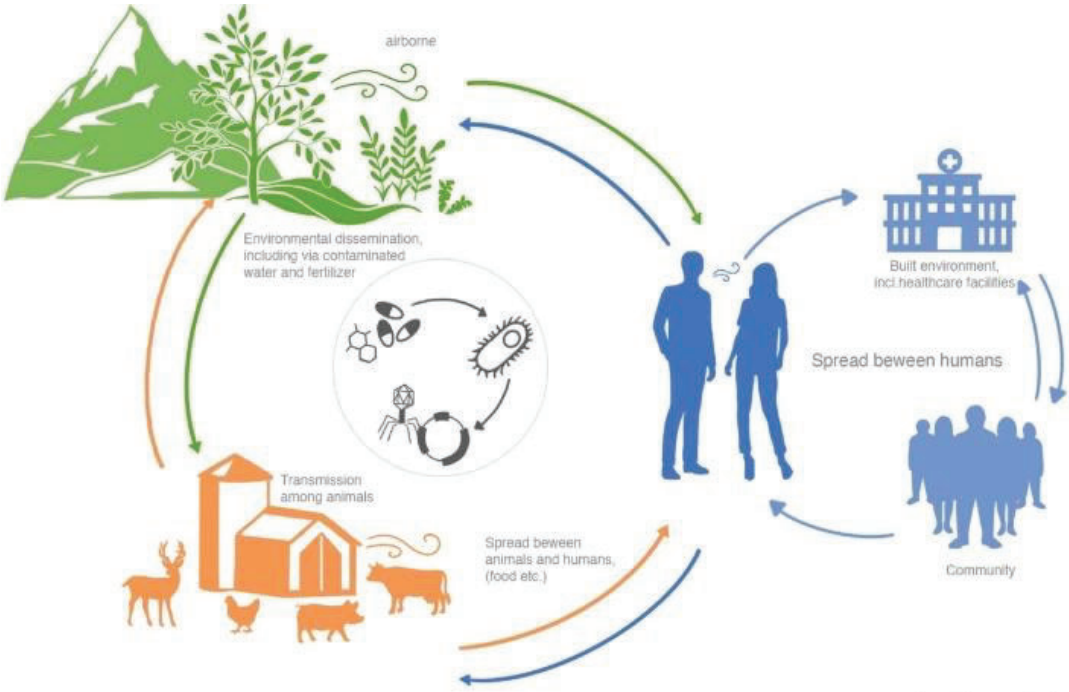


Figure 5. Mobilome-mediated AMR dissemination across different microbial reservoirs in One Health ecosystems (Despotovic *et al*., 2023).

Often time, AMR genes (ARGs) in bacteria are dessiminated across several hosts and the environment through mobile genetic elements by horizontal gene transfer (HGT) (Tao *et al.,* 2022). These ARGs can then be disseminated among many reservoirs, including humans, animals, and the environment (Despotovic *et al.*, 2023). To comprehend the distribution of ARGs and their associated microbial taxa, it is imperative to conduct a comprehensive analysis of the resistome across various microbial reservoirs (Wu *et al*., 2023). The interconnection of all ecosystems facilitates the transmission of resistant bacteria or ARGs across many reservoirs (Despotovic *et al*., 2023). The concept of One Health encompasses a transdisciplinary framework that redirects attention from the mere treatment and control of diseases towards a more proactive approach centred on illness prevention and surveillance (Despotovic *et al*., 2023). The integration of research on antimicrobial-resistant bacteria present in humans, animals, and the environment is of utmost importance in the One Health ecosystems. This approach plays a vital role in advancing our comprehension of the intricate epidemiology of AMR (Despotovic *et al*., 2023; Nadeem *et al*., 2020).

The dissemination of antimicrobial-resistant bacteria across several reservoirs, including humans, animals, and the environment, can occur at both local and global scales **(Figure 5).** The acquisition of ARGs has been attributed to various factors including

globalisation and increased mobility, expanding human population, proximity to animals and their habitats, intense agricultural practises, environmental pollution, deterioration of ecosystems, and climate change (Nadeem *et al*., 2020). These events possess the capacity to swiftly initiate a pandemic, in which AMR is not limited by geographical or interspecies boundaries (Nadeem *et al*., 2020).

1.3.2 Role of microbiome in AMR problem

Within the framework of One Health, it is noteworthy that natural microbial communities, commonly referred to as microbiomes, may possess a significant function in the propagation of AMR. The composition of human and animal microbiomes is influenced by various factors, such as the acquisition of microorganisms from external sources like animals and the environment, interactions between the host and microorganisms, and the consequences of competitive, cooperative, and predatory interactions, including those involving phages (Trinh *et al*., 2018). According to a recent study conducted by Stanton *et al*. (2020), there is emerging evidence indicating that commensal bacteria can transfer ARGs to human-associated and pathogenic bacteria at a rapid pace. This phenomenon presents a significant risk to human health. The significance of the resistome, which refers to the aggregation of ARGs inside a specific habitat or organism, and the variations observed across ecosystems, holds considerable epidemiological and ecological value (Yin *et al.*, 2023).

The investigation of AMR and its associated research in staphylococci has primarily focused on pathogenic strains that are clinically significant, such as methicillin-resistant *S. aureus* (MRSA) (Despotovic *et al*., 2023). Nevertheless, MRSA and MSSA have also been found in the human and animal nasal microbiome (Effelsberg *et al.,* 2023; Theophilus and Taft 2023; Islam *et al*., 2020). While the majority of bacteria that make up the human and animal microbiome are considered commensals, they have a significant role in the dissemination of AMR (Aslam *et al.,* 2021). The transmission of AMR can take place from pathogenic bacteria to commensal organisms, as well as from commensals or environmental bacteria to other members of the microbial community (Brinkac *et al*., 2017; Montassier *et al*., 2021). After the acquisition of ARGs, commensal organisms can facilitate the spread of AMR to microbes that possess pathogenic properties (Montassier *et al*., 2021).

The AMR potential within the microbiome is dependent on notable variations across different geographical locations, which can be attributed to disparities in antibiotic

usage, as well as factors associated with medicine and food production (Despotovic *et al*., 2023).

1.3.3 Use of antibiotics: Emergence and dissemination of AMR

Antibiotics are widely utilised in several contexts, including human medicine as well as livestock farming and aquaculture, particularly in the realm of food production (Pepi *et al.,* 20221). This widespread usage has resulted in alterations in the composition of microbial communities and the possible escalation of ARGs. The utilisation of antibiotics in the livestock industry for treatment purposes further amplifies the likelihood of the formation of antibiotic-resistant bacteria as a result of prolonged selective pressures. Skarżyńska *et al*. (2020) observed a higher prevalence of AMR in the gastrointestinal tract of domesticated farm animals (namely, chickens, turkeys, and pigs) as opposed to that of wild animals (including boars, foxes, and rodents). In a study conducted by Holman *et al*. (2020), it was shown that the prophylactic administration of antibiotics resulted in alterations in the microbiome of bovine faeces and nasopharynx. These changes were associated with an elevated presence of ARGS.

While the development of antibiotic-resistant pathogens is an important factor to consider, the transmission of ARGs from animals to the human microbiome is a more pressing issue. The dissemination of the pathogen can take place through various pathways, one of which is direct transmission facilitated by food products. A systematic review has documented that food animals can be a significant source of AMR in Africa, specifically non-*aureus* staphylococci (Ocloo *et al*., 2022). This highlights the idea that non-pathogenic bacteria, which are often not included in monitoring programmes, can potentially operate as a reservoir for AMR within food supply chains (Rossi *et al*., 2019).

The emergence of AMR is driven by selective pressures, primarily exerted by antibiotics, although other factors such as heavy metals or biocides can also contribute to the selection of AMR. This implies that the primary locations where AMR selection takes place are human-influenced habitats and non-natural and non-heavily contaminated ecosystems such as wildlife (Plaza-Rodríguez *et al*., 2021). Therefore, the presence of clinically significant ARGs and antibiotic-resistant bacteria in wild animals that have not been exposed to antibiotics should be seen as an indicator of AMR contamination, rather than a manifestation of AMR selection (Plaza-Rodríguez *et al*., 2021). Under this notion, a research investigation conducted on Australian sea turtles has demonstrated the presence of antibiotic-resistant Enterobacteriaceae in their microbiome. These resistant strains

include well-known human commensals/pathogens such as *Klebsiella* spp, *Citrobacter* spp, and *E. coli*. Furthermore, the study revealed that the prevalence of AMR was comparatively lower in sampling sites located at greater distances from urban areas (Ahasan *et al*., 2017). Moreover, the One-health-UR research group of the University of La Rioja (Spain) consistently reported the detection of AMR bacteria in different varieties of wild animals (Alonso *et al*., 2021; Gomez *et al.,* 2020; Rui-Ripa *et al*., 2020; Gomez *et al.,* 2016).

1.3.4 Is AMR something new? Role of wild animals and the environment

Following the notion that wild animals do not or rarely receive antibiotic treatment, there is a lack of definitive information regarding the contemporary occurrence of AMR in wildlife (Dolejska and Literak, 2019). However, it should be noted that the absence of clinically significant AMR does not imply that it did not exist before the advent of antibiotics. This is because ARGs are ancient components that evolved before the introduction of antibiotics in clinical use (Larson *et al*., 2021). In this regard, the study by Larson *et al* (2021) indicates that certain clinically significant mobile ARGs may have been acquired from natural environments due to selective pressures predating the antibiotic era. According to the study, it has been observed that certain lineages of MRSA were present in hedgehogs during the period before the discovery and widespread use of antibiotics in clinical practice **(Figure 6).**

According to existing evidence, it has been substantiated that wild animals serve as a reservoir for antibiotic-resistant bacteria and the corresponding resistance genes, hence contributing to the persistence of AMR in the One Health ecosystems. Laborda *et al*. (2022) suggest that migratory birds, such as storks and gulls, have the potential to facilitate the long-distance spread of AMR. Conversely, non-migratory animals, such as flies or cockroaches, may play a role in the transmission of antibiotic resistance across shorter distances **(Figure 6)**. The discovery that ARGs may be transmitted among bacteria found in the microbiota of insects, such as cockroaches, which have proximity to people, suggests that these species could potentially serve as vectors facilitating the dissemination of AMR among bacterial pathogens. In addition to their participation in the spread of antibiotic resistance, recent research findings confirmed that wild animals may play a role in the emergence of antibiotic resistance. This occurrence can be attributed to the hedgehog's exposure to b-lactam-producing microbes present in its microbiome (Larson *et al*., 2022).

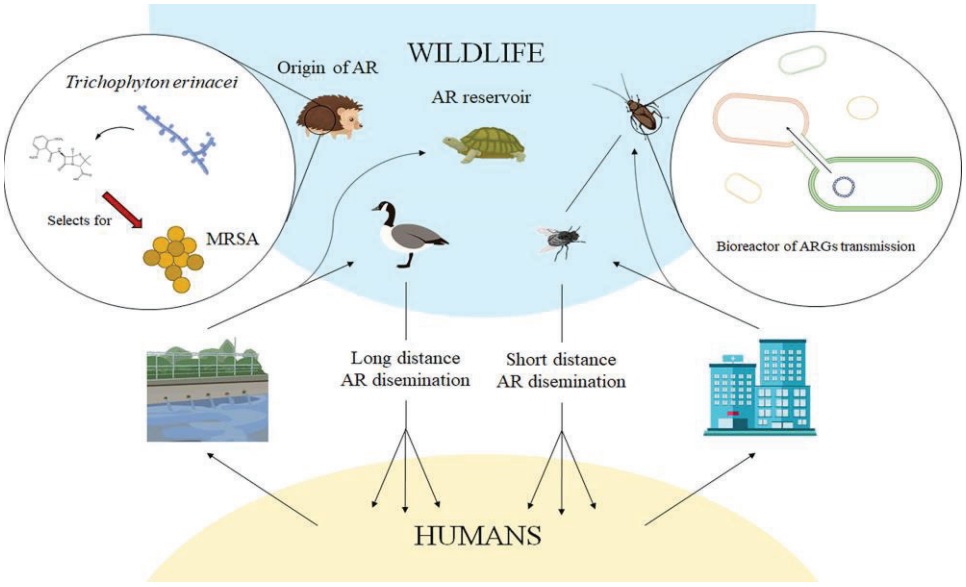


Figure 6. Contribution of wildlife to antibiotic resistance (Laborda *et al.,* 2022).

It is worth mentioning that *Trichophyton erinacei*, a dermatophyte found in hedgehogs, is capable of producing two b-lactams. These compounds have been suggested as a potential selective force contributing to the prevalence of MRSA in hedgehogs (Larsen *et al*., 2022). Nevertheless, despite these findings, there is a lack of quantitative analyses regarding the significance of wildlife pathways in the dissemination of AMR compared to other pathways such as human travellers versus migratory birds, or fomites and direct human-to-human contact versus flies and cockroaches coexisting with humans (Laborda *et al.,* 2022). In the present circumstances, wherein climate change has the potential to modify the global distribution of wildlife, including vectors implicated in the spread of infectious diseases (Agache *et al*., 2022), the provision of quantitative data about the involvement of wildlife in the emergence, development, and transmission of antimicrobial resistance (AR) is of significance in addressing AMR from the perspectives of One Health and Global Health.

The escalating utilisation and improper application of antimicrobial agents, with exposure to other microbial stressors such as environmental pollution could facilitate the emergence of microbial resistance in both human populations and the surrounding ecosystem (Samreen-Ahmad *et al*., 2021). Bacterial populations present in many environmental mediums, such as water, soil, and air, have the potential to develop resistance through interactions with other microorganisms that possess resistance traits **(Figure 7).** The potential for human exposure to AMR in the environment arises from various sources, including but not limited to polluted waters, contaminated food, inhalation

of fungal spores, and other channels that harbour microbes resistant to antimicrobial agents (Laborda *et al.*, 2022).

The dissemination of AMR in the environment often follows a sequential pattern, as depicted by the grey arrows in **Figure 7**. One such linkage involves the chromosomal ARG (red) being linked with insertion sequences (ISs; green), which facilitate intracellular mobility. The process of intracellular translocation, such as transferring to a plasmid, facilitates the horizontal movement of ARGs across different strains and species (Larsson *et al.*, 2022). The mobilised ARG can subsequently be transmitted to a pathogen through a series of one or more intermediary stages. In most critical instances, all genetic processes take place either inside the environment (uppermost) or within the microbiota of humans or domestic animals (lowermost). Nevertheless, it is important to note that bacteria harbouring the ARG have the potential to physically transfer from the surrounding environment to the microbiota of humans or domestic animals, as depicted by the distinctively coloured, bold arrows **(Figure 7)**.

The genetic reservoir inside the environment is notably more extensive, indicating that environmental bacteria frequently serve as the primary source for the emergence of novel ARGs (Larsson *et al.*, 2022). In contrast, people and domestic animals are more frequently exposed to recurring and intense antibiotic selection pressures, as well as close contact with pathogens. However, it is worth noting that certain external settings also exhibit these factors. The discharge of faecal bacteria into the environment can potentially enhance the evolutionary mechanism by introducing genetic components that are well-suited for acquiring and transmitting antibiotic-resistance genes (ARGs) (Larsson *et al.*, 2022).

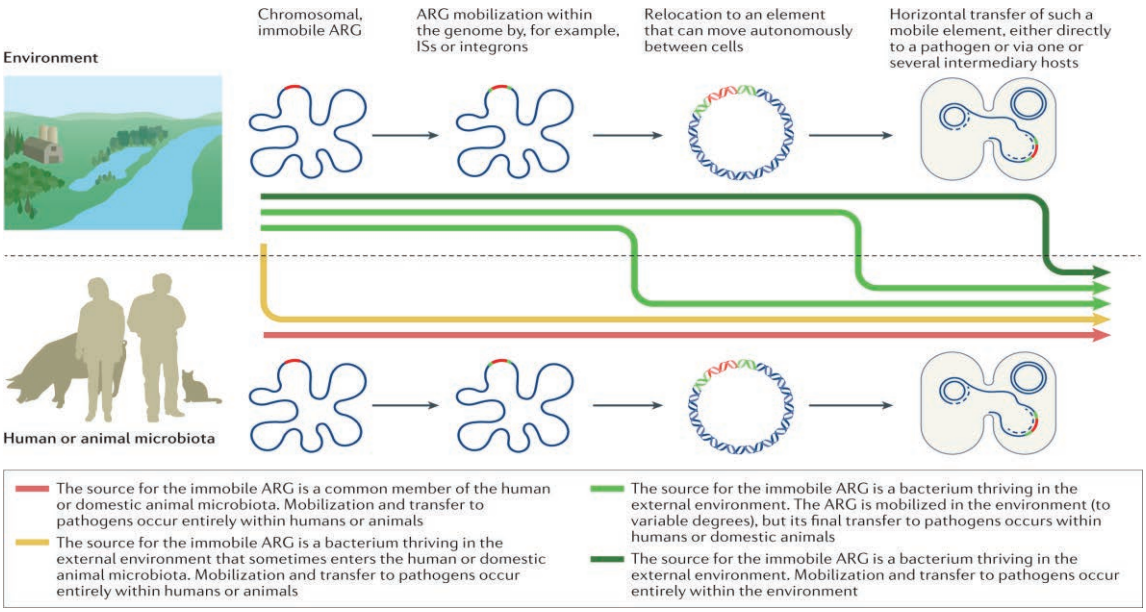


Figure 7. Conceptual description of the evolutionary stages that lead to the emergence of AMR genes (Larsson *et al.*, 2022).

1.4 *Staphylococcaceae* family and *Staphylococcus* genus

As per the "List of Prokaryotic names with Standing in Nomenclature (LPSN) was acquired in November 2019" (Parte *et al*., 2020), the Staphylococcaceae family has a total of 98 officially recognised species, which are distributed among nine distinct genera: *Abyssicoccus*, *Aliicoccus*, *Auricoccus*, *Corticicoccus*, *Jeotgalicoccus*, *Macrococcus*, *Nosocomiicoccus*, *Salinicoccus*, and *Staphylococcus* (Madhaiyan *et al*., 2020). The individuals belonging to this particular family exhibit characteristics such as being Gram-positive, lacking the ability to form spores, and having a spherical or coccoid shape (Madhaiyan *et al*., 2020). They have a size range of 0.5 to 2.5µm and are non-motile. These cells can be found either individually, in pairs, or tetrads. They are strictly aerobic or can function as facultative anaerobes. Additionally, they typically test positive for catalase, although their oxidase activity may vary. These organisms are chemoorganotrophs, capable of both aerobic respiration and fermentative metabolism (Schleifer *et al*., 2009).

Within this particular family, the genus that exhibits the highest population is *Staphylococcus*, which comprises a total of 55 officially recognised species and 23 subspecies **(Table 1).** The cells of this organism are arranged in clusters like grapes, which is a result of division planes that are perpendicular to each other. The cell wall of these cells is composed of peptidoglycan, with l-lysine being the main diamino acid. These cells are capable of growth in environments containing 10% NaCl (w/v) and within a temperature range of 18-40 °C (Lory, 2015).

Table 1. Staphylococci species based on coagulase reaction and predominant hosts

CoPS (hosts)	Coagulase-variable (hosts)	CoNS (host)
S. argenteus (humans and primates)	*S. agnetis* (dairy cattle, chicken) *chromogenes* (pigs and cattle)	*S. canis*, *S. caledonicus* (dogs and cats)
S. aureus subsp *anaerobius* (humans, sheep and goats)	*S. condimenti* (soy sauce mach, fish)	*S. arlattae* (chickens, goats and marine sponges)
S. aureus subspecies *aureus* (human, birds, cow, sheep, goat, horse)	*S. hyicus* subspecies *hyicus* (pigs and cattle)	*S. caeli* (air)
S. coagulans (Cats, dogs and horses)	*S. lutrae* (otters)	*S. carnosus* (dry sausage)
S. cornubiensis (humans, dogs)		*S. durrellii*, *S. lloydii* (fruit bats)
S. delphini (dolphins, horses, donkey, camel, minks, pigeons)		*S. devriesei*, *S. debuckii* (dairy animals)
S. intermedius (pigeon, cats and dogs)		*S. equorum* (fermented food products)
S. pseudintermedius (dogs, cats, parrots, and horses)		*S. edaphicus* (stones and soils), *S. epidermidis* (humans and primates)
S schweitzeri (bats and primates)		*S. felis* (cats), *S. fleurettii* (dairy animals)
S. singaporensis (humans)		*S. hominis* (humans, primates, pets and livestock)
S. roterodami (human)		*S. lentus* (poultry and diary animals)
S. ursi (black bears)		*S. muscae* (pets), *S. microti* (vole and cattle)
		S. nepalensis (domestic cats)
		S. pasteuri (food products, air and surfaces)
		S. piscifermentans (fish), *S. pseudoxylosus* (cattle)
		S. rostri (pigs), *S. ratti* (rodents)
		S. saprophyticus, *S. saccharolyticus*, *S. petrasii subsp. pragensis*, *S. petrasii S. pettenkoferi*, *S. massiliensis*, *S. jettensis*, *S. lugdunensis*, *S. cohnii*, *S. capitis*, *S. haemolyticus*, *S. taiwanensis*, *S. borealis* (humans)
		S. sciuri (ruminants, wild birds), *S. vitulinus* (beef, chicken, lamb, horses, voles, and whales)
		S. simulans (pigs, cows, sheep, goats, and horses)
		S. simiae (primates), *S. stepanovicii* (wild small mammals)
		S. succinus (Amber), *S. shinii* (chives), *S. warneri* (humans and primates)

In the study conducted by Chun *et al*. (2018), a new approach was introduced for precise bacterial categorization. This approach involved the utilisation of both 16S rRNA gene similarity and overall genome-related indices (OGRIs). The utilisation of various orthologous gene-based approaches, including digital DNA-DNA hybridization (dDDH), average nucleotide identity (ANI), average amino acid identity (AAI), and conserved signature proteins (CSPs), has been demonstrated to enhance the taxonomic resolution for taxa belonging to a specific family (Chun *et al*., 2018; Gupta *et al*., 2018).

Several studies (Luo *et al*., 2014; Thompson *et al*., 2013; Goris *et al*., 2007) have suggested threshold values of 95% for dDDH to distinguish between different species. The term "subspecies" was employed to distinguish between strains and genetically similar organisms that exhibit phenotypic divergence (Staley and Krieg, 1984). Therefore, the categorization of subspecies is constrained to a qualitative evaluation of phenotypic traits rather than evolutionary divergence or a specified threshold of similarity (Konstantinidis *et al*., 2018).

There are two primary classifications of staphylococci, which are distinguished by their coagulase activity in rabbit plasma (Carroll *et al*., 2021). These categories are known as coagulase-negative staphylococci (CoNS) and coagulase-positive staphylococci (CoPS) (Carroll *et al*., 2021). According to Carroll *et al*. (2021), the group known as CoPS demonstrates a higher degree of pathogenicity when compared to the other group. *S. aureus* serves as the representative example of the CoPS group, as indicated in **Table 1.** It is noteworthy that *S. aureus* is a significant pathogen affecting both animals and humans (Tong *et al*., 2015). Nevertheless, it is important to acknowledge the existence of other animal-associated CoPS strains that have been documented. One of the emerging clinically significant CoPS in canine medicine is *S. pseudintermedius*, which belongs to the *Staphylococcus intermedius* group (SIG) of five species **(Figure 8)**: *S. intermedius, S. pseudintermedius, S. delphini*, the recently identified *S. cornubiensis*, and *S. ursi* (Murray *et al*., 2018; Perreten *et al*., 2020; Carroll *et al*., 2021). Furthermore, apart from the aforementioned CoPS, *S. coagulans* is an additional species that was initially identified in 1990 as *S. schleiferi* subsp. *coagulans*. However, subsequent genomic investigations in 2020 led to its reclassification as a distinct species (Madhaiyan *et al*., 2020).

The CoNS comprise a substantial number of Gram-positive cocci that are characterised by their shared absence of the virulence component known as coagulase (Becker *et al*., 2020). Several species are classified within this category **(Table 1),** with the most recent addition being *S. borealis*, which was described in 2020 (Pain *et al*., 2020).

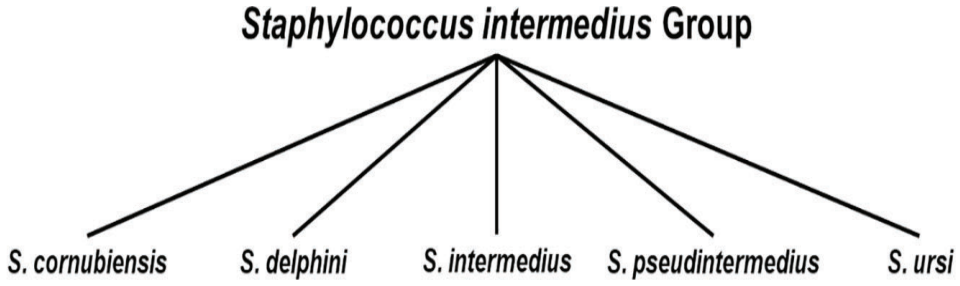


Figure 8. Members of the *S. intermedius* group and host associations (Carroll *et al.*, 2021).

1.4.1 The *Mammaliicoccus* genus, a recent offshoot from *Staphylococcus*

The taxonomic characterization of *Mammaliicoccus* is derived from the existing data presented by Madhaiyan *et al*. (2020). The cellular composition consists of Gram-positive, non-motile, non-spore-forming cocci, which are observed in singular form, as well as in pairs and irregular clusters. These organisms demonstrate the ability to develop under aerobic conditions, as well as under facultative anaerobic conditions. The tested samples exhibited good catalase activity, along with varying levels of oxidase activity. According to Madhaiyan *et al*. (2020), the DNA G+C content (mol%) varies between 31.6 and 35.7, while the genome size spans from 2.44 to 2.81 Mbp. The aforementioned description pertains to *M. sciuri* comb. nov., which serves as the designated type species. The differentiation of the genus from *Staphylococcus* has been achieved by the utilisation of various analytical techniques, including the examination of 16S rRNA gene sequences, the construction of phylogenetic trees using whole genome data, and the assessment of overall genome-related indices. These former *Staphylococcus* species include *M. fleurettii*, *M. lentus*, *M. sciuri*, *M. stepanovicii* and *M. vitulinus* (Madhaiyan *et al* (2020).

1.4.2 Coagulase-positive staphylococci (CoPS)

Staphylococcus species, including both CoPS and CoNS strains, are prevalent members of nasal microbial populations. The majority of staphylococcal species identified in the nasal cavity are predominantly commensal organisms (Parlet *et al*., 2019). However, it is important to note that certain species, particularly those belonging to the CoPS group, have the potential to act as opportunistic pathogens (Velázquez-Guadarrama *et al*., 2017). *S. aureus* is known to inhabit the nasal cavity of approximately 33% of individuals who are in good health condition. However, it is also associated with a wide range of illnesses, including but not limited to skin and soft tissue infections, as well as more severe manifestations (Sakr *et al*., 2018). MRSA is well-recognised as a highly significant

multidrug-resistant bacteria on a global scale, posing a substantial danger to public health (Lankhundi and Zhang, 2018).

Several genetic lineages of *S. aureus* have been identified in the human population, specifically concerning MRSA strains, with some linked with hospital settings and others with community settings (Aires-de-Sousa, 2017). Over the past decade, there has been an emergence of novel lineages within the animal species, with particular attention given to the livestock-associated (LA)-CC398 lineage (Laumay *et al*., 2021; Price *et al*., 2012). This lineage has exhibited extensive distribution among livestock species, particularly those involved in food production (Zarazaga *et al*., 2018). The transmission of MRSA-CC398 from animals to humans has been empirically established, resulting in a range of human illnesses, including both minor ailments such as skin and soft tissue diseases, as well as more serious or potentially fatal conditions (Sieber *et al.*, 2019; Anker *et al.*, 2018).

Furthermore, there have been concerning reports regarding the colonisation of wildlife animals by MRSA-CC398 (Ruiz-Ripa *et al*., 2019a; Gómez *et al*., 2016). The identification of a novel *mecA* gene variant, referred to as *mecC,* has been documented in methicillin-resistant MRSA strains, particularly those associated with animal hosts such as CC130 and ST425, as reported by García-Álvarez *et al*. (2011). The aforementioned lineages may serve as an exhibit significant zoonotic potential (Ruszkowski *et al.,* 2021; Paterson *et al*., 2014). Although the number of cases involving humans remains limited, there have been documented instances (Lozano *et al*., 2020; Larsen *et al*., 2017).

S. pseudintermedius, a CoPS species, is commonly observed within the microbial community of canines' skin (Moses *et al*., 2023). *S. pseudintermedius* is frequently identified as the predominant bacterial pathogen recovered from clinical specimens in canines, commonly associated with infections of the skin and surgical sites (Moses *et al*., 2023). In this regard, there has been a notable rise in the prevalence of methicillin-resistant *S. pseudintermedius* (MRSP), which has become a substantial concern within small animal veterinary medicine (Viegas *et al*., 2022). Furthermore, previous studies have primarily reported cases of human illnesses attributed to *S. pseudintermedius* among those who own or handle pets (Moses *et al*., 2023; Lozano *et al*., 2017). Companion animals appear to serve as a reservoir for this particular species, posing a significant concern for both animals and humans due to the possible zoonotic transmission (Moses *et al*., 2023).

1.4.2.1 Epidemiology of *S. aureus* and *S. pseudintermedius* in healthy humans and animals

To review the global epidemiology of nasal *S. aureus* and *S. pseudintermedius* carriage in healthy humans and animals, four systematic reviews and quantitative analyses were conducted throughout this thesis and published from 2021 to 2023 (Abdullahi *et al*., 2021a; Abdullahi *et al*., 2021b; Abdullahi *et al*., 2022; Abdullahi *et al*., 2023a). These studies could concisely be summarised as follows:

1.4.2.1.1 Healthy people with or without occupational contact with animals

The ecology and genetic lineages of *S. aureus* and MRSA nasal carriage in healthy people with various occupation risks were analysed (Abdullahi *et al*., 2021a). In this study, articles were thoroughly searched and eligible ones were grouped into those on healthy people with no reported risk of colonization (Group A), food handlers (Group B), veterinarians (Group C), and livestock farmers (Group D). A total of 166 eligible papers were evaluated for Groups A/B/C/D (n = 58/31/26/51). The pooled prevalence of *S. aureus* and MRSA in healthy humans of Groups A to D were 15.9%, 7.8%, 34.9%, and 27.1% (*S. aureus*); and 0.8%, 0.9%, 8.6%, and 13.5% (MRSA), respectively **(Table 2).** The pooled prevalence of MRSA-CC398 nasal carriage among healthy humans was as follows: Group A/B (<0.05%), Group C (1.4%), Group D (5.4%); and the following among Group D: pig farmers (8.4%) and dairy farmers (4.7%).

The pooled prevalence of CC398 lineage among the MSSA and MRSA strains from studies of the four groups were Group A (2.9 and 6.9%), B (1.5% and 0.0%), C (47.6% in MRSA), and D (11.5% and 58.8%). Moreover, MSSA-CC398 strains of Groups A and B were mostly of *spa*-t571 (animal-independent clade), while those of Groups C and D were *spa*-t011 and t034 (Abdullahi *et al*., 2021a). The MRSA-CC398 was predominately of t011 and t034 in all the groups (with few other *spa*-types, livestock-associated clades). The pooled prevalence of MSSA and MRSA strains carrying the PVL encoding genes were 11.5 and 9.6% (ranges: 0.0-76.9 and 0.0-28.6%), respectively. Moreover, one PVL-positive MSSA-t011-CC398 strain was detected in Group A. Contact with livestock and veterinary practice seems to increase the risk of carrying MRSA-CC398, but not in food handlers (Abdullahi *et al*., 2021a).

Table 2. Summary of the pooled global prevalence of *Staphylococcus aureus* and MRSA nasal carriages in the four studied groups (A to D).

Study Groups	Number of studies included	Pooled *S. aureus* nasal carriage rate (%) (range)	Number of *S. aureus* studies included	Total number of:		Pooled MRSA nasal carriage rate (%) (range)	Number of MRSA studies included	Total number of:	
				People	*S. aureus*			People	MRSA
A	58	15.9 (2.3-79.6)	55	133310	21133	0.8 (0.0-17.5)	52	131578	1020
B	31	7.8 (1.4-60.0)	31	35875	2803	0.9 (0.0-37.1)	21	18211	167
C	26	34.9 (19.4-50.8)	7	614	214	8.6 (0.7-38.4)	25	3735	343
D	51	27.1 (3.1-62.5)	25	4310	1169	13.5 (0.0-85.8)	49	7033	946

1.4.2.1.2 Healthy livestock

Given the central role of livestock in understanding the genomic epidemiology of *S. aureus*, eligible studies were systematically reviewed and data were extracted and synthesized to determined *S. aureus* epidemiological parameters. These eligible articles were grouped into those on healthy pigs (A), sheep and goats (B), cattle (C), poultry (D), camels (E) and buffaloes (F). Special focus was given to the prevalence of nasal MRSA, MRSA-CC398, MRSA-CC9, *mecC*-MRSA, MSSA-CC398, and resistance to linezolid (LZD^R), chloramphenicol (CLO^R) and tetracycline (TET^R) in *S. aureus* strains. Of the studied screened, 146 comprised groups A(83)/B(18)/C(33)/D(4)/E(5)/F(3) were found eligible **(Table 3).**

The overall pooled nasal prevalence of MRSA in healthy livestock was 13.8% (95% CI: 13.5–14.1) among a pooled 48,154 livestock population. Specifically, the pooled prevalence in groups A to F were: 16.0% (95% CI: 15.6–16.4), 3.7% (95% CI: 2.9–4.6), 13.6% (95% CI: 12.8–14.4), 5.8% (95% CI: 5.1–6.5), 7.1% (95% CI: 6.1–10.7), and 2.8% (95% CI: 1.5–4.8), respectively **(Table 3).** These values varied considerably by continent **(Figure 9).** Varied pooled prevalence of CC398 lineage concerning MRSA strains was obtained, with the highest from pigs and cattle (>70%) with various *spa* types **(Figure 10).** Moreover, other classical animal-adapted MRSA as well as MSSA-CC398-t1928 were reported. TET^R-MSSA was lowest in cattle (18.9%) and highest in pigs (80.7%). LZD^R-*S. aureus* was reported in 8 studies (mediated by *optrA* and *cfr*), mainly in pigs (n =4), while CLO^R-*S. aureus* was reported in 32 studies (Abdullahi *et al*., 2023a). The virulence genes *luk-S/F-PV, tst, etd, sea, see* were sparsely reported, and only in non-CC398-MRSA lineages. Certain *S. aureus* clones and critical AMR appeared to have predominance in some livestock, as in the case of pigs that are high nasal carriers of MRSA-CC398 and -CC9, and MSSA-CC398 (Abdullahi *et al*., 2023a).

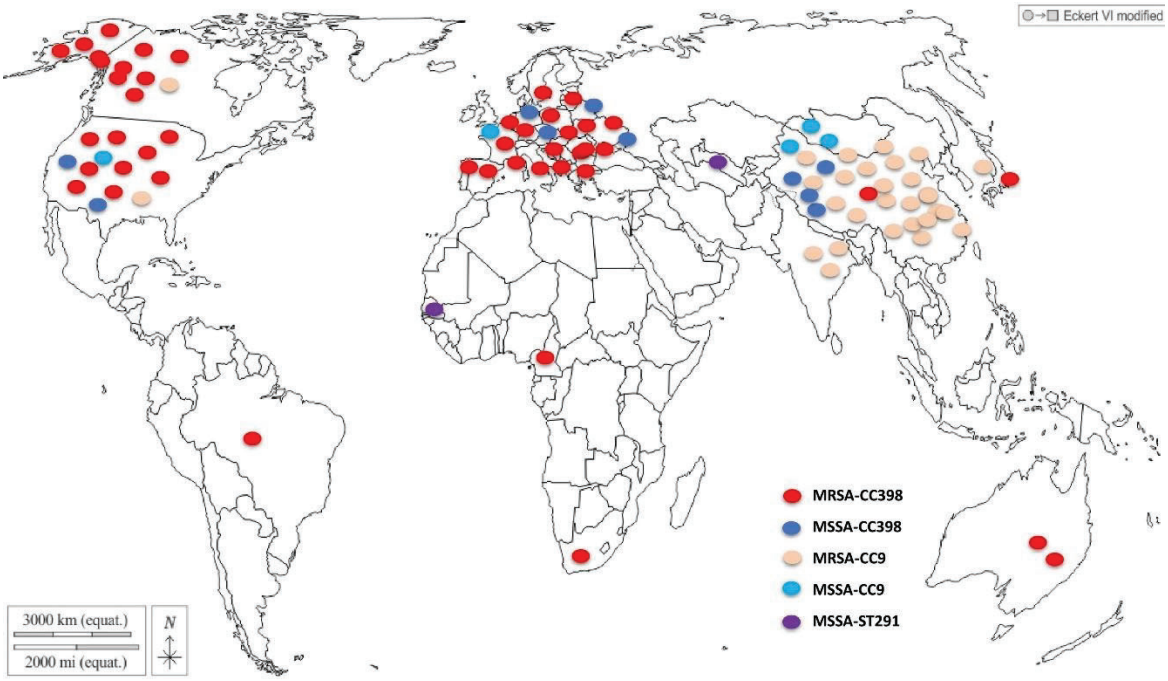


Figure 9. Geographical distribution of the major LA-MRSA and MSSA clonal complexes reported from nasal cavities of healthy livestock.

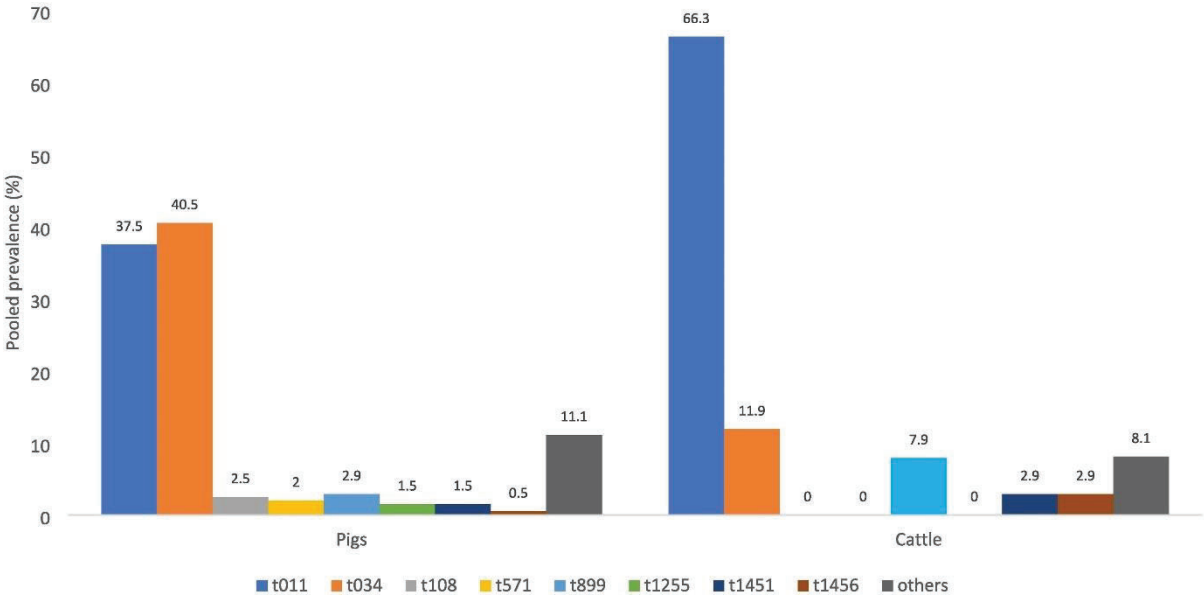


Figure 10. Distribution pattern of *spa* types of nasal MRSA strains from healthy pigs and cattle

Table 3. Global pooled prevalence of *S. aureus* and MRSA nasal carriages among the five studied livestock groups

Study groups	Number of *S. aureus* studies included	Total number of:		Pooled *S. aureus* carriage rate	Prevalence range	95% CI	Number of MRSA studies included	Total number of:		Pooled MRSA carriage rate (%) (95% CI)	Prevalence range	95% CI	Total number of studies included[a]
		Animals	*S. aureus*					Animals	MRSA				
Healthy pigs	82	32027	6784	21.2	0.5-99.0	20.7-21.6	81	31898	4964	15.6	0.0-99.0	15.2-15.9	82
Healthy sheep and goats	18	2405	559	23.2	7.8-46.7	21.6-24.9	16	2174	80	3.7	0.0-26.5	2.9-4.6	19
Healthy cattle	33	7751	1198	15.5	0.0-62.5	14.7-16.3	33	7751	1053	13.6	0.0-51.6	12.8-14.4	33
Healthy poultry	4	4708	1383	29.4	0.5-30.0	28.1-30.7	4	4708	272	5.8	0.0-30.0	5.1-6.5	4
Healthy camel	5	686	137	19.9	5.8-56.3	17.0-23.2	5	686	49	7.1	0.0-8.8	6.1-10.7	5
Healthy buffaloes	3	462	37	8.0	2.2-27.3	5.7-10.9	3	462	13	2.8	0.7-27.3	1.5-4.8	3
Total Livestock	145	48039	10098	20.9	0.0-99.0	20.7-21.4	142	47679	6431	13.5	0.0-99.0	13.2-13.8	145

1.4.2.1.3 Healthy dogs and cats

In healthy dogs and cats, the study focused on nasal *Staphylococcus aureus* and *S. pseudintermedius* carriage, their antibiotic resistance, virulence and genetic lineages of zoonotic relevance (Abdullahi *et al*., 2022). In this study, forty-nine studies were eligible and included in this systematic review **(Table 4).** The pooled prevalence of nasal carriage of *S. aureus*/methicillin-resistant *S. aureus* (MRSA) in healthy dogs and cats were 10.9% (95% CI: 10.1–11.9)/2.8% (95% CI: 2.4–3.2) and 3.2% (95% CI: 1.9–4.8)/0.5% (95% CI: 0.0–1.1), respectively (Abdullahi *et al*., 2022). Conversely, the pooled prevalence of *S. pseudintermedius*/methicillin-resistant *S. pseudintermedius* (MRSP) in healthy dogs and cats were 18.3% (95% CI: 17.1–19.7)/3.1% (95% CI: 2.5–3.7) and 1.3% (95% CI: 0.6–2.4)/1.2% (95% CI: 0.6–2.3), respectively **(Table 4).** The pooled prevalence of MRSA and MRSP in dogs varied across the continents of the world **(Figure 11).**

Although highly diverse genetic lineages of *S. aureus* were detected in healthy dogs and cats, MSSA-CC1/CC5/CC22/CC45/CC121/CC398 and MRSA-CC5/CC93/CC22/CC30 were mostly reported in dogs; and MSSA-CC5/CC8/CC15/CC48 and MRSA-CC22/CC30/CC80 in cats. Of note, MSSA-CC398 strains (*spa*-types t034 and t5883) were detected in dogs. Genetic lineages often associated with MSSP/MRSP were ST20/ST71, highlighting the frequent detection of the epidemic European MRSP-ST71 clone in dogs. *S. aureus* strains carrying the *luk-S/F-PV, tst, eta, etb* and *etd* genes were seldom detected in dogs, and *luk-S/F-PV* was the unique virulence factor reported in strains of cats. *S. pseudintermedius* strains harbouring the *luk-S/F-I, seint* and *expA* genes were frequently found, especially in dogs. High and diverse rates of AMR were noted, especially among MRSA/MRSP (Abdullahi *et al*., 2022).

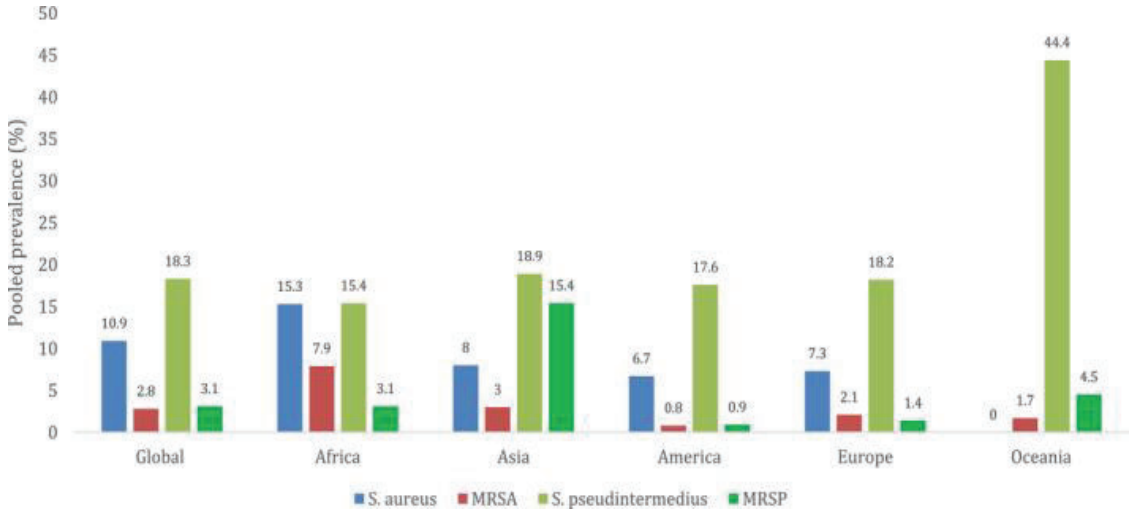


Figure 11. Pooled prevalence of *Staphylococcus aureus*, MRSA, *S. pseudintermedius* and MRSP nasal carriage among healthy dogs by continents.

Note: In each continent, the indicated pooled prevalence was analyzed independently. The number of studies per continent in *S. aureus*, MRSA, *S. pseudintermedius* and MRSP, respectively, were as follows: Africa (9, 8, 6, and 6); Asia (11, 10, 6, 5); America (3, 7, 4, and 5), Europe (9, 10, 8, and 8) and Oceania (0, 1, 1, and 3). MRSA, methicillin-resistant *S. aureus*; MRSP, methicillin-resistant *S. pseudintermedius.*

Table 4. Summary of the pooled global prevalence of *S. aureus*, *S. pseudintemedius*, MRSA and MRSP nasal carriage in healthy dogs and cats.

Animals	**Number of *S. aureus* publications included**	**Total number of:**		**Pooled *S. aureus* nasal carriage rate (%) (range)**	**Number of MRSA publications included**	**Total number of:**		**Pooled MRSA nasal carriage rate (%) (range)**	**Total number of publications included[a]**
		Animals	***S. aureus***			Animals	MRSA		
Dogs	33	4829	447	9.3 (0.0-76.7)	36	5395	156	2.9 (0.0-47.7)	45
Cats	9	586	21	3.6 (0.0-25.0)	10	972	5	0.5 (0.0-1.7)	12

Animals	**Number of *S. pseudintermedius* publications included**	**Total number of:**		**Pooled *S. pseudintermedius* nasal carriage rate (%) (range)**	**Number of MRSP publications included**	**Total number of:**		**Pooled MRSP nasal carriage rate (%) (range)**	**Number of publications included[a]**
		Animals	***S. pseudintermedius***			Animals	MRSP		
Dogs	25	3336	581	17.4 (0-92.4)	26	3296	113	3.4 (0.0-51.1)	45
Cats	7	677	10	1.5 (0.48-13.6)	7	664	9	1.4 (0.0-7.1)	12

1.4.2.1.4 Wild animals

Wild animals have been considered key players in the carriage and transmission of AMR as many of them, especially the migratory birds (such as storks), could be dynamic and move along distance across a variety of natural environments, landfills, hospital wastes, and livestock farms **(Figure 12).** Moreover, migratory birds occasionally come in contact with antibiotic residues in livestock carcasses or manure and they could carry and disseminate AMR bacteria such as MRSA of public health concerns (Abdullahi *et al*., 2021b).

In wild birds, the study determined the pooled prevalence of nasal, tracheal and/or oral (NTO) *S. aureus* and MRSA carriage in wild animals, with a special focus on *mecA* and *mecC* genes as well as the frequency of MRSA and MSSA of the lineages CC398 and CC130 in wild animals (Abdullahi *et al*., 2021b). This systematic review was executed on cross-sectional studies that reported *S. aureus* and MRSA in the NTO cavities of wild animals distributed in four groups: non-human primates (NHP), wild mammals (WM, excluding rodents and NHP), wild birds (WB) and wild rodents (WR). Of the 33 eligible and analysed studies, the pooled prevalence of NTO *S. aureus* and MRSA carriage was 18.5% (range: 0–100%) and 2.1% (range: 0.0–63.9%), respectively **(Table 5).** The pooled prevalence of *S. aureus*/MRSA in WM, NHP, WB and WR groups was 15.8/1.6, 32.9/2.0, 10.3/3.4 and 24.2/3.4%, respectively **(Table 5).**

The prevalence of *mecC*-MRSA among WM/NHP/WB/WR was 1.64/0.0/2.1/0.59%, respectively, representing 89.9/0.0/59.1/25.0% of total MRSA detected in these groups of animals. The MRSA-CC398 and MRSA-CC130 lineages were most prevalent in wild birds (0.64 and 2.07%, respectively); none of these lineages were reported in NHP studies. The MRSA-CC398 (mainly of *spa*-type t011, 53%), MRSA-CC130 (mainly of spa types t843 and t1535, 73%), MSSA-CC398 (*spa*-types t571, t1451, t6606 and t034) and MSSA-CC130 (*spa* types t843, t1535, t3625 and t3256) lineages were mostly reported (Abdullahi *et al*., 2021b).

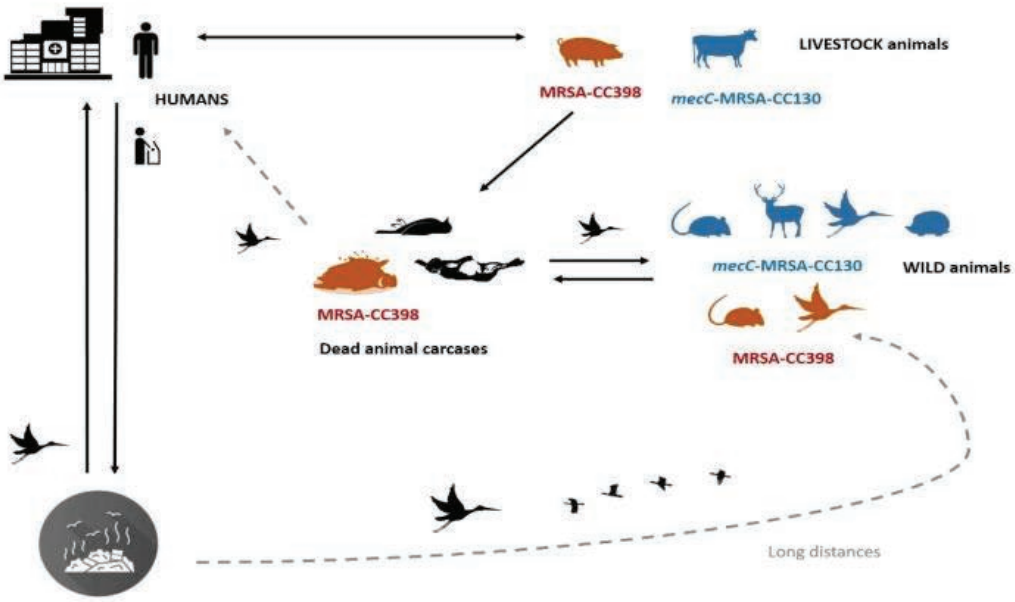


Figure 12. Transmission cycle of special MRSA clones across humans, animals (livestock and wild) and the environment (such as landfills and hospitals).

Note: In the silhouettes with colours, the animals in which MRSA-CC398 (red) and *mecC*-MRSA-CC130 (blue) strains have been detected in high prevalence were illustrated.

1.4.2.2 Epidemiology of *S. coagulans* in Humans and Animals

The bacterium *S. coagulans* was initially identified in 1990 as a subspecies of *S. schleiferi*, known as *S. schleiferi* subsp. *coagulans* (Ingimi *et al*., 1990). However, in 2020, based on genetic characteristics, it was reclassified as an independent species (Madhaiyan *et al*., 2020). *S. coagulans* predominantly acts as a commensal organism and an opportunistic pathogen in companion dogs (Paterson, 2021). *S. coagulans* has commonly been reported to be in the skin (Yamashita *et al*., 2005) and external ear canal (May *et al*., 2005) of healthy canines. Additionally, it has been linked to external ear otitis (Yamashita *et al*., 2005; May *et al*., 2005) and pyoderma (Hariharan *et al*., 2010). Although infrequent, there have been documented cases of *S. coagulans* producing opportunistic infections in humans (Nguyen *et al.*, 2023; Yarbrough *et al*., 2017; Tzamalis *et al.*, 2013). Similar to several species of staphylococci, *S. coagulans* exhibits a broad distribution across avian and mammalian hosts, and its prevalence among such hosts may surpass the current reported data (Paterson, 2021).

The presence of methicillin resistance in *S. coagulans* is determined by the *mecA* gene, as reported by Huse *et al*. (2018). Additionally, resistance to several additional antimicrobial agents has been documented (Paterson *et al*., 2021).

Table 5. Summary of the pooled global prevalence of *S. aureus* and MRSA NTO carriages in the four studied wild animal groups

Study groups	Number of *S. aureus* studies included	Total number		Pooled *S. aureus* carriage rate (%) (range)	OR (95% CI)	*p* value	Number of MRSA studies included	Total number		Pooled MRSA carriage rate (%) (range)	OR (95% CI)	*p* value	Total number of studies included [a]
		Animals	*S. aureus*					Animals	MRSA				
Wild Mammals (excluding rodents and NHP)	13	3031	479	15.8 (0.0–36.9)	Referent	Referent	17	6110	99	1.6 (0.0–63.6)	Referent	Referent	18
Non-human Primates	7	403	158	39.2 (0.0–100.0)	3.44 (2.78- 4.29)	<0.0001	7	403	8	2.0 (0.0–26.7)	1.23 (0.59-2.55)	0.578	7
Wild Birds	5	586	60	10.3 (5.0–34.8)	0.61 (0.46- 0.81)	0.0006	6	626	21	3.4 (0.0–4.0)	2.11 (1.31-3.40)	0.002	6
Wild Rodents	4	856	207	24.2 (15.3–41.0)	1.69 (1.41-2.04)	<0.0001	5	1452	49	3.4 (0.3–4.7)	2.12 (1.49-3.00)	<0.0001	5
Total Wild Animals	29	4876	905	18.5 (0.0-100)	NA	NA	35[a]	8601	177	2.1 (0.0-63.9)	NA	NA	36[a]

Key:
NA = Not applicable
OR = Odd Ratio
[a] Studies that analyse either *S. aureus*, MRSA or both.

Studies have demonstrated the transfer of antimicrobial resistance genes between different species of staphylococci (Michaelis and Grohmann, 2023). This raises concerns about the possibility of *S. coagulans* serving as a genetic reservoir for the transmission of resistance traits to other staphylococcal species, including more virulent strains such as *S. aureus* and *S. pseudintermedius* (Frosini *et al*., 2020).

Despite the high occurrence of *S. coagulans* as a pathogen that takes advantage of opportunities in companion dogs and its potential to be transmitted to humans, there is a limited number of genome sequences currently accessible. These sequences are crucial for enhancing our knowledge of *S. coagulans*, including its epidemiology, resistance to antimicrobial agents, and interactions between the bacterium and its hosts.

1.4.3 Coagulase-negative staphylococci (CoNS)

Over the last ten years, some coagulase-negative staphylococci (CoNS) species (*S. epidermidis*, *S. haemolyticus* and *S. hominis*) emerged and cause various forms of opportunistic infections such as septicaemia in children, patients with immunosuppression and medical implants (França *et al*., 2021; Heilmann *et al*., 2019). Most other species are rare human or animal pathogens and are often pan susceptible to antimicrobial agents (Merrild *et al*., 2023; Santoiemma *et al*., 2020; Argemi *et al*., 2019). However, there have been sporadic reports of some *S. pasteuri* causing endocarditis, whereas *S. hyicus, S. chromogenes, S. lentus,* and *S. sciuri* are considered etiological agents of exudative epidermitis with zoonotic potentials (Kirk *et al*., 2022; Kalai *et al*., 2021; Li *et al*., 2021). Moreover, *S. saprophyticus* strains contracted from contaminated food has long been implicated in urinary tract infections in young teenagers (Lawal *et al*., 2021a, 2021b).

The whole-genome sequence data of CoNS species have led to the identification and characterization of numerous putative virulence factors (Argemi *et al*., 2019). Furthermore, CoNS could acquire clinically relevant and critical ARGs and transmit them across other species and hosts through various mobile genetic elements (mobilome) (Rossi *et al*., 2020). Specifically, *S. haemolyticus* has been ranked as the most antibiotic-resistant species among the CoNS (Kranjec *et al*., 2021). The transferability of ARGs between different *Staphylococcus* spp has been strongly suggested by the sequence similarity of their associated mobilome especially plasmids (Souza-Silva *et al*., 2022).

1.4.3.1 Reclassification of *S. borealis* and separation from *S. haemolyticus*

In a comprehensive cohort study conducted by Pain *et al*. (2020), an investigation was carried out on *S. haemolyticus* obtained from the skin and blood of human subjects. The study included whole-genome sequencing techniques to analyse the genetic makeup of the strains. As a result of this analysis, five strains displaying atypical phenotypic and genotypic characteristics were found. The strains exhibited a characteristic yellow colouration that was deeper than usual and displayed essentially indistinguishable 16S rRNA gene sequences. Initially, these colonies were classified as *S. haemolyticus,* based on the analysis of their 16S rRNA gene sequence and MALDI-TOF MS. In contrast to *S. haemolyticus,* the five strains exhibited several distinguishing characteristics: (i) a significant evolutionary divergence, as indicated by an average nucleotide identity of less than 95% and inferred DNA-DNA hybridization of less than 70%; (ii) a pigmented phenotype; (iii) the ability to produce urease; and (iv) distinct fatty acid composition. The study concluded that the strains under investigation constituted a previously unidentified species based on their morphological and genotypic characteristics. Consequently, they proposed the name *S. borealis* sp. nov., which was subsequently accepted and recognised by the scientific community (Pain *et al*., 2020).

1.5 Distribution and types of mobile genetic elements in *Staphylococcus*

The ability of staphylococci to cause disease is attributed, in part, to their capacity for swift adaptation in response to selective pressures imposed by their hosts (Howden *et al*., 2023). Additionally, the horizontal transfer of genetic materials, either among or within bacterial species, contributes to their pathogenicity (Lima *et al*., 2023) **(Figure 13).**

Staphylococci have acquired multiple mobile genetic elements (MGEs) through horizontal gene transfer, including bacteriophages, transposons, plasmids, and pathogenicity islands (Haudiquet *et al*., 2022). According to Copin *et al*. (2018), approximately 20% of the genome of *S. aureus* is composed of mobile genetic elements (MGEs). Certain MGEs have been found to have a role in the dissemination of staphylococci, thereby significantly impacting their genome plasticity and evolutionary processes. This phenomenon has the potential to facilitate the transmission and long-term presence of resistance and virulence genes that are clinically significant in *S. aureus* (Giulieri *et al*., 2022).

MGEs are DNA fragments found in bacteria that cause the mobilization and dissemination of one or more AMR and/or virulence determinants (de Nies *et al*., 2022;

Panwar *et al*., 2023). These MGEs may also contain enzymes that facilitate their transfer and integration into other DNA molecules (Frost *et al*., 2005). MGEs are referred to as a "mobilome" when they exhibit the ability to move inside the same cell or between different cells (Siefert, 2009). MGEs can include different lengths of DNA sequences, including phages, transposons, pathogenicity islands, plasmids, and chromosome cassettes, through HGT (Alibayov *et al*., 2014).

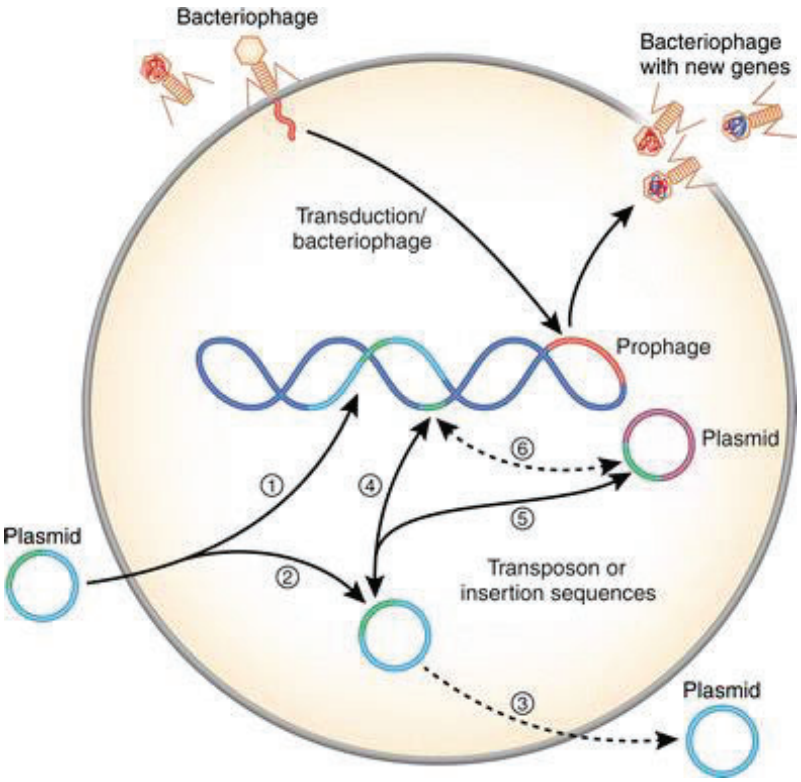


Figure 13. The process of obtaining mobile genetic elements by *Staphylococcus aureus.*
(1) Incorporation of plasmids or plasmid elements into genomic DNA. (2) Plasmids can be maintained as free circular DNA. (3) Suicide plasmid. (4) Transfer of a transposon or an insertion sequence between plasmid and genomic DNA. (5) Transfer of a transposon or an insertion sequence between plasmids within the cell. (6) Transfer of a transposon or an insertion sequence from genomic DNA to another plasmid (Malachowa and DeLeo, 2010).

1.5.1 Plasmids

Plasmids are small DNA molecules capable of undergoing semiautonomous replication within the genome of a compatible bacterial cell (Andreopoulos *et al*., 2022). Therefore, these entities can be classified as replicons. According to Andreopoulos *et al*. (2022), plasmids can facilitate the transfer of AMR and virulence determinants between bacteria through a process known as HGT. This transfer can occur not only between bacteria of the same species but also between distinct species and even genera. According to Neyaz *et al*. (2020), a significant proportion of staphylococci strains found in nature

possess one or many plasmids of varying sizes, ranging from 1 to 60 kilobase pairs (kbp). The plasmids of staphylococci have been categorised into three distinct classes according to their physical properties, genetic arrangements, and functional attributes (Firth *et al*., 2018). Class I consists of tiny plasmids (1.3–4.6 kbp) that exist in several copies (10–55 copies per cell). These plasmids can either be cryptic or carry a single (sometimes two) resistance determinant, such as *pT181*, *pC194*, *pSN2*, and *pE194* (Alibayov *et al*., 2014). According to Alibayov *et al*. (2014), multicopy plasmids lack transposons and prophages, and their replication occurs by an asymmetric rolling circle process.

Plasmids belonging to class II have greater sizes ranging from 15 to 46 kilobase pairs (kbp) and are present in lower quantities inside bacteria, often ranging from 4 to 6 copies per cell. Notably, this category encompasses a majority of the plasmids responsible for penicillinase as well as aminoglycoside/trimethoprim resistance, such as *pSK1* and pIP630 (Alibayov *et al*., 2014). The resistance to heavy metals, including cadmium, arsenate, and mercury, as well as the generation of beta-lactamases, are determined by penicillinase plasmids (Vats *et al*., 2022). Class III is comprised of plasmids that are of significant size, ranging from 30 to 60 kilobase pairs (kbp), and can transfer AMR genes through the process of conjugation (McCarthy *et al*., 2014).

According to Rossolini *et al*. (2017), it is typical for these plasmids to contain one or two transposons together with several copies of insertion sequences. Before integration into the host chromosome, staphylococcal plasmids are present as autonomous circular DNA molecules within the host bacterial cell (Malachowa and DeLeo, 2010).

1.5.2 Transposable elements

The staphylococcal genome harbours a diverse array of MGEs which include insertion sequences (IS), transposons (Tn), and transposon-like elements (Firth *et al*., 2018). These genetic elements under consideration play a role in the evolutionary process of staphylococci and can be located either on the chromosomal DNA or in conjunction with other MGEs as either singular or multiple copies (Malachowa *et al*., 2010). Insertion sequences (ISs) are genetic fragments that solely possess the necessary genetic information for the process of transposition ((Sheng *et al*., 2023; Malachowa *et al*., 2010). While insertion sequences (ISs) do not encode resistance themselves, they play a crucial role in the recombination and stabilisation of certain resistance genes (Sheng *et al*., 2023). Consequently, their existence has significant importance in the evolutionary dynamics of bacterial genomes (Sheng *et al*., 2023). Composite transposons frequently contain IS

elements. For instance, the combination of IS256 and IS257 is facilitated by Tn*4001* and Tn*4003*, resulting in a hybrid pair that enhances resistance to aminoglycosides (Babakhani *et al*., 2018). Alibayov *et al*. (2014) reported the occurrence of both contiguous and independent insertions of IS256 and IS257 elements into the staphylococcal chromosome. The presence of IS257 suggests a potential involvement of this element in molecular rearrangements of the genome.

Staphylococcal transposons are compact genetic elements capable of harbouring resistance genes, such as Tn*552* which carries the *blaZ* gene responsible for penicillinase production, and Tn*554* which carries the *ermA* gene conferring resistance to macrolide–lincosamide–streptogramin B antibiotics (Sarrou *et al*., 2019; Pérez-Roth *et al*., 2010).

1.5.3 Bacteriophages

Prophages are known to have significant implications in the evolutionary processes, adaptive mechanisms, and pathogenicity of bacterial genomes, particularly in the case of *Staphylococcus* (Sweet *et al*., 2023). In this regard, the double-stranded DNA phages of *S. aureus* can be categorised into three classes, namely lytic, temperate, and chronic phages, all of which fall under the Caudovirales order (Xia and Wolz, 2014). The phages can be categorised into three distinct families: *Podoviridae, Myoviridae,* and *Siphoviridae.* The *Podoviridae* family comprises both lytic and chronic phages, while the *Myoviridae* family also includes lytic and chronic phages (Jurado *et al*., 2023). On the other hand, the *Siphoviridae* family exclusively consists of temperate phages, which exhibit long-term persistence within the host organism (Feng *et al*., 2021). In this case, the phage's DNA can integrate into the staphylococcal genome as a prophage after bacterial lysis (Alibayov *et al*., 2014; Feng *et al*., 2021).

The prophage typically undergoes insertion and integration into the host chromosome or plasmids, as it possesses the ability to suppress the lytic actions of the phage (Gummalla *et al*., 2023). The majority of *S. aureus* strains typically possess one to two distinct prophages, primarily associated with the φ3 and MR11-like phage groups (Diene *et al*., 2017). The classification of *S. aureus* phages is determined by their response to polyclonal antiserum, resulting in the categorization of these phages into 11 serogroups, denoted as A to H and J to L (Xia *et al*., 2014). The majority of temperate phages have been categorised into serogroups A, B, and F. The F phages can be classified into two distinct subgroups, namely Fa and Fb (Xia *et al*., 2014). Presently, there has been a notable rise in the identification of a wide range of prophage sequences found within the genomes of *S.*

aureus strains. Consequently, this has led to the detection of a greater number of poly-lysogenic *S. aureus* strains (Gummalla, *et al*., 2023). Furthermore, a prior investigation revealed that *φ*Sa3-like prophages have limited genomic similarities with their structural genomic variations and have been associated with diverse bacterial virulence factors, which can be linked to the extensive diversity of virulent-related *S. aureus* prophages (Kashif *et al*., 2019).

Although the quantity of prophages identified in CoNS genomes is comparatively lower than that in *S. aureus*, there is a proposition that phages may also play a role in the pathogenesis and evolutionary processes of CoNS (Deghorain and Van Melderen, 2012). In this regard, the Siphoviridae family comprises the bulk of staphylococcal prophages, even in CoNS (Göller *et al.*, 2021). Prophages have been previously documented in other species, *including S. epidermidis, S. carnosus, S. hominis, S. capitis,* and *S. haemolyticus* (Schmelcher *et al.*, 2012; Deghorain *et al.*, 2012; Gutiérrez *et al.*, 2012; Rosenstein *et al.*, 2009; Daniel *et al.*, 2007).

In relation to genomic structure, there exists a notable similarity between CoNS and *S. aureus* prophages (França *et al.*, 2021). A notable distinction between these two entities lies in the fact that the presence of virulence factors in *S. aureus* is largely lacking in CoNS prophages (França *et al.*, 2021). The close association identified among staphylococci prophages may enhance the likelihood of HGT mediated by prophages between distinct staphylococcal species (Deghorain *et al.*, 2012). There has been speculation regarding the transmission of mobile elements from CoNS to *S. aureus* (Ray *et al.*, 2016; Otto *et al.*, 2013).

1.5.4 Pathogenicity islands

The presence of staphylococcal pathogenicity islands (SaPIs) extends beyond *S. aureus*, encompassing various other *Staphylococcus* species as well (Argemi *et al*., 2019). The SaPIs are MGEs ranging in size from 12 to 27 kilobases, which have been identified in various *Staphylococcus* species (Novick and Ram, 2017). These islands contain genes responsible for encoding integrase, resistance factors, virulence factors, and other determinants associated with superantigens but rarely with AMR (Partridge *et al*., 2017). They have been implicated in causing diseases such as food poisoning and facilitating host adaptation (Etter *et al*., 2020; Alibayov *et al*., 2014). Numerous exotoxins, such as staphylococcal enterotoxins (SE), are classified under the pyrogenic toxin superantigens (SAgs) family (Noli Truant *et al*., 2022).

Surface antigens (SAgs) can attach to the extracellular region of class II molecules, also known as major histocompatibility complex (MHC) molecules, in host animals. This interaction results in the formation of a complex when combined with the variable beta (Vb) chain of a T cell receptor (TCR). This compound induces non-specific T-cell proliferation, ultimately leading to suppression of the host immune system (Deacy *et al*., 2021). According to Hu *et al*. (2021), the SAgs genes associated with enterotoxins play a pivotal role in the development of distinct acute clinical syndromes, including toxic shock syndrome and food poisoning. According to Banaszkiewicz *et al*. (2022), the genetic encoding of Sags occurs on MGEs including plasmids, prophages, SaPI, genomic islands, and staphylococcal cassette chromosome (SCC) elements.

1.5.5 SCC*mec* and its classification system in methicillin-resistant staphylococci

A total of fourteen distinct types of SCC*mec* have been documented. These types are further categorised into broad groups, as shown in **Table 6.** The size of the SCC*mec* elements varies from 21 to 82 thousand nucleotides, as seen in **Table 6.** The typical configuration of SCC*mec* cassettes encompasses five distinct sections. The categorization of SCC*mec* into distinct types is determined by the specific *ccr* chromosomal recombinase gene complex, namely *ccrA, ccrB, and ccrC*, as discussed by Mlynarczyk-Bonikowska *et al*. (2022).

The classification of the *mec* gene complex represents a significant factor in the division of SCC*mec*. Several distinct classes can be identified, including A, B, B2, C1, C2, D, and E. The various classes exhibit variations in the extent of *mecI-mecR* gene deletion, as well as the relative positioning and distance from the entire or truncated IS431, IS1182, and IS1272 (Mlynarczyk-Bonikowska *et al*., 2022). The categorization of SCC*mec* subtypes is determined by the subclasses of the *mec* gene complex and the composition of the J1, J2, and J3 regions (Uehara *et al*., 2022).

1.5.5.1 *mec* Gene Complex

The *mec* gene complex is composed of *mecA,* its regulatory genes, and the accompanying insertion sequences. Currently, five classes of the *mec* gene complex have been described (Uehara, 2022). The *mec* gene complex types, subtypes and environments are shown in **Figure 14.** Epidemiologically, the SCC*mec* types carried by staphylococci could suggest the ecological niche of the strain such that certain SCC*mec* types have been largely considered hospital-associated (HA) or community-associated (CA) strains

(Asghar, 2014). However, both the so-called HA- and CA-SCC*mec* types can seldom be found in either niche (Bal *et al.*, 2016; Coll *et al.*, 2017).

1.6 AMR problem in staphylococci

The presence of methicillin-resistance in *Staphylococcus* (especially in *S. aureus*) has been considered a cause of numerous public health challenges (Lee *et al*., 2018a), which subsequently give rise to significant challenges in antibacterial chemotherapy (Mlynarczyk-Bonikowska *et al*., 2022). The initial emergence of MRSA strains occurred between 1960 and 1961, exhibiting high-level resistance to all beta-lactam antibiotics that were commonly employed for therapeutic purposes during that time (Mlynarczyk-Bonikowska *et al*., 2022).

The introduction of two cephalosporins, namely ceftobiprole and ceftaroline, which exhibit activity against MRSA and methicillin-resistant coagulase-negative staphylococci (MRCoNS) strains, did not occur until the year 2010 (Mlynarczyk-Bonikowska *et al*., 2022). In a brief period, there has been an emergence of strains that exhibit resistance to both of these medications (Chan *et al*., 2015). The dissemination of MRSA strains occurred during the 1970s and 1980s, coinciding with the widespread utilisation of cephalosporins inside hospital settings (Mlynarczyk-Bonikowska *et al*., 2022). Historically, MRSA strains were commonly associated with hospital-acquired MRSA (HA-MRSA), which was subsequently broadened to encompass healthcare-associated MRSA (Mlynarczyk-Bonikowska *et al*., 2022).

During the 1990s, there was an emergence of novel strains of methicillin-resistant *Staphylococcus aureus* (MRSA) that were observed to cause infections in individuals who were not hospitalised, commonly referred to as community-acquired CA-MRSA (Hryniewicz, 1999). Additionally, in the early 21st century, a distinct type of MRSA known as livestock-associated MRSA (LA-MRSA) was documented (Voss *et al*., 2005). The development of resistance to beta-lactams in MRSA and various MRCoNS is linked to the existence of transferable genomic islands known as staphylococcal chromosomal cassette mec (SCC*mec*) (Maree *et al*., 2022). These genomic islands contain the *mec* gene, which is responsible for conferring resistance to methicillin. These islands undergo fast evolutionary changes and harbour numerous mobile genetic components (Mlynarczyk-Bonikowska *et al*., 2022).

There were reports of strains exhibiting intermediate susceptibility to vancomycin and other glycopeptides, which were subsequently referred to as VISA (vancomycin-intermediate *S. aureus*) and GISA (glycopeptide intermediate *S. aureus*) (Spagnolo *et al*., 2014). Based on the prevailing European EUCAST criteria, strains that were previously categorised as VISA are presently reclassified as VRSA, denoting a minimum inhibitory concentration (MIC) of vancomycin exceeding 2 mg/L (Mlynarczyk-Bonikowska *et al*., 2022). The detection of the initial strains of vancomycin-resistant *S. aureus* (VRSA) occurred in 2002, wherein these bacteria were shown to exhibit the *vanA* operon within the Tn*1546* transposon (Sievert *et al*., 2008).

Linezolid, which belongs to the class of oxazolidones, has been identified as a highly significant antimicrobial agent in the treatment of MRSA infections (Timmermans *et al*., 2021). The phenomenon of linezolid resistance (LZD^R) holds significant importance in clinical infectious disease and epidemiology of Gram-positive cocci (Brenciani *et al*., 2022). The occurrence of LZD^R is primarily attributed to two mechanisms. Firstly, it could be mediated by point mutations in the domain V of the 23S rRNA gene, with G2576T and G2505A being the most prevalent mutations. Additionally, amino acid changes in ribosomal proteins L3, L4, and L22 also mediate LZD^R. Secondly, LZD^R can also result from the acquisition of transferable genes, namely *cfr, cfrB, cfrD, optrA,* and *poxtA,* which are commonly found on plasmids (Brenciani *et al*., 2022). These findings have been documented in various studies conducted by Prystowsky *et al*. (2001), Long *et al*. (2006), Wang *et al*. (2015), Antonelli *et al*. (2018), and Mališová *et al*. (2021).

It is noteworthy to remark that the *cfr* gene, responsible for encoding a methyltransferase enzyme that alters the A2503 location of the 23S ribosomal RNA, was initially identified in a calf-derived strain of *S. sciuri* in the year 2000 (Long *et al*., 2006). The *cfr* gene provides resistance to multiple classes of antibiotics, including lincosamides, streptogramin A, phenicols, linezolid, and pleuromutilins (Schwarz *et al*., 2000; Long *et al*., 2006), especially in staphylococci (Brenciani *et al*., 2022).

In the year 2006, the discovery of the initial class I integrons, which were previously observed in Gram-negative bacteria, was made in staphylococci. These integrons were originally identified in CoNS and subsequently in *S. aureus* (Shi *et al*., 2016; Xu *et al*., 2008). The cassette genes responsible for conferring resistance to streptomycin (*aadA2, aadA5*), chloramphenicol (*cmlA1*), and trimethoprim (*dfrA12, dfr17*) within these integrons have been previously documented in studies conducted by Xu *et al*. (2008) and Shi *et al*. (2006). The gene cassettes identified in these studies were identical to those observed in

Gram-negative bacilli (Xu *et al*., 2008; Shi *et al*., 2006). The potential dissemination of additional gene cassettes and integrons among staphylococci and other Gram-positive bacteria presents potential public health problem due to the rapid exchange of resistance genes between different species and genera, as well as the acquisition of genes from Gram-negative bacteria by Gram-positive bacteria (Mlynarczyk-Bonikowska *et al*., 2022). **Table 7** presents the mechanisms of antimicrobial resistance (AMR) and the related MGEs.

Table 6. Fourteen major types of SCC*mec* detected in methicillin-resistant *Staphylococcus* species

SCC*mec* Type	**Representative Strain**	**Isolated in**	**GenBank Accession**	**SCC*mec* size (kb)**	***ccr* Complex**	***mec* Complex**
I	NCTC10442 (JCSC9884)	England;1961	AB033763	34.4	1	B
II	N315 (JCSC9885)	Japan; 1981	D86934	53	2	A
III	85/2082 (JCSC9889)	New Zealand; 1985	AB037671	66.9	3	A
IV	CA05 (JCSC9890)	USA; 1999	AB063172	24.3	2	B
V	WIS (JCSC9897)	Australia; 1995	AB121219	27.6	5	C2
VI	HDE288 (JCSC9900)	Portugal; 1996	AF411935	23	4	B
VII	P5747/2002 (JCSC9900)	Sweden; 2002	AB373032	32.4	5	C1
VIII	C10682 (JCSC9902)	Canada; 2003	FJ390057	32.1	4	A
IX	JCSC6943 (JCSC9903)	Thailand; 2006	AB505628	43.7	1	C2
X	JCSC6945 (JCSC9904)	Canada; 2006	AB505630	50.8	7	C1
XI	LGA251 (JCSC9905)	England; 2007	FR821779	29.4	8	E
XII	BA01611	China; 2015	KR187111	49.3	9	C2
XIII	55-99-44	Denmark; 2018	MG674089	29.2	9	A
XIV	SC792 (JCSC11500)	Japan; 2013–2014	LC440647	81.5	5	A

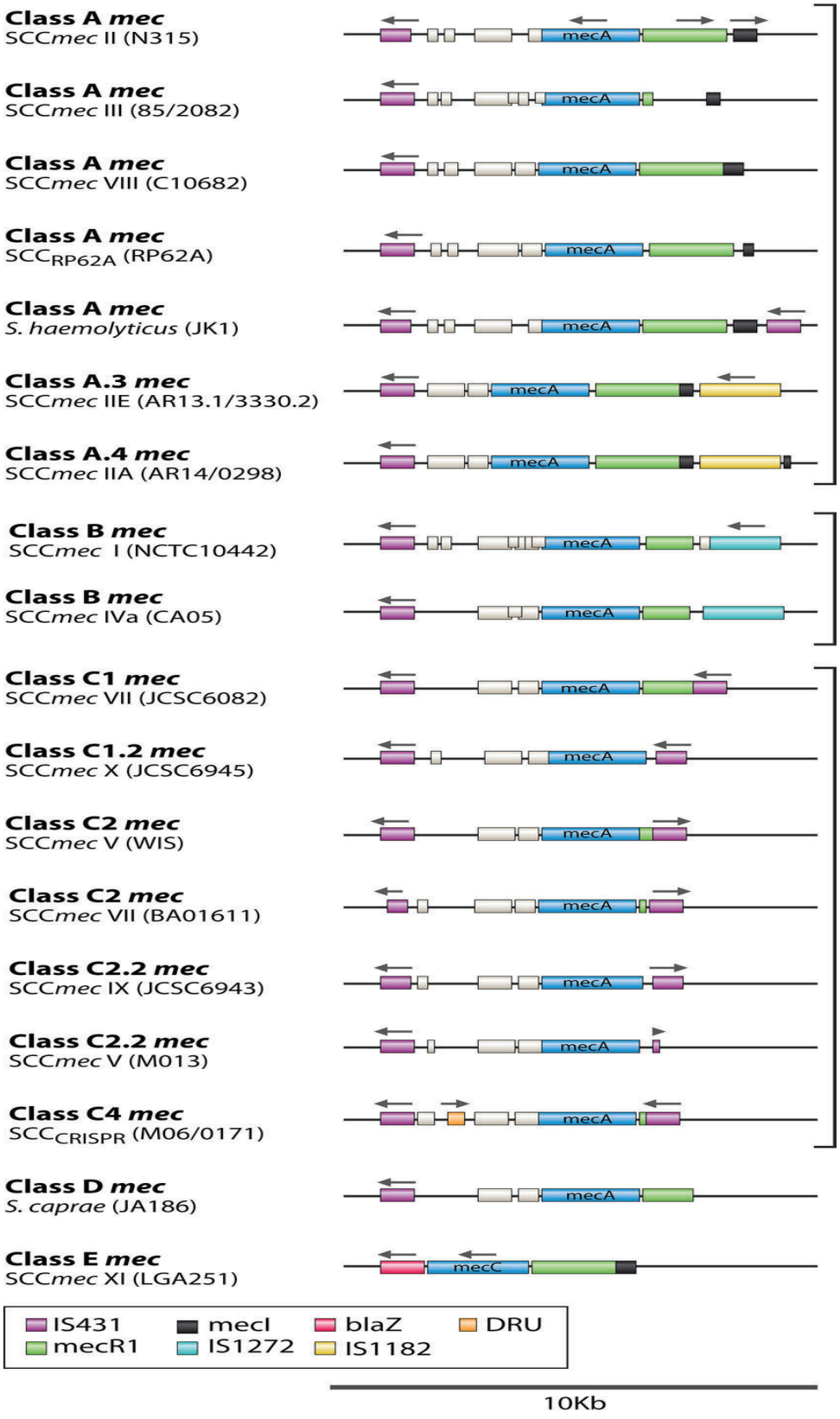


Figure 14. *mec* gene complex types and variants in staphylococci

Table 7. Mechanisms of action and resistance as well as the mobile genetic elements in various classes of antimicrobial agents that are relevant in staphylococci.

Class of antimicrobial agent	Mechanism of action in staphylococci	Resistance mechanism	Mobile Genetic Elements with resistance genes			References
			Plasmids	Transposons	Other MGEs	
Beta-lactams	Inhibit transpeptidases, transglycosylases and carboxypeptidases involved in peptidoglycan synthesis	*blaZ* (all species except *S. arlettae*)	*pI258*, *pII147*	Tn*552*, Tn*4002* and Tn*4201*	SCC*mec* type XI	García-Álvarez *et al* (2011), Shearer *et al.*, (2011), Olsen *et al* (2006), Rowland and Dyke (1990).
		bla_{ARL} (only *S. arlettae*)	None	None	None	Andreis *et al* (2017)
		mecA	None	None	Various SCC*mec* types	Miragaia (2018)
		mecA1 (*S. sciuri*), 85% homology with *mecA*	None	None	None	Miragaia (2018)
		mecA2 (*S. vitulinus*) 94% homology with *mecA*	None	None	None	Miragaia (2018)
		mecB (S. aureus) 69% homology with *mecA*	*pSAWWU4229_1*	None	None	Becker *et al* (2018)
		mecC (*S. aureus* LGA251 and many CoNS)	None	None	SCC*mec* XI and SCC*mec-mecC* hybrids	García-Álvarez *et al* (2011)
		mecC1 gene in *S. xylosus*	None	None	SCC*mec* XI	Harrison *et al* (2013)
		mecC2 gene in *S. saprophyticus*	None	None	SCC*mec-mecC* hybrid	Małyszko *et al* (2014)
		Mutations in genes encoding PBP2 and PBP4. Especially, on the genes *gdpP* and *yjbH* conditioning the overproduction of PBP4 protein and resistance to ceftobiprole.	None	None	None	Greninger *et al* (2016) Lee *et al* (2018b)
Macrolides, Lincosamides, Ketolides and Streptogramins B	Inhibit protein synthesis by union with four-nucleotide rRNA fragment (in the peptidyltransferase region) within the V domain of the 23S rRNA, in the 50S subunit of the ribosome	*ermA, ermB, ermC, ermT, erm45*: erythromycin-clindamycin resistance (constitutive or inducible)	*ermB* (*pI258*) *ermC* (*pE194*, *pT48*) *ermT* (*pUR2941*)	*ermA* (Tn*554*, Tn*6072*) *ermB* (*Tn*551)	None	Mlynarczyk-Bonikowska *et al* (2022)
		vgaA, vgaB, vga(A), vga(A)LC, lnuA and *lnuB:* clindamycin and lincomycin resistance				
		msrA, msrB: erythromycin resistance				

Table 7. Continuation

Class of antimicrobial agent	Mechanism of action in staphylococci	Resistance mechanism	Mobile Genetic Elements with resistance genes			References
			Plasmids	Transposposons	Other MGEs	
Aminoglycosides and Spectinomycin	Inhibit protein synthesis by interfering with the 30S subunit of the ribosome.	*aac2'-aph2"*: bifunctional gene for gentamycin, kanamycin, amikacin, netilmicin and tobramycin *aph3'*: kanamycin, neomycin, and lincomycin *ant4'*: tobramycin, kanamycin, neomycin *ant9'*: spectinomycin *ant6'*: streptomycin	*aac2'-aph2"* (*pSK1*) *ant6'* (*pS194*)	*aac2'-aph2"* (*Tn4001*) *ant9'* (Tn*6072*, Tn*554*) *aph3'* (Tn*3851*, Tn*4031* and Tn*5404*)	None	Weigel *et al* (2007)
Fluoroquinolones	Inhibit the activity of topoisomerase II (gyrase) and topoisomerase IV enzymes, responsible for DNA superspiralization and respiralization	Mutations in: a) *gyrA*/*gyrB* (topoisomerase II) b) *parC* (*grlA*)/*parE* (topoisomerase IV) c) Overproduction NorA, NorB, NorC and SdrM	None	None	None	Xu *et al* (2011), Ding *et al* (2008), Lowy (2003)
Tetracyclines	Inhibit protein synthesis by interfering with the 30S subunit of the ribosome.	*tet*(K), *tet*(L), *tet*(38), *tet*(42), *tet*(43), *tet*(45), *tet*(63), *tet*(M), *tet*(O), *tet*(W), *tet*(44), *tet*(U), and *tet*(S)	*tet*(K) (*pT181, pSTE2*) *tet*(L) (*pKKS825*)	*tet*(M) (Tn916, *Tn6014*)	None	Zhu *et al* (2021), Weigel *et al* (2008), de Vries, *et al* (2009), Mlynarczyk-Bonikowska *et al* (2022)
Mupirocin	Inhibits protein synthesis by inactivating isoleucyl-tRNA synthetase	*mupA* and *mupB*	*mupA* (*pUSA03*)	None	None	Dyke *et al* (1991) Seah *et al* (2012)
Fusidic Acid	Inhibits protein synthesis by interaction with elongation factor G	Mutations in *fusA* Overexpression of *fusB, fucC, fusD* and *fusE* genes	None	None	None	Lannergard *et al* (2009), Mairi *et al* (2021)

Table 7. Continuation

Class of antimicrobial agent	Mechanism of action in staphylococci	Resistance mechanism	Mobile Genetic Elements with resistance genes			References
			Plasmids	**Transposposons**	**Other MGEs**	
Daptomycin	Acts on the cytoplasmic membrane	Mutations in *mprF, dltABCD* operon, *vraSR* regulatory genes, *clpP, rpoC, vraG , spsB, fmtA, asp23, yycG* and *pgsA*	None	None	None	Mlynarczyk-Bonikowska *et al* (2022)
Streptogramins A and Quinupristin-Dalfopristin	Act on the 23S rRNA of the 50S subunit of the ribosome	*vatA, vatB, vatC, vatD, vatE, vgb(A)* and *vgb(B), vga(A), vga(Av), vga(B), vga(C)* and *vga(E)*	None	*vatB* (Tn*5406*) *vga(A), vga(Av)* (*Tn5406*) *vga(B), vga(C)* and *vga(E)* (Tn*6133*)	None	Allignet *et al* (1995)
Rifampicin	Inhibits transcription by interfering with the beta subunit of RNA polymerase.	Mutations in the *rpoB* gene	None	None	None	Aubry-Damon *et al* (1998)
Chloramphenicol	Inhibits protein synthesis by binding to the 50 S subunit of the 70 S ribosome	*catA, fexA, fexB,* cat_{PC194}, cat_{PC221}, cat_{PC223}	None	*fexA* (Tn*558*) *fexB* (Tn*6246*)	None	Kehrenberg and Schwarz (2005) Freitas *et al* (2020)
Trimethoprim-sulfamethoxazole	Inhibit folate synthesis pathways	*dfrA, dfrK, dfrD, dfrG, dfrA12* and *dfr15*	*dfrA* (*pSK1*) *dfrK* (*pKKS82*)	*dfrA* (Tn*4003*) *dfrK* (Tn*559*)	*dfrG* (SCC*mec* type V) *dfrA12* and *dfr17* (class I integron)	Ham *et al* (2023)
Glycopeptides and lipoglycopeptides	Inhibit peptidoglycan synthesis	*vanA* and *vanB*	None	*vanA* (Tn*1546*)	None	Lee *et al* (2018) Nepal *et al* (2023) Freitas *et al* (2016)
Oxazolidinones	Act on the 23S rRNA molecule in the 50S subunit of the ribosome (inhibition of protein synthesis)	Ribosomal mutations in L3, L4 and/or L22 ribosomal proteins Chromosomal point mutations in 23S rRNA *cfr, optrA,* and *poxtA*	*cfr* (*pLRSA417*) *optrA (pWo28-3*) *poxtA* (*pY80*)	*optrA* (T*n6823*) *poxtA* (Tn*6349*)	None	Brenciani *et al* (2022)

1.6.1 *mecC*-mediated methicillin resistance in *Staphylococcus aureus* and non-*aureus* staphylococci

The frequent association of methicillin resistance in staphylococci is attributed to the presence of *mec*-type genes (*mecA*, *mecB*, and *mecC*). These genes encode for penicillin-binding proteins (PBPs) that exhibit low affinity for β-lactams (Miragaia *et al*., 2018). The *mecA* gene exhibits a high prevalence on a global scale. In 2007, a gene known as *mecC* was identified in the United Kingdom as part of an epidemiological investigation of bovine mastitis (García-Álvarez *et al*., 2011; Shore *et al*., 2011). Subsequently, there have been consistent reports of *mecC*-mediated MRSA strains from humans and wild animals as systematically reviewed by Abdullahi *et al* (2021) and Lozano *et al* (2020). Notwithstanding, the *mecC* gene has been detected in several species of CoNS, including *S. sciuri*, *S. lentus*, *S. xylosus*, *S. stepanovicii*, *S. caprae*, and *S. warneri* (Harrison *et al*., 2014; Lakhundi and Zhang, 2018; Loncaric *et al*., 2019; Paterson, 2020, Schauer *et al*., 2021; de Moura *et al*., 2023; Belhout *et al*., 2023).

Remarkably, most of these *mecC*-carrying *Staphylococcus* species were identified from dairy animals (Harrison *et al*., 2014; Lakhundi and Zhang, 2018; Loncaric *et al*., 2019; Paterson, 2020, Schauer *et al*., 2021; de Moura *et al*., 2023; Belhout *et al*., 2023). Specifically, the classical SCC*mec* type XI of *mecC*-carrying *S. aureus* is a class E *mec* complex (*mecI-mecR1-mecC1-blaZ*) **(Figure 13).** This environment of *mecC* gene of most CoNS has been described to be encoded within a hybrid SCC*mec* element comprised of *mecA* encoding SCC*mec* type VII (de Moura *et al*., 2023; Belhout *et al*., 2023; Paterson *et al*., 2020).

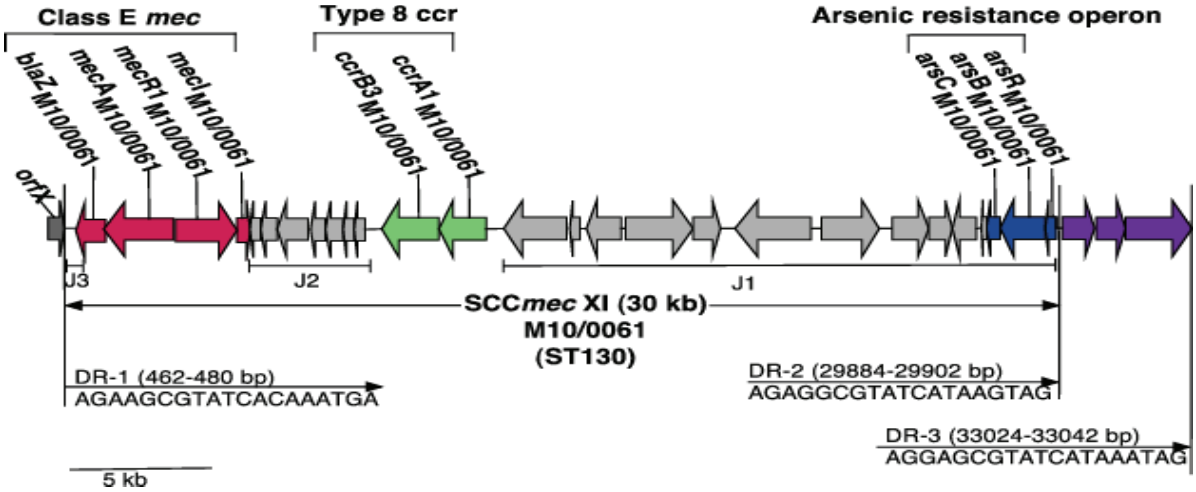


Figure 15. Genetic environment of the classical SCC*mec* type XI carrying *mecC* gene in MRSA-ST130 (Shore *et al*., 2011).

1.7 Virulence in staphylococci

The effectiveness of antimicrobial agents in addressing staphylococcal infections is not solely determined by the presence or absence of AMR, but can also be influenced by various factors. These factors include the growth phase of the bacterium (whether it is in the logarithmic growth or stationary phase), the specific site of infection (as drugs can attain different concentrations in different body compartments), the formation of biofilms, and numerous other considerations (Mlynarczyk-Bonikowska *et al*., 2022). The selection of an appropriate treatment approach should be closely associated with the specific type of infection, such as epidemic, nosocomial, or chronic, which may in turn be influenced by the presence or lack of a particular virulence factor that could potentially contribute to the pathogenicity of the staphylococci (Mlynarczyk-Bonikowska *et al*., 2022).

According to Heilmann *et al*. (2018), CoPS exhibit a higher degree of pathogenicity compared to CoNS. Concerning CoPS, *S. aureus* has an enormous potential for pathogenicity higher than other species. The virulence factors occur at different frequencies in different clones of this bacteria. However, the virulence potential of CoNS is mainly associated with their adhesion capacities to body tissues such as those mediated by biofilm genes (Marek *et al.,* 2021). Nevertheless, some CoNS could acquire unusual virulent genes which are *ab initio* classical genes that mediate the pathogenicity of *S. aureus* (França *et al.,* 2021). This suggests virulence *gene* exchange between CoNS *and S. aureus* that could lead to the emergence of a highly pathogenic CoNS strain, as seen in a recent case of toxic shock syndrome induced by *S. epidermidis* (Armeftis *et al.,* 2023).

Some pathogenicity factors exhibit several roles and can simultaneously be classified into multiple groups. For instance, staphylococcal enterotoxin genes could mediate both gastroenteritis and toxic shock syndrome (Etter *et al*., 2022). The co-occurrence of virulence and AMR genes on the same genomic elements is a common phenomenon (Darmancier *et al*., 2022). Nevertheless, it is important to note that the mere existence of a gene associated with pathogenicity does not necessarily guarantee its activation, as this process is influenced by one or more regulatory mechanisms within the bacterial cell (Mlynarczyk-Bonikowska *et al*., 2022). The virulence factors of staphylococci are very diverse and can be classified into several basic groups as presented in **Table 8.**

Table 8. Virulence determinants in the *Staphylococcus* genera

Staphylococci group	Species	Adherence	Exoenzymes	Exotoxin (unusual species)	Haemolysin	Immune evasion	Metal uptake	Secretory system
CoPS	*S. aureus*	*atl, clfA, clfB, cna, coa, epb, icaA-D, icaR, eap, ebh, ebp, efb, eno, epb, fnbA, fnbB, sdrC-H, map*	*aur, edinA-edinC, eta, etb, etd, etd2, geh, lip, hysA, sak, sspB, sspC, sspA, splA-F*	*lukD, lukE, lukM, lukF, lukG/LukH, lukS, luk-F/S-PV, sea-j, selK-r, selu-selx, vWF, tst,*	*hla, hld, hlg, hlgA-C*	*adsA, chp, efb, gala, sbi, scn*	*isd, htsBC*	*esaA-G, essa-C, esxA-D*
	S. pseud-intermedius/S. intermedius	*atl, bap, clfA, clfB, cna, coa, epb, icaA-D, icaR, eap, ebh, ebp, efb, eno, epb, fnbA, fnbB, sdrC-H*	*clpP, clpX, ebpS, expA, hrtA, lip, rrA, se-int, seccanine, spsD, spsO, SpsQ,*	*eta, luk-S/F-I, sea, seb, sec, sed, sei, sej, sek, ser, siet,*	*hlgB, hlgv*	*adsA, capA, capB, wbtP,*	None	None
CoNS		*atl, clfA, clfB, cna, epb, icaA-D, icaR, eap, ebh, ebp, efb, eno, epb, fnbA, fnbB, sdrC-H, map*	***eta*** (*S. cohnii*), *lip, sspB, sspC, hysA, geh, lip, sspA, splA-F, coa, sak,*	***luk-F/S-PV*** (*S. simulans*), *lukD, lukE, lukM, lukF, lukS,* ***sea-h*** (*S. epidermidis, S. haemolyticus, S. saprophyticus*), ***sell*** (*S. epidermidis*), ***tst*** (*S. sciuri, S. saprophyticus, S. haemolyticus*)	None	*adsA, capA, capB, wbtP*	*vctC,*	*esaA-G, essa-C, esxA-D*
Coagulase variable	*S. agnetis*	*sasF, sasH*	None	*seb,* ***sec, tst***	None	None	None	None

Unusual genes in bold

1.7.1 Virulence factors of coagulase-negative staphylococci

CoNS have become significant pathogens in healthcare-associated settings, with *S. epidermidis, S. haemolyticus,* and *S. lugdunensis* being the most medically significant species (Argemi *et al*., 2019). Although CoNS strains are often less virulent compared to the extensively researched *S. aureus,* there has been a continuous rise in the sequencing of CoNS species Consequently, this has led to an increasing identification of virulence components within these staphylococci. Specifically, the production of biofilms is regarded as a highly significant phenomenon that facilitates the virulence of CoNS (França *et al*., 2021). CoNS species possess various mechanisms to overcome unfavourable situations and, consequently, adapt and thrive in many ecological settings (França *et al*., 2021).

The process of biofilm creation starts with the attachment of planktonic cells to a surface, whether it be non-living or living, and subsequently progresses through cellular division and aggregation, resulting in the development of the distinctive multi-layered architecture (Schilcher and Horswill, 2020). Furthermore, the cells generate an additional polymeric protective matrix (Peng *et al*., 2022). The term "maturation phase" refers to this particular stage (França *et al*., 2021). The aforementioned process is facilitated by adhesins, as well as molecules possessing disruptive capabilities, such Phenol-soluble modulins (PSMs) since they play a crucial role in the formation of channels that facilitate the transportation of nutrients throughout all layers of the biofilm (França *et al*., 2021; Le *et al*., 2019). Furthermore, as anticipated, PSMs play a crucial role in the last stage of the biofilm life cycle, known as dispersion, by facilitating the release of biofilm cells and their subsequent colonisation of new locations (França *et al*., 2021). **Figure 16** offers a concise description of biomolecules utilised by CoNS to effectively respond to and survive external stimuli.

Certain CoNS have raised growing concerns in recent times, notably *S. lugdunensis,* which has been increasingly acknowledged as a pathogenic bacterium with significant virulence effects (Heilbronner and Foster, 2021). *S. lugdunensis* has the potential to induce more acute and detrimental episodes of infective endocarditis, resulting in greater fatality rates compared to other CoNS species, which often give rise to less severe infections.

In a recent investigation involving the analysis of over 1500 genomes of staphylococci, it was observed that genes responsible for producing enterotoxins were present in 97% of the genomes of *S. aureus* (857 out of 883) (Banaszkiewicz *et al*., 2019). Conversely, a significantly lower number of enterotoxin-encoding genes, specifically nine, were discovered in the

genomes of *S. epidermidis*. The nine strains of *S. epidermidis* identified in the study were found to possess genetic encoding for both *sec* and *sel* genes.

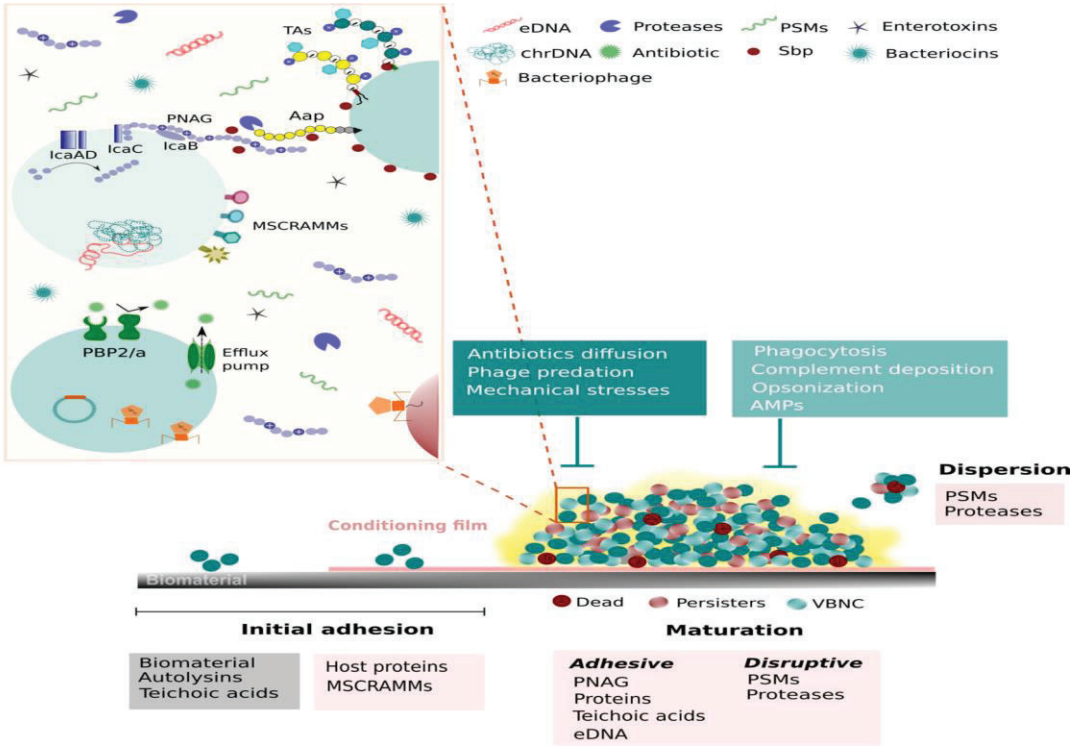


Figure 16. CoNS virulence factors summary illustration (França *et al.*, 2021).

Keys: Aap, accumulation associated protein; AMPs, Antimicrobial peptides; chrDNA, chromosomal DNA; eDNA, extracellular DNA; MSCRAMMs, Microbial surface components recognizing adhesion matrix molecules; PBP2/a, penicillin-binding protein 2 and 2a; PNAG, poly-N-acetylglucosamine; PSMs, phenol-soluble modulins; Sbp, Small basic protein; TAs, teichoic acids; VBNC, Viable but-non culturable cells

In the aforementioned study, Banaszkiewicz *et al*. (2019) conducted a phylogenetic analysis and noted that the nine enterotoxigenic *S. epidermidis* strains examined were classified into a distinct cluster consisting of 65 strains, which exhibited considerable divergence from the remaining 499 strains.

Given their shared ecological habitats, it is anticipated that there will be a substantial occurrence of gene flow between *S. aureus* and *S. epidermidis* (Lin *et al*., 2021). The aforementioned process has the potential to result in the development of toxigenic strains of *S. epidermidis*. Therefore, it is imperative to take into account the potential occurrence of staphylococcal food poisoning (SFP) resulting from CoNS strains in the future. In this regard, it is important to highlight the detection of *sec*- and *sel*-carrying *S. epidermidis* of the lineage ST595. Similar studies have previously reported these virulence genes and their associated pathogenicity islands in *S. epidermidis* (Lin *et al*., 2021; Nasaj *et al*., 2021; Banaszkiewicz *et*

al., 2019). Moreover, it has been suggested that only *S. epidermidis* from animals or food but not humans may typically produce *S. aureus*-related SEs (Nanoukon *et al*., 2018; Podkowik *et al*., 2016; Stach *et al*., 2015; Veras *et al*., 2008). However, some *sec* and *sel* genes have been identified in association with plasmids, phages and pathogenicity islands. Thus, they can be horizontally transmitted between any host, including humans.

It appears that the *sec* and *sel*-carrying *S. epidermidis* from nestling stork are not transferable as they were not associated with an MGE. Moreover, simultaneous colonization of the nostril by several *Staphylococcus* spp could promote the transfer of enterotoxin genes from *S. aureus* to commensal *S. epidermidis* (Nanoukon *et al*., 2018).

1.7.2 Host adaptation system: The case of *S. aureus*-CC398

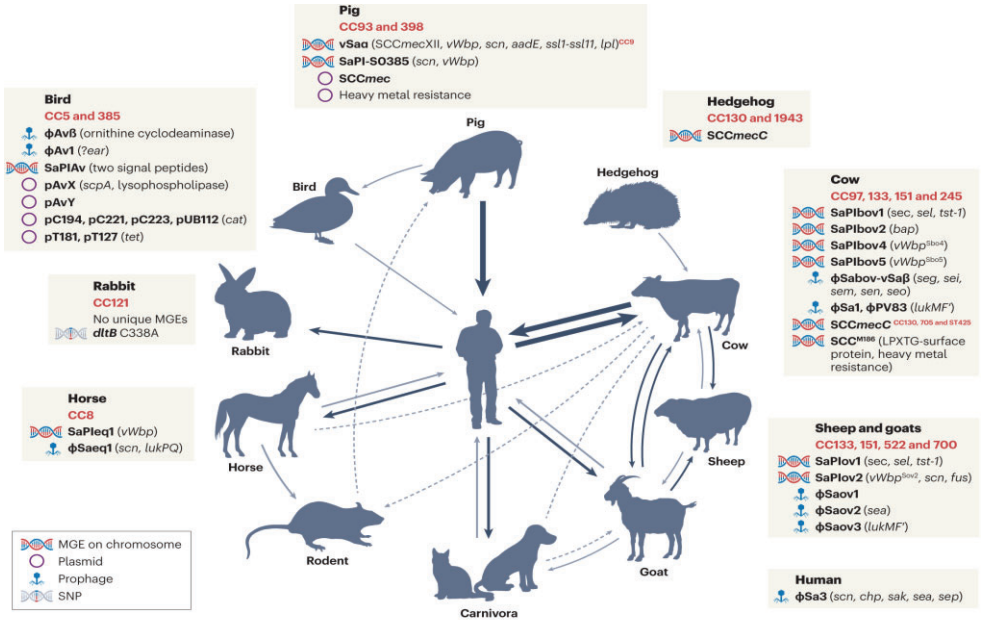
The occurrence of single-nucleotide mutations in bacteria has been linked to notable alterations in phenotype, such as the acquisition of host tropism in *S. aureus* (Chaguza *et al*., 2022). Furthermore, the association between the acquisition and loss of mobile genetic elements (MGEs), such as staphylococcal prophages and pathogenicity genomic islands, and the process of host adaptation in both humans and animals has been established (Price *et al*., 2012). An illustration of this phenomenon can be observed in the livestock-related *S. aureus* clones ST398 and ST9, which originated from humans and afterwards underwent adaptation in livestock. This adaptation has been connected with the loss of virulence genes that are associated with phages (Yu *et al*., 2022; Price *et al*., 2012). In a study conducted by Sieber *et al*. (2020), it was found that the acquisition of prophages by livestock-related clones was associated with heightened transmission and adaption to humans. Nevertheless, the significance of the varying abundance of these genes concerning host transmissibility and infection, as well as its true indication of host adaptation or potential barriers to gene flow between different hosts, such as restriction-modification systems (Park and Ronholm, 2021), remains uncertain. Hence, it is crucial to investigate the genetic variation that plays a pivotal role in the transmissibility of hosts, infection processes, switching mechanisms, and adaptive abilities of *S. aureus* and other pathogens with multiple species (Chugaza *et al*., 2022). These analyses have the potential to identify previously unknown genetic regions associated with disease-causing abilities. This knowledge can be used to develop efficient preventive and treatment strategies, to prevent and control the spread of epidermic strains that pose a significant risk to human and animal health.

The utilisation of genome-wide association studies (GWASs) has provided valuable knowledge concerning the genetic underpinnings of various aspects related to *S. aureus* and

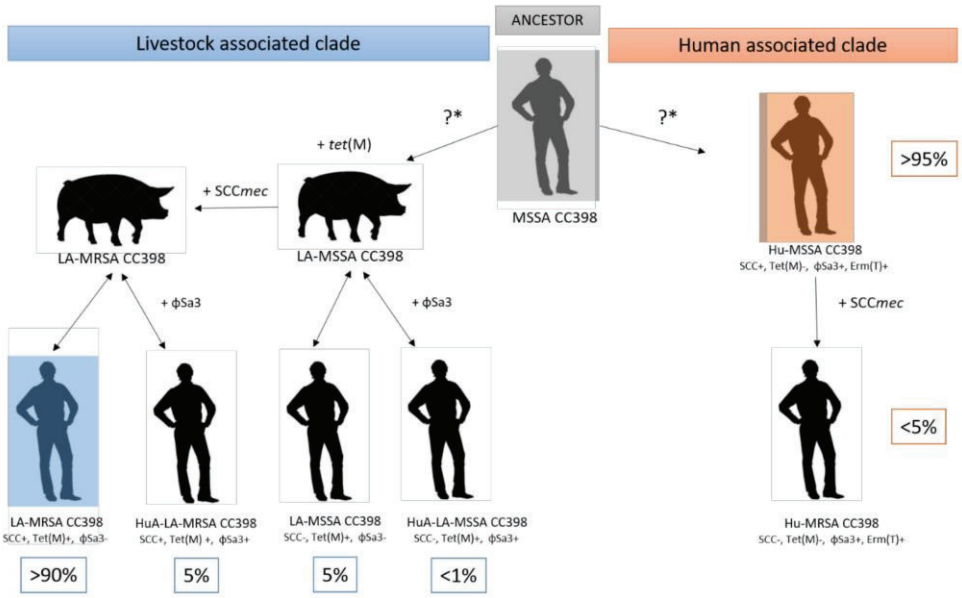
related species. Molecular studies have shown that livestock can act as intermittent carriers of *S. aureus* and reservoirs for zoonosis and dissemination of high-level AMR in farmers, the communities close to the farms and food derived from the animals (Gelbíčová *et al*., 2022; Golob *et al.,* 2022; Crespo-Piazuelo *et al*., 2021; Avberšek *et al.,* 2021; Anker *et al.,* 2018). In this regard, the major livestock-adapted lineage (MRSA-CC398) emerged from MSSA-CC398 following the loss of φSa3 and the acquisition of *tet*(M) and SCC*mec* elements and spread in the European pig production (EFSA 2009) and spillover back into humans in the community and healthcare settings (Sieber *et al.,* 2019; Anker *et al.,* 2018). Years later, the human-adapted and community-associated MSSA-CC398 reemerged to cause invasive infections in some European countries and China (Bouiller *et al*., 2022; Mama *et al*., 2021a; Bouiller *et al*., 2020; He *et al*., 2013). Epidemiologically, the *scn* and *tet(*M) genes are considered molecular markers that can be used to track these lineages (Price *et al.,* 2012).

Aside from the prophage φ3, other prophages such as φ2, φ6, and φ9 were also frequently identified in *S. aureus* strains of the nestling storks, pigs and pig farmers regardless of the methicillin susceptibility and genetic lineages (Laumay *et al*., 2021). Importantly, the presence of β-converting φ3-prophage variants carrying an immune evasion cluster (IEC) characterizes the MSSA-CC398 subclade. However, this subclade also harbours other prophages carrying integrase genes SebogoInt and Sa9int (Laumay *et al*., 2021)**.** Indeed, human-to-animal transmission is strongly correlated with the loss of φSa3, but it seems that LA-CC398 MRSA can, in rare cases, readapt to the human host through the regain of an IEC-harbouring φSa3 **(Figures 17a and 17b).**

Ward *et al*. (2014) provided evidence in favour of the presence of separate clades connected with humans and cattle. These clades developed around the same time, as indicated by the symbol "?*" in the **Figure 17b.** The utilisation of a double-arrow symbol was employed to depict the diverse subpopulations within the livestock-associated clade, as it has been observed that interspecies transmission occurs in both directions. The acquisition of the prophage φSa3 in the LA clade was likely facilitated by these transmissions. This event led to the differentiation of human (Hu) SA CC398 subpopulations from the adapted Humans (HuA) livestock-associated (LA) SA CC398 subpopulations.



(a)



(b)

Figure 17. *Staphylococcus aureus* host species adaptation over time (Howden *et al.*, 2023; Bouiller *et al.*, 2020).

The host-switching modelling study conducted on *S. aureus* revealed that humans play a significant role as a transmission hub between different species. The studies conducted by Howden *et al*. (2023) and Bouiller *et al.* (2020) present a comprehensive analysis of host-specific MGEs and their corresponding virulence genes across different animal species. The studies also investigate the primary associated CCs in these animals.

1.8 CRISPR-Cas system in staphylococci and its application against AMR

The CRISPR-Cas system, consisting of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas), serves as an RNA-

based adaptive immune mechanism employed by bacteria to defend themselves against the intrusion of MGEs (Tao *et al*., 2022). Therefore, it is plausible that the CRISPR–Cas system could serve as a viable strategy to impede the acquisition of plasmids and phage invasion, as well as hinder the horizontal transfer of AMR genes in staphylococci (Murugesan and Varughese, 2022).

The CRISPR-Cas system can be classified into two distinct types based on the structural characteristics, components, and mechanisms of action of their proteins (Nishimasu and Nureki, 2017). The Class 1 CRISPR-Cas system employs a variety of protein effector complexes to degrade nucleotides. This system can be further categorised into three categories, namely types-I, -III, and -IV **(Figure 18).** In contrast, the Class 2 CRISPR-Cas system employs effector complexes consisting of a single protein to degrade nucleotides. This system is further classified into types -II, -V, and -VI, as described by Shmakov *et al*. (2015) and Makarova *et al*. (2015). The Types II-CRISPR-Cas systems have been extensively investigated and have demonstrated efficacy in the targeted removal of antimicrobial resistance (AMR) genes, mostly attributed to their comparatively straightforward architectures (Tao *et al*., 2022). Furthermore, researchers have made advancements in the development and manipulation of Type I CRISPR-Cas systems to eradicate AMR genes (Tao *et al*., 2022).

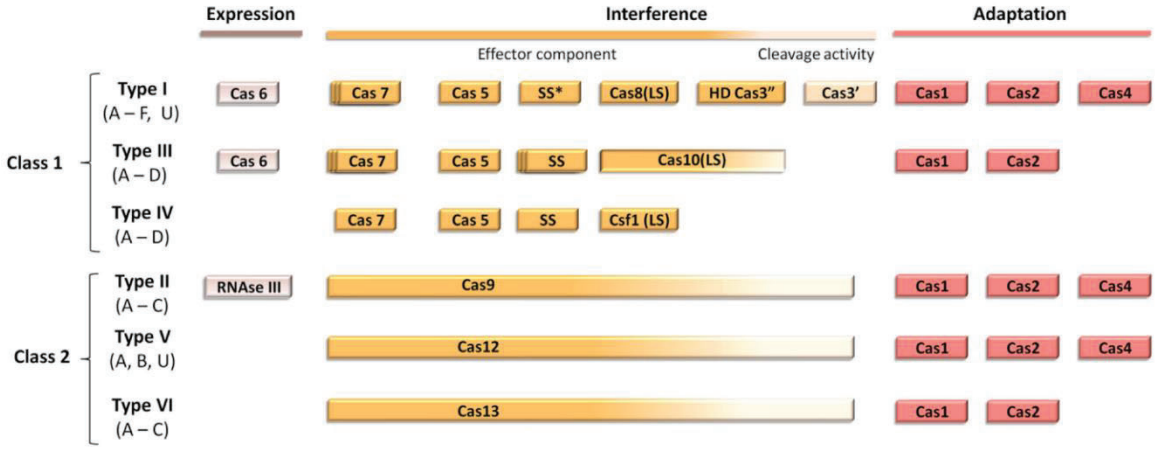


Figure 18. Modular organization of CRISPR-Cas classes (Ishino *et al.*, 2018).

The type III system possesses the capability to target both DNA and RNA and is considered the most ancient among the CRISPR-Cas systems (Mohanraju *et al*., 2016). The presence of the type III-A CRISPR-Cas system in *S. aureus* is limited, as indicated by a study conducted by Ao *et al*. (2016), which stated that only 0.94% (6/636) of clinical strains were found to possess this system. Holt *et al*. (2011) reported the presence of the CRISPR-Cas locus inside the SCC*mec* of the *S. aureus* strain 08BA02176 and *S. argenteus* MSHR1132. This

observation implies the potential mobility of the CRISPR-Cas system in both strains. Subsequent studies have provided evidence that indicates the predominant CRISPR-Cas type III-A systems in staphylococci are primarily located inside the SCC*mec* region (Li *et al*., 2021). According to a study conducted by Cao *et al*. (2016), clinical strains of *S. aureus* have been found to possess the type III-A CRISPR-Cas system, which confers resistance against plasmid invasion. The presence of Type III-A CRISPR-Cas systems has been identified in *S. epidermidis,* with a prevalence rate of 14% (Li *et al*., 2016). This system in *S. epidermidis* was found to confer resistance against both plasmid invasion and phage infection (Jiang *et al*., 2016).

The study conducted by Bikard *et al*. (2014) employed Cas-9 phagemid to eradicate MRSA strains from a heterogeneous bacterial community. According to Bikard *et al*. (2014), the RNA-guided Cas9 system did not cause lethality towards avirulent strains of *S. aureus*. However, it effectively targeted and deleted plasmids that harboured the *mecA* gene, without causing harm to the host bacteria. Synthetic CRISPR-Cas 9 with target specificity has been designed to detect and eliminate tetracycline-resistant plasmids, namely pUSA01 and pUSA02 (Bikard *et al*., 2014).

In a separate study, Kiga *et al*. developed a phage utilising Cas13a to target carbapenem-resistant *E. coli* and MRSA The findings of this study provide support for the potential use of CRISPR-Cas and phagemid as therapeutic approaches in addressing bacterial strains harbouring clinically significant AMR genes (Kiga *et al*., 2022). Nevertheless, the utilisation of phagemid is associated with two significant limitations. Firstly, it should be noted that phagemid does not exhibit phage amplification upon infection, hence necessitating a significantly larger quantity of phagemid for therapeutic purposes compared to the size of the target population. Furthermore, the limited host range and high population size of phagemids may hinder their widespread implementation (Juved *et al*., 2023).

One advantage of programmable Cas9-mediated killing is the capacity of a nuclease equipped with multiple crRNA guides to selectively activate separate plasmid and/or chromosomal sequences. This capability has the potential to reduce the occurrence of unaffected clones that abandon phagemid care due to the creation of target mutants. Also, it can broaden the scope of targeted cells that can be effectively manipulated. The introduction of the sequence-specific Cas9 nuclease and its reprogramming to targ*et al*ternative sequences leads to a reduction in plasmid content within a bacterial population without causing cell lysis. This approach effectively hinders the transfer of antibiotic-resistant and/or virulent plasmids among non-pathogenic strains (Juved *et al*., 2023).

1.9 The *Enterococcus* genus and its relationship with *Staphylococcus*

The indiscriminate and widespread utilisation of antibiotics in clinical settings and agricultural practices has led to a progressive emergence of resistance in enterococci (Ruiz-Ripa *et al*., 2020a; Torres *et al*., 2018).

According to a study conducted by Zhou *et al*. (2020), the primary phenotypic indicator of *E. faecium* strains in hospital settings is the emergence of ampicillin resistance. Furthermore, there is an increasing prevalence of resistance to vancomycin, particularly among strains bearing the *vanA* and *vanB* genes (Mirzaie *et al*., 2023). The *vanA*-mediated resistance is distinguished by a significant level of resistance caused by vancomycin and teicoplanin. The presence of this phenomenon is predominantly observed in strains of *E. faecium,* although it can also be found in *E. faecalis* and, to a lesser degree, in *E. durans, E. raffinosus, E. hirae, E. avium,* and *E. gallinarum* (Ahmed *et al*., 2018).

Moreover, linezolid resistance multidrug-resistant enterococci is a significant signal of critical and public health concern. The emergence of resistance to linezolid in clinical strains of *Enterococcus* genus has been documented for several years, primarily attributed to the presence of the *optrA* gene (Freitas *et al*., 2020). The *Staphylococcus* and *Enterococcus* genera share many genetic similarities in terms of AMR genes they can acquire and transfer between themselves (Brenciani *et al*., 2022). In this regard, the mechanisms of oxazolidinone resistance (ribosomal mutations and acquired resistance genes) are similar in both genera. Particularly, the wide flexibility of all the genetic elements carrying the oxazolidinone resistance genes makes their inter-genera transmission and transfer highly possible. Acquired resistance genes associated with MGEs pose a particular threat to the dissemination to all the One Health ecosystems (Brenciani *et al*., 2022).

OBJECTIVES OF THE THESIS
OBJETIVOS DE LA TESIS

1.10 Objectives of the thesis

1.10.1 Main objective

To deepen the understanding of staphylococcal diversity in the nasal cavities of healthy humans (with or without animal contact) and animals (healthy dogs, pigs and nestling storks) as well as to characterize their antimicrobial resistance mechanisms, virulence genes and genomic contents using culture-dependent strategies.

1.10.2 Specific objectives

1) To determine the diversity, co-carriage and frequencies of all the *Staphylococcus* species that colonize the noses of healthy humans (with/without animal contact), as well as of representative animals with different forms of contact with humans.

2) To characterize the antimicrobial resistance determinants and virulence genes in the recovered staphylococci in the four studied hosts, as well as to determine their genetic lineages.

3) To characterise the staphylococcal resistome, virulome and mobilome (plasmids, transposons, insertion sequences, bacteriophages and staphylococcal cassette chromosomes) using whole genome sequencing techniques.

4) To analyze possible biomarkers to be used as potential diagnostic strategies for antimicrobial resistance traits in staphylococci

5). To characterize the mechanisms of antimicrobial resistance in *Enterococcus* species from the four hosts, with a special focus on the linezolid resistance determinants and the environment of the detected resistance genes.

1.10.1 Objetivo principal

Profundizar en el conocimiento de la diversidad de estafilococos en las cavidades nasales de humanos sanos (con o sin contacto con animales) y animales sanos (perros, cerdos y polluelos de cigüeña), así como caracterizar sus mecanismos de resistencia a los antimicrobianos, genes de virulencia y contenidos genómicos empleando una estrategia cultivo dependiente.

1.10.2 Objetivos específicos

1) Determinar la diversidad, la colonización conjunta y la frecuencia de detección de especies de *Staphylococcus* en muestras nasales de humanos sanos (con/sin contacto animal), así como de animales con diferente grado de contacto con humanos.

2) Caracterizar los determinantes de resistencia antimicrobiana y los genes de virulencia en los estafilococos obtenidos de los cuatro hospedadores estudiados, así como determinar sus líneas genéticas.

3) Caracterizar el resistoma, viruloma y mobiloma de estafilococos (plásmidos, transposones, secuencias de inserción, bacteriófagos y casetes de estafilococos cromosómicos) mediante técnicas de secuenciación del genoma completo.

4) Analizar posibles biomarcadores que se utilizarán como posibles estrategias de diagnóstico de rasgos de resistencia a los antimicrobianos en cepas de estafilococos.

5). Caracterizar los mecanismos de resistencia antimicrobiana en cepas de *Enterococcus* obtenidas de los cuatro huéspedes, con especial enfoque en los determinantes de resistencia a linezolid y el entorno de los genes de resistencia detectados.

CHAPTER TWO

MATERIALS AND METHODS

2.0 SAMPLE COLLECTION, TRANSPORTATION AND PRESERVATION

2.1 SOURCES OF THE SAMPLES

The nasal samples obtained from healthy humans, healthy dogs and dog owners, were from the Autonomous Community of La Rioja while the nasal samples from healthy pigs and pig farmers were from pig farms in Aragon. Moreover, nasal and tracheal samples were collected from nestling storks from southern Spain **(Figure 19).**

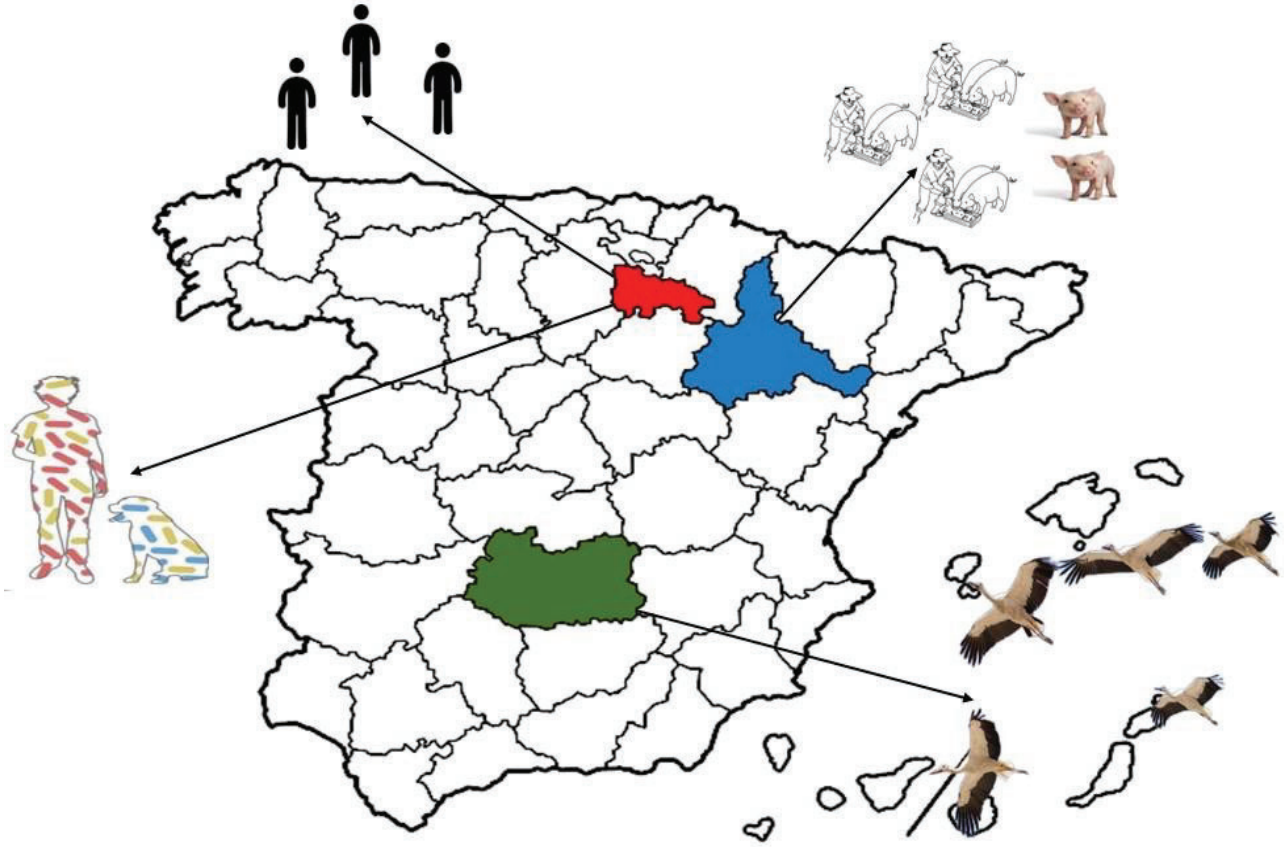


Figure 19. Locations where humans and animals were enrolled for sample collection

2.1.1 Healthy humans without animal contact

Fifty-seven (57) healthy people from La Rioja region (Northern Spain) were enrolled in this study, between July 2022 to March 2023. Their nasal samples were collected into Amies transport media and processed for staphylococci recovery. The inclusion criteria for participation in this study were; individuals who had no antibiotics or visited a hospital, had no contact with animals, and had no professional contact with health institutions and/or microbiology laboratories in the last 6 months.

2.1.2 Nestling storks

White stork nestlings (juvenile storks in the nest before fledging) were sampled in June 2021 at 45–55 days of age. Nasal and tracheal swab samples were collected from the stork nestlings from four different colonies based on the different foraging habits of their parents when raising their chicks. This study design took advantage of the fact that during the chick-raising period, parent storks are spatially bound to the nesting habitat (*i.e.*, forage primarily close to the nest) and thus a clear differentiation of the habitat in which food items are foraged is possible. Also, sampling of nestlings is less invasive and logistically less challenging than the capture of adult storks and is carried out during routine ringing procedures.

The storks corresponded to four different colonies with different foraging strategies (colonies 1 and 2: located and foraging in natural habitat; colonies 3 and 4: foraging in two different landfills). Nasal and tracheal samples from a total of 87 white stork nestlings were collected, which comprised 136 samples: 84 tracheal (T) and 52 nasal (N) **(Figure 20)**. Of these animals, 49 had both nasal and tracheal samples collected **(Figure 20).** The uneven distribution of samples was due to technical problems, as some samples could not be processed further due to contaminations. At least one full set (nasal and tracheal swabs) of samples of one of the siblings in each nest were collected.

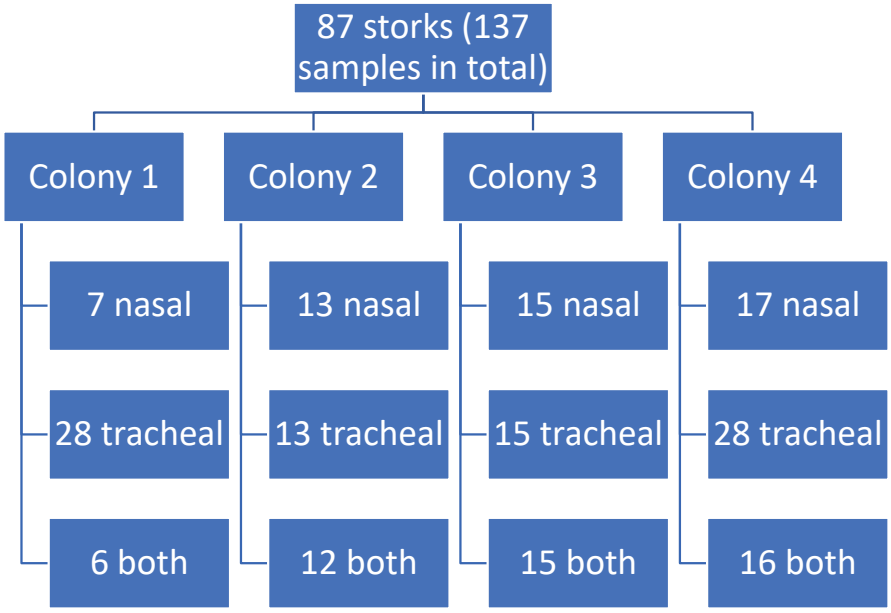


Figure 20. Number of nasal and tracheal samples collected from nestlings of parent storks.

The sampling was performed by expert researchers and veterinarians of the Instituto de Investigación en Recursos Cinegéticos (IREC) of the University of Castilla-La Mancha: Ciudad Real, Spain as follows:

a. Nestlings were extracted from the nest by gently wrapping them in a towel and lowering them to the floor by hand or in a large bag. Each bird was ringed with a metal and a PVC ring. The PVC ring is marked with a four-digit large alphanumeric code and allows identification of the individual stork from a distance using a telescope, for example during stork counts at landfills (visual recapture).
b. Nasal swabs were obtained using sterile cotton-tipped urethral swabs that were introduced into the left nasal opening on the beak of each individual, avoiding contact with the beak surface and external border of the cavity, and softly rotated twice to touch all nasal conchae surface. For tracheal swabs, sterile cotton-tipped swabs were used and briefly inserted into the trachea avoiding contact with the oral mucosa.
c. The swabs were transferred immediately to commercial Amies' transport medium tubes and stored at 4°C until arrival at the laboratory where they were frozen immediately at − 80°C until analysis. Nestlings were returned to the nest immediately after sampling.

2.1.3 Healthy dogs and dog owners

A total of 41 humans and 34 dogs from 27 households were prospectively studied to determine the nasal carriage of CoPS; the sampling was performed in La Rioja region (Northern Spain) between January to March 2022. Household density was classified into four, viz: (a) household with a dog and a human (b) household with >1 dog and a human, (c) household with 1 dog and >than 1 human, and (d) household with > than 1 dog and >than 1 human. The sampled humans and dogs did not have recent hospital stays prior to the study or received antibiotics (at least 3 months before sampling) and the humans had no professional contact with health institutions and did not work in microbiology laboratories. None of the participants had consultations or visits to hospitals in the last 3months before sample collection. Nasal samples were obtained using sterile swabs with conservation media (Amies BD Life sciences®, New Jersey, USA).

2.1.4 Healthy pigs and pig farmers

The study was performed in four pig farms (A-D) from the Aragon region (Spain) and were included 10 pigs from each farm (a total of 40 pigs) and 10 workers of the pig farms (2, 3, 2 and 3 humans in farms A, B, C and D, respectively). Farm A had a total of 6,000 piglets with an average weight of 20-22 kg and age of 9 weeks; Farm B had 15,000 piglets with an average weight of 9 kg and age range of 4–5 weeks; Farm C had 600 piglets with an average weight of 9 kg and age of 4–5 weeks; while Farm D had 400 piglets with an average weight of

10 kg and age of 6 weeks. All the pig farmers had no pets in their houses, except one from farm A who had a dog and cat. Nasal samples were collected (from January to March 2022) using sterile swabs with enrichment transport media (Amies).

The ethical committee of the Universities of Zaragoza and La Rioja (Spain) reviewed and approved all procedures which were carried out following all applicable national, and/or international guidelines for human experiments (as described in the revised Helsinki declaration). Concerning the ethical use of animals, this study adhered to specific directives: 2010/ 63/EU and Spanish laws 9/2003 and 32/2007, RD 178/2004 and RD 1201/2005. All procedures that involved nestling storks 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).

2.2 SAMPLES PROCESSING

The nasal and tracheal (in the case of nestling storks) samples were enriched in 6.5% supplemented brain heart infusion broth and incubated at 37^{o}C for 24 hours. Thereafter, the enriched nasal samples were diluted in sterile Milli-Q water as follows: 10ul of 1:1000000 and 1:1000 diluted broth were carefully dispensed onto blood agar (BA; Oxoid, ThermoFisher, UK) and mannitol salt agar (MSA; Condalab, Madrid, Spain), respectively. Fifty microliters of the crude enriched sample were also dispensed onto oxacillin screening agar base (ORSAB with 2 mg/L oxacillin; Oxoid, Hampshire, UK) and CHROMagar™ LIN plates (CHROMagar™ LIN; Paris, France). A sterile glass spreader was used to cover the entire area surface with the prepared sample starting from BA, MSA to ORSAB and CHROMagar™ LIN accordingly. The inoculated media plates were then incubated for bacterial recovery at 37^{o}C for 24 hours (BA and MSA) and 48 hours (for ORSAB and LIN).

2.3 IDENTIFICATION OF BACTERIAL STRAINS

After these incubations, between 4 and 12 different colonies were randomly selected based on colony morphology, colour, size and haemolysis per sample. All the selected colonies were passaged onto BHI agar at 37^{o}C for 24 hours to obtain pure colonies. Pure colonies were identified by the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Bruker Daltonics, Bremen, Germany) using the extended direct and extraction protocols as recommended by the manufacturer (Bruker Daltonics).

Briefly, from a pure culture grown for 24 hours at 37 ^{o}C in BHI agar medium, a small quantity of bacteria colony was transferred to the 96-well metal plate and allowed to dry at

room temperature. Afterwards, the wells were covered with 1 µL of alpha-cyano-4 hydroxy acid matrix cinnamic acid (HCCA; Bruker). In any case, if the strain could not be identified at the species level by this method, the extraction protocol as recommended by Bruker was done. Three pure colonies grown on BHI agar for 24 hours at 37 ºC were transferred to an Eppendorf tube and mixed with 300 µL of sterile Mili Q water and 900 µL of absolute ethanol. It was vortexed and centrifuged at 13,000 revolutions per minute (rpm) for 3 minutes. The supernatant was removed by decantation and centrifuged under the same conditions. The supernatant was removed and the precipitate was allowed to dry. Thereafter, it was resuspended in 15 µL of formic acid (70%) and 15 µL of acetonitrile. This suspension was vortexed and centrifuged at 13,000 rpm. for 3 minutes and 1 µL of the supernatant was deposited on the MALDI plate, allowed to dry at room temperature and covered with 1 µL of HCCA matrix. For the calibration of the spectrometer, the protein profile of the *E. coli* strain DH5 peptide was used (Bruker Daltonics).

2.4 ANTIMICROBIAL SUSCEPTIBILITY TESTS for staphylococci and enterococci

All identified staphylococci and enterococci were reisolated to obtain pure colonies which were later used to determine the antimicrobial susceptibility. The antimicrobial resistance (AMR) phenotype of staphylococci strains from healthy human and animal origins was determined against 12 antimicrobial agents by disc diffusion method using the following antibiotic discs (µg/disc): penicillin (1 unit for *S. aureus* and *S. lugdunensis*, and 10 units for CoNS), cefoxitin (30), oxacillin (1) to detect methicillin-resistant *S. pseudintermedius* or methicillin-resistant *S. coagulans*), mupirocin (200), gentamicin (10), tobramycin (10), clindamycin (2), erythromycin (15), ciprofloxacin (5), chloramphenicol (30), tetracycline (30), linezolid (10), and trimethoprim-sulfamethoxazole (1.25+23.75). In addition, the minimum inhibitory concentration (MIC) to linezolid and tedizolid was determined using E-test® (bioMérieux) (CLSI, 2022). For enterococci, the antimicrobial agents tested were as follows (µg/disk): penicillin (10), erythromycin (15), gentamicin (120), streptomycin (300), tetracycline (30), ciprofloxacin (5), chloramphenicol (30), linezolid (10), vancomycin (30) and teicoplanin (30).

For the disc difficusion test, a pure bacterial culture grown for 24 hours on BHI agar was suspended into sterile normal saline to produce a 0.5 McFarland concentration. Using a sterile swab stick, bacterial suspension was spread on Müeller-Hinton Agar (MHA, Condalab, Madrid, Spain) plates and antibiotic discs (Oxoid™) were carefully placed on the plates. After a 24-hour incubation at 37°C, the zones of inhibition (halo) produced against the bacteria by

the different antibiotics were measured. Moreover, detection of the inducible MLS_B (i.e., erythromycin-clindamycin-inducible) resistance phenotype in staphylococci was based on the double disk test (D-test) produced after placing the erythromycin and clindamycin discs at 15-20 mm apart (CLSI, 2019). If the strain presented the inducible MLS_B phenotype, a flattening of the inhibition halo appeared.

Once the antimicrobial resistance phenotype of all staphylococci and enterococci was determined, non-repetitive strains were selected for further characterization of their AMR genes.

2.4.1 Interpretation of Antimicrobial Resistance Phenotype

The interpretation and categorisation of results into resistant, intermediate resistance or susceptible was based on the breakpoints and recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), depending on the year the study was performed (2021, 2022 and 2023). For those antibiotics not considered in the EUCAST, the CLSI (Clinical and Laboratory Standards Institute) recommendations were used, depending on the year the study was performed (2021, 2022 and 2023) **(Tables 9 and 10).**

Table 9. Antibiotic discs concentration and breakpoints for staphylococci (EUCAST)

Antibiotic	Concentration (µg)	*Staphylococcus* spp	Susceptible (≥)	Intermediate	Resistant (≤)
PEN	1 U	*S. aureus/ S. lugdunensis*	26	-	25
PEN	10*	All staphylococci (except *S. aureus*/*S. lugdunensis*)	29	-	28
AMP	2	*S. saprophyticus*	18	-	17
FOX	30	*S. aureus* and other CoNS *S. epidermidis/S. lugdunensis*	22 27	- -	21 26
OXA	1	*S. pseudintermedius* S. *schleiferi/ S. coagulans*	20	-	19
ERY	15	All	21	18-20	17
CLI	2	All	22	-	21
TET	30	All	22	19-21	18
GEN	10	*S. aureus* CoNS	18 22	- -	17 21
TOB	10	*S. aureus* CoNS	18 20	- -	17 19
CIP	5	*S. aureus* CoNS	50 50	21-49 22-49	20 23
LZD	30*	All	21	-	20
SXT		All	17	-	13
CHL	30	All	18	-	17
MUP	200	All	30	18-29	17

PEN-: penicillin, FOX: cefoxitin, OXA: oxacillin, ERY: erythromycin, CLI: clindamycin, TET: tetracycline, CIP: ciprofloxacin, GEN: gentamicin, LZD: linezolid, SXT: trimethoprim-sulphametoxazole, TOB: tobramycin, CHL: chloramphenicol, MUP: mupirocin; * CLSI = breakpoint obtained from CLSI

Table 10. Antibiotic discs concentration and breakpoint for enterococci (EUCAST)

Antibiotic	Concentration (µg)	Susceptible (≥)	Intermediate	Resistant (≤)
PEN	10	15	-	14
ERY	15 *	23	14-22	13
TET	30*	19	15-18	14
GEN	120	8	-	7
CIP	5	15	-	14
LZD	30*	23	21-22	20
CLO	30*	18	13-17	12
VAN	30	12	-	11
TEC	30	16	-	15

PEN-: penicillin, ERY: erythromycin, TET: tetracycline, CIP: ciprofloxacin, GEN: gentamicin, LZD: linezolid, CLO: chloramphenicol, VAN: vancomycin, TEC: teicoplanin
* = breakpoint obtained from CLSI

2.5 DEFINITION OF KEY TERMS USED FOR CATEGORIZATION OF STRAINS

Based on the AMR profile of all the staphylococci and enterococci strains the following key terms were applied:

a. Non-repetitive or non-duplicated strains was defined as those of different samples or those from the same sample but with different antimicrobial resistance phenotypes.

b. Multi-drug resistance (MDR) was defined when an strain was resistant to ≥3 classes of the antimicrobial agents tested (Magiorakos *et al*., 2012).

c. Multiple antibiotic resistance (MAR) index was defined as the number of antibiotics to which an strain was resistant divided by the total number of antibiotics tested (Krumperman, 1983).

d. Within-host diversity (more than one species per host)

e. Intra-species AMR diversity (the same species with more than one AMR profile)

2.6 DNA EXTRACTION FOR STAPHYLOCOCCI AND ENTEROCOCCI

For staphylococci DNA extraction, strains were seeded on BHI agar and incubated for 24 hours at 37°C. An isolated colony was suspended in 45µL of sterile MiliQ water and 5µL of lysostaphin (1mg/mL) (Sigma) was added. The mixture was vortexed and incubated for 10 min at 37°C. Forty-five µL of sterile MiliQ water, 150µL of Tris–HCl (0.1M, pH 8) and 5µL of proteinase K (2mg/mL) (Sigma) were added. The final mixture was vortexed and incubated for 10 minutes at 60°C, then boiled for 5 minutes at 100°C. To separate and obtain the DNA (supernatant) from debris, the final mixture was centrifuged at 12,000 rpm for 3 minutes, and stored at −20°C.

The DNA extraction of enterococci strains of all origins was performed using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's

instructions. Briefly, pure and fresh isolated colonies were suspended in 1000 µL of sterile Milli-Q water, thoroughly mixed by vortex, and centrifuged at 12,000 rpm for 3 minutes. The supernatant was carefully eliminated and 200 µL of InstaGene matrix was added to the sediment, thoroughly mixed by vortexing and incubated in a bath for 20 minutes at 56 C. Later, reincubated for 8 minutes at 100°C and centrifuged at 12,000 rpm for 3 minutes. The DNA was stored at - 20°C.

2.6.1 Bacterial DNA quantification

After DNA extraction, their concentration and purity were measured using the spectrophotometer and the Nano-Drop™ software (Thermo Scientific™). The concentration was calculated from the absorbance (optical density) at 260 nm, while the purity was obtained by the A260/A280 absorbance ratio. In this sense, a pure bacterial DNA sample must have a ratio between 1.8 and 2.0.

2.7 POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) was used to amplify small fragments of DNA using a pair of specific primers based on the use of DNA polymerase. The entire reaction comprised of three phases (denaturation phase, annealing phase with the primers and elongation phase). The PCR technique was used for molecular typing and characterization of AMR genes, virulence factors and genetic lineages. The PCRs were carried out in the T3 and T3000 Thermocycler (Biometra). The reagents and amount used for the reactions to produce a final volume of 25µL were as follows: 0.5µL each for forward and reverse primer (Sigma Aldrich), 0.5µL of dNTP, 0.75µL of $MgCl_2$ (Bioline), 15.1µL of miliQ water, 0.15 µL of BioTaq DNA polymerase (Bioline), 0.15µL of NH4 buffer (Bioline) and 5µL of pure bacterial DNA. Positive and negative controls were included in all the reactions performed. The primers used for individual and multiplex PCRs are presented in **Tables 11 – 31.**

2.7.1 Agarose Gel Electrophoresis

For the visualization of the DNA fragments amplified by PCR, agarose gel electrophoresis was used. This makes it possible to separate DNA fragments based on their size by the action of an electric field. Agarose gel (D-1 agarose; Conda, Madrid, Spain) was prepared at a concentration of 1-2% in 1X Tris-borate-EDTA (TBE) buffer (5X TBE: 54 g/L Tris-Base; 27.5 g/L boric acid; 20 mL 0.5 M EDTA pH 8) and Midori Green (Nippon Genetic) was added at a final concentration of 0.6 µg/mL to allow visualization of the DNA fragments.

About 10 µL of the PCR product was loaded with 1 µL of loading buffer (sucrose 40%; bromophenol blue 0.25%; xylene cyanol 0.25%).

The electrophoresis conditions were 45 minutes at 96 V and 120 mA. The gel was visualized with the ChemiDoc™ ultraviolet light transilluminator (Bio-Rad) and photographed with Image Lab™ software (Biorad, USA).

2.8 IDENTIFICATION OF *S. pseudintermedius nuc* GENE

As the MALDI-TOF system is not always capable of distinguishing between species of the *Staphylococcus intermedius* group (SIG), a multiplex PCR that targets the *nuc* gene specific for the three species was analysed **(Table 11).**

Table 11. Genes, primers' sequences and PCR conditions utilized for the identification of the SIG species.

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
nuc Group A *S. delphini* (661 pb)	F: TGAAGGCATATTGTAGAACAA R: CGRTACTTTTCGTTAGGTCG	95 ºC 2 min 1 cycle 95 ºC 30 sec 56 ºC 35 sec 30 cycles 72 ºC 1 min 72 ºC 2 min 1 cycle	Sasaki et *al* (2010)
nuc Group B *S. delphini* (1135 pb)	F: GGAAGRTTCGTTTTTCCTAGA R: TATGCGATTCAAGAACTGA	95 ºC 2 min 1 cycle 95 ºC 30 sec 56 ºC 35 sec 30 cycles 72 ºC 1 min 72 ºC 2 min 1 cycle	Sasaki et *al* (2010)
nuc *S. pseudintermedius* (926 pb)	F: TRGGCAGTAGGATTCGTTAA R: CTTTTGTGCTYCMTTTTGG	95 ºC 2 min 1 cycle 95 ºC 30 sec 56 ºC 35 sec 30 cycles 72 ºC 1 min 72 ºC 2 min 1 cycle	Sasaki et *al* (2010)
nuc *S. intermedius* (430 pb)	F: CATGTCATATTATTGCGAATG R: AGGACCATCACCATTGACATATTGAAACC	95 ºC 2 min 1 cycle 95 ºC 30 sec 56 ºC 35 sec 30 cycles 72 ºC 1 min 72 ºC 2 min 1 cycle	Sasaki et *al* (2010)

2.9 MOLECULAR TYPING OF THE GENETIC LINEAGE OF *Staphylococcus aureus*

2.9.1 *spa*-type

All the non-repetitive *S. aureus* strains obtained from this PhD thesis were characterized to determine their lineage by *spa* typing. In this regard, the *spa* gene was amplified, and the amplicons of the polymorphic variable-number tandem repeat was sequenced using the primers and PCR conditions as presented in **Table 12**. The sequence obtained was analysed with the

program Ridom® Staph-Type (Ridom GmbH), which automatically assigns *spa*-type based on repetitions detected. In the case of detecting a new *spa*-type, the sequence was uploaded to the Ridom server *Spa*Server (https://www.spaserver.ridom.de) and, after being checked, assigned a new *spa* type.

Table 12. Primers' sequences and PCR conditions utilized for *spa* typing of *S. aureus*.

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
spa Hypervariable	F: AGACGATCCTTCGGTGAGC R: GCTTTTGCAATGTCATTTACTG	80 ºC 5 min 1 cycle 94 ºC 45 sec 50 ºC 45 sec 35 cycles 72 ºC 1.5 min 72 ºC 10 min 1 cycle	Harmsen *et al* (2003)

2.9.2 Specific PCR for *S. aureus*-CC398 lineage

The detection of *S. aureus* strains that belonged to the CC398 lineage was performed using a specific PCR that amplifies the *sau1-hsdS1* gene based on the primers and conditions as presented in **Table 12.**

Table 12. Primers' sequences and PCR conditions utilized for the amplification of the *sau1-hsdS1* gene

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
sau1-hsdS1 (296 bp)	F: AGGGTTTGAAGGCGAATGGG R: CAGTATAAAGAGGTGACATGACCCCT	95 ºC 12 min 1 cycle 95 ºC 30 sec 61 ºC 30 sec 35 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Stegger *et al* (2011)

2.10 MULTILOCUS SEQUENCING TYPING OF STAPHYLOCOCCI AND ENTEROCOCCI

To perform the multilocus sequence typing (MLST), the highly conserved seven housekeeping genes were amplified by PCR and the amplicons were sequenced. The sequences obtained from each gene were analyzed to assign an allele to each one of the genes. The sequence type (ST) of each strain was determined according to the allelic combination obtained from the seven genes. The comparison of the allelic profiles was made using the NCBI database for individual species (https://pubmlst.org).

2.10.1 *S. aureus*

To determine the ST of *S. aureus*, the amplification of *arcC* (carbamate kinase), *aroE* (shikimate dehydrogenase), *glpF* (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triosephosphate isomerase) and *yqiL* (acetyl coenzyme A acetyltransferase) genes was done, and their amplicons were sequenced based on the primers and PCR conditions used as presented on **Table 13.**

Table 13. Primers' sequences of the seven genes and PCR condition utilized for MLST typing of *S. aureus.*

Gene (amplicon size)	**Primers' oligonucleotide (5'⇒3')**	**PCR conditions**	**Reference**
arcC (456 pb)	F: TTGATTCACCAGCGCGTATTGTC R: AGGTATCTGCTTCAATCAGCG	95 ºC 5 min 1 cycle 95 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Enright *et al* (2000)
aroE (456 bp)	F: ATCGGAAATCCTATTTCACATTC R: GGTGTTGTATTAATAACGATATC	95 ºC 5 min 1 cycle 95 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Enright *et al* (2000)
glpF (465 pb)	F: CTAGGAACTGCAATCTTAATCC R: TGGTAAAATCGCATGTCCAATTC	95 ºC 5 min 1 cycle 95 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Enright *et al* (2000)
gmk (429 pb)	F: ATCGTTTTATCGGGACCATC R: TCATTAACTACAACGTAATCGTA	95 ºC 5 min 1 cycle 95 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Enright *et al* (2000)
pta (474 bp)	F: GTTAAAATCGTATTACCTGAAGG R: GACCCTTTTGTTGAAAAGCTTAA	95 ºC 5 min 1 cycle 95 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Enright *et al* (2000)
tpi (402 bp)	F: TCGTTCATTCTGAACGTCGTGAA R: TTTGCACCTTCTAACAATTGTAC	95 ºC 5 min 1 cycle 95 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Enright *et al* (2000)
yqiL (516 bp)	F: CAGCATACAGGACACCTATTGGC R: CGTTGAGGAATCGATACTGGAAC	95 ºC 5 min 1 cycle 95 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Enright *et al* (2000)

2.10.2 *S. pseudintermedius*

To determine the sequence type of *S. pseudintermedius*, the amplification of a*ck* (acetate kinase), *cpn60* (chaperonin 60), *fdh* (formate dehydrogenase), *pta* (phosphate acetyltransferase), *purA* (adenylsuccinate synthetase), *sar* (sodium sulfate symport) and *tuf* (elongation factor

Tu) genes was done, and their amplicons were sequenced based on the primers and PCR conditions used as presented on **Table 14.**

Table 14. Primers' sequences of the seven genes and PCR condition utilized for MLST typing of *S. pseudintermedius*

Gene (amplicon size)	**Primers' oligonucleotide (5'⇒3')**	**PCR conditions**	**Reference**
ack (680 pb)	F: CACCACTTCACAACCCAGCAAACT R: AACCTTCTAATACACGCGCACGCA	95 ºC 1.5 min 1 cycle 52 ºC 30 sec 72 ºC 1 min 94 ºC 1 min 35 cycles 52 ºC 30 sec 72 ºC 5 min 1 cycle	Solyman *et al* (2013)
fdh (408 bp)	F: TGCGATAACAGGATGTGCTT R: CTTCTCATGATTCACCGGC	95 ºC 1.5 min 1 cycle 52 ºC 30 sec 72 ºC 1 min 94 ºC 1 min 35 cycles 52 ºC 30 sec 72 ºC 5 min 1 cycle	Solyman *et al* (2013)
purA (490 pb)	F: GATTACTTCCAAGGTATGTTT R: TCGATAGAGTTAATAGATAAGTC	95 ºC 1.5 min 1 cycle 52 ºC 30 sec 72 ºC 1 min 94 ºC 1 min 35 cycles 52 ºC 30 sec 72 ºC 5 min 1 cycle	Solyman *et al* (2013)
sar (521 pb)	F: GGATTTAGTCCAGTTCAAAATTT R: GAACCATTCGCCCCATGAA	95 ºC 1.5 min 1 cycle 52 ºC 30 sec 72 ºC 1 min 94 ºC 1 min 35 cycles 52 ºC 30 sec 72 ºC 5 min 1 cycle	Solyman *et al* (2013)
pta (470 bp)	F: GTGCGTATCGTATTACCAGAAGG R: GCAGAACCTTTTGTTGAGAAGC	95 ºC 2 min 1 cycle 95 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Bannoehr *et al* (2007)
cpn60 (552 bp)	F: GCGACTGTACTTGCACAAGCA R: AACTGCAACCGCTGTAAATG	95 ºC 2 min 1 cycle 95 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Bannoehr *et al* (2007)
tuf (550 bp)	F: CAATGCCACAAACTCG R: GCTTCAGCGTAGTCTA	95 ºC 2 min 1 cycle 95 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Bannoehr *et al* (2007)

2.10.3 *S. epidermidis*

To determine the sequence type of *S. epidermidis*, the amplification of *arcC* (carbamate kinase), *aroE* (shikimate dehydrogenase), *gtr* (ABC transporter), *mutS* (pDNA error repair protein), *pyrR* (pyrimidine operon regulatory protein), *tpi* (triosephosphate isomerase), and *yqiL* (acetyl coenzyme A acetyltransferase) genes was done, and their amplicons were sequenced based on the primers and PCR conditions used as presented on **Table 15.**

Table 15. Primers' sequences of the seven genes and PCR conditions utilized for MLST typing of *S. epidermidis*

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
arcC (465 pb)	F: TGTGATGAGCACGCTACCGTTAG R: TCCAAGTAAACCCATCGGTCTG	95 ºC 3 min 1 cycle 95 ºC 30 sec 50 ºC 1 min 34 cycles 72 ºC 1 min 72 ºC 8 min 1 cycle	Thomas *et al* (2007)
aroE (420 bp)	F: CATTGGATTACCTCTTTGTTCAGC R: CAAGCGAAATCTGTTGGGG	95 ºC 3 min 1 cycle 95 ºC 30 sec 50 ºC 1 min 34 cycles 72 ºC 1 min 72 ºC 8 min 1 cycle	Thomas *et al* (2007)
gtr (438 pb)	F: CAGCCAATTCTTTTATGACTTTT R: GTGATTAAAGGTATTGATTTGAAT	95 ºC 3 min 1 cycle 95 ºC 30 sec 50 ºC 1 min 34 cycles 72 ºC 1 min 72 ºC 8 min 1 cycle	Thomas *et al* (2007)
mutS (412 pb)	F: GATATAAGAATAAGGGTTGTGAA R: GTAATCGTCTCAGTTATCATGTT	95 ºC 3 min 1 cycle 95 ºC 30 sec 50 ºC 1 min 34 cycles 72 ºC 1 min 72 ºC 8 min 1 cycle	Thomas *et al* (2007)
pyrR (428 bp)	F: GTTACTAATACTTTTGCTGTGTTT R: GTAGAATGTAAAGAGACTAAAATGAA	95 ºC 3 min 1 cycle 95 ºC 30 sec 50 ºC 1 min 34 cycles 72 ºC 1 min 72 ºC 8 min 1 cycle	Thomas *et al* (2007)
tpi (424 bp)	F: ATCCAATTAGACGCTTTAGTAAC R: TTAATGATGCGCCACCTACA	95 ºC 3 min 1 cycle 95 ºC 30 sec 50 ºC 1 min 34 cycles 72 ºC 1 min 72 ºC 8 min 1 cycle	Thomas *et al* (2007)
yqiL (416 bp)	F: CACGCATAGTATTAGCTGAAG R: CTAATGCCTTCATCTTGAGAAATAA	95 ºC 3 min 1 cycle 95 ºC 30 sec 50 ºC 1 min 34 cycles 72 ºC 1 min 72 ºC 8 min 1 cycle	Thomas *et al* (2007)

2.10.4 *E. faecalis*

To determine the ST of *E. faecalis*, the amplification of *aroE* (shikimate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *gki* (glucokinase), gyd (glyceraldehyde-3-phosphate dehydrogenase), *pstS* (ATP-phosphate binding cassette transporter), *xpt* (xanthine phosphoribosyltransferase) and *yqiL* (acetyl coenzyme A acetyltransferase genes was done, and their amplicons were sequenced based on the primers and PCR conditions used as presented on **Table 16.**

Table 16. Primers' sequences of the seven genes and PCR conditions utilized for MLST typing of *E. faecalis*

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
aroE (459 pb)	F: TGGAAAACTTTACGGAGACAGC R: GTCCTGTCCATTGTTCAAAAGC	94 ºC 5 min 1 cycle 94 ºC 30 sec 52 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Ruiz-Garbajosa *et al* (2006)
gdh (530 bp)	F: GGCGCACTAAAAGATATGGT R: CCAAGATTGGGCAACTTCGTCCCA	94 ºC 5 min 1 cycle 94 ºC 30 sec 52 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Ruiz-Garbajosa *et al* (2006)
gki (438 pb)	F: GATTTTGTGGGAATTGGTATGG R: ACCATTAAAGCAAAATGATCGC	94 ºC 5 min 1 cycle 94 ºC 30 sec 52 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Ruiz-Garbajosa *et al* (2006)
mutS (395 pb)	F: CAAACTGCTTAGCTCCAAGGC R: CATTTCGTTGTCATACCAAGC	94 ºC 5 min 1 cycle 94 ºC 30 sec 52 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Ruiz-Garbajosa *et al* (2006)
pstS (428 bp)	F: CGGAACAGGACTTTCGC R: ATTTACATCACGTTCTACTTGC	94 ºC 5 min 1 cycle 94 ºC 30 sec 52 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Ruiz-Garbajosa *et al* (2006)
xpt (424 bp)	F: AAAATGATGGCCGTGTATTAGG R: AACGTCACCGTTCCTTCACTTA	94 ºC 5 min 1 cycle 94 ºC 30 sec 52 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Ruiz-Garbajosa *et al* (2006)
yqiL (436 bp)	F: CAGCTTAAGTCAAGTAAGTGCCG R: GAATATCCCTTCTGCTTGTGCT	94 ºC 5 min 1 cycle 94 ºC 30 sec 52 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Ruiz-Garbajosa *et al* (2006)

2.10.5 *E. faecium*

In the case of *E. faecium*, the MLST was performed by the amplification of *adk* (adenylate kinase), *atpA* (ATP synthase), *ddl* (d-alanine-d-alanine ligase), *gdh* (glucose-6-phosphate dehydrogenase), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *pstS* (cassette binding transporter of ATP-phosphate) and *purK* (phosphoribosyl-aminoimidazole carboxylase subunit ATPase) genes . The amplicons were sequenced based on the primers and PCR conditions used as presented on **Table 17.**

Table 17. Primers' sequences of the seven genes and PCR condition utilized for MLST typing of *E. faecium*

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
adk (437 pb)	F: TATGAACCTCATTTTAATGGG R: GTTGACTGCCAAACGATTTT	95 ºC 3 min 1 cycle 93 ºC 30 sec 50 ºC 30 sec 30 cycles 72 ºC 30 sec 72 ºC 5 min 1 cycle	Homan *et al* (2002)
atpA (556 bp)	F: CGGTTCATACGGAATGGCACA R: AAGTTCACGATAAGCCACGG	95 ºC 3 min 1 cycle 93 ºC 30 sec 50 ºC 30 sec 30 cycles 72 ºC 30 sec 72 ºC 5 min 1 cycle	Homan *et al* (2002)
ddl (465 pb)	F: GAGACATTGAATATGCCTTAT R: AAAAAGAAATCGCACCG	95 ºC 3 min 1 cycle 93 ºC 30 sec 50 ºC 30 sec 30 cycles 72 ºC 30 sec 72 ºC 5 min 1 cycle	Homan *et al* (2002)
gdh (395 pb)	F: GGCGCACTAAAAGATATGGT R: CCAAGATTGGGCAACTTCGTCCCA	95 ºC 3 min 1 cycle 93 ºC 30 sec 50 ºC 30 sec 30 cycles 72 ºC 30 sec 72 ºC 5 min 1 cycle	Homan *et al* (2002)
gyd (395 bp)	F: CAAACTGCTTAGCTCCAAGGC R: CATTTCGTTGTCATACCAAGC	95 ºC 3 min 1 cycle 93 ºC 30 sec 50 ºC 30 sec 30 cycles 72 ºC 30 sec 72 ºC 5 min 1 cycle	Homan *et al* (2002)
pstS (586 bp)	F: TTGAGCCAAGTCGAAGCTGGA R: CGTGATCACGTTCTACTTCC	95 ºC 3 min 1 cycle 93 ºC 30 sec 50 ºC 30 sec 30 cycles 72 ºC 30 sec 72 ºC 5 min 1 cycle	Homan *et al* (2002)
purK (492 bp)	F: GCAGATTGGCACATTGAAAGT R: TACATAAATCCCGCCTGTTTY	95 ºC 3 min 1 cycle 93 ºC 30 sec 50 ºC 30 sec 30 cycles 72 ºC 30 sec 72 ºC 5 min 1 cycle	Homan *et al* (2002)

2.11 SCC*mec* TYPING OF METHICILLIN-RESISTANT STAPHYLOCOCCI

The SCC*mec* types of all the non-repetitive MRSA and MRCoNS were determined by multiplex PCRs based on the primers and conditions in **Table 18.**

Table 18. Primers' sequences of the genes and PCR conditions utilized for SCC*mec* typing based on Zhang *et al* (2015)

Gene (amplicon size)	**Primers' oligonucleotide (5'⇒3')**	**PCR conditions**	**Reference**
SCC*mec* type I (613 pb)	F: GCTTTAAAGAGTGTCGTTACAGG R: GTTCTCTCATAGTATGACGTCC	94 ºC 5 min 1 cycle 94 ºC 45 sec 65 ºC 45 sec 10 cycles 72 ºC 1.5 min 72 ºC 7 min 1 cycle 94 ºC 45 sec 55 ºC 45 sec 25 cycles 72 ºC 1.5 min 72 ºC 10 min 1 cycle	Zhang *et al* (2015)
SCC*mec* type II (398 bp)	F: CGTTGAAGATGATGAAGCG R: CGAAATCAATGGTTAATGGACC	94 ºC 5 min 1 cycle 94 ºC 45 sec 65 ºC 45 sec 10 cycles 72 ºC 1.5 min 72 ºC 7 min 1 cycle 94 ºC 45 sec 55 ºC 45 sec 25 cycles 72 ºC 1.5 min 72 ºC 10 min 1 cycle	Zhang *et al* (2015)
SCC*mec* type III (280 pb)	F: CCATATTGTGTACGATGCG R: CCTTAGTTGTCGTAACAGATCG	94 ºC 5 min 1 cycle 94 ºC 45 sec 65 ºC 45 sec 10 cycles 72 ºC 1.5 min 72 ºC 7 min 1 cycle 94 ºC 45 sec 55 ºC 45 sec 25 cycles 72 ºC 1.5 min 72 ºC 10 min 1 cycle	Zhang *et al* (2015)
SCC*mec* type IVa (776 pb)	F: GCCTTATTCGAAGAAACCG R: CTACTCTTCTGAAAAGCGTCG	94 ºC 5 min 1 cycle 94 ºC 45 sec 65 ºC 45 sec 10 cycles 72 ºC 1.5 min 72 ºC 7 min 1 cycle 94 ºC 45 sec 55 ºC 45 sec 25 cycles 72 ºC 1.5 min 72 ºC 10 min 1 cycle	Zhang *et al* (2015)
SCC*mec* type IVb (493 bp)	F: TCTGGAATTACTTCAGCTGC R: AAACAATATTGCTCTCCCTC	94 ºC 5 min 1 cycle 94 ºC 45 sec 65 ºC 45 sec 10 cycles 72 ºC 1.5 min 72 ºC 7 min 1 cycle 94 ºC 45 sec 55 ºC 45 sec 25 cycles 72 ºC 1.5 min 72 ºC 10 min 1 cycle	Zhang *et al* (2015)

Table 18. Continuation

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
SCC*mec* type IVc (200 bp)	F: ACAATATTTGTATTATCGGAGAGC R: TTGGTATGAGGTATTGCTGG	94 ºC 5 min 1 cycle 94 ºC 45 sec 65 ºC 45 sec 10 cycles 72 ºC 1.5 min 72 ºC 7 min 1 cycle 94 ºC 45 sec 55 ºC 45 sec 25 cycles 72 ºC 1.5 min 72 ºC 10 min 1 cycle	Zhang *et al* (2015)
SCC*mec* type IVd (881 bp)	F: CTCAAAATACGGACCCCAATACA R: TGCTCCAGTAATTGCTAAAG	94 ºC 5 min 1 cycle 94 ºC 45 sec 65 ºC 45 sec 10 cycles 72 ºC 1.5 min 72 ºC 7 min 1 cycle 94 ºC 45 sec 55 ºC 45 sec 25 cycles 72 ºC 1.5 min 72 ºC 10 min 1 cycle	Zhang *et al* (2015)
SCC*mec* type V (325 bp)	F: GAACATTGTTACTTAAATGAGCG R: TGAAAGTTGTACCCTTGACACC	94 ºC 5 min 1 cycle 94 ºC 45 sec 65 ºC 45 sec 10 cycles 72 ºC 1.5 min 72 ºC 7 min 1 cycle 94 ºC 45 sec 55 ºC 45 sec 25 cycles 72 ºC 1.5 min 72 ºC 10 min 1 cycle	Zhang *et al* (2015)

2.12 CHARACTERIZATION OF MECHANISMS OF ANTIMICROBIAL RESISTANCE

2.12.1 Resistance by beta-lactamase

In all the non-repetitive methicillin-resistant staphylococci strains the presence of *mecA*, *mecB* and *mecC* genes was determined. Also, in the strains that presented resistance to penicillin, the presence of the *blaZ* gene was determined. For *mecC*-carrying staphylococci, the presence of *blaZ* associated with *SCCmec* type XI (i.e., *blaZ*-SCC*mec* XI) was investigated. Moreover, the presence of bla_{ARL} gene that is specific to penicillin-resistant- *S. arlettae* was determined. The primers and PCR conditions utilized for these experiments are presented in **Table 19.**

Table 19. Genes, primers' sequences and PCR conditions utilized for the detection of beta-lactam resistance mechanisms in staphylococci

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
mecA (527 pb)	F: GGGATCATAGCGTCATTATTC R: AACGATTGTGACACGATAGCC	94 ºC 5 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Paulsen et *al* (2003)
mecB (456 pb)	F: TTAACATATACACCCGCTTG R: TAAAGTTCATTAGGCACCTCC	95 ºC 5 min 1 cycle 95 ºC 30 sec 57 ºC 30 sec 35 cycles 72 ºC 2.5 min 72 ºC 7 min 1 cycle	Becker et *al* (2018)
mecC (304 pb)	F: GCTCCTAATGCTAATGCA R: TAAGCAATAATGACTACC	95 ºC 2 min 1 cycle 95 ºC 30 sec 56 ºC 35 sec 30 cycles 72 ºC 1 min 72 ºC 2 min 1 cycle	Cuny et *al* (2011)
blaZ (772 bp)	F: CAGTTCACATGCCAAAGAG R: TACACTCTTGGCGGTTTC	94 ºC 3 min 1 cycle 94 ºC 1 min 50 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 2 min 1 cycle	Schnellmann *et al* (2006)
bla_{ARL} (378 bp)	F: CTATCTTTGTCTTACTCTGTGT R: GCMTGACGTGCTGCTTTGTGC	94 ºC 7 min 1 cycle 94 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Andreis *et al* (2017)
blaZ-SCC*mec* XI (809 bp)	F: AGTCGTGTTAGCGTTGATATTAA R: CAATTTCAGCAACCTCACTTACTA	94 ºC 5 min 1 cycle 94 ºC 1 min 58 ºC 1 min 30 cycles 72 ºC 2 min 72 ºC 5 min 1 cycle	García-Álvarez *et al* (2011)

2.12.2 Resistance to tetracyclines

The presence of *tet*(K), *tet*(L), *tet(*M) and *tet*(O) genes was investigated on staphylococci and enterococci that were resistant to tetracycline **(Table 20).**

Table 20. Genes, primers' sequences and PCR conditions utilized for the detection of genes that mediate resistance to tetracyclines

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
tet(K) (697 pb)	F: TTAGGTGAAGGGTTAGGTCC R: GCAAACTCATTCCAGAAGCA	94 ºC 1 min 1 cycle 94 ºC 1 min 55 ºC 2 min 30 cycles 72 ºC 2 min 72 ºC 10 min 1 cycle	Aarestrup *et al* (2000)

Table 20. Continuation

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
tet(L) (576 pb)	F: GTTAAATAGTGTTCTTGGAG R: CTAAGATATGGCTCTAACAA	94 ºC 1 min 1 cycle 94 ºC 1 min 50 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Aarestrup *et al* (2000)
tet(M) (546 pb)	F: CATTTGGTCTTATTGGATCG R: ATTACACTTCCGATTTCGG	94 ºC 1 min 1 cycle 94 ºC 1 min 55 ºC 2 min 30 cycles 72 ºC 2 min 72 ºC 10 min 1 cycle	Aarestrup *et al* (2000)
tet(O) (615 pb)	F: GATGGCATACAGGCACAGAC R: CAATATCACCAGAGCAGGCT	94 ºC 1 min 1 cycle 94 ºC 1 min 50 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Aarestrup *et al* (2000)

2.12.3 Flouroquinolone resistance

To study the mechanism of resistance to fluoroquinolones, amino acid changes in specific genes of the **quinolone resistance-determining *regions*** (***QRDRs***) were analysed. Specifically, amino acid changes in GyrA and GrlA of *S. aureus* and *S. epidermidis* were studied (Table 21). In this regard, the *gyrA* and *grlA* genes were amplified and sequenced, and the sequences were compared with the reference sequences: *S. aureus* NCTC 8325 (GenBank accession number: CP000253) and *S. epidermidis* ATCC® 12228 (GenBank accession number: CP022247).

Table 21. Genes, primers' sequences and PCR conditions utilized for the detection of amino acid changes in *gyrA* and *grlA* genes in *S. aureus* and *S. epidermidis*

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
gyrA *S. aureus* (222 pb)	F: AATGAACAAGGTATGACACC R: TACGCGCTTCAGTATAACGC	94 ºC 10 min 1 cycle 94 ºC 20 sec 55 ºC 20 sec 25 cycles 72 ºC 50 sec 72 ºC 5 min 1 cycle	Schmitz (1998)
grlA *S. aureus* (559 pb)	F: ACTTGAAGATGTTTTAGGTGAT R: TTAGGAAATCTTGATGGCAA	94 ºC 10 min 1 cycle 94 ºC 20 sec 55 ºC 20 sec 25 cycles 72 ºC 50 sec 72 ºC 5 min 1 cycle	Schmitz (1998)

Table 21. Continuation

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
gyrA *S. epidermidis* (284 pb)	F: ATGCGTGAATCATTCTTAGACTATGC R: GAGCCAAAGTTACCTTGACC	94 ºC 3 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Yamada *et al* (2008)
grlA *S. epidermidis* (197 pb)	F: TCGCAATGTATTCAAGTGGG R: ATCGTTATCGATACTACCATT	94 ºC 3 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Yamada *et al* (2008)

2.12.4 Chloramphenicol resistance

The presence of the genes *fexA, fexB, catA,* cat_{PC194}, cat_{PC221} and cat_{PC223} was investigated on all staphylococci and enterococci strains that presented resistance to chloramphenicol **(Table 22).**

Table 22. Genes, primers' sequences and PCR conditions utilized for the detection of genes that mediate resistance to chloramphenicol in staphylococci and enterococci

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
fexA (1272 pb)	F: GTACTTGTAGGTGCAATTACGGCTGA R: CGCATCTGAGTAGGACATAGCGTC	94 ºC 1 min 1 cycle 94 ºC 1 min 48ºC 2 min 34 cycles 72 ºC 3 min 72 ºC 7 min 1 cycle	Kehrenberg and Schwarz (2005)
fexB (816 pb)	F: TTCCCACTATTGGTGAAAGGAT R: GCAATTCCCTTTTATGGACGTT	94 ºC 7 min 1 cycle 94 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	H. Liu et al (2012)
catA (505 pb)	F: GGATATGAAATTTATCCCTC R: CAATCATCTACCCTATGAAT	94 ºC 5 min 1 cycle 94 ºC 1 sec 55 ºC 1 sec 30 cycles 72 ºC 2.5 min 72 ºC 7 min 1 cycle	Aarestrup *et al* (2000)
cat_{PC194} (570 pb)	F: CGACTTTTAGTATAACCACAGA R: GCCAGTCATTAGGCCTAT	94 ºC 3 min 1 cycle 94 ºC 1 min 50 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Schnellmann *et al* (2006)
cat_{PC221} (434 pb)	F: ATTTATGCAATTATGGAAGTTG R: TGAAGCATGGTAACCATCAC	94 ºC 3 min 1 cycle 94 ºC 1 min 50 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Schnellmann *et al* (2006)

Table 22. Continuation

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
cat_{PC221} (434 pb)	F: ATTTATGCAATTATGGAAGTTG R: TGAAGCATGGTAACCATCAC	94 ºC 3 min 1 cycle 94 ºC 1 min 50 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Schnellmann *et al* (2006)

2.12.5 Linezolid resistance

For the chloramphenicol-resistant strains, the presence of *optrA, poxtA, cfr* and *cfr(D)* genes was investigated whether they were linezolid-resistant or not. However, for strains that were phenotypically resistant to linezolid, the presence of mutations in domain V of the 23S rRNA and amino acid changes in the genes encoding ribosomal proteins L3 (*rplC*), L4 (*rplD*) and L22 (*rplV*) were tested by PCR and sequencing **(Table 23).** The sequences obtained were compared with the reference sequences: *S. aureus* NCTC 8325 (GenBank accession number: CP000253), *S. epidermidis* ATCC® 12228 (GenBank CP022247), *E. faecalis* ATCC® 29212 (GenBank accession number: CP008816) and *E. faecium* DO (GenBank accession number: CP003583) **(Table 23).**

Table 23. Genes, primers' sequences and PCR conditions utilized for the detection of genes and mutations associated with linezolid resistance in staphylococci and enterococci

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
cfr (746 pb)	F: TGAAGTATAAAGCAGGTTGGGAGTCA R: ACCATATAATTGACCACAAGCAGC	94 ºC 3 min 1 cycle 94 ºC 1 min 56 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Kehrenberg and Schwarz (2006)
cfrB (293 pb)	F: TGAGCATATACGAGTAACCTCAAGA R: CGCAAGCAGCGTCTATATCA	94 ºC 5 min 1 cycle 94 ºC 30 min 58 ºC 30 min 35 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Lee et al (2017)
cfrD (595 pb)	F: AGAAGTCGCAACAAGTGAGGA R: GCAACTGCATGAGTCAAAGAA	94 ºC 7 min 1 cycle 94 ºC 1 sec 60 ºC 1 sec 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Ruiz-Ripa *et al* (2020a)
optrA (1395 pb)	F: AGGTGGTCAGCGAACTAA R: ATCAACTGTTCCCATTCA	94 ºC 7 min 1 cycle 94 ºC 1 min 48 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Wang *et al* (2015)

Table 23. Continuation

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
poxtA (791 pb)	F: TCAATGCAGAGCAGGAAGCA R: GGTGGATTTACCGACACCGT	94 ºC 7 min 1 cycle 94 ºC 1 min 60 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Ruiz-Ripa *et al* (2020a)
23S rRNA (420 pb)	F: GCGGTCGCCTCCTAAAAG R: ATCCCGGTCCTCTCGTACT	94 ºC 5 min 1 cycle 94 ºC 1 min 54 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Dibo *et al* (2004)
rplC *Staphylococcus* spp. (799 pb)	F: ACCCTGATTTAGTTCCGTCTA R: GTTGACGCTTTAATGGGCTTA	94 ºC 5 min 1 cycle 94 ºC 1 min 52 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Miller *et al* (2008)
rplD *Staphylococcus* spp. (1080 pb)	F: TCGCTTACCTCCTTAATG R: GGTGGAAACACTGTAACTG	94 ºC 5 min 1 cycle 94 ºC 1 min 54 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Miller *et al* (2008)
rplV *Staphylococcus* spp. (468 pb)	F: CAACACGAAGTCCGATTGGA R: GCAGACGACAAGAAAACAAG	94 ºC 5 min 1 cycle 94 ºC 1 min 52 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Mendes *et al* (2010)
rplC *Enterococcus* spp. (618 pb)	F: ATGACCAAAGGAATCTTAGGG R: CACAGCTGATTTGATWGTGATT	94 ºC 7 min 1 cycle 94 ºC 1 min 55 ºC 1 min 35 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Diaz *et al* (2012)
rplD *Enterococcus* spp. (617 pb)	F: GCCGAATGTAGCATTATTCAA R: CAAGCACCTCCTCAATTTGAGT	94 ºC 7 min 1 cycle 94 ºC 1 min 55 ºC 1 min 35 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Diaz *et al* (2012)
rplV (L22) *E. fecalis* (476 pb)	F: GCCACGTTGCTGACGATAA R: ACCCACTGATTGTCCCTCCT	94 ºC 3 min 1 cycle 94 ºC 45 sec 54 ºC 45 sec 35 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Lee *et al* (2017)
rplV *E. faecium* (486 pb)	F: GGACATGCTGCTGACGATA R: ACCATTTAGCATCCCAGTCG	94 ºC 3 min 1 cycle 94 ºC 45 sec 54 ºC 45 sec 35 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Lee *et al* (2017)

2.12.6 Vancomycin resistance

The presence of the *vanA* and *vanB* genes was investigated in strains that presented vancomycin resistance or intermediate resistance to vancomycin **(Table 24).**

Table 24. Genes, primers' sequences and PCR conditions utilized for the detection of genes that mediate vancomycin resistance

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
vanA (399 pb)	F: ATGGCAAGTCAGGTGAAGATGG R: TCCACCTCGCCAACAACTAACG	94 ºC 2 min 1 cycle 94 ºC 30 sec 50 ºC 30 sec 35 cycles 72 ºC 1 sec 72 ºC 10 min 1 cycle	Woodford *et al* (1993)
vanB (484 pb)	F: CAAAGCTCCGCAGCTTGCATG R: TGCATCCAAGCACCCGATATAC	94 ºC 3 min 1 cycle 94 ºC 30 sec 55 ºC 2 min 35 cycles 72 ºC 2 min 72 ºC 6 min 1 cycle	Dahl *et al* (1999)

2.12.7 Mupirocin resistance

For staphylococcal strains that presented high-level resistance to mupirocin, the *mupA* gene was investigated **(Table 25).**

Table 25. Genes, primers' sequences and PCR conditions utilized for the amplification of genes that mediate mupirocin resistance

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
mupA (419 pb)	F: CCCATGGCTTACCAGTTGA R: CCATGGAGCACTATCCGAA	94 ºC 5 min 1 cycle 94 ºC 3 min 60 ºC 45 sec 35 cycles 72 ºC 1 sec 72 ºC 2 min 1 cycle	Udo *et al* (2003)

2.12.8 Sulfamethoxazole-trimethoprim resistance

For staphylococcal strains that presented resistance to sulfamethoxazole-trimethoprim, the genes *dfrA, dfrD, dfrG* and *dfrK* were analyzed **(Table 26).**

Table 26. Genes, primers' sequences and PCR conditions utilized for the amplification of genes that confer resistance to sulfamethoxazole-trimethoprim

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
dfrA (374 pb)	F: CCTTGGCACTTACCAAATG R: CTGAAGATTCGACTTCCC	94 ºC 3 min 1 cycle 94 ºC 1 min 50 ºC 1 min 25 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Schnellmann et al (2006)
dfrD (582 pb)	F: TTCTTTAATTGTTGCGATGG R: TTAACGAATTCTCTCATATATATG	94 ºC 3 min 1 cycle 94 ºC 1 min 50 ºC 1 min 25 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Schnellmann et al (2006)
dfrG (323 pb)	F: TCGGAAGAGCCTTACCTGACAGAA R: CCCTTTTTGGGCAAATACCTCATTCCA	94 ºC 3 min 1 cycle 94 ºC 1 min 58 ºC 1 min 25 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Gómez-Sanz *et al* (2010)
dfrK (423 bp)	F: GAGAATCCCAGAGGATTGGG R: CAAGAAGCTTTTCGCTCATAAA	94 ºC 3 min 1 cycle 94 ºC 30 sec 56 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Gómez-Sanz *et al* (2010)

2.12.9 Resistance to Macrolides, Lincosamides and/or Streptogramins

All staphylococcal strains that showed resistance to erythromycin, clindamycin or both (either constitutive or clindamycin inducible) were investigated for the *ermA*, *ermB*, *ermC*, *ermT*, *erm45*, *msrA*, *mphC*, *lnuA*, *lnuB*, and/or *vgaA* genes based on the primers and conditions presented on **Table 27**. Moreover, all CoNS strains that presented clindamycin resistance were investigated for *sal*A gene (that confers intrinsic resistance to *S. sciuri*). In this regards, all *S. sciuri* strains were screened for the *salA* gene, whether they showed clindamycin resistance or not. Nevertheless, all enterococcal strains that presented erythromycin resistance were investigated for *ermA*, *ermB*, *ermC*, *ermT* based on the primers and conditions presented on **Table 27.**

Table 27. Genes, primers' sequences and PCR conditions utilized for the amplification of genes that mediate resistance to macrolides, lincosamides and/or streptogramin

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
*erm*A (645 pb)	F: TCTAAAAAGCATGTAAAAGAA R: CTTCGATAGTTTATTAATATTAG	93 ºC 3 min 1 cycle 93 ºC 1 min 52 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Sutcliffe *et al* (1996)

Table 27. Continuation

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
*erm*B (639 pb)	F: GAAAAGTACTCAACCAAATA R: AGTAACGGTACTTAAATTGTTTA	93 ºC 3 min 1 cycle 93 ºC 1 min 52 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Sutcliffe *et al* (1996)
*erm*C (642 pb)	F: TCAAAACATAATATAGATAAA R: GCTAATATTGTTTAAATCGTCAAT	93 ºC 3 min 1 cycle 93 ºC 1 min 52 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Sutcliffe *et al* (1996)
ermT (200 pb)	F: CCGCCATTGAAATAGATCCT R: TTCTGTAGCTGTGCTTTCAAAAA	94 ºC 3 min 1 cycle 94 ºC 1 min 55 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Gómez-Sanz *et al* (2010)
erm43 (609 pb)	F: TACAGCAGATGATAACATTG R: GTTGTTTCGATATTTTATTTAAG	94 ºC 3 min 1 cycle 94 ºC 30 min 50 ºC 30 sec 30 cycles 72 ºC 40 sec 72 ºC 5 min 1 cycle	Schwendener and Perreten (2012)
msrA (399 pb)	F: GCAAATGGTGTAGGTAAGACAACT R: ATCATGTGATGTAAACAAAAT	95 ºC 3 min 1 cycle 93 ºC 30 min 55 ºC 2 min 30 cycles 72 ºC 1.5 min 72 ºC 5 min 1 cycle	Wondrack *et al* (1996)
mphC (900 pb)	F: ATGACTCGACATAATGAAAT R: CTACTCTTTCATACCTAACTC	94 ºC 3 min 1 cycle 94 ºC 1 min 45 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Schnellmann *et al* (2006)
lnuA (323 pb)	F: GGTGGCTGGGGGGTAGATGTATTAACTGG R: GCTTCTTTTGAAATACATGGTATTTTTCGATC	94 ºC 2 min 1 cycle 94 ºC 30 sec 57 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Lina *et al* (1999a)
lnuB (944 pb)	F: CCTACCTATTGTTTGTGGAA R: ATAACGTTACTCTCCTATTC	94 ºC 5 min 1 cycle 94 ºC 45 sec 54 ºC 45 sec 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Bozdogan *et al* (1999)
vgaA (1264 pb)	F: AGTGGTGGTGAAGTAACACG R: GGTTCAATACTCAATCGACTGAG	94 ºC 3 min 1 cycle 94 ºC 1 min 56 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Lozano *et al* (2012)
salA (610 pb)	F: CTATTAATCGATGAACCAACAAACC R: TTGATTTACCTGTACCATTTCTGC	94 ºC 7 min 1 cycle 94 ºC 1 min 52 ºC 1 min 30 cycles 72 ºC 2 min 72 ºC 7 min 1 cycle	Hot *et al* (2014)

2.12.10 Resistance to aminoglycosides

Concerning staphylococcal strains that presented resistance to gentamicin, tobramycin or both, the genes *aac2'-aph6"* and/or *ant4'* genes were investigated. Moreover, enterococcal strains that presented high-level resistance to gentamicin and streptomycin were investigated for the presence of *aac2'-aph6", ant6',* and/or *str* genes, accordingly **(Table 28).**

Table 28. Genes, primers' sequences and PCR conditions utilized for the amplification of genes that confer resistance to aminoglycosides

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
*aac*6'-aph2" (220 pb)	F: CCAAGAGCAATAAGGGCATA R: CACTATCATAACCACTACCG	94 ºC 3 min 1 cycle 94 ºC 1 min 50 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Schnellmann *et al* (2006)
ant4' 165 pb)	F: GCAAGGACCGACAACATTTC R: TGGCACAGATGGTCATAACC	94 ºC 3 min 1 cycle 94 ºC 30 sec 60 ºC 45 sec 30 cycles 72 ºC 2 min 72 ºC 5 min 1 cycle	van de Klundert and Vliegenthart, (1993)
ant6' (597 pb)	F: ACTGGCTTAATCAATTTGGG R: TTATTGATAATTTTGGTT	94 ºC 5 min 1 cycle 94 ºC 1 min 58 ºC 1 min 30 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Clark *et al* (1999)
str (646 pb)	F: TATTGCTCTCGAGGGTTC R: CTTTCTATATCCATTCATCTC	94 ºC 3 min 1 cycle 94 ºC 30 sec 60 ºC 45 sec 30 cycles 72 ºC 2 min 72 ºC 5 min 1 cycle	Schnellmann *et al* (2006)

2.13 CHARACTERIZATION OF VIRULENCE DETERMINANTS IN STAPHYLOCOCCI

2.13.1 Virulence factors of *S. aureus*

For the *S. aureus* strains, the genes *tst, luk-F/S*-PV, *eta*, *etb*, *etd*, *sea*, *seb, sec*, *sed, see* that encode for the toxic shock syndrome, Panton-Valentine leucocidin, exfoliative toxins, and enterotoxins, respectively, were analysed by PCR. Furthermore, all *S. aureus* strains that were positive for any of these genes were confirmed by Sanger sequencing of the PCR amplicons. The analyses were based on the primers and PCR conditions presented in **Table 29.**

Table 29. Genes, primers' sequences and PCR conditions utilized for the amplification of genes associated with virulence traits in *S. aureus*

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
luk-F/S-PV (443 pb)	F: ATCATTAGGTAAAATGTCTGGACATGATCCA R: GCATCAAGTGTATTGGATAGCAAAAGC	94 ºC 5 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Lina *et al* (1999b)
eta (190 bp)	F: ACTGTAGGAGCTAGTGCATTTGT R: TGGATACTTTTGTCTATCTTTTTCATCAAC	94 ºC 5 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Jarraud *et al* (2002)
etb (612 bp)	F: CAGATAAAGAGCTTTATACACACATTAC R: AGTGAACTTATCTTTCTATTGAAAAACACTC	94 ºC 5 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Jarraud *et al* (2002)
etd (376 bp)	F: AACTATCATGTATCAAGG R: CAGAATTTCCCGACTCAG	94 ºC 5 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Jarraud *et al* (2002)
tst (180 bp)	F: TTCACTATTTGTAAAAGTGTCAGACCCACT R: TACTAATGAATTTTTTTATCGTAAGCCCTT	94 ºC 5 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 1 min 72 ºC 10 min 1 cycle	Yamaguchi *et al* (2002)
sea (344 bp)	F: ATGGTTATCAATGTGCGGGTGIIIIICCAAAC AAAAC R: TGAATACTGTCCTTGAGCACCAIIIIIATCGTAA TTAAC	94 ºC 10 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 30 sec 72 ºC 10 min 1 cycle	Hwang *et al* (2007)
seb (196 bp)	F: TGGTATGACATGATGCCTGCACIIIIIGATAAA TTTGAC R: AGGTACTCTATAAGTGCCTGCCTIIIIIACTAA CTCTT	94 ºC 10 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 30 sec 72 ºC 10 min 1 cycle	Hwang *et al* (2007)
sec (399 bp)	F: GATGAAGTAGTTGATGTGTATGGATCIIIIIACT ATGTAAAC R: AGATTGGTCAAACTTATCGCCTGGIIIIIGCATCAT ATC	94 ºC 10 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 30 sec 72 ºC 10 min 1 cycle	Hwang *et al* (2007)
sed (190 bp)	F: CTGAATTAAGTAGTACCGCGCTIIIIIATATGAA AC R: TCCTTTTGCAAATAGCGCCTTGIIIIIGCATCTAA TTC	94 ºC 10 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 30 sec 72 ºC 10 min 1 cycle	Hwang *et al* (2007)
see (286 bp)	F: CGGGGGTGTAACATTACATGATIIIIICCGATTG ACC R: CCCTTGAGCATCAAACAAATCATAAIIIIICGTGG AC CCTTC	94 ºC 10 min 1 cycle 94 ºC 30 sec 55 ºC 30 sec 30 cycles 72 ºC 30 sec 72 ºC 10 min 1 cycle	Hwang *et al* (2007)

2.13.2 Virulence factors of *S. pseudintermedius*

For the *S. pseudintermedius* strains, the presence of the *lukS/F-I, siet,* and *sient* genes was analysed by PCR using the primers and conditions presented in **Table 30.**

Table 30. Genes, primers' sequences and PCR conditions utilized for the amplification of virulence genes associated with *S. pseudintermedius*

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
lukF-I (572 pb)	F: CCTGTCTATGCCGCTAATCAA R: AGGTCATGGAAGCTATCTCGA	94 ºC 5 min 1 cycle 94 ºC 1 min 57 ºC 1 min 35 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Futagawa-Saito *et al* (2004a)
lukS-I (332 pb)	F: TGTAAGCAGCAGAAAATGGGG R: GCCCGATAGGACTTCTTACAA	94 ºC 3 min 1 cycle 94 ºC 1 min 50 ºC 1 min 25 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Futagawa-Saito *et al* (2004a)
siet (359 pb)	F: ATGGAAAATTTAGCGGCATCTGG R: CCATTACTTTTCGCTTGTTGTGC	94 ºC 3 min 1 cycle 94 ºC 30 min 56 ºC 30 min 30 cycles 72 ºC 1 min 72 ºC 5 min 1 cycle	Lautz *et al* (2006)
se-int (147 pb)	F: GCAAGCATATCATTACATTTG R: ACTTGATATACCCTGTTTCGT	94 ºC 5 min 1 cycle 94 ºC 1 min 55 ºC 1 min 35 cycles 72 ºC 1 min 72 ºC 7 min 1 cycle	Futagawa-Saito *et al* (2004b)

2.14 DETECTION AND CLASSIFICATION OF IMMUNE EVASION CLUSTERS

The presence of the immune evasion cluster (IEC) was determined by PCR analysis of the *scn* gene. Regardless of the presence or absence of the *scn* gene in the *S. aureus* strains, four additional IEC genes (*chp, sak, sea,* and *sep*) were analyzed as presented on **Table 31.** Furthermore, the combination of the analysed genes enabled the classification of the immune evasion cluster (IEC) types into A–G as previously described (van Wamel *et al*., 2006).

Table 31. Genes, primers' sequences and PCR conditions utilized for the amplification of the IEC genes

Gene (amplicon size)	Primers' oligonucleotide (5'⇒3')	PCR conditions	Reference
scn (257 pb)	F: AGCACAAGCTTGCCAACATCG R: TTAATATTTACTTTTTAGTGC	94 ºC 3 min 1 cycle 94 ºC 30 sec 53 ºC 30 sec 30 cycles 72 ºC 2 min 72 ºC 6 min 1 cycle	van Wamel *et al* (2006)
chp (366 pb)	F: TTTACTTTTGAACCGTTTCCTAC R: CGTCCTGAATTCTTAGTATGCATATTCATTAG	94 ºC 3 min 1 cycle 94 ºC 30 sec 53 ºC 30 sec 30 cycles 72 ºC 2 min 72 ºC 6 min 1 cycle	van Wamel *et al* (2006)
sak (223 pb)	F: AAGGCGATGACGCGAGTTAT R: GCGCTTGGATCTAATTCAAC	94 ºC 3 min 1 cycle 94 ºC 30 sec 53 ºC 30 sec 30 cycles 72 ºC 2 min 72 ºC 6 min 1 cycle	van Wamel *et al* (2006)
sea (344 pb)	F: AGATCATTCGTGGTATAACG R: TTAACCGAAGGTTCTGTAGA	94 ºC 3 min 1 cycle 94 ºC 30 sec 53 ºC 30 sec 30 cycles 72 ºC 2 min 72 ºC 6 min 1 cycle	van Wamel *et al* (2006)
sep (196 pb)	F: AATCATAACCAACCGAATCA R: TCATAATGGAAGTGCTATAA	94 ºC 3 min 1 cycle 94 ºC 30 sec 53 ºC 30 sec 30 cycles 72 ºC 2 min 72 ºC 6 min 1 cycle	van Wamel *et al* (2006)

There are seven IEC types (A to G) depending on the combination of *scn, chp, sak, sea/sep* genes: IEC-type A (*sea, sak, chp, scn*), IEC-type B (*sak, chp, scn*), IEC-type C (*chp, scn*), IEC-type D (*sea, sak, scn*), IEC-type E (*sak, scn*), IEC-type F (*sep, sak, chp, scn*) and IEC-type G (*sep, sak, scn*).

2.15 SANGER SEQUENCING

The PCR product sequencing technique was used for *spa* and MLST typing, for confirming the presence of certain resistance and virulence genes, for the detection of mutations and for studying the genetic environments of resistance genes. The sequencing was carried out by the biotech company Genewiz (Leipzig, Germany), which uses the Sanger method with the automatic system ABI 3730xl DNA Analyzers. For the analysis of the sequences obtained, different computer tools and software were used:

a. NCBI BLAST (Basic Local Alignment SearchTool): to compare the sequences obtained with the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

b. EMBOSS (European Molecular Biology Open Software Suite) Transeq: to translate sequences from nucleotides to amino acid sequences. https://www.ebi.ac.uk/Tools/st/emboss_transeq/

c. EMBOSS Needle: to align two nucleotide or protein sequences. https://www.ebi.ac.uk/Tools/psa/emboss_needle/

d. MLST: to compare the alleles obtained with the alleles previously described and assigns a type sequence depending on the allelic combination. https://pubmlst.org

e. Ridom® Staph-Type version 2.2.1 (Ridom GmbH): to analyse the polymorphic region of the *spa* gene and assigns the corresponding *spa*-type.

2.16 WHOLE GENOME SEQUENCING (WGS)

One hundred and seven (107) strains of different species of *Staphylococcus* and *Enterococcus* were analysed by WGS. The strains that were analysed by WGS were selected based on the following criteria:

a. From the healthy carriers that had no contact with animals, and hospital facilities, seven MSSA-CC398 were selected. This represents one per healthy carrier.
b. From the healthy dogs and dog owners, 13 selected strains (11 *S. aureus* and two *S. pseudintermedius*): (a) all MSSA-CC398 strains, (b) all strains from households with two or more humans and/or dogs' carriers of either *S. aureus* or *S. pseudintermedius*, (c) the only MRSA strain.
c. Twenty-three *S. aureus* strains from nestlings of white stork parents foraging in natural areas and landfills were selected to represent one *S. aureus* strain from each of the nestling storks, although more than one strain was included when they presented different clonal complexes (CC).
d. Seventeen *S. aureus* strains from healthy pigs and 12 from pig farmers were selected from farms A to D. The selection was based on the similarity in their ST and AMR genes in each farm and the similarity of genetic lineages and AMR genes from strains of pigs and pig farmers which were previously determined.
e. Twenty-six CoNS strains were selected. For these strains, 4, 11, 3, 3, 1 and 6, respectively were selected from nestling storks, pigs, pig farmers, dog owners, dog and healthy people who had no contact with animals, respectively. The selection criteria of the strains was: (i) CoNS that presented a MDR phenotype for four or more classes of antibiotics, selecting one species each per host carrying the MDR; (ii) MDR-CoNS

strains with similar AMR genes detected from humans and animals in the same ecological niche.

f. Nine linezolid-resistant enterococci (LRE) (seven *E. faecalis*, one *E. faecium* and one *E. casseliflavus*) obtained from the nares of healthy dogs, pigs, pig farmers and tracheal of nestling storks were selected. The *E. faecalis* strains from the pig farms were selected based on their origins, STs and AMR genes. While the other species were selected solely because they harboured the linezolid resistance genes

2.16.1 Genome assembly and phylogenetic analyses

Whole genome sequencing of the selected strains was carried out on the Illumina NextSeq platform. The MagNA Pure 96 DNA Multi-Sample Kit (Life Technologies, Carlsbad, CA, USA, 4413021) was used to extract genomic DNA according to instructions provided by the manufacturers. The Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, Scoresby, VIC, Australia) was used for DNA quantification, while Sequencing libraries were prepared using the Illumina Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA, FC-131-1096) and sequenced on the NextSeq 500 platform (Illumina, San Diego, CA, USA) using a 300-cycle kit to obtain paired-end 150bp reads, as previously described (Saidenberg *et al.*, 2022).

All the genomes analysed in this study were *de novo* assembled using SPAdes (v.3.15.5). *In silico* typing was performed on raw sequencing reads using the *kma* algorithm (Clausen *et al.*, 2018) with a minimum of 90% coverage, 80% identity and minimum 6× depth. Core-genome single nucleotide polymorphisms (SNPs) between the 52 *S. aureus* strains in this study were detected with the NASP pipeline v.1.0.0 (Sahl *et al*., 2016) and presented on a phylogenetic tree. Briefly, the raw sequencing data from all CC398 strains were mapped together with 88 previously published *S. aureus* CC398 genomes (Bioproject number: PRJNA514245) against the chromosome of ST398 strain S0385 (GenBank accession no. AM990992) as reference to obtain a CC398 phylogeny. GATK (v.4.2.2) was used to call SNPs and excluded positions featuring <90% unambiguous variant calls and <10 depth. IQ-TREE (v.2.1.2), was used to construct the phylogenetic trees using ModelFinder with 100 bootstrap replicats. The graphical data was added to the phylogenies with iTOL v.6.6 (Letunic and Bork, 2021).

2.16.2 Genome annotation, typing and *in-silico* analysis

The sequence types (STs) were determined with MLST v.2.16 (Jolley *et al*., 2018). Virulence factors, plasmid replicons, and antimicrobial resistance genes were identified using ABRicate v.0.9.0 (https://github.com/tseemann/abricate) and the respective databases VFDB, Plasmidfinder, and Resfinder databases from the Center for Genomic Epidemiology. Mutations associated with AMR were identified using ResFinder v4.1 (Bortolaia *et al*., 2020) and PointFinder (Zankari *et al*., 2017). Biocide and heavy metal resistance genes were identified using BACMET (Pal *et al*., 2014). Phaster was used to identify all prophage elements (Arndt *et al*., 2016). Moreover, manual mapping of all the strains against sets of prophage integrases and IEC genes was done using Geneious prime with following genes and their corresponding GenBank accession numbers; sa12int (NC_010147), Sa9int (NC_007057), Sa8int (NC_007622), Sa7int (NC_007049), Sa6int (NC_005356), Sa6int (M27965), Sa5int (NC_004615), Sa4int (NC_002953), Sa3int (NC_009641), Sa3int (NC_004617), Sa3int (DQ530361), Sa2int (NC_004616), Sa2int (NC_002321), Sa1int (NC_003288), phiJB_int (NC_028669), SebagoInt (MK618716), IEC_chp (NC_009641), IEC_sak (NC_009641), IEC_scn (NC_009641), IEC_sea (NC_009641), and IEC_sep (BA000018). The *spa*Typer v1.0 tool was used to confirm the *spa* types (Bartels *et al*., 2014). The SCC*mec* types were assigned using SCC*mec*Finder 1.2 (https://cge.food.dtu.dk/services/SCCmecFinder/). The genetic environment of the *ermT* gene was illustrated in comparison with the reference strains; MRSA pUR1902 (GenBank accession number: HF583291.1), MRSA pUR2941 (GenBank accession number: HF583290.1) and MRSA AV4_1 (GenBank accession number: SAMN00828682) using the EasyFig Software**.**

For the genomes of CoNS, first, core-genome single nucleotide polymorphisms (SNPs) between the eight *S. epidermidis* strains in this study were detected with the NASP pipeline v.1.0.0 (Sahl *et al*., 2016) after they were mapped together with previously 30 published *S. epidermidis* genomes from different countries with similar genetic lineages from the PubMLST database (https://pubmlst.org/bigsdb?db=pubmlst_sepidermidis_strains&page=query&genomes=1) (identification numbers: 32110, 32113, 32116, 41749, 42109, 43340, 43421, 43426, 43427, 43436, 43455, 43466, 43518, 43568, 43636, 43643, 43656, 43697, 43720, 43770, 43771, 43774, 43786, 43800, 43816, 43921, 44294, 44298, 44496, 44521) to obtain an *S. epidermidis* phylogenetic trees. GATK (v.4.2.2) was used to call SNPs and excluded positions featuring <90% unambiguous variant calls and <10 depth. IQ-TREE (v.2.1.2), was used to construct the

phylogenetic trees using ModelFinder with 100 bootstraps. The graphical data was added to the phylogenies with iTOL v.6.6 (Letunic and Bork, 2021). To determine the relatedness of the *S. saprophyticus* from a pig and pig farmer, a web-based CSI phylogeny database was used to obtain the SNPs by mapping the genomes to a reference *S. saprophyticus* ATCC 15305 (GenBank accession no. AP008934.1) with the default parameter, except for that the minimum distance between SNPs which was disabled. Also, the SNPs of the *S. borealis* from two pigs were determined by comparing them with 13 additional *S. borealis* strains available from NCBI (GenBank Accession numbers: GCA_003042555.1, GCA_003580835.1, GCA_009735325.1, GCA_013345165.1, GCA_013345175.1, GCA_013345185.1, GCA_013345195.1, GCA_013345205.1, GCA_030362875.1, GCA_030501495.1, GCF_004343675.1, GCF_013345185.1, GCF_013345195.1) by using the web-based CSI phylogeny database following settings similar to the ones used for *S. saprophyticus.*

2.16.3 Oxford Nanopore Sequencing (Long reads technology)

The *E. casseliflavius* (X4962) and *S. saprophyticus* (X4944) strains were further sequenced on the MinION platform (Oxford Nanopore Technologies (ONT), Oxford, United Kingdom) as described here. Single colonies were obtained from a fresh over-night blood agar plating and resuspended in enzymatic lysis buffer [Proteinase K (Roche); Lysozyme (Sigma)] and incubated at 37°C (30 min) and 55°C (1 h). The MagNA Pure 96 DNA Multi-Sample Kit (Life Technologies, Carlsbad, CA, USA, 4413021) was used to extract genomic DNA according to the manufacturer's instructions. Quant-iT dsDNA BR and HS Assay Kits (Thermo Fisher Scientific, Scoresby, VIC, Australia) were used for DNA quantification.

2.16.4 Genetic Environment of antimicrobial resistance genes

The genetic environment of *optrA, poxtA* and *cfrD* genes was illustrated using the reference strains; *E. faecalis* (GenBank accession number: KP399637), *E. faecium* plasmid pGZ8 (GenBank accession number: CP038162) and *E. faecium* (GenBank accession number: MN831413) and *E. faecalis* (GenBank accession number: CP097040) in EasyFig Software. Also, *ermT* sequences of two MRSA strains from a pig (X4905) and a pig farmer (X5473) and five MSSA strains from nestling storks (X3906, X3913, X4603, X4630, X4703) were used to construct their environment in comparison with the reference strains; MRSA pUR1902 (GenBank accession number: HF583291.1), MRSA pUR2941 (GenBank accession number: HF583290.1) and MRSA AV4_1 (GenBank accession number: SAMN00828682). Also, the environments of *ermT* gene of MSSA-CC398 strains from healthy people with animal contact were construct in comparison with the reference MSSA-CC398 strains (Gene Bank Accession

numbers: LNJ000000000, SRR15903552, SRR15903562, SRR15903539, SRR15903541, SRR15903551, SRR15903559, SRR15903563, SRR15903567). Moreover, the genetic environment of the *ermC* gene in one of the MSSA-CC398 strains was illustrated in comparison with the reference MSSA-CC398 strains (Gene Bank Accession numbers: ERR3306808, SRR445274).

The genetic environment of the *ermT, cfr* and *mecC* genes in CoNS was illustrated in comparison with reference strains as follows: (a) for *ermT*, reference strains with GenBank accession numbers ERX9972358, ERX9972359, HF583290, and HF583291 were used, (b) in relation to the *cfr* gene, reference strains with GenBank accession numbers CP065195, KR230047, MN642001 were used, (c) concerning *mecC* gene, the reference strains with GenBank accession numbers FR821779, JAPNQM000000000, JAPNQN01000000, HE993884, and SAMN33407026 were used as reference strains.

2.17 DETERMINATION OF THE CRISPR-Cas SYSTEM OF STAPHYLOCOCCI

The CrisprCasFinder (https://crisprcas.i2bc.paris-saclay.fr/) was used to identify the numbers of CRISPR, Cas proteins and spacers of all the MDR-CoNS (Couvin *et al*., 2018). Specifically, the size of the flanking region and other parameters were set to default values. Moreover, three CoNS trains that contained larger sequences than CrisprCasFinder could handle were analyzed by the CRISRPCasMeta (https://crisprcas.i2bc.paris-saclay.fr/CrisprCasMeta/Index) applying all the default settings.

2.18 GENOME AVAILABILITY

a. All genome reads of the MSSA-CC398 strains generated from healthy people without animal contact have been deposited at the European Nucleotide Archive under the study accession number PRJEB63134.
b. All the raw genome reads generated from the CoPS strains from dogs and dog owners have been deposited at the European Nucleotide Archive under Study Accession no. PRJEB57210.
c. All the raw genome reads generated from *S. aureus* strains from nestling storks, pigs and pig farmers have been deposited at the European Nucleotide Archive under Study accession no. PRJEB66351.
d. All the raw genome reads generated from the CoNS strains have been deposited at the European Nucleotide Archive under Study Accession no. PRJNA1023081
e. All the raw genome reads of the linezolid-resistant enterococci have been deposited at the European Nucleotide Archive under Study Accession number PRJEB62654. The

optrA-associated plasmid in *E. casseliflavus* (pURX4962) was deposited in GenBank with the accession number OR069652.

2.19 STATISTICAL ANALYSIS

a. In nestling storks, to assess the effect of the use of landfills as a food resource on the frequency of appearance of the different bacteria, 94 linear mixed models with binomial distributed dependent variables were constructed (47 for each type of sample, nasal or tracheal). Of these, 26 were discarded because all values were equal to 0 (16 nasal and 10 tracheal). In these models, natural or landfill was included as a factor, and the nest was included as a random factor to avoid pseudoreplication. In addition, to evaluate if the presence of a microorganism differs between the nasal and tracheal cavity, 47 models with binomial distributed dependent variables were constructed. In these models, nasal or tracheal was included as a fixed factor, and nest of origin of the nestlings and natural or landfill habitat were included as random factors. Finally, to check if a correlation between the appearance of the different microorganisms exists, the Jaccard Similarity Index for all bacteria by sample type was calculated (nasal or tracheal). These models were performed in R 4.1.3 (R Core Team 2022) using the R packages lme4 (1.1–28), car (3.0–12) and vegan (2.6–2) (Bates *et al*., 2015; Fox and Weisberg 2019). The package ggplot2 (3.3.5) was used to create the figures (Wickham 2016). Data were subjected to univariate logistic regression to compute Odds Ratio (OR) at a 95% confidence interval (95% CI) between the prevalence rate of *S. aureus* and the foraging habit of the colonized and non-colonized storks. Statistical significance was set at $p < 0.05$ for all analyses.

b. In healthy people, univariate logistic regression was to compute the odd ratio (OR) at a 95% confidence interval (95%CI) between the co-carriage rate of *S. aureus* and *S. epidermidis* with significant association at $p<0.05$.

c. In dogs and dog owners, data were subjected to univariate logistic regression to compute odd ratio (OR) at a 95% confidence interval (95%CI) between the carriage rate of *S. aureus/S. pseudintermedius* and the household densities with significant association ($p<0.05$).

d. In pigs and pig farmers, data were subjected to univariate logistic regression to compute the Odd Ratio (OR) at a 95% confidence interval (95%CI) of the association between the presence of MRSA, MSSA and the number of CoNS species in pigs and pig-farmers.

All the statistical analyses from bivariate logistic regression from sections b to d were performed by *MedCalc Version* 22.009 (Ostend, Belgium).

CHAPTER THREE

RESULTS

3.0 RESULTS

3.1 DIVERSITY AND FREQUENCY OF BACTERIAL SPECIES AND MOLECULAR CHARACTERIZATION of *Staphylococcus*

3.1.1 NESTLING WHITE STORKS

3.1.1.1 Frequency of Bacteria Species and Genera Recovered from the Nasal (N) and Tracheal (T) Samples of nestling storks

A total of 806 strains were recovered (up to 12/sample), and 703 of them (87.2%) were identified by MALDI-TOF-MS: 398 from T-samples (56.6%) and 305 from N-samples (43.4%) **(Table 32).** A total of 46 species were detected. Of all the identified bacteria, 408 strains were *Staphylococcus* (T = 218, N = 190), 144 *Enterococcus* (T = 74, N = 70), 34 *Macrococcus* (T = 24, N = 10), 30 *Bacillus* (T = 15, N = 15), 19 *Corynebacterium* (T = 13, N = 6), 22 *Proteus* (T = 19, N = 3), 11 *Lactococcus* (T = 9, N = 2), 7 *Enterobacter* (T = 6, N = 1), 3 *Arthrobacter* (T = 3), 6 *Streptococcus* (T = 4, N = 2), 5 *Acinetobacter* (T = 3, N = 2), 4 *Escherichia coli* (T = 4) and *Providencia* spp (T = 4), 2 *Citrobacter* spp (N = 2), and one each *Micrococcus* spp (T = 1) and *Klebsiella* spp (N = 1) **(Table 32).**

Of all the bacteria genera identified, there were significant associations of *Enterococcus* and *Proteus* with the sample type collected from the storks (N or T respectively) ($p < 0.05$) **(Table 32).** Out of the 408 staphylococci strains, the most frequently identified species were *S. sciuri* (n = 251, 61.5%), *S. aureus* (n = 67, 16.4%), *S. chromogenes* (n = 20, 5.0%), *S. epidermidis* (n = 17, 4.1%) and *S. xylosus* (n = 11, 2.7%). Out of the 144 enterococci strains, the most frequently detected were *E. faecalis* (n = 78, 54.2%), *E. faecium* (n = 47, 32.6%), then *E. cecorum* (n = 8, 5.6%) and *E. casseliflavus* (n = 5, 3.5%) **(Table 33).** Among other genera with few species identified, *Macrococcus caseolyticus* (4.8%), *Lactococcus garvieae* (1.6%), *Micrococcus luteus* (0.1%), *Streptococcus gallolyticus* (0.9%), *Arthrobacter cretinolyticus* (0.4%), *Corynebacterium falsenii* (0.4%), *Escherichia coli* (0.6%), *Klebsiella pneumoniae* (0.1%) and *Acinetobacter baumannii* (0.3%) were found in low frequencies **(Table 33).**

Table 32. Distribution pattern of bacteria genera identified from tracheal and nasal samples of storks analysed

Bacterial genera	№ (%) in tracheal samples (n=85)	№ (%) in nasal samples (n=52)	χ^2	*p*	Total number of strains of: Tracheal	Nasal	Total number of strains in tracheal and nasal samples
			Gram-positive cocci				
Staphylococcus	76 (89.4)	51 (98.1)	3.58	0.058	218	190	408
Enterococcus	37 (43.5)	36 (69.2)	8.65	0.003*	74	70	144
Macrococcus	15 (17.6)	7 (13.5)	0.42	0.517	24	10	34
Lactococcus	7 (8.2)	1 (1.9)	2.24	0.126	9	2	11
Streptococcus	3 (3.5)	2 (3.8)	0.01	0.924	4	2	6
Micrococcus	0 (0.0)	1 (1.9)	2.04	0.153	0	1	1
Vagococcus	1 (1.2)	1 (1.9)	0.13	0.723	1	1	2
			Gram-positive bacilli				
Bacillus	15 (17.6)	13 (22.4)	1.07	0.300	15	15	30
Arthrobacter	3 (8.2)	0 (0.0)	1.88	0.170	3	0	3
Corynebacterium	4 (4.7)	3 (5.8)	0.08	0.784	13	6	19
			Gram negative bacteria: Enterobacterales				
Proteus	16 (18.8)	2 (3.8)	6.34	0.012*	19	3	22
Enterobacter	4 (4.7)	1 (1.9)	0.71	0.399	6	1	7
Escherichia	3 (3.5)	0 (0.0)	1.88	0.171	4	0	4
Providencia	4 (4.7)	0 (0.0)	2.52	0.112	4	0	4
Klebsiella	1 (1.2)	0 (0.0)	0.62	0.433	1	0	1
Citrobacter	0 (0.0)	1 (1.9)	1.62	0.204	0	2	2
			Gram negative bacteria-non-fermenting				
Acinetobacter	3 (3.5)	1 (1.9)	0.29	0.588	3	2	5
Total strains					398	305	703

Note: The number of viable samples from each source is as follows:
a. Both tracheal and nasal = 49
b. Total animals tested = 87
* Significant association determined by Chi-squared test at 95% CI.

Table 33. Number of strains of each species recovered from the nasal and tracheal samples of storks

Bacteria genera and species	№ (%) of strains from tracheal samples (n=85)	№ (%) of strains from nasal samples (n=52)	Total number (%) of strains from tracheal/nasal samples	Percentage of strains of species per genus
Staphylococcus				
S. sciuri	146 (36.7)	105 (34.4)	251 (35.7)	61.5
S. aureus	26 (6.5)	41 (13.4)	67 (9.5)	16.4
S. chromogenes	6 (1.5)	14 (4.6)	20 (2.8)	5.0
S. epidermidis	13 (3.3)	4 (1.3)	17 (2.4)	4.1
S. xylosus	2 (0.5)	9 (3.0)	11 (1.6)	2.7
S. lentus	7 (1.8)	3 (1.0)	10 (1.4)	2.5
S. simulans	1 (0.3)	7 (2.3)	8 (1.1)	1.9
S. hominis	7 (1.8)	0 (0.0)	7 (1.0)	1.7
S. saprophyticus	5 (1.3)	1 (0.3)	6 (0.9)	1.5
S. hyicus	1 (0.3)	4 (1.3)	5 (0.7)	0.6
S. haemolyticus	2 (0.5)	0 (0.0)	2 (0.3)	0.5
S. arlettae	0 (0.0)	2 (0.7)	2 (0.3)	0.5
S. capitis	1 (0.3)	0 (0.0)	1 (0.1)	0.2
S. pasteuri	1 (0.3)	0 (0.0)	1 (0.1)	0.2
Total	218	190	408	100.0
Enterococcus				
E. faecalis	44 (11.1)	34 (11.1)	78 (11.1)	54.2
E. faecium	19 (4.8)	28 (9.2)	47 (6.7)	32.6
E. cecorum	8 (2.0)	0 (0.0)	8 (1.1)	5.6
E. casseliflavus	0 (0.0)	5 (1.6)	5 (0.7)	3.5
E. gallinarum	1 (0.3)	1 (0.3)	2 (0.3)	1.4
E. durans	0 (0.0)	2 (0.7)	2 (0.3)	1.4
E. canis	1 (0.3)	0 (0.0)	1 (0.1)	0.7
E. hirae	1 (0.3)	0 (0.0)	1 (0.1)	0.7
Total	74	70	144	100.0
Macrococcus caseolyticus	24 (6.0)	10 (3.3)	34 (4.8)	100.0
Lactococcus garvieae	9 (2.3)	2 (0.7)	11 (1.6)	100.0
Streptococcus gallolyticus	4 (1.0)	2 (0.7)	6 (0.9)	100.0
Micrococcus luteus	0 (0.0)	1 (0.3)	1 (0.1)	100.0
Vagococcus lutrae	1 (0.3)	1 (0.3)	2 (0.3)	100.0
Bacillus				
Bacillus spp	14 (3.5)	9 (2.9)	23 (3.3)	76.7
B. cereus	0 (0.0)	4 (1.3)	4 (0.6)	13.3
B. licheniformis	1 (0.3)	1 (0.3)	2 (0.3)	6.7
B. subtilis	0 (0.0)	1 (0.3)	1 (0.1)	3.3
Total	15	15	30	100.0

Table 33. Continuation

Bacteria genera and species	№ (%) of strains from tracheal samples (n=85)	№ (%) of strains from nasal samples (n=52)	Total number (%) of strains from tracheal/nasal samples	Percentage of strains of species per genus
Arthrobacter cretinolyticus	3 (0.8)	0 (0.0)	3 (0.4)	100.0
Corynebacterium				
Corynebacterium spp	12 (3.0)	3 (1.0)	15 (2.1)	78.9
C. falsenii	1 (0.3)	2 (0.7)	3 (0.4)	15.8
C. auromucosum	0 (0.0)	1 (0.3)	1 (0.1)	5.3
Total	13	6	19	100.0
Proteus				
Proteus spp	18 (4.5)	3 (1.0)	21 (3.0)	95.5
P. vulgaris	1 (0.3)	0 (0.0)	1 (0.1)	4.5
Total	19	3	22	100.0
Enterobacter				
E. cloacae	6 (1.5)	0 (0.0)	6 (0.9)	85.7
E. asburea	0 (0.0)	1 (0.3)	1 (0.1)	14.3
Total	6	1	7	100.0
Escherichia coli	4 (1.0)	0 (0.0)	4 (0.6)	100.0
Providencia				
P. stuartii	3 (0.8)	0 (0.0)	3 (0.4)	75.0
P. retgerii	1 (0.3)	0 (0.0)	1 (0.1)	25.0
Total	4	0	4	100.0
Klebsiella pneumoniae	1(0.3)	0 (0.0)	1 (0.1)	100.0
Citrobacter				
C. braakii	0 (0.0)	1 (0.3)	1 (0.1)	50.0
C. freundii	0 (0.0)	1 (0.3)	1 (0.1)	50.0
Total	0	2	2	100.0
Acinetobacter				
A. junii	1 (0.3)	2 (0.7)	3 (0.4)	60.0
A. baumannii	2 (0.5)	0 (0.0)	2 (0.3)	40.0
Total	3	2	5	100.0
Total strains (%)	398 (56.6)	305 (43.4)	703 (100.0)	100.0

3.1.1.2 Diversity of Bacterial Species from Nasal and Tracheal Cavities of Nestlings Based on Foraging Habits of Parent Storks

Of the 52 nasal and 85 tracheal samples collected from 87 storks, about 88.1% of nestlings from parent storks foraging in natural habitats and 81.4% nestlings of parent storks foraging in landfills had at least one *Staphylococcus* species in their tracheal samples. However, all the stork nestlings from parents foraging in natural habitats (100%) and 90.6% of those foraging in landfills had at least one *Staphylococcus* species in their nasal samples **(Table 34).** On the other hand, 55.0% and 68.8% of stork nestlings from parents foraging in natural habitats and landfills, respectively, were enterococcal nasal carriers. In contrast, 38.1% and 46.5% of nestlings of parent storks foraging in natural habitats and landfills, respectively, had enterococcal tracheal carriage **(Table 34).**

Table 34. Diversity pattern of nasal and tracheal staphylococci and enterococci of stork nestlings in relation to the foraging habits of adult storks

Bacterial genera and species	**Tracheal**		**Nasal**	
	Nº (%) of positive animals foraging in:		**Nº (%) of positive animals foraging in:**	
	Natural areas (n=42)	**Landfills (n=43)**	**Natural areas (n=20)**	**Landfills (n=32)**
Staphylococci	37 (88.1)	35 (81.4)	20 (100.0)	29 (90.6)
S. sciuri	36 (85.7)	23 (53.4)	18 (90.0)	28 (87.5)
S. aureus	3 (7.1)	7 (16.3)	7 (35.0)	12 (37.5)
S. epidermidis	0 (0.0)	8 (18.6)	1 (5.0)	4 (12.5)
S. hominis	0 (0.0)	7 (16.3)	0 (0.0)	0 (0.0)
S. lentus	2 (4.8)	4 (9.3)	1 (5.0)	3 (9.4)
S. chromogenes	1 (2.4)	2 (4.7)	1 (5.0)	5 (15.6)
S. xylosus	2 (4.8)	0 (0.0)	5 (25.0)	1 (3.1)
S. capitis	1 (2.4)	0 (0.0)	0 (0.0)	0 (0.0)
S. hyicus	0 (0.0)	1 (2.3)	0 (0.0)	0 (0.0)
S. simulans	0 (0.0)	7 (16.3)	2 (10.0)	2 (6.2)
S. saprophyticus	2 (4.8)	3 (7.0)	0 (0.0)	1 (3.1)
S. haemolyticus	1 (2.4)	2 (4.7)	0 (0.0)	0 (0.0)
S. pasteuri	1 (2.4)	0 (0.0)	0 (0.0)	0 (0.0)
S. arlettae	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.1)
Enterococci	16 (38.1)	20 (46.5)	11 (55.0)	22 (68.8)
E. faecalis	10 (23.8)	10 (23.3)	8 (40.0)	11 (34.4)
E. faecium	5 (11.9)	8 (18.6)	1 (5.0)	14 (43.8)
E. cecorum	1 (2.4)	6 (13.9)	0 (0.0)	0 (0.0)
E. canis	1 (2.4)	0 (0.0)	0 (0.0)	0 (0.0)
E. hirae	1 (2.4)	0 (0.0)	0 (0.0)	0 (0.0)
E. casseliflavus	0 (0.0)	0 (0.0)	4 (20.0)	0 (0.0)
E. gallinarum	0 (0.0)	1 (2.3)	0 (0.0)	1 (3.1)
E. durans	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)

Note:
The number of viable tracheal and nasal samples from each group follows, respectively:

a. Foraging in natural areas = 42 T versus 20 N

b. Foraging in landfills = 43 T versus 32

In most cases, stork nestlings with parents foraging in landfills had a relatively higher prevalence of various species of *Staphylococcus* and *Enterococcus*. For the tracheal samples, *S. sciuri* was significantly higher among nestlings of storks foraging in natural habitats than those in landfills (χ^2 = 8.568, d.f. = 1, p = 0.0034). In the nasal samples, a significantly higher prevalence of *E. faecium* was identified in nestlings of storks foraging in landfills than in those in the natural habitat (χ^2 = 5.594, d.f = 1, p = 0.018) **(Tables 34-35, Figure 21).** Regarding the other groups of bacteria in each of the samples, *M. caseolyticus* (χ^2 = 4.623, d.f. = 1, p = 0.032) was detected significantly more frequently in the tracheal cavity of nestlings of storks foraging in natural habitat in contrast to those foraging on landfills **(Figure 21, Table 35).** In contrast, *Bacillus* spp was more frequently present in samples from the tracheal cavity of nestlings of storks foraging in landfills than those in the natural habitat (χ^2 = 8.023, d.f. = 1, p = 0.0046) **(Figure 21, Table 35).** There was no significant association between all other species identified (either from nasal or tracheal cavity) with the foraging habits of the parent storks **(Table 35).**

Table 35. Presence of each species based on foraging habitat (Natural/Landfill) and type of sample (Nasal/Tracheal)

Species	Nasal / Tracheal	χ^2 (d.f.)	p value	Presence[a]
Staphylococcus aureus	Nasal	0.033 (1)	0.855	
	Tracheal	0.0753 (1)	0.7838	
S. sciuri	Nasal	0.075 (1)	0.784	
	Tracheal	8.568 (1)	0.0034*	Natural (86%) > Landfill (53%)
S. chromogenes	Nasal	0.087 (1)	0.769	
	Tracheal	0.382 (1)	0.537	
S. epidermidis	Nasal	0.111 (1)	0.739	
	Tracheal	0 (1)	1	
S. xylosus	Nasal	0.457 (1)	0.499	
	Tracheal	0.001 (1)	0.991	
S. hominis	Nasal	All 0		
	Tracheal	0.001 (1)	0.989	
S. lentus	Nasal	0.322 (1)	0.571	
	Tracheal	0.108 (1)	0.743	
S. simulans	Nasal	0.240 (1)	0.625	
	Tracheal	0.001 (1)	0.994	
S. saprophyticus	Nasal	0 (1)	0.995	
	Tracheal	0.264 (1)	0.607	
S. haemolyticus	Nasal	All 0		
	Tracheal	0.001 (1)	0.972	
S. capitis	Nasal	All 0		
	Tracheal	0.001 (1)	0.994	
S. hyicus	Nasal	All 0		
	Tracheal	0 (1)	1	
S. pasteuri	Nasal	All 0		
	Tracheal	0.001 (1)	0.994	
S. arlettae	Nasal	0 (1)	1	
	Tracheal	All 0		
Enterococcus faecalis	Nasal	0.180 (1)	0.671	
	Tracheal	0.010 (1)	0.919	
E. faecium	Nasal	5.594 (1)	0.018*	Natural (5%) < Landfill (44%)
	Tracheal	0.508 (1)	0.476	
E. cecorum	Nasal	All 0		
	Tracheal	0.500 (1)	0.48	

Table 35. Continuation

Species	Nasal / Tracheal	χ^2 (d.f.)	*p* value	Presence[a]
E. cecorum	Nasal	All 0		
	Tracheal	0.500 (1)	0.48	
E. casseliflavus	Nasal	0.001 (1)	0.989	
	Tracheal	All 0		
E. gallinarum	Nasal	0.001 (1)	0.994	
	Tracheal	0.001 (1)	0.992	
E. durans	Nasal	0.001 (1)	0.982	
	Tracheal	All 0		
E. hirae	Nasal	All 0		
	Tracheal	0.001 (1)	0.985	
E. canis	Nasal	All 0		
	Tracheal	0 (1)	0.999	
Lactococcus garvieae	Nasal	0.001 (1)	0.982	
	Tracheal	0.064 (1)	0.8	
Streptococcus gallolyticus	Nasal	0.004 (1)	0.952	
	Tracheal	0.001 (1)	0.972	
Proteus spp	Nasal	0.115 (1)	0.735	
	Tracheal	0.455 (1)	0.5	
P. vulgaris	Nasal	All 0		
	Tracheal	0 (1)	1	
Bacillus spp	Nasal	2.794 (1)	0.095	
	Tracheal	8.023 (1)	0.0046*	Natural (5%) < Landfill (30%)
B. subtilis	Nasal	0 (1)	1	
	Tracheal	All 0		
B. cereus	Nasal	0.001 (1)	0.989	
	Tracheal	All 0		
B. licheniformis	Nasal	0 (1)	0.997	
	Tracheal	0.001 (1)	0.985	
Macrococcus caseolyticus	Nasal	0.344 (1)	0.558	
	Tracheal	4.623 (1)	0.032*	Natural (29%) > Landfill (7%)
Corynebacterium spp	Nasal	0 (1)	1	
	Tracheal	0 (1)	1	
C. falsenii	Nasal	0 (1)	1	
	Tracheal	1.272 (1)	0.259	
C. auromucosum	Nasal	0 (1)	0.997	
	Tracheal	All 0		
Micrococcus luteus	Nasal	0.227 (1)	0.634	
	Tracheal	All 0		
Arthrobacter cretinolyticus	Nasal	All 0		
	Tracheal	1.091 (1)	0.296	
Vagococcus lutrae	Nasal	0.032 (1)	0.858	
	Tracheal	0.001 (1)	0.987	
Escherichia coli	Nasal	All 0		
	Tracheal	0.288 (1)	0.592	
Acinetobacter junii	Nasal	0 (1)	0.999	
	Tracheal	0.002 (1)	0.967	
A. baumannii	Nasal	All 0		
	Tracheal	0.001 (1)	0.992	
Klebsiella pneumoniae	Nasal	All 0		
	Tracheal	0.001 (1)	0.98	
Enterobacter cloacae	Nasal	All 0		
	Tracheal	0.872 (1)	0.351	
E. asburea	Nasal	0 (1)	0.999	
	Tracheal	All 0		
Providencia stuartii	Nasal	All 0		
	Tracheal	0.001 (1)	0.992	
P. retgerii	Nasal	All 0		
	Tracheal	0 (1)	1	
Citrobacter freundii	Nasal	0.001 (1)	0.988	
	Tracheal	All 0		
C. braakii	Nasal	0.001 (1)	0.988	
	Tracheal	All 0		

*Significant association determined by Chi-squared test at 95% CI

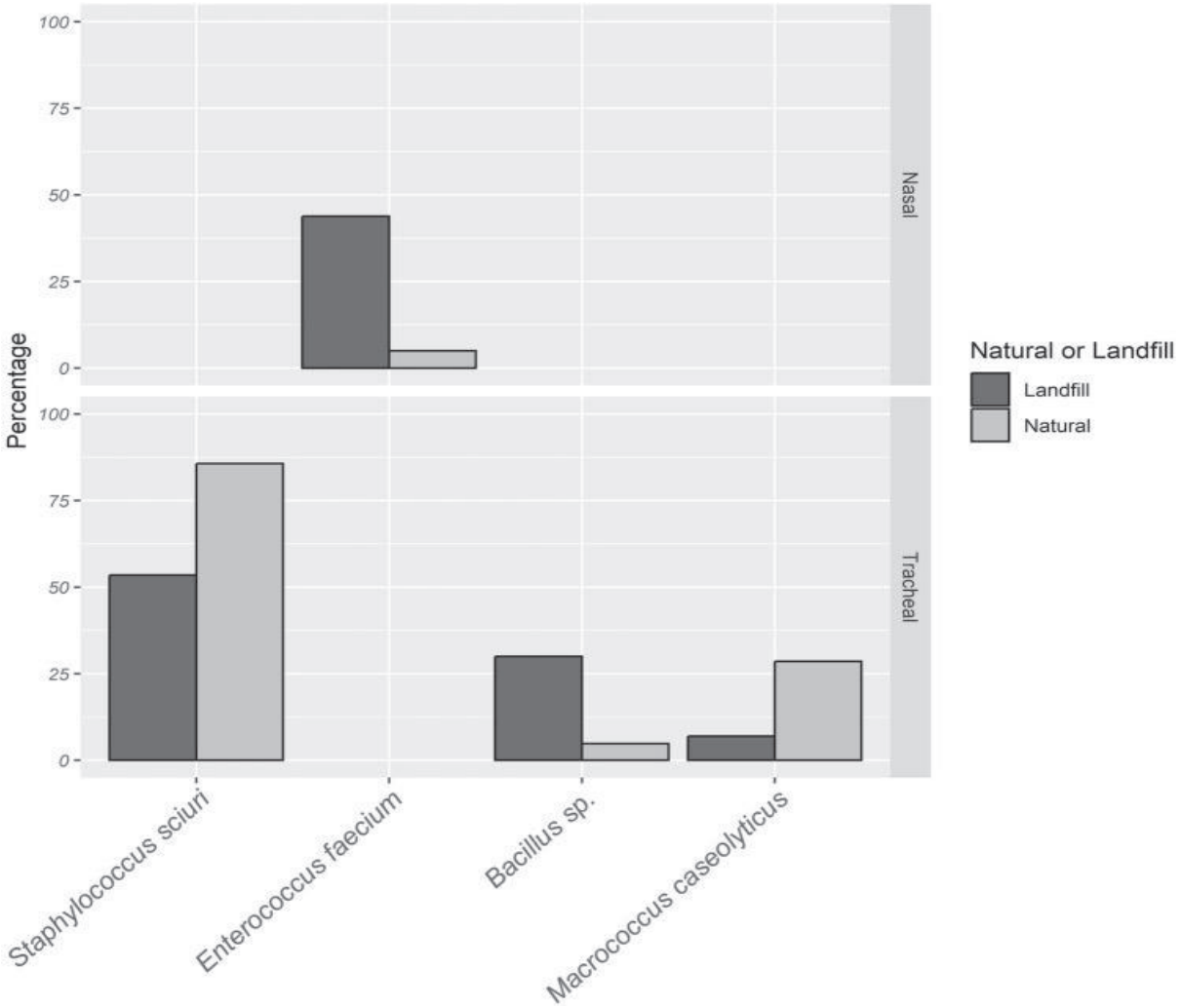


Figure 21. Bacterial species with significant association with foraging habitat of adults in either nasal or tracheal cavities of nestling storks

3.1.1.3 Distribution Pattern of Bacterial Species Based on the Sample Types of White Stork Nestlings

In most cases, the bacteria recovery rates were relatively higher from the nasal than the tracheal cavities **(Table 36).** Significantly higher associations were found in *S. aureus, S. sciuri, S. chromogenes, S. xylosus* with the nasal than the tracheal cavities of the storks (χ^2 test all at d.f. = 1, $p < 0.05$, χ^2 = 10.69, 6.732, 5.644 and 5.433, respectively) **(Figure 22, Table 36).** However, a significantly higher association was obtained in *Proteus* species. with the tracheal than in nasal cavities of the storks (χ^2= 7.131, d.f. = 1, p = 0.0075) **(Table 36).** There was no significant association between all other species identified with the type of samples analysed **(Table 36).**

Table 36. Differences in the presence of each species based on the type of sample (Nasal or Tracheal).

Species	χ^2 (d.f.)	*p* value	Presence
Staphylococcus aureus	10.69 (1)	0.001*	Nasal (37%)> Tracheal (12%)
S. sciuri	6.732 (1)	0.009*	Nasal (88%)> Tracheal (69%)
S. chromogenes	5.644 (1)	0.019*	Nasal (12%)> Tracheal (4%)
S. epidermidis	0.013 (1)	0.909	
S. xylosus	5.433 (1)	0.019*	Nasal (12%)> Tracheal (2%)
S. hominis	0 (1)	0.998	
S. lentus	0.007 (1)	0.936	
S. simulans	2.842 (1)	0.092	
S. saprophyticus	1.163 (1)	0.281	
S. haemolyticus	0 (1)	0.999	
S. capitis	0 (1)	0.999	
S. hyicus	0.001 (1)	0.97	
S. pasteuri	0 (1)	0.999	
S. arlettae	0 (1)	1	
Enterococcus faecalis	2.667 (1)	0.103	
E. faecium	2.888 (1)	0.089	
E. cecorum	0 (1)	1	
E. casseliflavus	0 (1)	1	
E. gallinarum	0.004 (1)	0.951	
E. durans	0 (1)	1	
E. hirae	0 (1)	0.999	
E. canis	0 (1)	1	
Lactococcus garvieae	3.690 (1)	0.055	
Streptococcus gallolyticus	0.214 (1)	0.644	
Proteus spp	7.131	0.008*	Nasal (4%) < Tracheal (18%)
P. vulgaris	0.003 (1)	0.956	
Bacillus spp	0.239 (1)	0.625	
B. subtilis	0 (1)	1	
B. cereus	0 (1)	1	
B. licheniformis	0.105 (1)	0.75	
Macrococcus caseolyticus	0.323 (1)	0.57	
Corynebacterium spp	0.105 (1)	0.746	
C. falsenii	2.325 (1)	0.127	
C. auromucosum	0.001 (1)	0.987	
Micrococcus luteus	0 (1)	1	
Arthrobacter cretinolyticus	0.149 (1)	0.7	
Vagococcus lutrae	0.105 (1)	0.75	
Escherichia coli	0 (1)	0.999	
Acinetobacter junii	0.001 (1)	0.98	
A. baumannii	0.001 (1)	0.987	
Klebsiella pneumoniae	0 (1)	0.999	
Enterobacter cloacae	0 (1)	0.998	
E. asburea	0 (1)	1	
Providencia stuartii	0 (1)	0.999	
P. retgerii	0 (1)	1	
Citrobacter freundii	0 (1)	1	
C. braakii	0 (1)	1	

*Significant association determined by Chi-squared test at 95% CI

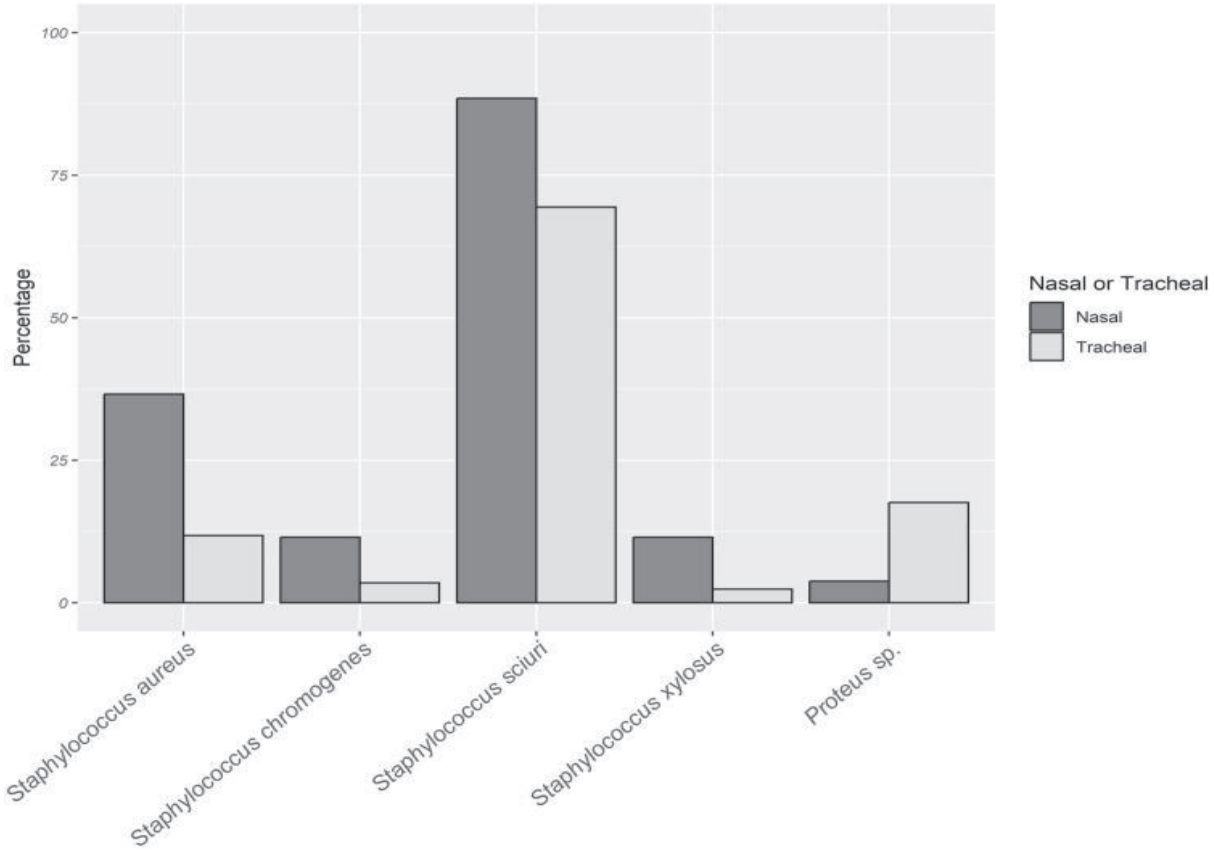


Figure 22. Bacterial species with significant association with sample type of nestling storks.

3.1.1.4 Co-Colonization of Bacteria Species in the Nasal and Tracheal Samples of White Stork Nestlings

In the tracheal cavities, the vast majority of the bacterial species had 1–10% correlation with one another **(Figure 23).** In the remaining species, the highest correlation was between *B. lichenformis* versus *E. hirae* (100.0%), *K. pneumoniae* versus *A. baumanni* (50.0%), *S. haemolyticus* versus *K. pneumoniae* (33.3%), *A. baumanni* versus *S. haemolyticus* (25.0%) and *L. garvieae* versus *E. coli* (25.0%) **(Figure 23).** In the nasal cavities of storks, the majority of the bacterial species had 1–10% correlation between them **(Figure 24).** In the others, the highest correlation was between *S. aureus* versus *E. faecalis* (46.2%), then *S. aureus* versus *S. sciuiri* (35.4%), *S. scuiri* versus *E. faecalis* (32.7%), and *M. caseolyticus* versus *S. chromogenes* (30.0%). Those with between 20.1 and 29.9% correlation included *S. simulans* versus *E. durans* (25.0%), *S. simulans* versus *C. auromucosum* (25.0%), *S. saprophyticus* versus *S. falsenii* (25.0%), *S. sciuri* versus *E. faecium* (27.1%), *L. garvieae* versus *E. casseliflavus* (25.0%), *E. casseliflavus* versus *C. freundii* (25.0%), and *E. casseliflavus* versus *C. braakii* (25.0%) **(Figure 24).**

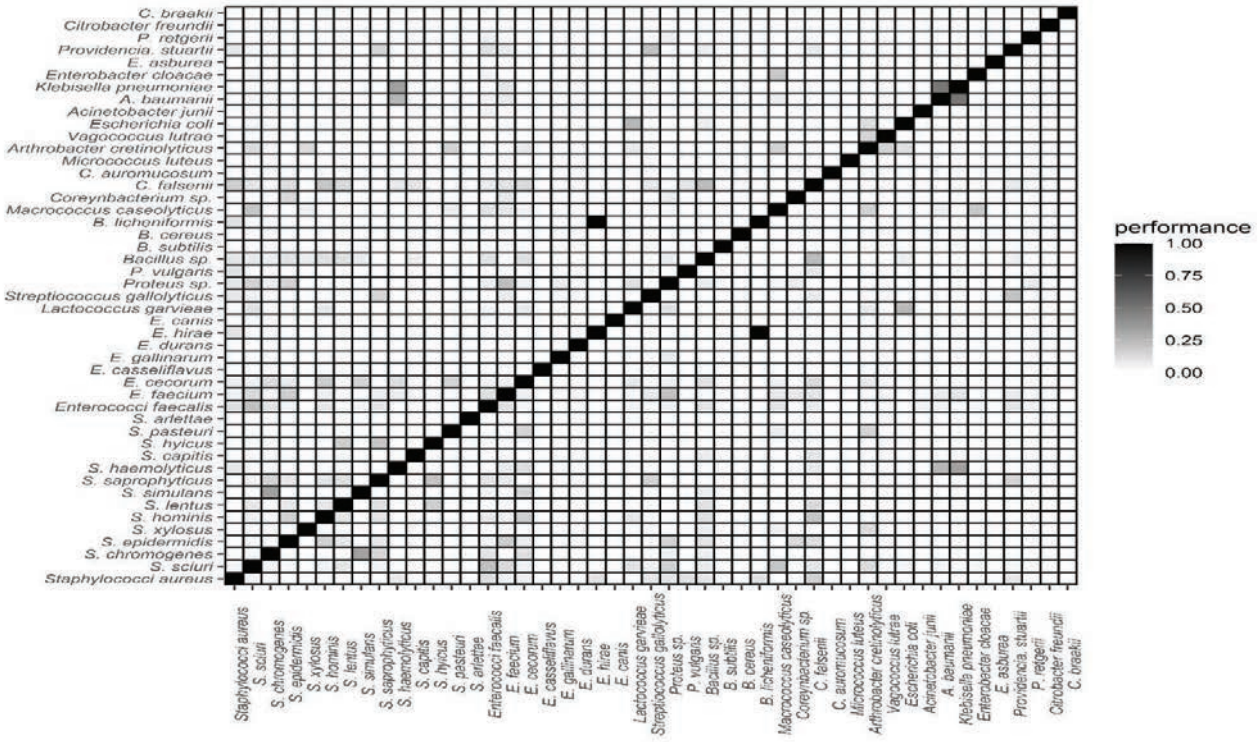


Figure 23. Correlation matrix of bacteria in the tracheal cavity of nestling storks.
NB: The co-colonization index of two bacteria species is directly proportional to the performance level

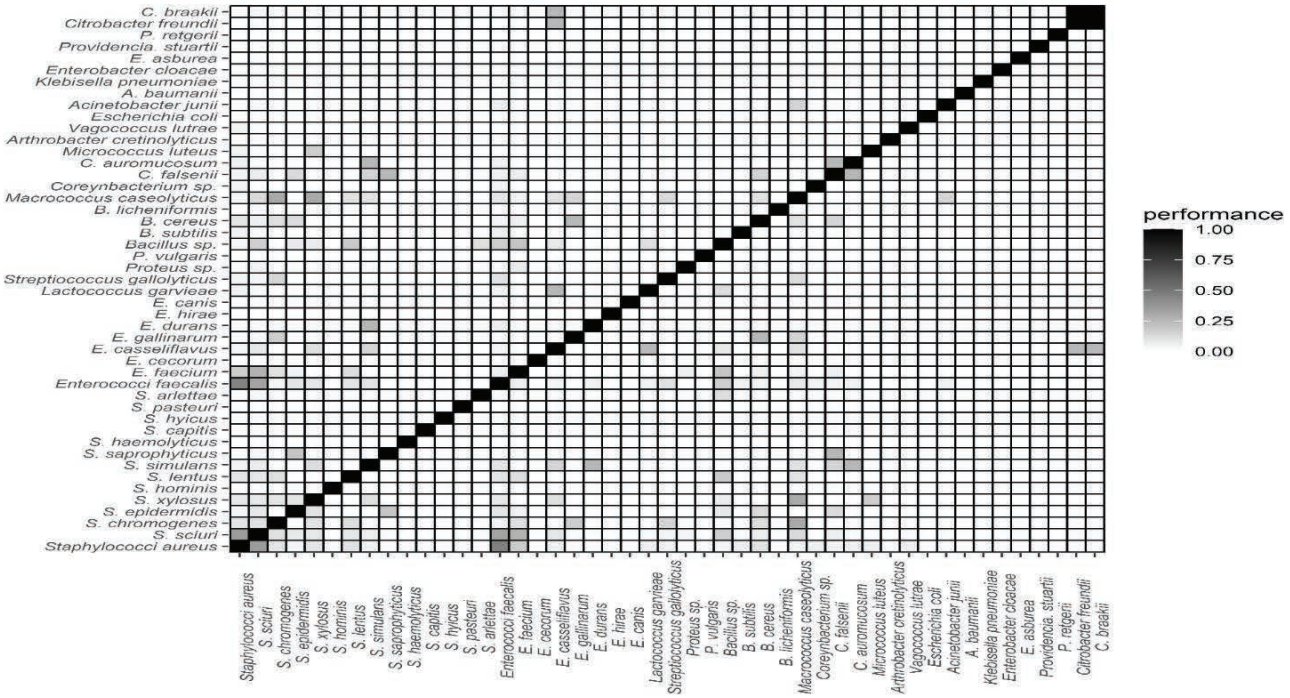


Figure 24. Correlation matrix of bacteria in the nasal cavity of nestling storks.
NB: The co-colonization index of two bacteria species is directly proportional to the performance level.

3.1.1.5 Antimicrobial resistance phenotypes and genotypes and virulence determinants among nasotracheal *S. aureus* strains from stork nestlings

All the 67 *S. aureus* strains recovered of nasal and tracheal samples of white stork nestlings were MSSA, and 7 of them (8.8%) were susceptible to all the antibiotics tested **(Figure 25).** The most frequently identified AMR was to penicillin (PEN^R) (*n* = 53, 79.1%) with *blaZ* detected in 90.6% of PEN^R strains. Other AMR-phenotypes/percentage/genes detected were as follows: erythromycin-clindamycin inducible/ 19.1%/*ermA*, *ermT*; tetracycline/11.9%/*tet*(K); clindamycin/4.5%/*lnuA* and ciprofloxacin/4.5%. Multidrug resistance (resistance to at least 3 different families of antibiotics) was identified in one strain (1.5%) **(Figure 25).** None of the 67 strains tested carried the *luk-S/F-PV* gene but the *tst*, *eta* and *etb* genes were detected in strains of 4 nestling storks.

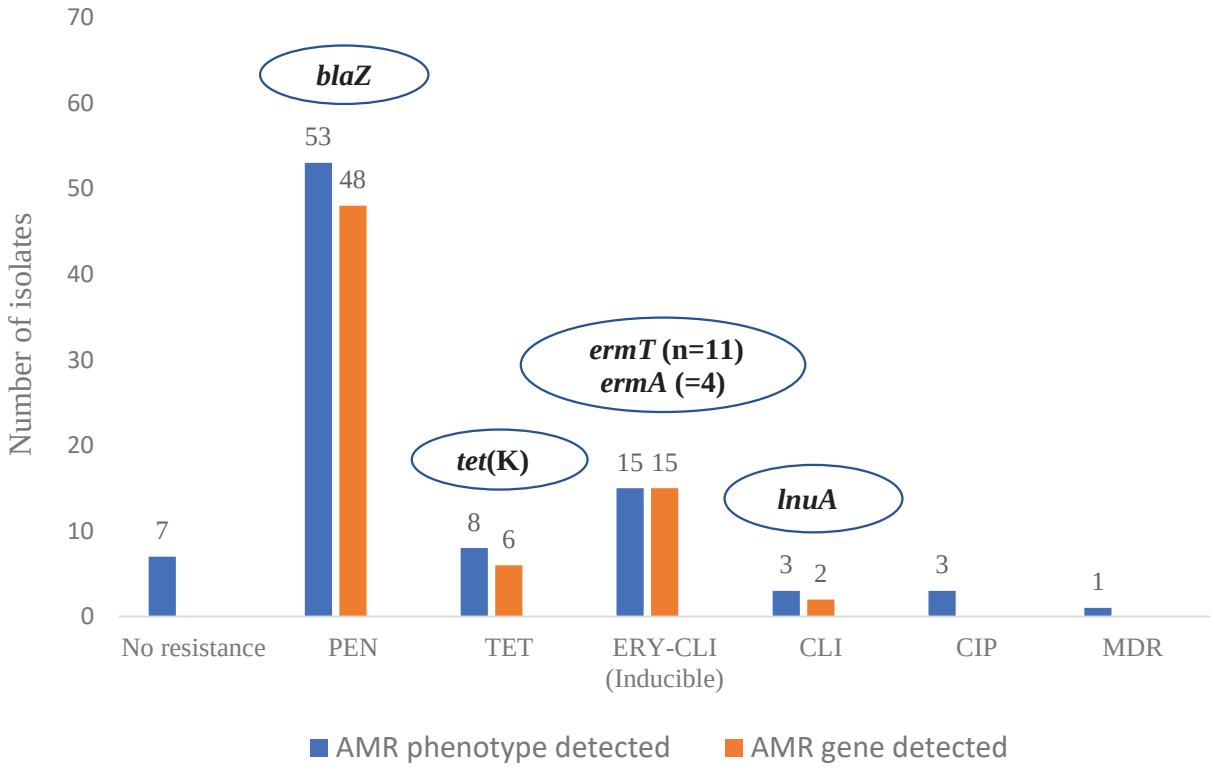


Figure 25. Antimicrobial resistance phenotypes and genotypes in the 67 *S. aureus* strains recovered from white stork nestlings.
Abbreviation: CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; MDR: multi-drug resistance; PEN: penicillin; TET: tetracycline

3.1.1.6 Genetic lineages, antimicrobial resistance genotypes and virulence content

Of all the 67 MSSA strains, 18 different *spa* types were detected including 2 new *spa* types (t7778-ST15-CC15 and t18009-ST26-CC25), and they were ascribed to 11 clonal complexes (CCs): CC1, CC5, CC9, CC15, CC22, CC25, CC30, CC45, CC97, CC130 and CC398 **(Table**

37). Twelve of the MSSA strains (from 7 storks), belonged to CC398 lineage and *spa* types t571 (*n* = 5), t1451 (*n* = 3) and t1456 (*n* = 4); all of them were of the IEC-type C. Six of these CC398 strains were penicillin-resistant and presented the erythromycin-clindamycin inducible resistance phenotype with the corresponding genes *blaZ* and *ermT*. Moreover, two of the MSSA-CC398 strains were ciprofloxacin-resistant **(Table 37).** In addition, 11 strains obtained from six storks corresponded to the lineage MSSA-ST291-t2313/IEC type B, being ST291 a double-locus variant of ST398. Putting together, 14.1% of storks carried MSSA-CC398 or MRSA-ST291 (closely related lineages). Also, two MSSA-CC130 (*spa*-type t6220) strains were identified, and they were susceptible to all antimicrobial agents.

Other genetic lineages detected in high number were the following ones (number of strains): (a) MSSA-CC5-t227/IEC-B (*n* = 6) and CC5-t1094/IEC negative (*n* = 1); (b) MSSA-CC15-t774/IEC type-E (*n* = 5); (c) MSSA-CC15-t085/t335/IEC-C (*n* = 5); (d) MSSA-CC9-t209/IEC negative (*n* = 4) and (e) MSSA-CC45-t015/IEC-B (*n* = 3). All genetic lineages and their corresponding AMR phenotypes are presented in **Table 37.** One *S. aureus* strain showed a multidrug-resistant (MDR) phenotype (PEN-ERY-CLI^{inducible}-CIP) and was typed as MSSA-CC398-t571. Interestingly, two of the storks carried *tst*-positive *S. aureus* strains of different lineages (MSSA-CC22-t223-IEC-B and MSSA-CC30-t1654-IEC-negative, both of them PEN^{R}). Also, *eta*-positive (MSSA-CC9-t209-*scn* negative) and *etb*-positive strains (MSSA-CC45-t015-IEC-B) were detected in two additional storks. However, all strains were *luk-F/S-PV*-negative **(Table 37).**

Stork nestlings whose parents foraged in landfills presented relatively more genetically diverse *S. aureus* strains (10 CCs and lineage ST291) than those of parents foraging in natural habitats (only 3 CCs and lineage ST291) **(Table 38)**. Moreover, all *tst*-, *eta*- and *etb*-positive strains were recovered from nestlings whose parents foraged in landfills. Of the 12 MSSA-CC398 strains obtained from seven positive storks **(Figure 25)**, five strains were from nestlings of parents that foraged in natural habitat while the other seven were obtained from stork nestlings of parents foraging in landfills **(Table 38)**. Aside from MSSA-CC398 strains that were found in high frequency, 11 MSSA-ST291-*spa*-type t2313 strains were also found in 5 storks. Others include MSSA-CC15, MSSA-CC5 and CC97 in 5, 3 and 3 storks, respectively. However, MSSA of the CC9, CC22, CC25, CC30, CC45 and CC130 lineages were all found in one stork each **(Figure 26)**.

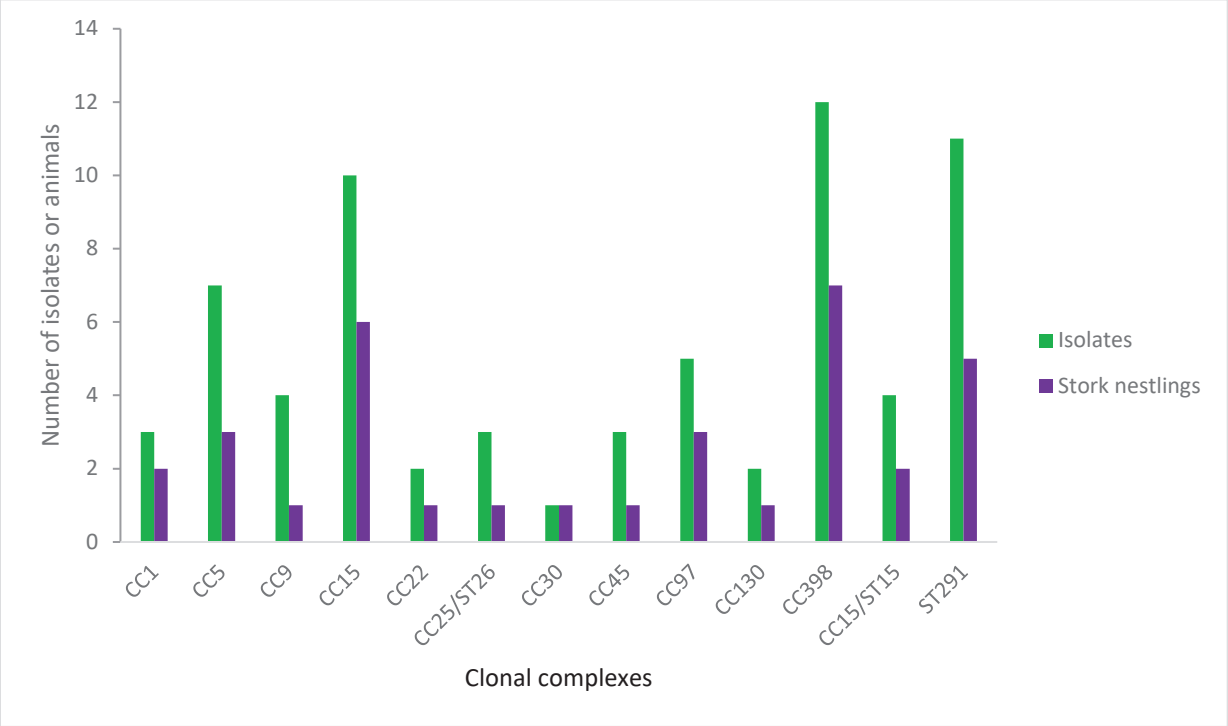


Figure 26. Number of strains and white stork nestlings in which the different *S. aureus* clonal complexes were detected

Table 37 Antimicrobial resistance phenotype and genotype of the 67 MSSA strains in relation to their *spa*-type and clonal complexes

***spa*-type**	**CC[a]**	**N: strains/ N-storks**	**Virulence genes[b]**	**IEC type (N. strains)**	**AMR phenotypes[c]**	**AMR genes[c]**
t571	CC398	5/ 3	ND	C (5)	PEN^{3}-CIP^{2}-ERY^{4}-CLI^{ind4}	*blaZ*^{1}, *ermT*^{4}
t1451	CC398	3/2	ND	C (3)	PEN^{1}-ERY^{3}-CLI^{ind3}	*ermT*^{3}
t1456	CC398	4/2	ND	C (4)	PEN^{2}-ERY^{4}-CLI^{ind4}	*blaZ*^{2}, *ermT*^{4}
t127	CC1	3/2	ND	Negative (3)	CLI^{3}-TET^{3}	*tet*(K)^{3}, *lnuA*^{3}
t227	CC5	6/2	ND	B (6)	PEN^{6}-CLI^{1}	*blaZ*^{6}
t1094	CC5	1/1	ND	Negative	PEN^{1}-CIP^{1}	*blaZ*^{1}
t209	CC9	4/1	*eta*$^{4+}$	Negative	PEN^{4}-ERY^{4}-CLI^{4}	*blaZ*^{4}, *ermA*^{4}
t085	CC15	5/2	ND	C (5)	PEN^{5}	*blaZ*^{5}
t774	CC15	5/3	ND	E (5)	PEN^{5}-TET^{5}	*blaZ*^{4}, *tet*(K)^{4}
t7778[d]	CC15/ST15	4/2	ND	C (1), negative (3)	PEN^{3}	*blaZ*^{3}
t223	CC22	2/1	*tst*$^{2+}$	B (2)	PEN^{2}	*blaZ*-negative
t18009[d]	CC25/ST26	3/1	ND	B (3)	PEN^{3}	*blaZ*^{3}
t1654	CC30	1/1	*tst*$^{1+}$	Negative	PEN	*blaZ*^{1}
t015	CC45	3/1	*etb*$^{3+}$	B (3)	PEN^{3}	*blaZ*^{3}
t521	CC97	2/1	ND	E (2)	Susceptible	NT[f]
t3380	CC97	3/2	ND	E (3)	PEN^{1}	*blaZ*^{1}
t6220	CC130	2/1	ND	Negative (2)	Susceptible	NT[f]
t2313	ST291[e]	11/5	ND	B (12)	PEN^{11}	*blaZ*^{12}

[a] CC assigned according to the *spa*-type, except for CC398 (determined by specific PCR) and CC25/ST26, CC15/ST15 and ST291 (by MLST); [b] All *tst*, *eta*, *etb* positive strains were confirmed by sequencing; [c] In superscript is the number of strains that presented the specific phenotype/genotype; [d]New *spa*-types. ;[e]ST291 is a double locus variant of ST398.;[f]NT: Not tested. ; ND: Not detected (negative for *lukS-PV/lukF-PV*, *tst*, *eta*, *etb*); Abbrev: CLI^{ind}: Clindamycin inducible; CLI: Clindamycin; CIP: Ciprofloxacin; ERY: Erythromycin; PEN: Penicillin; TET: Tetracycline.

Table 38. Genetic lineage variation of *S. aureus* strains from white stork nestlings according to the foraging habits of their parents.

Genetic lineage	*spa*-types (number) detected in nesting of parent foraging in:			
	Natural Habitat		Landfills	
	Nasal samples	Tracheal samples	Nasal samples	Tracheal samples
CC1	ND	ND	t127 (3)	ND
CC5	ND	ND	t1094 (1)	t227 (6)
CC9	ND	ND	ND	t209 (4)
CC30	ND	ND	t1654 (1)	ND
CC15	ND	ND	t085 (4), t774 (1), t335 (1)	t774 (4)
CC15/ST15	ND	ND	t085 (1), t7778 (2)	t7778 (2)
CC22	ND	ND	t309 (2)	ND
CC25/ST26	ND	ND	ND	t18009 (3)
CC45	ND	ND	t015 (3)	ND
CC97	t3380 (1)	t3380 (1)	t521 (2), t3380 (1)	ND
CC130	ND	t6220 (2)	ND	ND
CC398	t1456 (3)	t571 (1), t1456 (1)	t571 (4), t1451 (3)	ND
ST291	t2313 (4)	t2313 (1)	t2313 (5)	t2313 (2)
Total	8	5	33	21

ND: Not detected

3.1.1.7 Intra-sample variation of genetic lineages or AMR genotypes of *S. aureus* strains from stork nestlings

Five of 27 storks positive for *S. aureus* (18.5%) harboured *S. aureus* strains with diverse *spa*-types or AMR genotypes in the same animal. Between 2 to 5 genetically distinct *S. aureus* strains were detected in these animals **(Table 39)**. Two storks had genetic lineage variation and AMR phenotypes/genotypes in the tracheal *S. aureus* carriages: (1) MSSA-ST291-t2313-PENR-*blaZ* and MSSACC130-t6620-PEN^S and (2) two MSSA-CC15-t774 and one MSSA-t7778 strain **(Table 39).** Moreover, a stork with nasal carriage of MSSA-CC398 had 2 distinct *spa* types (t571 and t1451): one of the MSSA-CC398 strains was completely susceptible to antibiotics, whereas the other carried the *ermT* gene **(Table 39)**.

3.1.1.8 Antimicrobial resistome of coagulase-negative staphylococci

Out of the 341 CoNS strains previously identified of white storks, 268 were considered non-repetitive when the antimicrobial resistance phenotype was determined: they corresponded to one strain of each species per sample or more than one if they presented different AMR phenotypes **(Table 40).** This collection of 268 non-repetitive CoNS strains were used in further analyses**.** For the study of the rates of antibiotic resistance, the collection of non-repetitive *S. sciuri* strains (n = 191) and non-*S. sciuri* (NSc)-CoNS strains (n = 77) were analysed independently.

Table 39. Intra-sample and intra-host variation of genetic lineages or AMR genotypes of *S. aureus* strains

Stork's ID	Sample type	Number of strains	IEC type	AMR phenotypes (gene detected)	*spa*-type/ CC
436	Tracheal	1	B	PEN (*blaZ*)	t2313
	Tracheal	2	Negative	Susceptible	t6620/CC130
489	Nasal	1	C	PEN (*blaZ*)	t7778/ST15/CC15
	Nasal	1	Negative	PEN (*blaZ*)	t1094/CC5
	Nasal	1	C	Susceptible	t7778/ST15/CC15
490	Tracheal	2	Negative	PEN (*blaZ*)	t7778/ST15/CC15
	Tracheal	1	E	PEN (*blaZ*)	t774/CC15
505	Nasal	1	C	PEN-ERY-CLI^{ind} (*ermT*)	t571/ CC398
	Nasal	2	C	PEN-ERY-CLI^{ind} (*ermT*)	t1451/CC398
	Nasal	1	C	Susceptible	t571/ CC398
546	Nasal	3	Negative	CLI-TET (*tet*(K), *lnuA*)	t127/CC1
	Nasal	1	E	Susceptible	t3380/ CC97
	Nasal	1	E	PEN-TET (*blaZ*, *tet*(K))	t774/CC15
	Tracheal	3	B	PEN (*blaZ*)	t227/CC5

Abbreviation: CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; PEN: penicillin; TET: tetracycline; IEC: Immune Evasion Cluster;
AMR: Antimicrobial Resistance

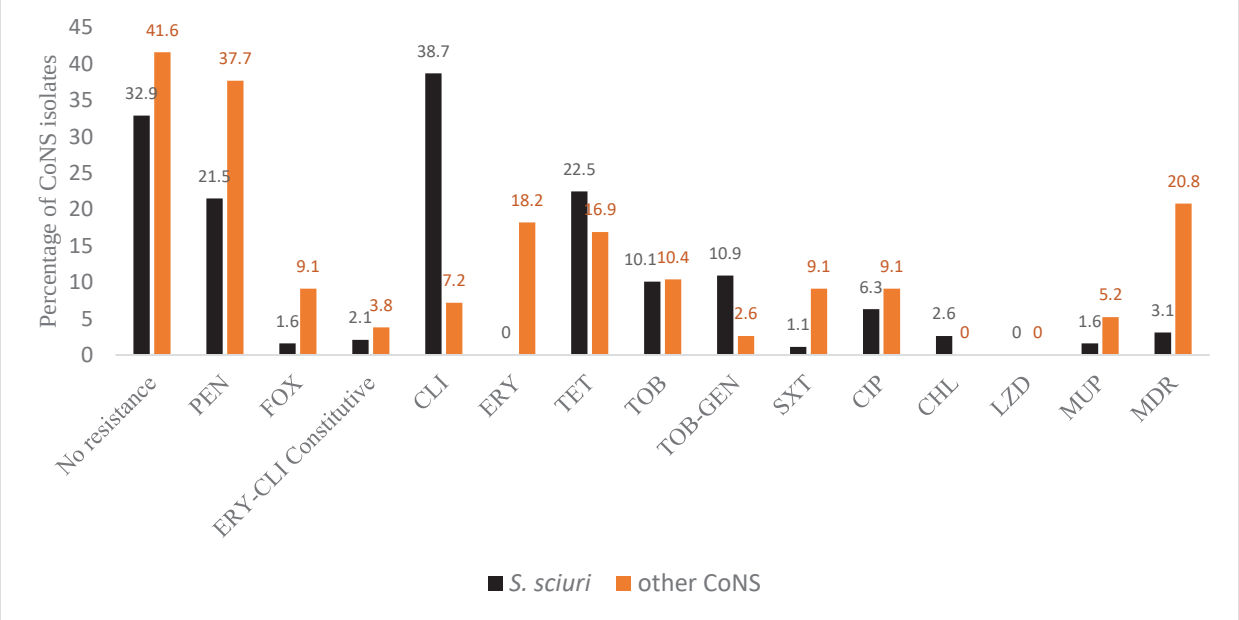


Figure 27. Frequency of antimicrobial resistance exhibited by the coagulase-negative staphylococci strains from nasal and tracheal samples of stork nestlings.
Note: The number of *S. sciuri* and non-*sciuri*-CoNS strains were 191 and 77, respectively.
Abbreviation: CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; LZD: linezolid; MUP: mupirocin; MDR: multi-drug resistance (resistance to at least three families of antibiotics; in the case of *S. sciuri*, clindamycin resistance was not considered); PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin.

Table 40. Number of coagulase-negative staphylococci species from all stork nestlings and those with distinct AMR and MDR phenotype

CoNS species	Total CoNS strains	Non-repetitive CoNS strains[a]			
		Total number	Strains susceptible to all antibiotics tested	Strains resistant to only one antibiotic	Strains with MDR[b] phenotype
S. sciuri	251	191	137	27	6
S. epidermidis	17	16	6	1	4
S. hominis	7	7	1	1	1
S. lentus	10	10	0	1	7
S. chromogenes	20	16	12	1	0
S. saprophyticus	6	5	3	0	1
S. xylosus	11	11	4	5	1
S. capitis	1	1	0	1	0
S. hyicus	5	1	1	0	0
S. simulans	8	6	4	2	0
S. haemolyticus	2	2	0	1	1
S. pasteuri	1	1	1	0	0
S. arlettae	2	1	0	0	1
Total (%)	**341**	**268 (78.6)**	**169 (63.1)**	**40 (14.9)**	**22 (8.2)**

[a]Non-repetitive strains: one strain of each species/sample or more than one if they presented different AMR phenotypes
[b]MDR: resistance to at least 3 families of antibiotics. In *S. sciuri*, clindamycin resistance was not considered for MDR analyses (this species has an intrinsic mechanism of clindamycin resistance, *salA* gene).

3.1.1.8.1 Phenotypes and genotypes of resistance of *S. sciuri* strains

The phenotypes of resistance of the 191 *S. sciuri* strains are shown in **Figure 27**. All *S. sciuri* strains carried the intrinsic *salA* gene (associated with clindamycin resistance) and 39% and 33% of them were clindamycin-resistant and clindamycin-intermediate, respectively; 28% of the strains showed clindamycin susceptibility, even with the *salA* gene **(Figure 28).** As this mechanism of resistance is intrinsic in *S. sciuri* strains, we did not consider this antibiotic for the analysis of the phenotypes of resistance or the MDR phenotype in this species. Two clindamycin-resistant strains carried another clindamycin resistance gene (*lnuA*), in addition to *salA* **(Figure 28).** About 71.7% of the *S. sciuri* strains showed susceptibility to all antimicrobials tested (without consideration of clindamycin) and 27 (14.1%) strains showed resistance to only one antimicrobial (mainly penicillin, tobramycin or tetracycline) **(Table 40 and Figure 29a).** The following AMR phenotypes and genotypes were detected among the *S. sciuri* strains (percentage of resistant strains/ genes detected): penicillin (21.5/ *blaZ*), erythromycin-clindamycin-constitutive (2.1/ *ermA*, *ermC*), tetracycline (22.5/ *tet*(K), *tet*(L), *tet*(M)), tobramycin (10.1/ *ant4′*), tobramycin-gentamicin (10.9/ *aac6′-aph2″*),

sulfamethoxazole-trimethoprim (1.1/ *dfrA, dfrG, dfrK*), chloramphenicol (2.6/ *fexA, fexB,* cat_{PC221}), mupirocin (1.6/ *mupA*) and ciprofloxacin (6.3/ non tested) **(Figure 27 and Table 41).** None of the CoNS strains showed linezolid resistance **(Figure 27),** whereas three *mecA*-positive methicillin-resistant *S. sciuri* strains were recovered **(Table 41 and Figure 27).**

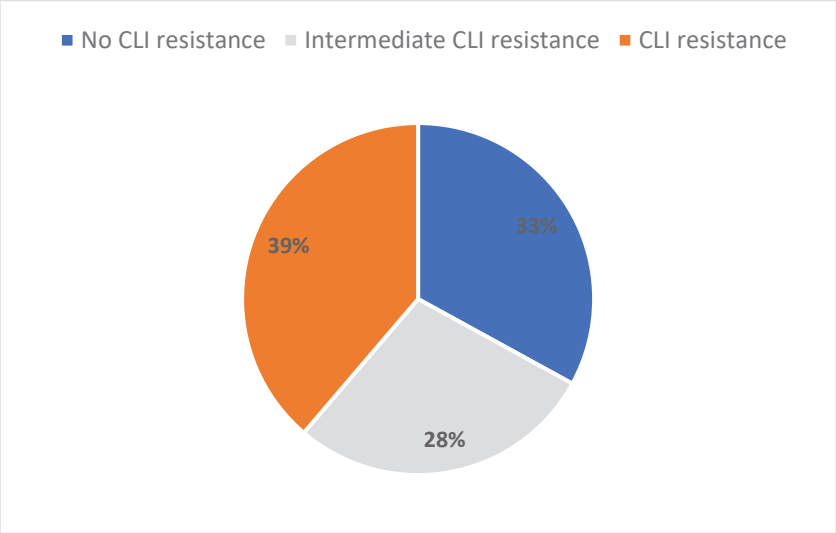


Figure 28. Phenotypes of clindamycin resistance among all the *S. sciuri* strains with *salA* gene
Note: Two clindamycin-resistant *S. sciuri* co-harboured *lnuA* gene in addition to the *salA* gene.

3.1.1.8.2 Phenotypes and genotypes of resistance of NSc-CoNS

Of the 77 NSc-CoNS strains, 41.6% were susceptible to all antibiotics tested and 16.9% showed resistance to only one antimicrobial agent (mainly penicillin, erythromycin, tetracycline or clindamycin) **(Table 40 and Figure 29b).** The rate of AMR phenotypes and genes detected among the NSc-CoNS strains were as follows (percentage of strains/ genes detected): penicillin (37.7/ *blaZ*, bla_{ARL}), methicillin (9.1/*mecA*, *mecC*), erythromycin-clindamycin-constitutive (3.8/*ermA*, *ermC*, *ermT*), clindamycin (7.2/*lnuA*, *vgaA*), erythromycin (18.2/*msrA*, *mphC*), tetracycline (16.9/ *tet*(K), *tet*(L), *tet*(M)), tobramycin (10.4/ *ant4′*), tobramycin-gentamicin (2.6/ *aac6′-aph2″*), sulfamethoxazole-trimethoprim (9.1/ *dfrA, dfrG, dfrK*), mupirocin (5.2/ *mupA*), and ciprofloxacin (9.1/ not tested) **(Figure 27 and Table 41).** The bla_{ARL} gene was identified in the unique *S. arlettae* strain of this collection **(Table 41).** Interestingly, one *S. epidermidis* strain carried the *ermT* gene **(Table 41)** and one methicillin-resistant *S. lentus* strain carried both *mecA* and *mecC* genes. None of the strains was resistant to chloramphenicol or linezolid **(Figure 27),** whereas six *mecA*-positive methicillin-resistant NSc-CoNS strains were recovered (3 *S. epidermidis*, 2 *S. hominis*, and 1 *S. haemolyticus*) **(Table 42 and Figure 27).**

a) b)

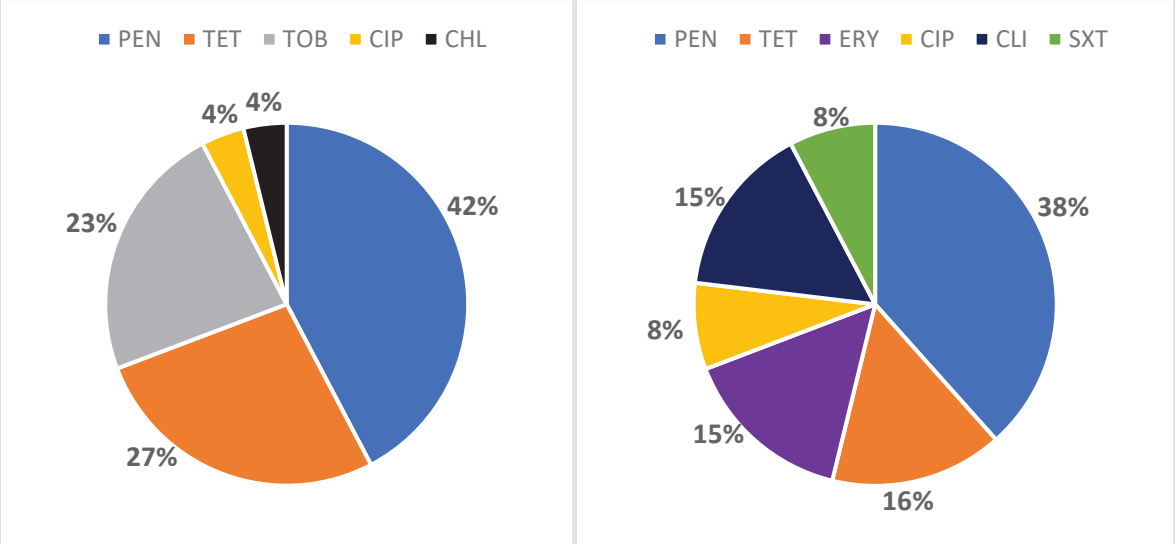


Figure 29. (a) Frequency of *S. scuiri* and (b) NSc-CoNS showing resistance to only one of the antibiotics tested.
Abbreviation: CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin: ERY: erythromycin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline; TOB: tobramycin.
Note: The number of *S. scuiri* and NSc-CoNS strains showing resistance to only one antibiotic were 27 and 14, respectively

Table 42. SCC*mec* types and AMR genes in methicillin-resistant CoNS strains from stork nestlings

Strain	Stork ID/type of sample	*SCCmec* type	AMR genes detected
S. hominis X3726	531/ Tracheal	III	*mecA, blaZ*
S. hominis X3764	507/ Tracheal	IV	*mecA, blaZ, msrA*
S. sciuri X3904	469/ Tracheal	IV	*mecA, blaZ, ant4', lnuA, salA*
S. sciuri X3905	469/ Tracheal	Non-typeable	*mecA, blaZ*
S. sciuri X4574	536/ Nasal	IV	*mecA, tet*(K), *lnuA, salA*
S. epidermidis X3736	439/ Tracheal	Non-typeable	*mecA, blaZ*
S. epidermidis X3727	532/ Tracheal	Non-typeable	*mecA, blaZ*
S. epidermidis X3834	558/ Tracheal	IV	*mecA, blaZ, msrA, mupA*
S. *haemolyticus* X3784	546/ Tracheal	V	*mecA, blaZ, tet*(K), *dfrG, ant4'*
S. lentus/ X4638	507/Nasal	Hybrid VII	*mecA, mecC, blaZ, mphC, tet*(K)

SCC*mec*: Staphylococcal chromosomal cassette *mec*

Table 41. Antimicrobial resistance phenotypes and genotypes in coagulase-negative *Staphylococcus* species identified from nasal and tracheal samples of stork nestlings.

Species with antimicrobial resistance (number of strains)	**Antimicrobial resistance**	
	Phenotypes (number strains)	**Genotypes (number strains)**
S. sciuri (41), *S. epidermidis* (9), *S. xylosus* (5), *S. lentus* (7), *S. simulans* (1), *S. hominis* (4), *S. saprophyticus* (2), *S. haemolyticus* (1)	PEN (70)	*blaZ* (43)
S. arlettae (1)	PEN (1)	bla_{ARL} (1)
S. sciuri (3), *S. epidermidis* (3), *S. hominis* (2), *S. haemolyticus* (1), *S. lentus* (1)	FOX (10)	*mecA* (10), *mecC* (1)*
S. sciuri (1), *S. lentus* (1), *S. arlettae* (1)	ERY-CLI constitutive (3)	*ermA* (3), *ermC* (2)
S. hominis (10), *S. saprophyticus* (2), *S. haemolyticus* (1), *S. lentus* (1)	ERY (13)	*msrA* (13), *mphC* (11)
S. epidermidis (1)	ERY-CLI constitutive (1)	*ermT* (1)
S. epidermidis (1), *S. xylosus* (1), *S. lentus* (4)	CLI (7)	*lnuA* (3), *vgaA* (3)
S. sciuri (74)	CLI (74)	*lnuA* (2), *salA* (74)
S. sciuri (21), *S. chromogenes* (1), *S. lentus* (1)	GEN-TOB (23)	*aac6'-aph2"* (23)
S. sciuri, S. epidermidis, S. lentus, S. hominis,S. saprophyticus, S. arlettae	TOB (29)	*ant4'* (29)
S. sciuri (12), *S. lentus* (2), *haemolyticus* (1), *S. arlettae* (1)	CIP (16)	NT
S. sciuri (23), *S. epidermidis* (2), *S. xylosus* (3), *S. lentus* (3), *S. haemolyticus* (1), *S. arlettae* (1), *S. saprophyticus* (1)	TET (34)	*tet*(K) (34), *tet*(L) (10), *tet*(M) (5)
S. sciuri (3), *S. epidermidis* (3), *S. saprophyticus* (1), *S. haemolyticus* (1), *S. lentus* (1)	SXT (9)	*dfrA* (9), *dfrG* (1), *dfrK* (3)
S. sciuri (5)	CHL (5)	*fexA* (3), *fexB* (1), cat_{PC221} (2)

CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; MUP: mupirocin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin
NT: non-tested.
*: An *S. lentus* strain carried both *mecA* and *mecC* gene

3.1.1.9 Multidrug-resistant strains and SCC*mec* mobile element types

Of the 268 CoNS strains, 6 (3.1%) and 16 (20.8%) of *S. sciuri* and NSc-CoNS strains, respectively, showed multidrug resistance **(Tables 42 and 43, Figure 27)**. Of the MRCoNS, one *S. hominis* and one *S. haemolyticus* strains carried the SCC*mec* types III and V, respectively. Moreover, one each of *S. hominis* and *S. epidermidis* and two *S. sciuri* strains carried the SCC*mec* type IV element **(Table 42).** Remarkably, one methicillin-resistant *S. lentus* was positive for the *blaZ*-SCC*mec* XI gene. However, no consensus SCC*mec* type was determined for other MR-CoNS, either because they were not ascribed to a previously known SCC*mec* type, or because they were non-typeable with the primers utilized in this study.

A high diversity of resistance genes was detected among the CoNS with MDR phenotype, carrying up to five acquired resistance genes conferring resistance to up to 4 families of antibiotics (**Table 43**). MDR-*S. sciuri* was significantly more frequent in nestlings of parent storks foraging in natural areas than foraging in landfills ($\chi 2$ =7.59, p = 0.006). However, MDR in NSc-CoNS strains was significantly more frequent in nestlings of parent storks foraging in landfills than those in natural areas ($\chi 2$ = 7.845, p = 0.005). Collectively, MDR in all the CoNS was relatively higher in nestlings of parent storks foraging in landfills than those in natural areas; however, there was no significant association between MDR phenotype among all the CoNS of the nestlings and the foraging habit of the parent storks ($\chi 2$ = 3.421, df= 1, p = 0.064) **(Figure 30).**

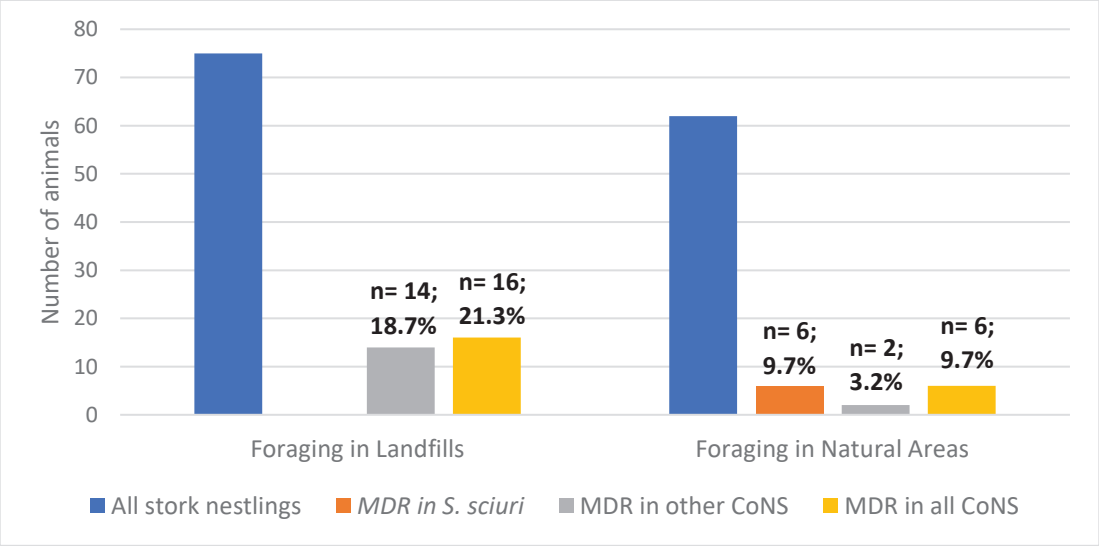


Figure 30. Frequency of stork nestlings carrying multidrug-resistant *S. sciuri* and other coagulase-negative staphylococci based on foraging habitat.

Test statistics for *S. sciuri:* χ^2 = 7.59, df= 1, p= 0.006; Test statistics for non-*sciuri*-CoNS: χ^2 = 7.845, df= 1, p= 0.005; Test statistics for all CoNS: χ^2 = 3.56, df= 1, p= 0.064

Table 43. Antimicrobial resistance determinants identified in CoNS carrying multi-drug resistant (MDR) phenotype in stork nestlings

Stork's ID code	Strains	Stork's foraging habit	Sample type	MDR phenotype	AMR genes detected
427	*_S. sciuri_/ X3969	Natural	Tracheal	SXT-CHL-TOB-GEN-TET	*salA, dfrA, dfrG, fexA, aac6'-aph2", tet*(K)
444	*_S. sciuri_/ X4074	Natural	Tracheal	CLI-CIP-TOB-GEN-TET	*salA, aac6'aph2", tet*(K)
449	*_S. sciuri_/ X4087	Natural	Tracheal	CHL-CLI-TET-CIP	*salA, lnuA, fexA, cat*$_{PC221}$*, tet*(K), *tet*(L),
453	*_S sciuri_/ X4092	Natural	Tracheal	CLI -TOB-TET-MUP-CIP	*salA,lnuA, ant4', tet*(K), *mupA*
477	*_S. sciuri_/ X4534	Natural	Nasal	PEN-FOX-ERY-CLI-TOB-GEN-TET- CIP	*salA,blaZ, mecA, ermA, aac6'-aph2", tet(K)*
476	*_S. sciuri_ / X3937	Natural	Tracheal	PEN-TOB-MUP	*salA, blaZ, ant4', mupA*
439	_S. lentus_/ X4118	Natural	Tracheal	PEN-CLI-SXT-TOB-GEN-TET	*blaZ, lnuA, dfrG, aac6'aph2", tet*(K), *tet*(L)
533	_S. lentus_/ X4586	Landfill	Nasal	ERY-CLI-TOB-TET	*ermC, ant4', tet*(K)
538	_S. lentus_/ X3751	Landfill	Tracheal	PEN-ERY-CLI-CIP	*blaZ, ermA, lnuA*
539	_S. lentus_/ X3753	Landfill	Tracheal	PEN-ERY-CLI-CIP	*blaZ, ermA*
507	_S. lentus_/ X4638	Landfill	Nasal	PEN-FOX-CLI-TET	*blaZ, mecA, mecC, mphC, tet*(K)
542	_S. lentus_/ X3863	Landfill	Tracheal	PEN-CLI-CIP	*blaZ, vgaA*
487	_S. epidermidis_/ X4430	Landfill	Nasal	PEN-ERY-SXT- MUP	*blaZ, msrA, mupA, dfrA*
488	_S. epidermidis_/ X4615	Landfill	Nasal	PEN-ERY-CLI-TET-MUP	*blaZ. ermA, ermC, tet*(K), *mupA*
489	_S. epidermidis_/ X3875	Landfill	Tracheal	PEN-TOB-TET	*blaZ, ant4', tet*(K)
558	_S. epidermidis_/ X3834	Landfill	Tracheal	PEN-FOX-ERY-MUP	*blaZ, mecA, msrA, mupA*
546	_S. haemolyticus_/ X3784	Landfill	Tracheal	PEN-FOX-SXT-CIP-TOB-TET	*blaZ, mecA, tet*(K), *dfrG, ant4'*
510	_S. hominis_/ X3892	Landfill	Tracheal	PEN-ERY-TOB	*blaZ, msrA, ant4'*
505	_S. saprophyticus_/ X3866	Landfill	Tracheal	PEN-ERY-TOB-TET	*blaZ, msrA, ant4', tet*(K)
444	_S. xylosus_/ X4076	Natural	Tracheal	CLI- TET- CIP	*lnuA, tet*(K)
535	_S. arlettae_/ X4721	Landfill	Nasal	PEN-ERY-CLI-TOB-TET- CIP	*bla*$_{ARL}$*, ermA, lnuA, ant4', tet*(K)

CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; MUP: mupirocin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin

*MDR in *S. sciuri* is defined by ≥4 AMR phenotypes including CLI. For other species, MDR was defined by ≥ 3 AMR phenotypes.

3.1.1.10 Co-occurrence of CoNS species, intra-host species and intra-species AMR diversity in the same nestling stork

Twenty-six of the 87 storks (29.9%) harboured more than one non-repetitive CoNS (with intra-host species AMR and intra-host species diversities) either in the nasal, tracheal or both samples. In twelve nestling storks, *S. sciuri* strains were found with other NSc-CoNS (intra-host species diversity) such as *S. epidermidis*, *S. lentus*, *S. xylosus* and *S. chromogenes*. Whereas intra-species AMR diversity in *S. sciuri* (2–4 AMR profiles) was detected in ten nestling storks **(Table 44)**.

Table 44. Intra-host species and intra-species AMR diversity of CoNS by foraging habit and sample types of white storks

Stork ID code	Sample type	Foraging habit	CoNS Species	AMR Phenotypes	AMR genes detected
426	Trachea	Natural	*S. sciuri*	CLI	*salA, vgaA*
			S. sciuri	TOB	*salA, ant4'*
427	Trachea	Natural	*S. sciuri*	SXT-TOB-GEN	*dfrA, dfrG, aac6'-aph2"*
			S. sciuri	SXT-TOB-CHL-GEN-TET	*salA, dfrA, dfrG, aac6'-aph2", fexA, tet*(K)
			S. sciuri	TET	*salA, tet*(K)
			S. capitis	TET	*tet*(K), *tet*(M)
432	Trachea	Natural	*S. sciuri*	TET	*salA, tet*(K)
			S. sciuri	CLI	*salA*
			S. chromogenes	TOB-GEN	*aac6'-aph2"*
			S. saprophyticus	SXT-ERY	*dfrA, msrA*
433	Trachea	Natural	*S. sciuri*	PEN-CIP	*salA, blaZ*
			S. sciuri	CLI-TOB-TET	*salA, ant4'. tet*(K)
			S. sciuri	TOB-GEN	*salA, aac6'-aph2"*
435	Trachea	Natural	*S. sciuri*	CLI-GEN	*salA, aac6'-aph2"*
			S. sciuri	CLI-TOB-GEN	*salA, lnuA, aac6'-aph2"*
			S. sciuri	PEN-CLI-TET	*salA, blaZ, tet*(K)
436	Trachea	Natural	*S. sciuri*	CLI-TOB-GEN	*salA, aac6'-aph2"*
			S. sciuri	TET-MUP	*salA, tet(K), mupA*
437	Trachea	Natural	*S. lentus*	PEN-CLI	*blaZ, lnuA*
			S. sciuri	PEN-TOB-CLI	*salA, blaZ, ant4'*
441	Trachea	Natural	*S. sciuri*	PEN-CLI-TOB-GEN	*salA, blaZ, lnuA, aac6'-aph2"*
			S. sciuri	CLI	*salA*
443	Trachea	Natural	*S. sciuri*	CLI-GEN	*salA, aac6'-aph2"*
			S. sciuri	PEN	*blaZ*
	Nasal	Natural	*S. xylosus*	TET	*tet*(K)
			S. sciuri	TOB	*salA, ant4'*
444	Trachea	Natural	*S. sciuri*	CLI-TOB-GEN-CIP	*salA, aac6'-aph2"*
			S. sciuri	CLI- TOB-GEN-TET- CIP	*salA, lnuA, aac6'-aph2", tet*(K)
			S. sciuri	CLI-CIP	*salA*
			S. sciuri	TOB-GEN-TET	*salA, aac6'-aph2", tet*(K), *tet*(L)
			S. xylosus	CLI-TET- CIP	*lnuA, tet*(K)
453	Trachea	Natural	*S. sciuri*	TOB-GEN-TET	*salA, aac6'-aph2", tet(K)*
			S. sciuri	CLI-TET	*salA, tet(K)*
			S. sciuri	CLI-CIP-TOB-TET-MUP	*salA, lnuA, ant4', tet(K), mupA*
469	Trachea	Natural	*S. sciuri*	PEN	*salA, blaZ*
			S. sciuri	TET	*salA, tet(K)*
			S. sciuri	PEN-CLI	*salA, blaZ, lnuA, salA*
			S. sciuri	PEN-FOX-CLI-TOB	*salA, blaZ, mecA, salA, ant4'*
473	Trachea	Natural	*S. sciuri*	PEN-FOX-CLI-TOB	*salA, blaZ, mecA, ant4'*
			S. sciuri	PEN-TOB-MUP	*salA, blaZ, ant4', mupA*
			S. sciuri	PEN	*salA, blaZ*
477	Trachea	Natural	*S. sciuri*	PEN-CLI	*salA, blaZ*
			S. sciuri	PEN-CLI-TOB	*salA, blaZ, ant4'*

Table 44. Continuation

Stork ID code	Sample type	Foraging habit	CoNS Species	AMR Phenotypes	AMR genes detected
505	Trachea	Landfill	*S. saprophyticus* *S. epidermidis*	PEN-ERY-TOB-TET PEN-ERY	*blaZ, msrA, ant4'. tet*(K) *blaZ, mphC*
507	Trachea	Landfill	*S. hominis* *S. sciuri*	PEN-FOX-ERY PEN-CLI-TOB	*blaZ, mecA, msrA* *salA, blaZ, ant4'*
	Nasal	Landfill	*S. lentus* *S. sciuri*	PEN-FOX-CLI-TET PEN-CLI	*blaZ, mecA, mecC, mphC, tet*(K) *salA, blaZ*
531	Trachea	Landfill	*S. hominis* *S. lentus*	PEN-FOX PEN-ERY-CLI	*blaZ, mecA* *blaZ, ermA*
538	Trachea	Landfill	*S. lentus* *S. sciuri*	PEN-ERY-CLI-CIP TET-CLI	*blaZ, ermA, lnuA* *tet(K), lnuA*
539	Trachea	Landfill	*S. sciuri* *S. epidermidis* *S. lentus*	CLI PEN-FOX PEN-ERY-CLI-CIP	*salA* *blaZ, mecA* *blaZ, ermA*
429	Nasal	Natural	*S. xylosus* *S. sciuri*	PEN, TET TOB-GEN	*blaZ, tet*(K) *salA, aac6'- aph2"*
487	Nasal	Landfill	*S. epidermidis* *S. xylosus*	PEN-ERY- SXT-MUP PEN	*blaZ, msrA, dfrA, mupA* *blaZ*
480	Nasal	Natural	*S. simulans* *S. xylosus*	PEN PEN	*blaZ* *blaZ*
488	Nasal	Landfill	*S. epidermidis* *S. sciuri*	PEN-ERY-CLI-TET-MUP PEN	*blaZ. ermA, ermC, tet*(K), *mupA* *salA, blaZ*
532	Nasal	Landfill	*S. sciuri* *S. sciuri* *S. sciuri*	CHL-TOB-GEN PEN-GEN TOB	*salA, fexA, aac6'-aph2"* *salA, blaZ, aac6'-aph2"* *salA, ant4'*
538	Nasal	Landfill	*S. epidermidis* *S. sciuri*	ERY-CLI-MUP CLI	*ermT, mupA* *salA*
548	Nasal	Landfill	*S. sciuri* *S. sciuri*	CLI-TOB-GEN PEN-CLI	*salA, mphC, aac6'-aph2"* *salA, blaZ*

CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; MUP: mupirocin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin

3.1.2 HEALTHY HUMANS WHO HAD NO CONTACT WITH ANIMALS AND HOSPITALS

3.1.2.1 Frequency of bacteria Species and Genera Recovered from the nasal cavities of healthy humans

A total of 241 strains were recovered (up to 12/sample) and were identified by MALDI-TOF-MS. Eighteen species were detected including species of *Staphylocccus*, *Escherichia* and *Corynebacterium* among others **(Table 45).** Of all the identified bacteria, *Staphylococcus* was the most frequent and diverse genus **(Table 45).**

3.1.2.2 Frequency and species diversity of nasal staphylococci from healthy humans

Staphylococci could be detected from 56 of the 57 healthy people tested (98.2%), of which a total of two hundred and fourteen (214) staphylococci (*S. aureus* and CoNS) were recovered from the healthy humans tested (up to eight strains of similar or different species per sample). The distribution of *Staphylococcus* species is presented in **Table 46**. After species identification and AMR phenotype determination, one hundred and forty-three (143) non-

repetitive strains (27 *S. aureus* and 116 CoNS) were genetically characterized and considered in this study. These 143 strains corresponded to one strain of each species per sample or more than one if they presented different AMR phenotypes **(Table 46a).** The 27 non-repetitive *S. aureus* strains were identified from 21 (36.8%) healthy people. Moreover, the non-repetitive CoNS strains identified from 56 healthy people were of six species: *S. epidermidis* (87.7%), *S. aureus* (36.8%), *S. hominis* (7%), *S. haemolyticus* (5.3%), *S. warneri* (5.3%), *S. lugdunensis* (1.8%), and *S. pasteuri* (1.8%) **(Table 46a).**

Table 45. Number of strains and frequency of carriers of species recovered from the nose of healthy humans

Bacteria genera and species	Nº (%) of strains from nasal samples	Number (%) of healthy human carriers (n=57)
Staphylococcus		
S. epidermidis	137	50 (87.7)
S. aureus	62	21 (36.8)
S. haemolyticus	5	3 (5.3)
S. hominis	4	4 (7.2)
S. warneri	4	4 (7.2)
S. lugdunensis	1	1 (1.8)
S. pasteuri	1	1 (1.8)
E. faecalis	7	2 (3.6)
Streptococcus salivarius	1	1 (1.8)
Pontea agglomerans	1	1 (1.8)
Citrobacter youngae	2	1 (1.8)
Bacillus pumilus	1	1 (1.8)
Pseudomonas		
P. montelli	2	1 (1.8)
P. oryzihabitans	1	1 (1.8)
E. coli	4	3 (5.3)
Klebsiella		
K. oxytoca	2	1 (1.8)
K. aerogenes	1	1 (1.8)
Corynebacterium		
C. accolens	4	3 (5.3)
C. propinquum	1	1 (1.8)
Total	241	

Table 46a. Number of strains and carriage rate of each staphylococci species recovered from the nasal samples of healthy adults.

Staphylococcus **species**	**Total strains recovered**	**Non-repetitive strains[a]**	**Nº of carriers (%)**
S. epidermidis	137	103	50 (87.7)
S. aureus	62	27	21 (36.8)
S. haemolyticus	5	3	3 (5.3)
S. hominis	4	4	4 (7)
S. warneri	4	4	4 (6.8)
S. lugdunensis	1	1	1 (1.8)
S. pasteuri	1	1	1 (1.8)
Total (%)	214	143	56 (98.2)[b]

Note: Between 2 to 6 different staphylococci colonies were randomly selected per sample

[a]non- repetitive strains = one strain of each species per sample or more than one if they presented different AMR phenotypes

[b]at least one *Staphylococcus* species

Table 46b. Co-carriage rate of *S. aureus* and *S. epidermidis* in **the nasal samples of healthy adults**

***S. aureus* and *S. epidermidis* co-carriage**	**Presence of *S. epidermidis* (%)**	**OR (95% CI)**	***p* value**
MSSA-CC398 (n=7)	2 (33.3)	0.707 (0.119-4.177)	0.7027
Non-CC398-MSSA (n= 15)	9 (60)	2.654 (0.771-9.141)	0.1219
Presence of *S. aureus* (all lineages) (n=21)	11 (52.4)	1.608 (0.539-4.801)	0.3944
Absence of *S. aureus* (n= 36)	13 (36.1)	Referent	Referent

Statistical association determined by bivariate regression at 95% Confidence interval (CI)

3.1.2.3 Antimicrobial resistance determinants of the non-repetitive nasal staphylococci from healthy humans

Of the 27 non-repetitive *S. aureus* strains, no methicillin resistance was detected but 14.8% presented a multidrug resistance phenotype. The following AMR rates were found (percentage of strains/ genes or mutations detected): penicillin (81.5/*blaZ*), erythromycin-clindamycin-inducible (25.9/*ermC, ermT*), erythromycin (7.4/ *msrA*), clindamycin (3.7/*lnuA*), ciprofloxacin (14.8/ GrlA [S80F], GyrA [S84L]), tobramycin (7.4/*ant4''*), tetracycline (3.7/*tet(K)*), sulfamethoxazole-trimethoprim (3.7/ *dfrG*), mupirocin (3.7/*mupA*) **(Figure 31).**

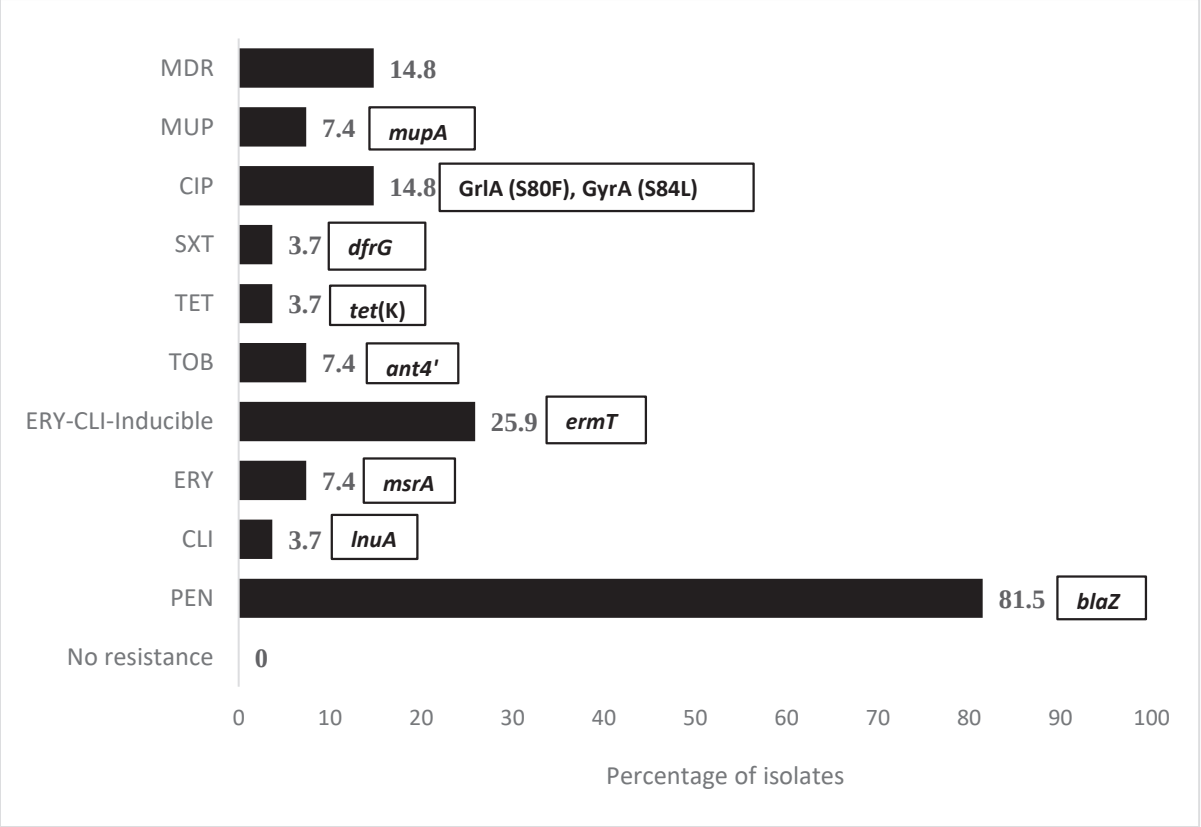


Figure 31. Antimicrobial resistance phenotypes and their determinants in the 27 *S. aureus* strains recovered from healthy humans. Abbreviation: CLI: clindamycin, CIP: ciprofloxacin, ERY: erythromycin, MDR: multidrug resistance, PEN: penicillin; TET tetracycline; TOB: tobramycin; SXT: sulfamethoxazole-trimethoprim

Of the 116 non-repetitive CoNS strains, 12.9% were susceptible to all antibiotics tested, 28.4% were resistant to only one antibiotic and 30.2% presented the MDR phenotype. The following AMR rates were detected (percentage of strains/ genes detected): penicillin (66.4/*blaZ*), cefoxitin (26.7/*mecA*), erythromycin-clindamycin-constitutive (12.1/*ermA*, *ermB*, *ermC*, *erm43*, *vgaA*, *mphC*), erythromycin-clindamycin-inducible (4.3/*ermB*, *ermC*, *erm43*), erythromycin (24.1/*msrA*, *mphC*), clindamycin (6/*lnuA*, *vgaA*), ciprofloxacin (4.3), tobramycin (6/*ant4"*), gentamicin-tobramycin (7.8/*aac6'-aph2"*), tetracycline (18.9/*tet(K)*), sulfamethoxazole-trimethoprim (14.7/ *dfrA*, *dfrG*), mupirocin (30.1/*mupA*), chloramphenicol (0.9/*catA*) **(Table 47).** No linezolid resistance gene was detected in all the staphylococci (**Table 47**).

Among the 35 MRCoNS, SCC*mec* type IV and V elements were the predominant (28.6% each), then SCC*mec* type III (8.5%), while others were non-typeable (34.1%) **(Figure 32).** The multiple antibiotic resistance (MAR) index of most *S. aureus* strains (70.4%) was less than 2 (range: 0.08-0.5). However, the MAR index of the CoNS strains ranged from 0-0.67 **(Tables 48 and 49).**

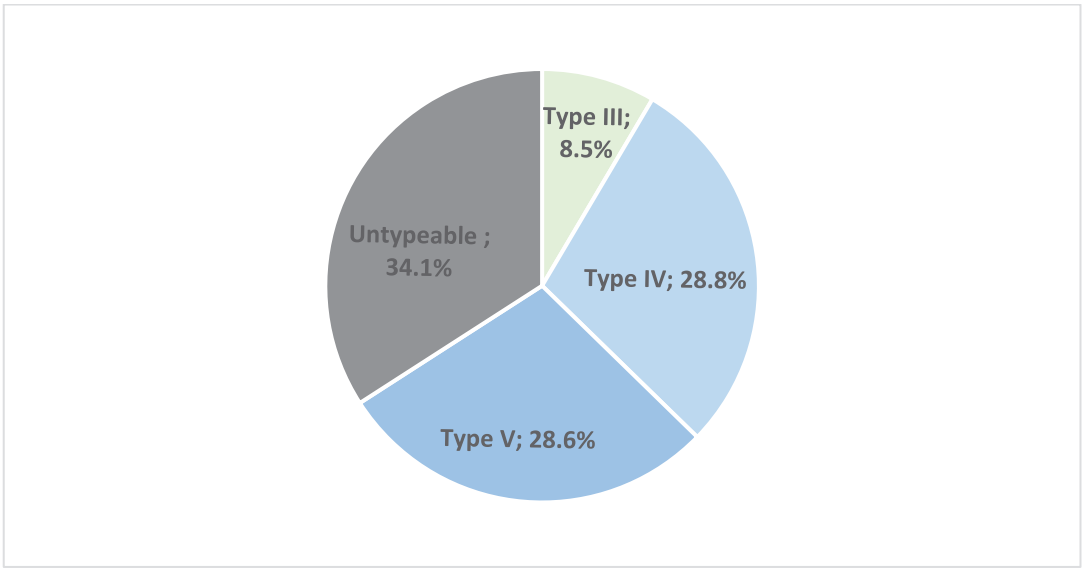


Figure 32. Frequency of the SCC*mec* mobile elements identified among the 35 non- repetitive methicillin-resistant coagulase-negative staphylococci strains in healthy humans.

Table 47. Number of strains of the different coagulase-negative staphylococci species from healthy humans and those with distinct AMR and MDR phenotype

CoNS Species	Non-repetitive CoNS strains[a]				% of antimicrobial resistance/ genes detected												
	Total number	**N. strains susceptible to all antibiotics tested (%)[b]**	**N. strains resistant to only one antibiotic (%)[c]**	**N. strains with MDR phenotype (%)[d]**	**PEN**	**FOX**	**ERY**	**CLI**	**ERY-CLI^{cons}**	**ERY-CLI^{ind}**	**TET**	**TOB**	**GEN-TOB**	**SXT**	**CIP**	**CLO**	**MUP**
S. epidermidis	103	12 (11.8)	27 (26.5)	31 (30.1)	77.5/ *blaZ*	24.3/ *mecA*	22.3/*msrA, mphC*	4.8/ *lnuA, vgaA*	12.7/*ermA, ermB, ermC, erm43, vgaA, mphC*	4.9/ *ermB, ermC, erm43*	20.9/ *tet* (K)	5.8/ *ant4'*	6.9/*aac6'-aph2, ant4'*	14.7/ *dfrA, dfrG*	4.9	0.9/ *cat*	40.2/ *mupA*
S. haemolyticus	3	0	1 (33.3)	2 (66.7)	66.7/ *blaZ*	66.7/ *mecA*	66.7/*msrA, mphC*	33.3/ *vgaA*	33.3/*ermC*	S	33.3/ *tet*(K)	33.3/ *ant4'*	33.3/*aac6'-aph2"*	33.3/ *dfrG*	S	S	33.3/ *mupA*
S. hominis	4	0	2 (50)	2 (50)	75/ *blaZ*	50/ *mecA*	25/*msrA, mphC*	S	S	S	S	S	25/*aac6'-aph2, ant4'*	25/ *dfrA*	S	S	25/ *mupA*
S. warneri	4	2 (50)	2 (50)	0	25/ *blaZ*	25/ *mecA*	S	25/ *vgaA*	S	S	S	S	S	S	S	S	S
S. lugdunensis	1	0	1 (100)	0	100/ *blaZ*	S	S	S	S	S	S	S	S	S	S	S	S
S. pasteuri	1	1 (100)	0	0	S	S	S	S	S	S	S	S	S	S	S	S	S
Total strains (%)	116	15 (13)	33 (28)	35 (30)	77 (66)	31 (27)	28 (24)	7 (6)	14 (12)	5 (4)	22 (19)	7 (6)	9 (8)	17 (15)	5 (4)	1 (1)	43 (30)

[a]Non-repetitive strains: one strain of each species/sample or more than one if they presented different AMR phenotypes.
[b]MAR index = 0
[c]MAR index = 0.1
[d]MDR: resistance to at least 3 families of antibiotics.
S: Susceptible
CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; MUP: mupirocin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin

Table 48. Molecular typing, AMR and virulence determinant of the 27 *S. aureus* strains from healthy humans.

Host ID Nº	*spa* type	[a]CC/ST	AMR phenotypes	MAR index	AMR genes or mutations detected	IEC type	Virulence genes detected
H4	t571	CC398	ERY[1]-CLI[ind1]	0.17	*ermT*[2]	C	Negative
H5	t571	CC398	ERY[2]-CLI[ind2]	0.17	*ermT*[2]	C	*sec*
H7	t4390*	CC121/ST51	PEN[1]-ERY[1]-MUP[1]	0.25	*blaZ*[1], *msrA*[1], *mupA*[1]	E	*eta, etb, sed*
H8	t012	CC30	PEN[2]	0.08	*blaZ*[2]	E	*sec, sed*
	t4390*	CC121/ST51	PEN[1]-CIP[1]	0.17	*blaZ*[1], GrlA (p.S80F) [1], GyrA (p.S84L) [1]	E	*eta, etb, sec, sed, see*
H9	t355	CC152	PEN[3]	0.08	*blaZ*[3]	D	*lukF/S-PV, sea, sed, see*
H14	t571	CC398	PEN[4]	0.08	*blaZ*[4]	C	Negative
H15	t571	CC398	PEN[4]-ERY[4]-CLI[ind4]	0.25	*blaZ*[4], *ermT*[4]	C	Negative
H17	t091	CC7	PEN[4]	0.08	*blaZ*[4]	G	*sea, sec, sed, sep*
	t091	CC7	PEN[2]-ERY[2]	0.17	*blaZ*[2], *msrA*[2]	G	*see, sep*
H18	t7521*	CC15/ST15	PEN[4]	0.08	*blaZ*[4]	C	*sec*
H19	t091	CC7	PEN[3]	0.08	*blaZ*[3]	A	*sea, sed, see*
H22	t3200*	CC5/ST7476	PEN[1]-CLI[1]-TET[1]- SXT[1]-TOB[1]-CIP[1]	0.5	*blaZ*[1], *lnuA*[1], *tet*(K)[1], *dfrG*[1], *ant4'*[1], GrlA (S80F) [1], GyrA (S84L) [1]	B	*sec, see*
H25	t1994	CC159	PEN[1]-TOB[1]-MUP[1]	0.25	*blaZ*[1], *ant4'*[1], *mupA*[1]	E	*eta, etb, sed, see*
	t1451	CC398	PEN[1]-ERY[1]-CLI[Ind1]	0.25	*blaZ*[1], *ermT*[1]	C	*sec, see*
	t1451	CC398	ERY[1]-CLI [Ind1]	0.17	*ermT*[1]	C	Negative
	t1077*	CC121/ ST4244	MUP	0.08	*mupA*[1]	B	*eta, etb, sed, see*
H33	t1998	CC398	PEN[3]-ERY[3]-CLI[ind3]-CIP[3]	0.33	*blaZ*[3], *ermT*[3], GrlA (p.S80F) [3], GyrA (S84L) [3]	C	Negative
	t1451	CC398	PEN[2]-ERY[2]-CLI[ind2]	0.25	*blaZ*[2], *ermC*[2]	C	Negative
H41	t1451	CC398	PEN[3]-ERY[3]-CLI[ind3]	0.25	*blaZ*[3], *ermT*[3]	C	Negative
H43	t6389	CC121	PEN[1]	0.08	*blaZ*[1]	G	*sep*
H46	t223	CC22	PEN[3]	0.08	*blaZ*[3]	F	*sec, sed, see, sep, tst*
H47	t223	CC22	PEN[3]	0.08	*blaZ*[3]	F	*sec, sed, see, sep, tst*
H50	t223	CC22	PEN[3]	0.08	*blaZ*[3]	B	*eta, sec, see, tst*
H51	t159	CC121	PEN[3]	0.08	*blaZ*[3]	B	*sec*
H55	t005	CC22	PEN[1]	0.08	*blaZ*[1]	F	*sec, sep, tst*
H63	t078	CC5	PEN[1]	0.08	*blaZ*[1]	B	*etd, sec, sed, see*

Sequence Type: ST; * new *spa*-types; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline; TOB: tobramycin; ERY-CLI[ind]: erythromycin-clindamycin inducible

[a]CC assigned according to the *spa*-type, except for CC398 (determined by specific PCR). The ST of strains with new *spa* types were determined by MLST

Table 49. Antimicrobial resistance determinants identified in CoNS carrying multi-drug resistant (MDR) phenotype of healthy humans

Host ID number	Strain/ code	*MDR phenotype	MAR index	AMR genes detected	SCC*mec* type	Genetic lineage[a]
H1	*S. epidermidis*/ X6411	PEN-ERY-CLI-MUP	0.33	*blaZ, ermA, msrA, vgaA, mupA*	-	NT
H4	*S. epidermidis*/ X6380	PEN-FOX-ERY-CLI-MUP	0.42	*blaZ, mecA, msrA, mupA*	IV	ST59
H5	*S. epidermidis*/ X6412	PEN-FOX-ERY-SXT-MUP	0.42	*blaZ, mecA, msrA, dfrA, dfrG, mupA*	IV	ST59
	S. epidermidis/ X6473	PEN-FOX-ERY-MUP	0.33	*blaZ, mecA, msrA, mupA*	IV	ST5
H6	*S. epidermidis*/ X6414	PEN-FOX-CLI-SXT-CIP	0.42	*blaZ, mecA, dfrA, dfrG, vgaA*	V	ST2
H7	*S. epidermidis*/ X6406	PEN-FOX-ERY-CLI-SXT	0.42	*blaZ, mecA, erm43, vgaA*	IV	ST22
H13	*S. haemolyticus*/ X6461	PEN-FOX-CLI-MUP	0.33	*blaZ, mecA, vgaA, mupA*	V	NT
H16	*S. epidermidis*/ X6630	PEN-ERY-CLI-MUP	0.33	*blaZ, ermC, vgaA, mupA*	-	NT
	S. epidermidis/ X6827	PEN-FOX-TET-TOB-GEN	0.42	*blaZ, mecA, tet*(K), *aac6'-aph2", ant4'*	IV	ST87
H19	*S. epidermidis*/ X6590	PEN-ERY-CLI- MUP	0.33	*blaZ, ermC, lnuA, vgaA, mupA*	-	NT
H21	*S. epidermidis*/ X6602	PEN-FOX-ERY-CLI-TET	0.42	*mecA, erm43, vgaA, tet*(K)	III	ST49
H22	*S. epidermidis*/ X6599	PEN-ERY-CLI-SXT-TOB	0.42	*blaZ, ermC, lnuA, dfrG, ant4'*	-	ST210
	S. epidermidis/ X6601	PEN-FOX-SXT-CIP-CHL	0.42	*blaZ, mecA, dfrA, dfrG, catA*	V	ST210
H24	*S. epidermidis*/ X6752	PEN-ERY-TET	0.25	*blaZ, mphC, msrA, tet*(K)	-	NT
	S. epidermidis/ X6711	PEN-FOX-ERY-SXT-MUP	0.42	*blaZ, mecA, dfrA, mupA*	IV	ST969
H25	*S. hominis*/ X6712	PEN-FOX-ERY-MUP	0.33	*mecA, mphC, msrA, mupA*	IV	NT
H26	*S. epidermidis*/ X6759	PEN-ERY-CLI-TOB-CIP	0.42	*blaZ, mphC, msrA, dfrA, dfrG, ant4', mupA*	-	ST24
	S. epidermidis/ X6737	ERY-SXT-CIP	0.25	*mphC, msrA, dfrA*	-	NT
	S. epidermidis/ X6738	PEN-ERY-CLI^{ind}-TET-SXT	0.42	*blaZ, ermB, tet*(K), *dfrA*	-	ST59
	S. epidermidis/ X6739	PEN-FOX-ERY-MUP	0.33	*mecA, mphC, msrA, mupA*	V	ST59
	S. epidermidis/ X6740	PEN-ERY-SXT-MUP	0.33	*blaZ, mphC, msrA, dfrA, mupA*	-	-
	S. epidermidis/ X6741	PEN-FOX-ERY-CLI^{ind}-MUP	0.42	*blaZ, mecA, ermC, erm43, dfrA*	V	ST5
H29	*S. epidermidis*/ X6837	PEN-ERY-TOB-GEN	0.33	*blaZ, msrA, aac6'-aph2"*	-	NT
	S. epidermidis/ X6821	PEN-ERY-MUP	0.25	*blaZ, mphC, msrA, mupA*	-	NT
H32	*S. epidermidis*/ X6832	PEN-ERY-TOB	0.25	*blaZ, mphC, msrA, ant4'*	-	NT
H33	*S. haemolyticus*/ X7059	PEN-FOX-ERY-TET-TOB-GEN-SXT	0.58	*blaZ, mecA, msrA, mphC, tet*(K), *aac6'-aph2", ant4', dfrG*	V	NT
H35	*S. epidermidis*/ X8987	PEN-FOX-TOB-GEN MUP	0.42	*blaZ, mecA, aac6'-aph2", mupA*	IV	ST8
H39	*S. epidermidis*/ X8993	PEN-ERY-CLI-MUP	0.33	*blaZ, ermC, vgaA, mupA*	-	NT

Table 49. Continuation

Host ID number	Strain/ code	*MDR phenotype	MAR index	AMR genes detected	SCC*mec* type	Genetic lineage[a]
H46	*S. epidermidis*/ X9066	PEN-FOX-CLI-TET-SXT-TOB-GEN-CIP	0.67	*blaZ, mecA, lnuA, tet*(K), *aac6'-aph2", ant4'*	V	ST173
	S. epidermidis/ X9097	PEN-ERY-CLI-MUP	0.33	*blaZ, ermC, vgaA, mupA*	-	NT
	S. epidermidis/ X9092	PEN-ERY-CLI^{ind}-MUP	0.33	*blaZ, ermC, vgaA, mupA*	-	-
H47	*S. hominis*/ X9052	PEN-FOX- SXT-CIP	0.33	*blaZ, mecA, dfrA*	IV	-
H60	*S. epidermidis*/ X9084	PEN-ERY-CLI^{ind}- TET-TOB-MUP	0.5	*blaZ, erm43, aac6'-aph2", mupA*	-	ST5
	S. epidermidis/ X9085	ERY-TET-TOB	0.25	*msrA, tet*(K), *ant4'*	-	NT
H64	*S. epidermidis*/ X9086	PEN-ERY-TOB-GEN-MUP	0.42	*blaZ, vgaA, aac6'-aph2", mupA*	-	ST22

CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; MUP: mupirocin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin

* MDR was defined by ≥ 3 AMR phenotypes for different families of agents

[a]: MLST of all MDR-MR-*S. epidermidis* with an MDR phenotype of ≥3 classes were conducted. While the genetic lineages of methicillin susceptible-*S. epidermidis* with an MDR phenotype of ≥4 classes was determined.

3.1.2.4 Genetic lineages, virulence genes and IEC types of the *S. aureus* strains.

Eight clonal complexes (CCs) were identified among the *S. aureus* strains based on the 17 different *spa* types of the MSSA strains, of which CC398 (*spa* types t571, t1451 and t1998) was the predominant (33.3%), followed by CC121 (18.5%), then CC22 (14.8%) and CC7 (11.1%) **(Table 48).** About 85.1% of the *S. aureus* strains carried one or more of the *luk-S/F-PV, tst, eta, etb, etd, sea, seb, sec, sed, see* and *sep* genes. All seven IEC types were identified among the MSSA strains. Interestingly, the IEC-type C was the only one detected in the MSSA-CC398 strains **(Table 48).** The predominant virulent determinants detected (genes, frequencies, and genetic lineages) were those that encode: enterotoxin (*sea to see* and *sep*, 81.5%, all the CCs), followed by exfoliatin (*eta, etb, etd,* 22.2%, CC5, CC22, CC121, CC159), and then toxic shock syndrome (*tst*, 11.1%, CC22). However, only one MSSA strain carried the Panton-Valentine Leucocidin (*lukF/S-PV*, 3.7%, CC152) **(Table 48).**

About 33.3% of healthy humans with MSSA-CC398 had *S. epidermidis* nasal co-carriage, whereas 60% of those with non-CC398-MSSA strains had *S. epidermidis* co-carriage **(Table 46b).** Collectively, 52.4% of those with *S. aureus* (both CC398 and non-CC398) had *S. epidermidis* co-carriage. However, there was no significant association between the presence of nasal MSSA, MSSA-CC398 and *S. epidermidis* co-carriage in healthy people ($p > 0.05$) **(Table 46b).**

3.1.2.5 Genetic diversity of nasal staphylococci from healthy humans

Of the 21 *S. aureus* carriers, about 19% harboured varied strains with different genetic lineages and/or AMR genes in the same host **(Table 48, Figure 33).** Intra-host CoNS species diversity (more than one CoNS species in a sample) was detected in 12.5% of healthy human staphylococci carriers **(Figure 34)**. Moreover, healthy human carriers of CoNS with similar species with diverse AMR genotypes (2-6 AMR profile) were detected in 76.8% **(Figure 33)**. For instance, in one of the *S. epidermidis* carriers (H26), six different AMR profiles were identified, viz: (i) *blaZ, mphC, msrA, dfrA, dfrG, ant4', mupA;* (ii) *mphC, msrA, dfrA;* (iii) *blaZ, ermB, tet*(K), *dfrA;* (iv), *blaZ, mecA, msrA, mupA;* (v) *blaZ, mphC, msrA, dfrA, mupA;* and (vi) *blaZ, mecA, ermC, erm43, dfrA* **(Table 49).**

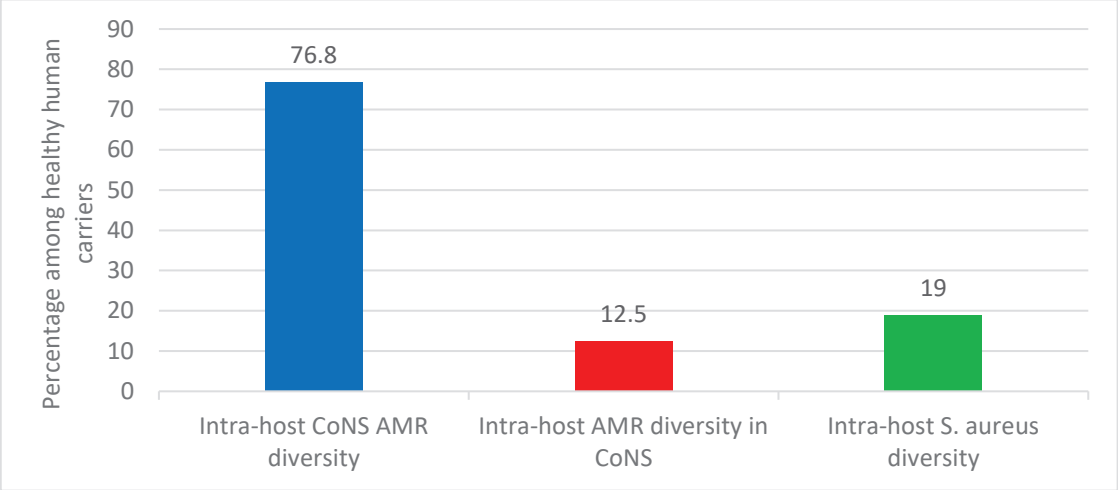


Figure 33. Frequency of intra-host species and intra-species AMR diversity among non-repetitive staphylococci from healthy humans.

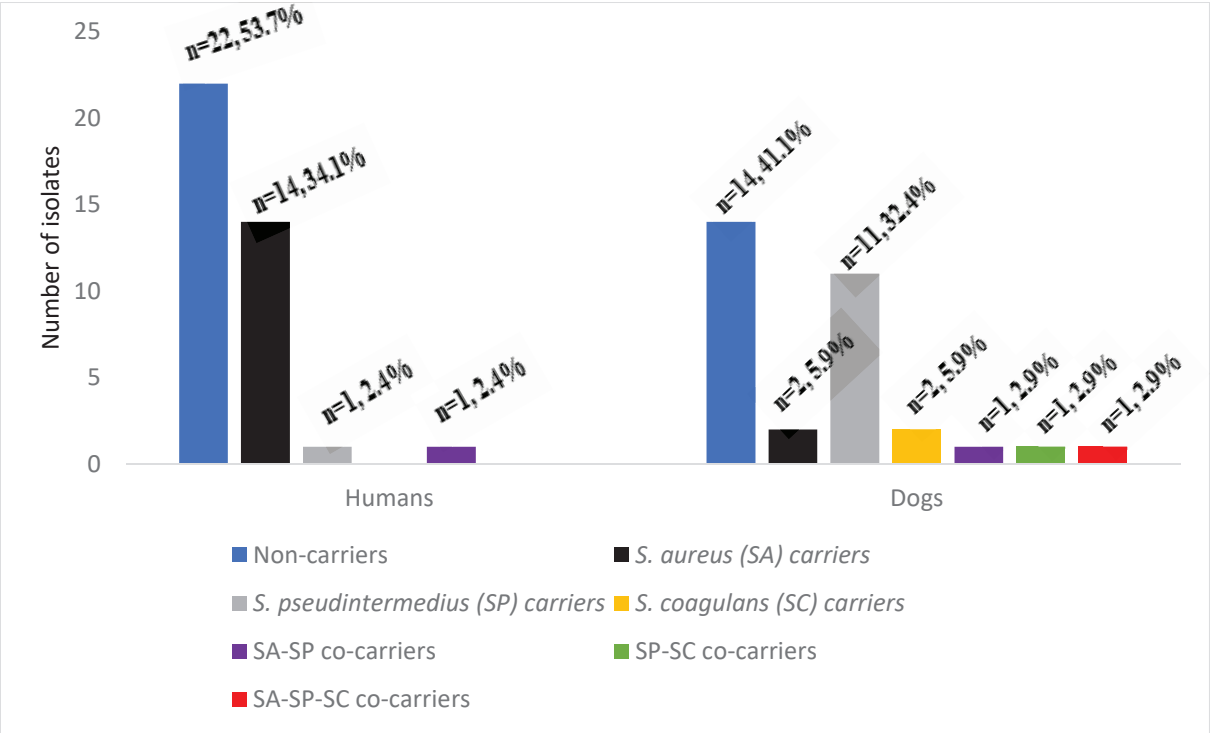


Figure 34. Number and prevalence of *S. aureus*, *S. pseudintermedius*, *S. coagulans* strains and co-carriage detected in the nasal cavities of healthy humans and dogs

3.1.2.6 Genetic characteristics of the 35 MDR-CoNS strains from healthy human carriers.

Of the 35 non-repetitive MDR-CoNS strains (*S. epidermidis*, *S. haemolyticus* and *S. hominis*) from 22 carriers (38.6%), the predominant genetic lineage among *S. epidermidis* was ST59 (12.1%), followed by ST5 and ST210 (6.1% each). Other genetic lineages detected but

in low frequencies include ST2, ST8 ST24, ST49, ST87 and ST173 **(Table 49).** Of the 22 MDR-CoNS carriers, 8 (36.4%) had varied AMR genotypes and/ or SCC*mec* types **(Table 49).**

3.1.3 HEALTHY DOGS AND DOG OWNERS

3.1.3.1 Frequency of bacteria Species and Genera Recovered from the nasal cavities of healthy dogs and dog owners

A total of 376 strains were recovered (up to 8 per sample) and were identified by MALDI-TOF- MS: 121 from dogs and 255 from dog owners. Thirthy-one species were detected including species of *Staphylocccus*, *Enterococcus*, *Streptococcus*, *Micrococcus* and *Enterobacter*, among others **(Table 50).** Of all the identified bacteria, the species of *Staphylococcus*, *Enterococcus* and *Streptococcus* were the most frequent and diverse **(Table 50).**

Table 50. Number of strains and frequency of carriers of bacteria species recovered from the nose of healthy dogs and dog owners

Bacteria genera and species	Nº (%) strains from dog nasal samples	Number (%) dog carriers (n=34)	Nº (%) strains from dog owner nasal samples	Number (%) of dog owner carriers (n=41)
Staphylococcus				
S. aureus	2	2 (5.9)	71	14 (34.1)
S. pesudintermedius	30	12 (35.3)	1	1 (2.4)
S. coagulans	2	1 (2.9)	0	0
S. epidermidis	17	9 (26.5)	150	33 (80.5)
S. hominis	8	3 (8.8)	5	4 (9.8)
S. cohnii	4	3 (8.8)	0	0
S. lugdunensis	0	0	4	4 (9.8)
S. pasteuri	1	1 (2.9)	6	3 (7.3)
S. waeneri	5	2 (5.9)	3	2 (4.8)
S. xylosus	2	1 (2.9)	2	1 (2.4)
S. haemolyticus	4	2 (5.9)	0	0
S. simulans	2	2 (5.9)	0	0
S. muscae	1	1 (2.9)	0	0
Enterococcus				
E. faecalis	7	2 (5.9)	4	2 (4.8)
E. faecium	18	5 (14.7)	0	0
E. raffisonsus	2	1 (2.9)	0	0
Micrococcus				
M. lutrae	1	1 (2.9)	0	0
M. luteus	2	1 (2.9)	0	0
Paenibacillus glucanolyticus	0	0	2	1 (2.4)
Streptococcus				
S. gallolyticus	1	1 (2.9)	0	0
S. pluranimalium	1	1 (2.9)	0	0
S. salivarius	0	0	1	1 (2.4)
S. canis	2	2 (5.9)	0	0
S. hyovaginalis	3	1 (2.9)	0	0
S. oralis	0	0	1	1 (2.4)

Table 50. Continuation

Bacteria genera and species	Nº (%) strains from dog nasal samples	Number (%) dog carriers (n=34)	Nº (%)strains from dog owner nasal samples	Number (%)dog owner carriers (n=41)
Corynebacterium pseudodiphtheriticum	0	0	1	1 (2.4)
Pontea agglomerans	5	2 (5.9)	0	0
Exiguobacterium mexicanum	1	1 (2.9)	0	0
Klebsiella oxytoca	0	0	2	2 (4.8)
Enterobacter				
E. cloacae	0	0	1	1 (2.4)
E. asburea	0	0	1	1 (2.4)
Total	**121**		**255**	

3.1.3.2 CoPS nasal carriage in healthy dogs' households

A total of 73 *S. aureus*, 31 *S. pseudintermedius* and two *S. coagulans* strains were recovered from 75 nasal samples of humans and dogs. After AMR phenotype determination, 52 non-repetitive strains were selected for further characterization (31 *S. aureus*, 19 *S. pseudintermedius* and 2 *S. coagulans*), corresponding to one strain per sample or more than one if they presented different species and/or different AMR phenotype. *Staphylococcus aureus* was found in 14 humans (34.1%) (including one individual with MRSA) and two dogs (5.9%) **(Figure 34).** *S. pseudintermedius* was identified in one human (2.4%) and 11 dogs (32.4%). However, *S. coagulans* was solely identified in two dogs (5.9%) of the same household. Apart from these three species, no other CoPS species were detected in the cultures. Remarkably, one human presented *S. aureus*/*S. pseudintermedius* co-carriage (2.4%) while a dog had co-carriage of all three CoPS species (2.9%) **(Figure 34).** In total, 14 humans and 12 dogs carried CoPS. Household density was significantly associated with *S. pseudintermedius* carriage in households with > than 1 dog and >than 1 human (OR = 18.10, 95% CI: 1.24–260.93, $p = 0.034$) **(Table 51)**

Table 51. Association of household density with nasal *S. aureus* and *S. pseudintermedius* carriage

Household density	1 dog &1 human (n=10)	> 1 dog and a human (n=3)	1 dog and > than 1 human (n=8)	> than 1 dog and > than 1 human (n=6)
S. aureus carriers (%)	3 (30)	3 (100)	2 (25)	5 (83.3)
S. aureus non-carriers (%)	7 (70)	0 (0)	6 (75)	1 (16.7)
OR (95% CI)	Referent	15.0 (0.59-376.7)	0.8 (0.09-6.32)	11.7 (0.92-147.57)
p value	Referent	0.099	0.814	0.057
S. pseudintermedius carriers	1 (10)	1 (33.3)	2 (25)	4 (66.7)
S. pseudintermedius non-carriers	9 (90)	2 (66.7)	6 (75)	2 (33.3)
OR (95% CI)	Referent	4.5 (0.19-106.8)	3.0 (0.22-40.93)	18.0 (1.24-260.93)
p value	Referent	0.352	0.410	0.034*

*Significant association determined by bivariate regression at 95% confidence interval (CI)

3.1.3.3 Phenotypic and genetic characteristics of CoPS strains

The 31 distinct *S. aureus* strains harboured AMR as follows [percentage of resistant strains/resistance genotype]: penicillin [77.4/*blaZ*], cefoxitin [9.7/*mecA*], erythromycin-clindamycin-inducible [19.4/*ermT*], erythromycin [9.7/*msrA, mphC*], clindamycin [3.2/*lnuA*], gentamicin-tobramycin [22.6/*aac6′-aph2″*], tetracycline [3.2/*tet*(K)], sulfonamide [3.2/*dfrA*], fluoroquinolones [22.5/amino acid changes in GrlA: S80F, GyrA: S84L], mupirocin (3.2/*mupA*) and linezolid [3.2/G2261A & T1584A point mutations in 23S rDNA] **(Figure 35; Table 52).** Moreover, the 19 distinct *S. pseudintermedius* strains harboured AMR as follows [percentage of resistant strains/resistance genotype]: penicillin [57.9/*blaZ*], erythromycin-clindamycin-constitutive [26.3/*ermB*], tobramycin [15.8/*ant4′*], tetracycline [26.3/*tet*(M)], trimethoprim-sulfamethoxazole [63.2/*dfrA, dfrD, dfrG, dfrK*], and chloramphenicol [5.3/*catA*] **(Figure 35; Table 52).** No resistance markers were detected in the *S. coagulans* strains, that were susceptible to all antimicrobial agents tested **(Figure 35).**

Regarding the genetic lineages of *S. aureus* strains, the three MRSA strains from humans (same individual but different AMR phenotypes/genotypes) belonged to the *spa* type t222, associated with CC5. All other strains were methicillin-susceptible *S. aureus* (MSSA) with 19 different *spa*-types assigned to 10 different CCs. The MSSA-CC398 clone (t1451 and t571) was the most frequently identified (18.8% of *S. aureus* carriers); these strains were all IEC-type C. Other CCs (*spa*-types) detected were as follows: CC5 (t041), CC7 (t091), CC8 (t121, t126, t1070, t3092), CC15 (t084, t2013), CC30 (t012, t1824), CC45 (t015, t065, t505, t1689), CC97 (t267), CC133 (t4735) and CC152 (t355) **(Table 52)**. For *S. pseudintermedius* strains, all of them were methicillin susceptible (MSSP) (including two ST1115) **(Table 53).**

Clonally related *S. aureus* or *S. pseudintermedius* strains were found in humans or dogs among 11.1% of households (n = 3). Two of the 16 households (household N^{os}11 and 21) positive for nasal *S. aureus* had human carriers with similar clonal complexes (CCs), *spa*-types and IEC types **(Table 52).** In one of these households (No 11), MSSA-CC30-*spa*-type t1070 strains (*scn*-negative) were identified in two humans, however, a different lineage, MSSA-CC8 of the *spa*-type t121 (IEC type-D), was identified in their dog **(Table 52).** In the second household (N° 21), two humans carried MSSA-CC398 strains of different *spa*-types (t1451 and t571), although the dog was not *S. aureus* carrier. Moreover, in another household (Nº 10), a dog and a human were carriers of the same genetic lineage of *S. pseudintermedius* (MSSP-ST1115); in this household, the human also carried MSSA-CC97-t267 and a dog MSSA-t2013-CC15 **(Table 52).** All the *S. aureus* strains were negative for *lukS/F*-PV, *tst*, *eta* and *etb* genes

(Table 52). However, all the *S. pseudintermedius* strains were positive for *lukS/F-I, siet,* and *sient* virulence genes, but one was only *sient*-positive **(Table 53).**

3.1.3.4 Intra-host variation of genetic lineages or AMR genotypes of CoPS

Nine of the 16 *S. aureus* (56.3%) carriers harboured diverse *spa*-types or AMR genotypes in the same individual (dog or human). Of these, two to four genetically distinct *S. aureus* strains were detected in these hosts **(Table 52).** In one human (ID number 3) with both MSSA and MRSA-SCC*mec* type-IV (2B) nasal carriage, three different MRSA-CC5-t2220 strains with different AMR phenotypes/genes were detected: PEN-FOX-ERY-CLI-CIP-TOB-MUP-LZD/*blaZ, mecA, lnuA, msrA, mphC, mupA,* G2261A point mutation in 23S rDNA; PEN-FOX-ERY-CIP-GEN-TOB/*blaZ, mecA, aac6'-aph2", msrA, mphC*; and PEN-FOX-ERY-CIP/*blaZ, mecA, msrA, mphC*, respectively; moreover, the MSSA strain was typed as CC30-t012 and showed resistance only to PEN (*blaZ* positive) **(Table 52).** Two other humans (ID numbers 57 and 58) from the same household (Nº 21) carried *S. aureus* strains both with similar genetic lineage (CC398) but different *spa* types (t571 and t1451) and similar AMR phenotypes (PEN-ERY-CLI^{inducible}-GEN-TOB) **(Table 52).** In another human *S. aureus* carrier (ID number 60) from a different household (Nº 22), strains with different genetic lineages (CC15 and CC152) were detected **(Table 52).**

In the *S. pseudintermedius* strains, 6 of the 12 carriers showed differences and intra-host variations in the AMR phenotypes or AMR genotypes. For instance, one of the dogs (ID number 52) harboured two different MSSP strains (PEN-SXT/*blaZ, dfrA, dfrG* and PEN-SXT-TOB/*blaZ, dfrG, ant4'*) **(Table 53).** About 31.7% of the *S. pseudintermedius* had an MDR phenotype **(Figure 35).** All three MRSA strains and some MSSA strains (20.0%) presented an MDR profile **(Table 52).**

Table 52. Molecular characterization and antimicrobial resistance profile of the *S. aureus* strains from humans and dogs[a]

Host/ ID[b]	Household ID/ population	Nº strains	*spa* type	CC	AMR phenotypes	Methicillin susceptibility	MDR	AMR genes detected[c]	IEC genes/type
H/3	2/1H&1D	3	t2220	CC5	PEN^{3}-FOX^{3}-ERY^{3}-CIP^{3}-GEN^{2}-TOB^{2}-MUP^{1}-LZD^{1}	MRSA-SCC*mec* type-IV (2B)	Yes	*blaZ*^{3}, *mecA*^{3}, *aac6'-aph2''*^{2}, *msrA*^{3}, *mphC*^{3}, *mupA*^{1}-G2261A/T1584A 23S rDNA^{1}-*grlA* (S80F) ^{1}/*gyrA* (S84L)^{1}	Negative
		1	t012	CC30	PEN	MSSA	No	*blaZ*	Negative
H/9	5/1H&2Ds	1	t012	CC30	PEN-CIP-TET	MSSA	Yes	*blaZ*, *tet*(K)	Negative
		1	t012	CC30	PEN-CIP	MSSA	No	*blaZ*	Negative
		1	t1824	CC30	PEN	MSSA	No	*blaZ*	*scn*, *sak*, *sep*/G
H/17	8/2Hs&2Ds	1	t4735	CC133	Susceptible	MSSA	No	NT	Negative
		1	t4735	CC133	CIP-SXT	MSSA	No	*dfrA*	Negative
H/20	9/1H&1D	1	t3092	CC8	PEN	MSSA	No	*blaZ*	Negative
		1	t068	CC8	PEN	MSSA	No	*blaZ*	*scn*, *sak*, *sep*/G
H/23	10/2Hs&2Ds	1	t267	CC97	Susceptible	MSSA	No	NT	*scn*, *sak*/ E
		1	t267	CC97	Susceptible	MSSA	No	NT	*scn*, *sak*/ E
D/24		1	t2013	CC15	PEN	MSSA	No	*blaZ*	*scn*, *chp*/ C
H/26	11/2Hs&2Ds	1	t1070	CC30	PEN	MSSA	No	*blaZ*	Negative
H/27		1	t1070	CC30	PEN	MSSA	No	*blaZ*	Negative
D/29		1	t121	CC8	PEN-CIP	MSSA	No	*blaZ*, *grlA* (S80F)	*scn*, *sak*, *sea*/ D
H/ 30	12/1H&1D	1	t041	CC5	Susceptible	MSSA	No	NT	*scn*, *sak*/ E
H/38	15/1H&2Ds	1	t505	CC45	PEN	MSSA	No	*blaZ*	*scn*, *sak*/ E
		1	t065	CC45	PEN	MSSA	No	*blaZ*	*scn*, *sak*/ E
		1	t015	CC45	PEN	MSSA	No	*blaZ*	*scn*, *sak*, *sep*/ G
H/44	17/1H&1D	1	t571	CC398	ERY-CLI^{ind}	MSSA	No	*ermT*	*scn*, *chp*/ C
H/50	19/2Hs&2Ds	1	t1689	CC45	PEN	MSSA	No	*blaZ*	*scn*, *sak*/ E
		1	t091	CC7	PEN	MSSA	No	*blaZ*	*scn*, *sak*, *sep*/ G
H/57	21/2Hs&1D	1	t1451	CC398	PEN-ERY-CLI^{ind}-GEN-TOB	MSSA	Yes	*blaZ*, *ermT*, *aac6'-aph2''*	*scn*, *chp*/ C
		1	t1451	CC398	PEN-ERY-CLI^{ind}-GEN-TOB	MSSA	Yes	*ermT*, *aac6'-aph2''*	*scn*, *chp*/ C
		1	t571	CC398	PEN-ERY-CLI^{ind}-GEN-TOB	MSSA	Yes	*blaZ*, *ermT*, *aac6'-aph2''*	*scn*, *chp*/ C
H/58		1	t1451	CC398	PEN-ERY-CLI^{ind}-GEN-TOB	MSSA	Yes	*blaZ*, *ermT*, *aac6'-aph2''*	*scn*, *chp*/ C
		1	t571	CC398	PEN-ERY-CLI^{ind}-GEN-TOB	MSSA	Yes	*blaZ*, *ermT*, *aac6'-aph2''*	*scn*, *chp*/ C
H/ 60	22/IH&1D	1	t084	CC15	PEN	MSSA	No	*blaZ*	*scn*, *chp*/ C
		1	t355	CC152	PEN	MSSA	No	*blaZ*	*scn*, *sak*/ E

[a]All strains were negative for the virulence genes *lukS/F-PV*, *tst*, *eta*, *etb*; [b]H, human; D, dog; [c]In superscript is the number of strains that present the specific characteristic
ST: Sequence Type; NT: not tested CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; GEN; gentamicin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline; TOB: tobramycin; ERY-CLI^{ind}: erythromycin-clindamycin inducible;

Table 53. Intra-host variation of AMR determinant and virulence factors of *S. pseudintermedius* strains

Host/ID[a]	Household ID/ population[a]	Nº strains	AMR phenotypes	MDR	AMR genes detected[b]	Methicillin Susceptibility/ ST	Virulence genes detected
D/2	1/1H&1D	4	PEN^{4}-ERY^{4}-CLI^{4}-TET^{4}-SXT^{1}-TOB^{2}	Yes	*blaZ*^{4}, *ermB*^{4}, *tet*(M)^{3}, *ant4'*^{2}	MSSP/NT	*lukS/F-I*^{3}, *siet*^{3}, *sient*^{4}
H/23	10/2Hs&2Ds	1	Susceptible	No	NT	MSSP/ ST1115	*lukS/F-I*^{1}, *siet*^{1}, *sient*^{1}
D/25	10/2Hs&2Ds	1	Susceptible	No	NT	MSSP/ ST1115	*lukS/F-I*^{1}, *siet*^{1}, *sient*^{1}
D/28	11/2Hs&2Ds	1	SXT^{1}	No	*dfrK*^{1}	MSSP/ NT	*lukS/F-I* ^{1}, *siet*^{1}, *sient*^{1}
D/29	11/2Hs&2Ds	1	SXT^{1}	No	ND	MSSP/ NT	*lukS/F-I* ^{1}, *siet*^{1}, *sient*^{1}
D/43	16/2Hs&1D	1	PEN^{1}	No	*blaZ*^{1}	MSSP/ NT	*lukS/F-I* ^{1}, *siet*^{1}, *sient*^{1}
D/39	15/1H&2Ds	1	PEN^{1}-SXT^{1}	No	*blaZ*^{1}, *dfrA*^{1}, *dfrG*^{1}	MSSP/ NT	*lukS/F-I* ^{1}, *siet*^{1}, *sient*^{1}
D/39	15/1H&2Ds	1	SXT^{1}	No	*dfrA*^{1}, *dfrG*^{1}	MSSP/ NT	*lukS/F-I* ^{1}, *siet*^{1}, *sient*^{1}
D/49	18/2Hs&2Ds	1	SXT^{1}	No	*dfrA*^{1}, *dfrG*^{1}	MSSP/ NT	*lukS/F-I* ^{2}, *siet*^{2}, *sient*^{2}
D/48	18/2Hs&2Ds	1	TET^{1}	No	*tet*(M)^{1}	MSSP/NT	*lukS/F-I* ^{4}, *siet*^{4}, *sient*^{4}
D/52	19/2Hs&2Ds	3	PEN^{3}-SXT^{3}-TOB^{1}	Yes	*blaZ*^{3}, *dfrA*^{1}, *dfrG*^{1}, *blaZ*^{1}, *dfrG*, *ant4'*^{1}	MSSP/ NT MSSP/ NT	*lukS/F-I* ^{3}, *siet*^{3}, *sient*^{3}
D/53	19/2Hs&2Ds	2	PEN^{2}-SXT^{2}	No	*blaZ*^{1}, *dfrG*^{2}, *dfrK*^{2},	MSSP/ NT	*lukS/F-I* ^{2}, *siet*^{2}, *sient*^{2}
D/60	22/1H&1D	1	PEN^{1}-ERY^{1}-CLI^{1}-CHL^{1}-SXT^{1}	Yes	*ermB*^{1}, *catA*^{1}, *dfrD*^{1}	MSSP/ NT	*lukS/F-I* ^{1}, *siet*^{1}, *sient*^{1}

[a]H, human; D, dog; [b]In superscript is the number of strains that present the specific characteristic
ST; Sequence Type; NT: Not tested; ND: not detected; CHL: Chloramphenicol; CLI: clindamycin; ERY: erythromycin; PEN: penicillin; OXA: oxacillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline; TOB: tobramycin

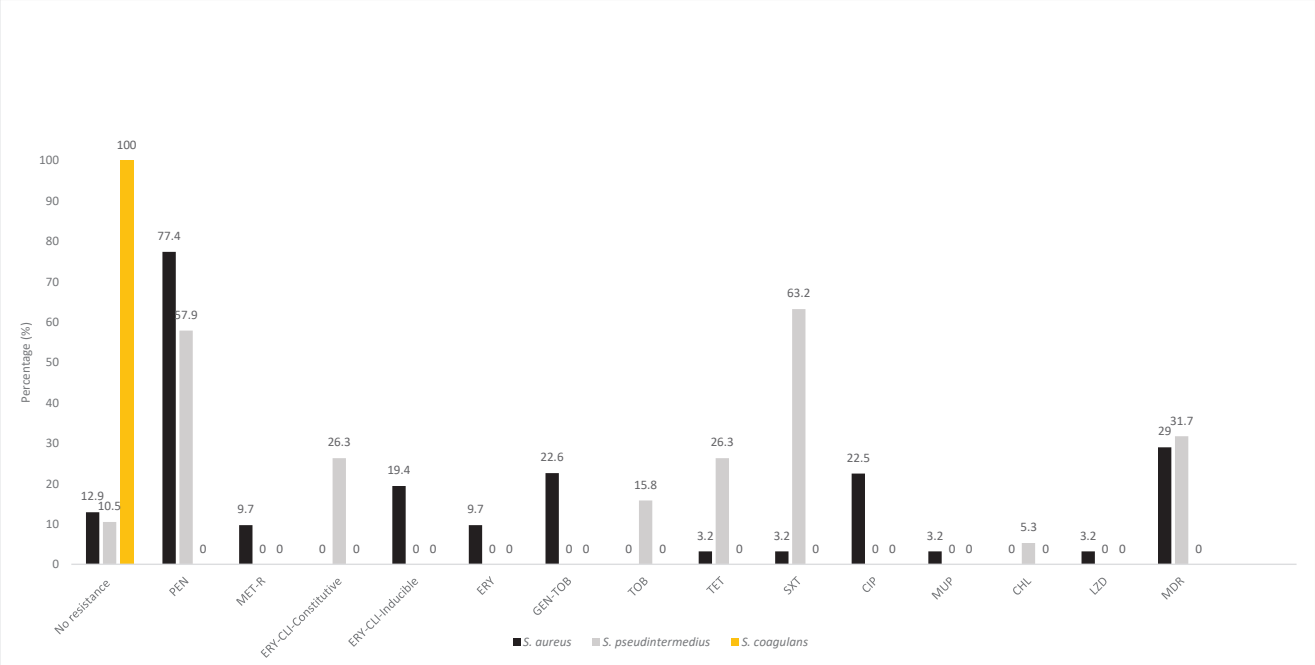


Figure 35. Antimicrobial resistance rates in *S. aureus*, *S. pseudintermedius* and *S. coagulans* strains.
Percentages were based on the collection of CoPS (31 *S. aureus*, 19 *S. pseudintermedius* and 2 *S. coagulans*) obtained from different samples or those of the same sample but with different species and/or AMR phenotype.
Abbreviation: CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: Erythromycin; GEN; gentamicin; MET-R: methicillin-resistant; MDR: multidrug resistance (resistance to three or more classes of antibiotics); PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin.

3.1.3.5 Frequencies and species diversity of coagulase-negative staphylococci in healthy dogs and dog-owners

A total of 216 CoNS were recovered from the 34 dogs and 41 dog-owners of the 27 households tested in this study (up to six strains per positive sample) and the distribution of species is indicated in **Table 54**. After species identification and AMR phenotype determination, a collection of 130 non-repetitive CoNS strains was obtained and they were genetically characterized and considered in this study. These 130 CoNS strains corresponded to one strain of each species per sample or more than one if they presented different AMR phenotypes **(Table 54).**

A total of 32 non-repetitive CoNS strains were identified from 16 of the 34 dogs (of nine species: *S. epidermidis, S. hominis, S. cohnii, S. pasteuri, S. warneri, S. xylosus, S. haemolyticus, S. simulans* and *S. muscae*) **(Table 54).** In addition, 98 non-repetitive strains were identified from 37 of 41 dog-owners (of six species: *S. epidermidis, S. lugdunensis, S. hominis, S. pasteuri, S. warneri* and *S. xylosus*) **(Table 54).** About 41.7% and 90.2% of dogs and dog-owners carried at least one CoNS species, respectively. The predominant species from dog carriers were *S. epidermidis* (26.5%), *S. hominis* (8.8%), *S. cohnii* (8.8%); whereas, in the human carriers, the predominant ones were *S. epidermidis* (80.4%), *S. lugdunensis* (9.8%) and *S. hominis* (9.8%) **(Table 54)**.

3.1.3.6 Antimicrobial resistance phenotypes and genotypes of non-repetitive CoNS strains

Of the 32 and 98 non-repetitive CoNS from dogs and dog-owners, 21.9% and 19.4% were susceptible to all antimicrobial agents tested, respectively. Also, 34.4% and 26.5% of the dogs and dog-owners strains were resistant to only one antibiotic tested **(Table 54)**. However, 28.1% and 32.7% of the dogs and dog-owners strains presented an MDR phenotype. Collectively, 20% were susceptible to all antibiotics tested while 31.5% carried multidrug resistance (MDR) phenotype **(Table 54)**.

The rates of resistance detected in the collection of 130 CoNS strains were as follows (percentage of resistance/detected genes or mutations): penicillin (50/*blaZ*), cefoxitin (17.4/*mecA*), erythromycin-clindamycin-inducible (8.5/*ermA*), erythromycin-clindamycin-constitutive (8.5/*ermC, ermT*), erythromycin (32.3/ *mphC, msrA*), clindamycin (5.4/ *vgaA, lsaB*), tobramycin (9.2/ *ant4'*), gentamicin-tobramycin (5.4/*aac6'-aph2"*), tetracycline (10/*tet*(K), *tet*(M)), sulfamethoxazole-trimethoprim (13.1/*dfrA, dfrG*), chloramphenicol (4.6/cat_{PC221}), mupirocin (20.8/*mupA*), linezolid (0.8/four mutations in L3 and one in L4

ribosomal proteins) and ciprofloxacin (3.8) **(Tables 55 and 56, and Figures 36, 37a &b).** The *ermT* gene was detected only in *S. epidermidis* and *S. hominis* strains of dog-owners **(Table 54)**. Moreover, one of the four *S. lugdunensis* strains identified in four dog-owners carried an MDR phenotype (PEN-ERY-MUP/*blaZ, ermA, mupA*), other two were only resistant to PEN (*blaZ*) while the remaining one was susceptible to all antibiotics tested **(Tables 55 and 56).**

The linezolid-resistant *S. epidermidis* strain had an MIC for this antibiotic of 16µg/mL and resistance was mediated by mutations on 50S ribosomal protein L3 (Ile188Val, Gly218Val, Asp219Ile, Lue220Asp) and L4 (Asn158Ser) **(Figures 37a & b).** Among the 33 MRCoNS, SCC*mec* type V element was the predominant (24.2%), followed by SCC*mec* type III (18.1%), SCC*mec* type IVc (12.1%) and SCC*mec* type II (6.1%), while others were non-typeable (39.5%) **(Figure 38).**

3.1.3.7 Intra-host species diversity of coagulase-negative staphylococci in healthy dogs and dog-owners

Intra-host species diversity (more than one CoNS species in a sample) was detected in 37.5% of dogs and 21.6% of dog-owners **(Figure 39 and Table 57)**. In one of the dog-owners (number 26 in household 11) carrying diverse strains, three heterogeneous *S. epidermidis* and two *S. hominis* strains carrying different resistome were identified **(Table 57).** Also, in dog-owner 66 of household 25, three heterogeneous *S. epidermidis* and one *S. lugdunensis* strain carrying different antimicrobial resistance genes were identified **(Table 57)**. In dog 18 of household 8, five different strains of *S. epidermidis, S. haemolyticus* and *S. warneri* carrying both methicillin-resistant and methicillin-susceptible traits were identified **(Table 57)**. Conversely, 50% of dogs and 70.3% of dog-owners had intra-species AMR diversity (2-4 varied AMR profiles) **(Figure 39).**

3.1.3.8 Intra-household carriers of similar *S. epidermidis* strains and their genetic lineages

Dogs and dog-owners' carriers of *S. epidermidis* with similar AMR patterns and genetic lineages were detected in three households (11.1%). In two of these households, the *S. epidermidis* were susceptible to all antibiotics tested while the other *S. epidermidis* strains from the 3rd household were resistant to only clindamycin **(Table 58).** The genetic lineage of *S. epidermidis* in the carriers in households 3, 6, 11, 13 and 19 were ST59, ST61, and ST278.

Table 54. Frequencies of coagulase-negative staphylococci from healthy dogs and dog-owners and characteristics of non-repetitive strains[a].

CoNS species	Total strains recovered of dogs and dog-owners	Non-repetitive strains		Nº of carriers (%)		Non-repetitive strains with the following characteristics:						
						Susceptible to all antibiotics (%)		Resistance to only one antibiotic (%)		MDR phenotype (%)		
		Dogs	Dog-owners	Dogs	Dog-owners	Dogs	Dog-owners	Dogs	Dog-owners	Dogs	Dog-owners	Total
S. epidermidis	167	12	82	9 (26.5)	33 (80.4)	3 (25)	14 (17.1)	4 (33.3)	22 (26.8)	4 (33.3)	29 (35.6)	33 (35.1)
S. hominis	13	3	5	3 (8.8)	4 (9.8)	1 (33.3)	1 (20)	1 (33.3)	1 (20)	2 (66.7)	2 (40)	4 (50)
S. cohnii	4	3	0	3 (8.8)	0	0	0	0	0	2 (66.7)	0	2 (66.7)
S. lugdunensis	5	0	4	0	4 (9.8)	0	1 (25)	0	1 (25)	0	1 (25)	1 (25)
S. pasteuri	7	1	4	1 (2.9)	3 (7.3)	1 (100)	2 (50)	0	1 (25)	0	0	0
S. warneri	9	5	2	2 (5.7)	2 (4.9)	0	1 (50)	2 (40)	0	1 (20)	0	1 14.3)
S. xylosus	4	2	1	1 (2.9)	1 (2.4)	0	0	1 (50)	1 (100)	0	0	0
S. haemolyticus	3	3	0	2 (5.7)	0	1 (33.3)	0	1 (33.3)	0	0	0	0
S. simulans	3	2	0	2 (5.7)	0	1 (50)	0	1 (50)	0	0	0	0
S. muscae	1	1	0	1 (2.9)	0	0	0	1 (100)	0	0	0	0
Total (%)	216	32	98	16 (47.1)	37 (90.2)	7 (21.9)	19 (19.4)	11 (34.4)	26 (26.5)	9 (28.1)	32 (32.7)	41 (31.5)

[a]Non-repetitive strains are those of different individuals or of different species or different AMR phenotypes.

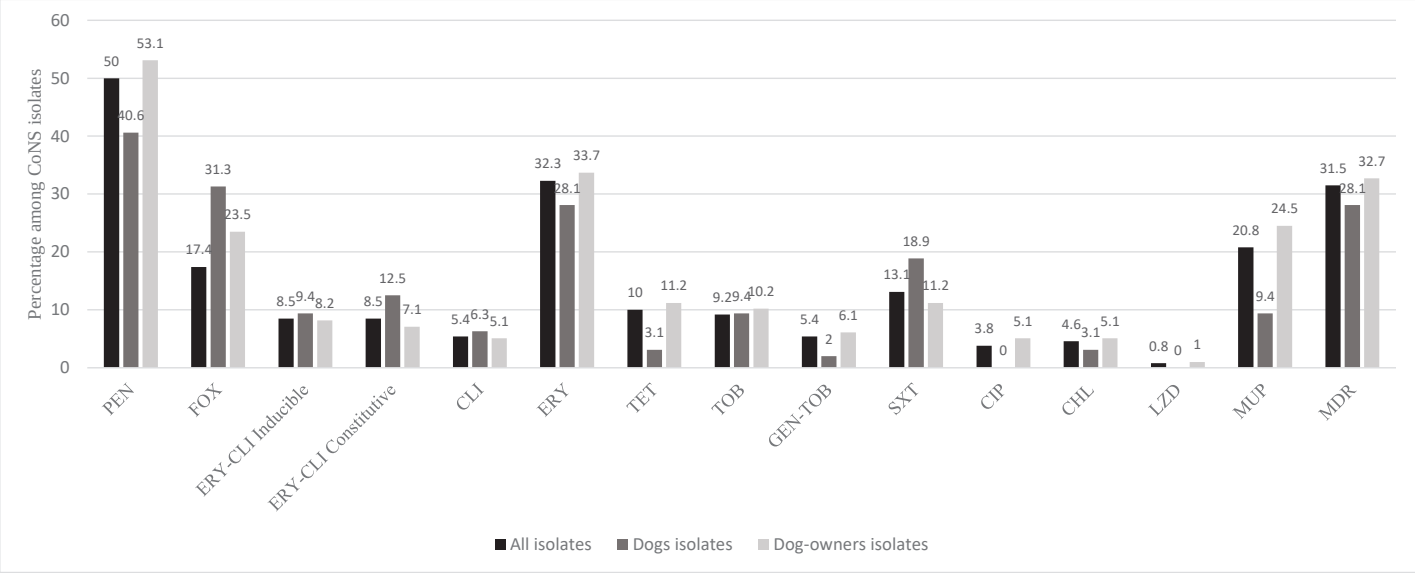


Figure 36. Frequency of antimicrobial resistance among non-repetitive coagulase-negative staphylococci strains from nasal cavities of healthy dogs and dog-owners.

Abbreviation: CHL: Chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; LZD: linezolid; MUP: mupirocin; MDR: multi-drug resistance phenotype; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin.

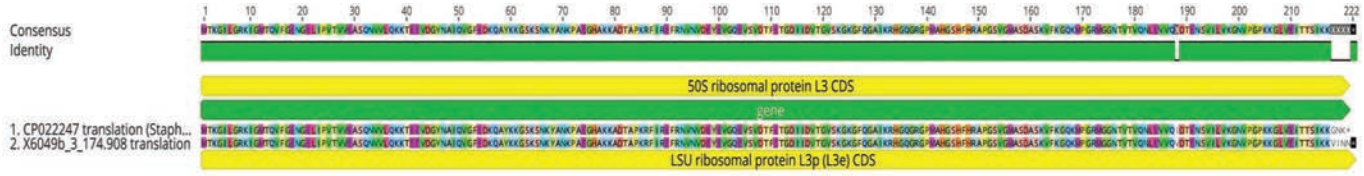
Table 55. Antimicrobial resistance phenotypes and genotypes in non-repetitive coagulase-negative staphylococci identified from healthy dog-owners.

Species with antimicrobial resistance in dog-owners	Antimicrobial resistance among strains from dog-owners	
	Phenotype (number)	**Genes or mutations detected (number)**
S. epidermidis, S. pasteuri, xylosus	PEN (49)	*blaZ* (46)
S. lugdunensis	PEN (3)	*blaZ* (3)
S. epidermidis, S. hominis	FOX (23)	*mecA* (23)
S. epidermidis, S. hominis	ERY-CLI constitutive (5)	*ermA* (5)
S. epidermidis, S. hominis	ERY-CLI constitutive (2)	*ermT* (2)
S. epidermidis	ERY-CLI inducible (8)	*ermC* (8)
S. epidermidis, S. pasteuri, S. hominis	ERY (33)	*msrA* (33), *mphC* (6)
S. lugdunensis	ERY (1)	*msrA* (1)
S. epidermidis	CLI (5)	*vgaA* (5), *lsaB* (1)
S. epidermidis, hominis	GEN-TOB (6)	*aac6'-aph2"* (6)
S. epidermidis	TOB (9)	*ant4'* (9)
S. epidermidis	CIP (5)	NT
S. epidermidis, S. hominis	TET (11)	*tet*(K) (11), *tet*(M) (1)
S. epidermidis	SXT (11)	*dfrA* (10), *dfrG* (5)
S. epidermidis	CHL (5)	cat_{PC221} (5)
S. epidermidis-ST35	LZD (1)	MIC of 16 μg/mL and mediated by mutations in L3 (I188V, G218V, N219I, L220D) and L4 (N158S)
S. epidermidis, S. hominis, S. warneri	MUP (23)	*mupA* (23)
S. lugdunensis	MUP (1)	*mupA* (1)

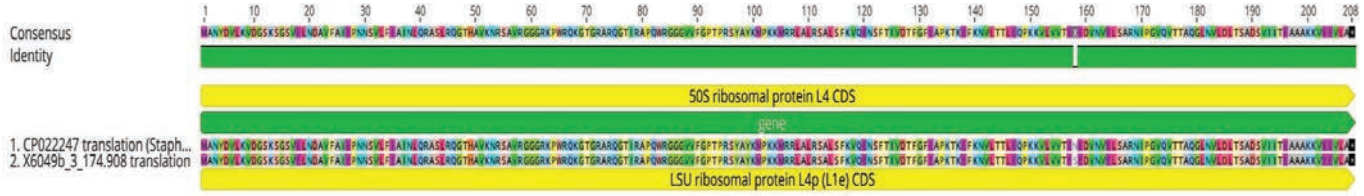
Table 56. Antimicrobial resistance phenotypes and genotypes in non-repetitive coagulase-negative staphylococci identified from healthy dogs

Species with antimicrobial resistance in dogs	Antimicrobial resistance among strains from dog	
	Phenotype (number)	**Genes or mutations detected (number)**
S. epidermidis, S. cohnii, S. simulans, xylosus	PEN (13)	*blaZ* (13)
S. epidermidis, S. cohnii, S. hominis	FOX (10)	*mecA* (10)
S. epidermidis, S. cohnii	ERY-CLI constitutive (4)	*ermA* (4)
S. epidermidis, S. hominis	ERY-CLI inducible (3)	*ermC* (3)
S. epidermidis, S. warneri, S. hominis	ERY (9)	*msrA* (9)
S. epidermidis	CLI (2)	*vgaA* (2)
S. haemolyticus	GEN-TOB (1)	*aac6'-aph2"* (1)
S. hominis, S. xylosus	TOB (3)	*ant4'* (3)
S. haemolyticus	TET (1)	*tet*(K) (1)
S. epidermidis, S. warneri, S. hominis	SXT (6)	*dfrA* (6)
S. warneri	CHL (1)	cat_{PC221} (1)
S. epidermidis	MUP (3)	*mupA* (3)

Abbreviation: CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; LZD: linezolid; MUP: mupirocin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin
NT: Not tested; ND: Not detected.



(a)



(b)

Figure 37. Consensus of the ribosomal protein L3 and L4 sequences of *S epidermidis*-ST35 (X6049b) showing various amino acids point mutations

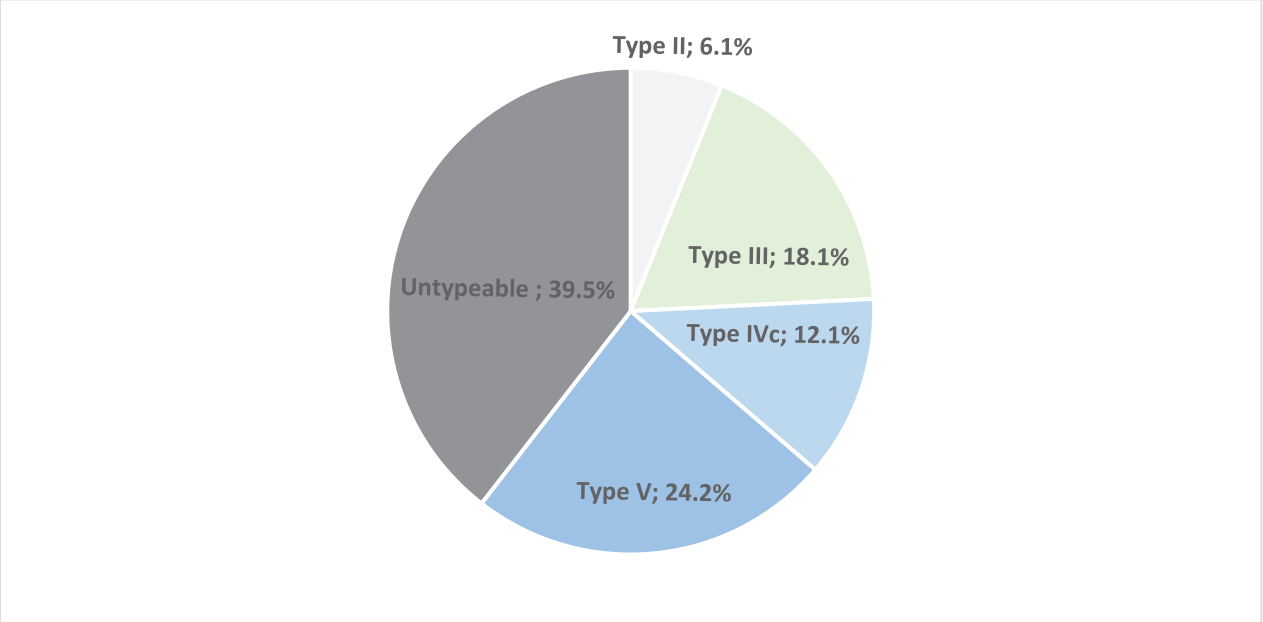


Figure 38. Frequency of the SCC*mec* types identified among the 33 non-repetitive methicillin-resistant coagulase-negative staphylococci strains in healthy dogs and dog-owners

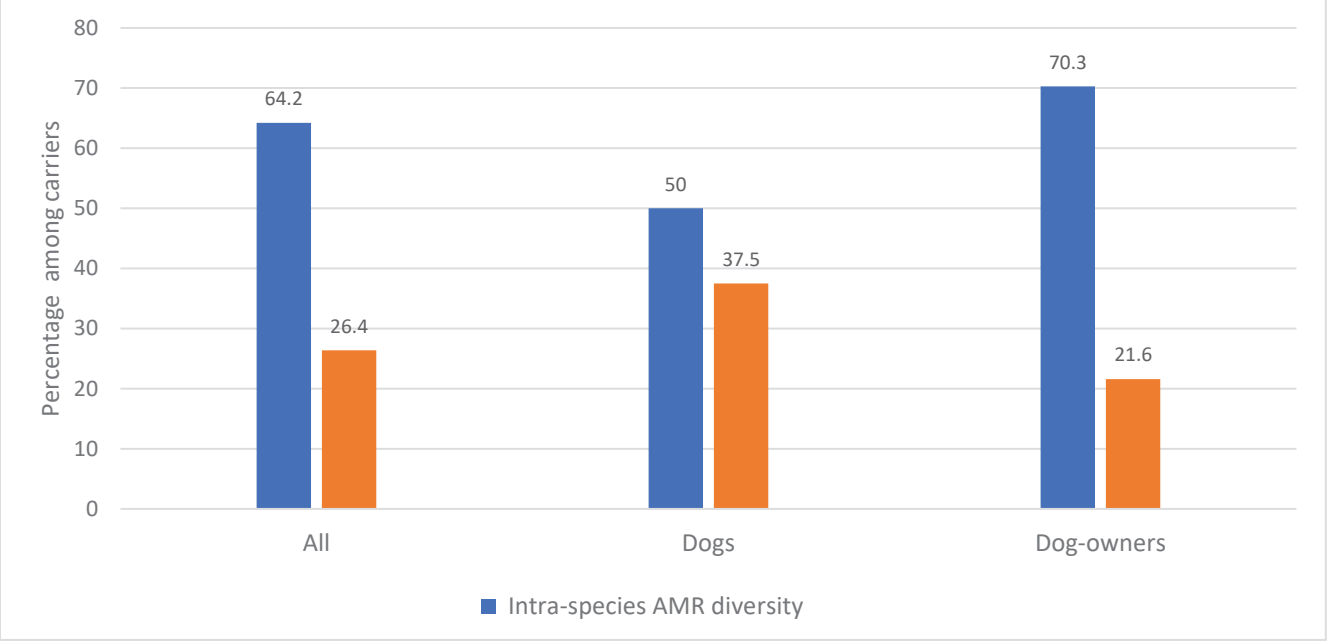


Figure 39. Frequency of intra-host species and intra-species AMR diversity among non-repetitive coagulase-negative staphylococci from healthy dogs and dog-owners.
Note: The number of hosts with nasal carriage of more than one CoNS species were 16 dogs and 37 dog-owners.

Table 57. Intra-host species and intra-species AMR diversity in coagulase-negative staphylococci from healthy dogs and dog-owners

Host	Host ID/ household	Species	AMR Phenotype	AMR genes detected
Human	22/ 10	*S. pasteuri*	Susceptible	NT
		S. pasteuri	PEN-ERY	*blaZ, msrA*
		S. epidermidis	PEN-TOB	*blaZ, ant4'*
		S. epidermidis	MUP	*mupA*
	26/ 11	*S. epidermidis*	PEN	*blaZ*
		S. epidermidis	PEN-ERY-CLI^{ind}	*blaZ, ermC*
		S. epidermidis	PEN-ERY-CLI-MUP-LZD	*blaZ, mecA, msrA, mphC, mupA*
		S. hominis	PEN-FOX-ERY-CLI-TET-MUP	*blaZ, mecA, ermA, tet*(K), *mupA*
		S. hominis	PEN-ERY-GEN-MUP	*blaZ, mecA, msrA, aac6'-aph2", mupA*
	27/ 11	*S. epidermidis*	PEN-ERY-CLI-MUP	*blaZ, ermC, mupA*
		S. lugdunensis	PEN	*blaZ*
		S. hominis	PEN	*blaZ*
	35/ 14	*S. pasteuri*	ERY	*msrA*
		S. epidermidis	CLI-FOX	*lnuA, mecA*
	64/ 24	*S. xylosus*	PEN	*blaZ*
		S. epidermidis	Susceptible	NT
	66/ 25	*S. epidermidis*	FOX-TET-MUP	*mecA, tet*(K), *mupA*
		S. epidermidis	PEN-FOX-ERY-MUP	*blaZ, mecA, msrA, mphC, mupA*
		S. epidermidis	PEN-FOX-GEN-TOB-CIP	*blaZ, mecA, aac6'-aph2"*
		S. lugdunensis	PEN-ERY-MUP	*blaZ, msrA, mupA*
	72/ 27	*S. epidermidis*	PEN-ERY	*blaZ, msrA, mphC*
		S. hominis	Susceptible	NT
	74/ 27	*S. epidermidis*	PEN-SXT-CIP	*blaZ, dfrA, dfrG,*
		S. epidermidis	PEN-FOX-ERY-CIP	*blaZ, mecA, msrA*
		S. lugdunensis	PEN	*blaZ*
Dog	4/ 2	*S. hominis*	PEN-ERY-TOB	*blaZ, msrA, ant4'*
		S. epidermidis	ERY	*msrA*
	18/ 8	*S. haemolyticus*	TET	*tet*(K), *tet*(M)
		S. warneri	ERY-SXT	*msrA, dfrA*
		S. warneri	ERY-SXT-CHL	*msrA, dfrA, cat*$_{PC221}$
		S. epidermidis	PEN-FOX-ERY-SXT	*blaZ, mecA, msrA, dfrA*
		S. epidermidis	PEN-FOX	*blaZ, mecA*
	28/ 11	*S. simulans*	Susceptible	NT
		S. epidermidis	PEN	*blaZ*
		S. pasteuri	Susceptible	NT
	29/ 11	*S. cohnii*	PEN-FOX-ERY-CLI^{ind}	*blaZ, mecA, ermA*
		S. simulans	PEN	*blaZ*
	32/ 13	*S. haemolyticus*	GEN-TOB	*aac6'-aph2"*
		S. epidermidis	FOX-ERY-CLI^{ind}-MUP	*mecA, ermA, mupA*
		S. epidermidis	Susceptible	NT
		S. epidermidis	FOX-ERY-MUP	*mecA, msrA, mupA*
		S. haemolyticus	FOX-ERY-CLI^{ind}-SXT-MUP	*mecA, ermA, dfrA, mupA*
	59/ 21	*S. hominis*	PEN	*blaZ*
		S. warneri	PEN-ERY	*blaZ, msrA*
		S. wareneri	PEN	*blaZ*

Abbreviation: CHL: Chloramphenicol; CLI: clindamycin; CLI^{ind}: clindamycin inducible CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; LZD: linezolid; MUP: mupirocin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin.
NT: Not tested.

Table 58. Intra-household carriers of coagulase-negative staphylococci with similar AMR pattern and sequence type

Household code	Host ID code	Species	AMR Phenotype	AMR gene detected	Sequence Type
3	Human 5	*S. epidermidis*	Susceptible	NT	ST59
	Dog 6	*S. epidermidis*	Susceptible	NT	ST59
6	Human 13	*S. epidermidis*	Susceptible	NT	ST166
	Dog 12	*S. epidermidis*	Susceptible	NT	ST166
11	Human 26	*S. epidermidis*	PEN	*blaZ*	ST5
	Dog 28	*S. epidermidis*	PEN	*blaZ*	ST88
13	Dog 32	*S. epidermidis*	Susceptible	NT	ST61
	Human 33	*S. epidermidis*	Susceptible	NT	ST61
	Human 34	*S. epidermidis*	Susceptible	NT	ST73
19	Human 50	*S. epidermidis*	Susceptible	NT	ST85
	Hunan 51	*S. epidermidis*	CLI	*vgaA*	ST278
	Dog 53	*S. epidermidis*	CLI	*vgaA*	ST278

Abbreviation: CLI: clindamycin; PEN: penicillin; NT: not tested.

3.1.4 HEALTHY PIGS AND PIG FARMERS

3.1.4.1 Frequency of bacteria Species and Genera Recovered from the nasal cavities of healthy pigs and pig farmers

A total of 355 strains were recovered (up to 14 per sample) and were identified by MALDI-TOF-MS. Of this, 289 were from pigs while 66 were from pig farmers. A total of 30 species were detected including species of *Staphylocccus*, *Enterococcus*, *Streptococcus* and *Escherichia (E coli),* among others **(Table 59).** Of all the identified bacteria, *Staphylococcus* spp, *Enterococcus* spp and *E coli* were the most frequent and diverse **(Table 59).**

Table 59. Number of strains and frequency of carriers of bacteria species recovered from the nose of healthy pigs and pig farmers

Bacteria genera and species	Nº (%)strains from pigs nasal samples	Number (%) pig carriers (n=40)	Nº (%)strains from pig farmers nasal samples	Number (%)pig farmers carriers (n=10)
Staphylococcus				
S. aureus	106	26 (65)	36	8 (80)
S. chromogenes	13	9 (22.5)	2	1 (10)
S. haemolyticus	15	8 (20)	2	1 (10)
S. hyicus	10	8 (20)	1	1 (10)
S. sciuri	29	6 (15)	0	0
S. epidermidis	5	5 (12.5)	8	4 (40)
S. saprophyticus	6	3 (7.5)	1	1 (10)
S. xylosus	3	2 (5)	0	0
S. pasteuri	2	2 (5)	0	0
S. simulans	1	1 (2.5)	3	2 (20)

Table 59. Continuation

Bacteria genera and species	Nº (%)strains from pigs nasal samples	Number (%) pig carriers (n=40)	Nº (%)strains from pig farmers nasal samples	Number (%)pig farmers carriers (n=10)
Enterococcus				
E. faecalis	34	15 (37.5)	4	3 (30)
E. faecium	4	3 (7.5)	4	3 (30)
E. casseliflavus	1	1 (2.5)	0	0
E. gallinarum	2	1 (2.5)	0	0
E. hirae	2	1 (2.5)	0	0
Citrobacter				
C. braakii	4	4 (10)	0	0
C. freundii	1	1 (2.5)		
Klebsiella pneumoniae	1	1 (2.5)	0	0
Streptococcus				
S. suis	1	1 (2.5)	0	0
S. dysgalactiae	1	1 (2.5)	0	0
S. pluranimalium	0	0	1	1 (10)
S. hyovaginalis	1	1 (2.5)	0	0
Corynebacterium glutamicuum	5	4 (10)	0	0
Pontea agglomerans	1	1 (2.5)	0	0
Vagococcus lutrae	2	2 (5)	1	1 (10)
Bacillus licheniformis	1	1 (2.5)	0	0
E. coli	26	17 (42.5)	2	2 (20)
Rothia nasimurium	11	8 (20)	0	0
Aerococcus viridians	1	1 (2.5)	0	0
Morganella morganii	0	0	1	1 (10)
Total	289		66	

3.1.4.2 Nasal staphylococci diversity in healthy pigs and pig farmers

A total of 243 staphylococci were isolated and identified from the nasal samples of healthy pigs and pig-farmers and they were distributed into 10 species. Of this, 142 *S. aureus*, 29 *S. sciuri*, 17 *S. haemolyticus*, 15 *S. chromogenes*, 13 *S. epidermidis*, 11 *S. hyicus*, 7 *S. saprophyticus*, 4 *S. simulans*, 3 *S. xylosus* and 2 *S. pasteuri* strains were recovered from 38 nasal samples of pigs and 9 of pig-farmers **(Table 60)**. Concerning the nasal staphylococcal species in the pigs, 65% of the animals were *S. aureus* carriers, and the carriage rate for other species were: *S. chromogenes* (22.5%), *S. haemolyticus* (20%), *S. hyicus* (20%), *S. sciuri* (15%), *S. epidermidis* (12.5%), *S. saprophyticus* (7.5%), *S. xylosus* (5%), *S. pasteuri* (5%) and *S. simulans* (2.5%). Whereas the nasal staphylococci carriage in pig-farmers was highest for *S. aureus* (80%), *S. epidermidis* (40%), *S. simulans* (20%), and 10% each for *S. chromogenes, S. saprophyicyus, S. hyicus* and *S. haemolyticus*. None of the pig-farmers had nasal carriage of *S. xylosus, S. sciuri* and *S. pasteuri* **(Table 60)**.

Table 60. Number of strains and carriage rate of each staphylococci species recovered from the nasal samples of pigs and pig-farmers in four Spanish farms (A-D)

Species	**Nº of strains from pigs in farm A**	**Nº (%) of pigs from farm A**	**Nº of strains from pigs in farm B**	**Nº (%) of pigs from farm B**	**Nº of strains from pigs in farm C**	**Nº (%) of pigs from farm C**	**Nº of strains from pigs in farm D**	**Nº (%) of pigs from farm D**	**Nº of strains from pigs in all farms**	**No. (%) of pigs from all farms**
S. aureus	18	6 (60)	31	7 (70)	14	3 (30)	43	10 (100)	106	26 (65)
S. chromogenes	9	5 (50)	1	1 (10)	2	2 (20)	1	1 (10)	13	9 (22.5)
S. haemolyticus	7	4 (40)	7	3 (30)	0	0 (0)	1	1 (10)	15	8 (20)
S. hyicus	3	3 (30)	5	3 (30)	2	2 (20)	0	0 (0)	10	8 (20)
S. sciuri	10	6 (60)	0	0 (0)	19	9 (90)	0	0 (0)	29	6 (15)
S. epidermidis	4	4 (40)	1	1 (10)	0	0 (0)	0	0 (0)	5	5 (12.5)
S. saprophyticus	5	2 (20)	1	1 (10)	0	0 (0)	0	0 (0)	6	3 (7.5)
S. xylosus	0	0 (0)	0	0 (0)	3	2 (20)	0	0 (0)	3	2 (5)
S. pasteuri	2	2 (20)	0	0 (0)	0	0 (0)	0	0 (0)	2	2 (5)
S. simulans	1	1 (10)	0	0 (0)	0	0 (0)	0	0 (0)	1	1 (2.5)
Species	**Nº of strains from pig-famers in farm A**	**Nº (%) of pig-famers from farm A**	**Nº of strains from pig-famers in farm B**	**Nº (%) of pig-famers from farm B**	**Nº of strains from pig-famers in farm C**	**Nº (%) of pig-famers from farm C**	**Nº of strains from pig-famers in farm D**	**Nº (%) of pig-famers from farm D**	**Nº of strains from pig-famers in all farms**	**No. (%) of pig-farmers from all farms**
S. aureus	5	1 (50)	15	3 (100)	4	2 (100)	12	2 (66.7)	36	8 (80)
S. chromogenes	0	0 (0)	0	0 (0)	0	0 (0)	2	1 (33.3)	2	1 (10)
S. haemolyticus	0	0 (0)	0	0 (0)	0	0 (0)	2	1 (33.3)	2	1 (10)
S. hyicus	0	0 (0)	1	1 (33.3)	0	0 (0)	0	0 (0)	1	1 (10)
S. sciuri	0	0 (0)	0	0 (0)	0	0 (0)	0	0 (0)	0	0 (0)
S. epidermidis	5	1 (50)	2	2 (66.6)	1	1 (50)	0	0 (0)	8	4 (40)
S. saprophyticus	0	0 (0)	1	1 (33.3)	0	0 (0)	0	0 (0)	1	1 (10)
S. xylosus	0	0 (0)	0	0 (0)	0	0 (0)	0	0 (0)	0	0 (0)
S. pasteuri	0	0 (0)	0	0 (0)	0	0 (0)	0	0 (0)	0	0 (0)
S. simulans	0	0 (0)	0	0 (0)	1	1 (50)	2	1 (33.3)	3	2 (20)

Note: Between 4 to 9 different staphylococci colonies were randomly selected per sample

3.1.4.3 Phenotypic and genetic characteristics of *S. aureus* strains

After AMR phenotype determination of all the 142 *S. aureus* strains, 51 distinct strains were selected for further characterization that corresponded to one per sample or more than one if they showed different AMR phenotypes. Of all the 51 distinct *S. aureus* strains, only 6 (11.8%, 4 from pigs and 2 from pig-farmers) were methicillin-susceptible (MSSA) and were all from farm-C. Essentially, the MRSA strains from pigs (*n* = 33) harboured AMR as follows (percentage of resistant strains/resistance genes detected): penicillin (100/*blaZ*), cefoxitin (100/*mecA*), erythromycin-clindamycin-constitutive (90.1/*ermB, ermC, ermT*), clindamycin (9.1/*lnuB*), gentamicin-tobramycin (63.6/*aac6'-aph2"*), tobramycin (9.1/*ant4'*), tetracycline (100/*tet*(K), *tet*(L), *tet*(M)), ciprofloxacin (60.1), sulfamethoxazole-trimethoprim (87.9/*dfrA, dfrG, dfrK*), and chloramphenicol (39.4/*fexA*, cat_{PC221}). Moreover, the 12 distinct MRSA strains from pig-farmers harboured AMR as follows: penicillin (100/*blaZ*), cefoxitin (100/*mecA*), erythromycin-clindamycin-constitutive (69.2/*ermC, ermT*), clindamycin (16.7/*lnuB*), gentamicin-tobramycin (41.6/*aac6'-aph2"*), tobramycin (23.1/*ant4'*), tetracycline (100/*tet*(K), *tet*(M)), ciprofloxacin (58.3), sulfamethoxazole-trimethoprim (66.7/*dfrA, dfrG, dfrK*), and chloramphenicol (25/*fexA*, cat_{PC221}) **(Table 61, Figure 40)**.

Regarding the 4 MSSA strains from pigs, they harboured AMR as follows (percentage of resistance/detected genes): penicillin (100/*blaZ*), erythromycin-clindamycin-constitutive (75/*ermC*), clindamycin (25/*lnuB*), gentamicin-tobramycin (100/*aac6'-aph2"*), tetracycline (100/*tet(K), tet(M)*), ciprofloxacin (100), trimethoprim-sulfamethoxazole (75/*dfrA, dfrK*) and chloramphenicol (75/*fexA*) (Table 61, Figure 39)**.** However, one of the two MSSA strains from the pig-farmers was resistant to only penicillin, while the other harboured *dfrA, dfrG, tet(K), tet(M), aac6'-aph2"* and *fexA* resistance genes **(Table 61)**.

Table 61. With-hosts and -farm variations of resistomes and genetic lineages of *S. aureus* strains from all pigs and pig-farmers of the four analysed farms (A-D).

Farm	Host/ ID	N. strains	AMR Phenotypes	AMR genes detected	*spa*/CC [a]	IEC
A	Pig 1	1	PEN-FOX-SXT-ERY-CLI-TET-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M)	t011/CC398	Negative
	Pig 2	1	PEN-FOX-SXT-ERY-CLI-TET-TOB	*blaZ*, *mecA*, *dfrA*, *ermB*, *ermC*, *tet*(M), *ant4'*, *aac6'-aph2''*	t011/CC398	Negative
	Pig 4	3	PEN-FOX-SXT-ERY-CLI-TET-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *ermT*, *tet*(K), *tet*(L), *tet*(M)	t011/CC398	Negative
	Pig 5	4	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(M), *aac6'-aph2''*	t011/CC398	Negative
	Pig 5	3	PEN-FOX-SXT-ERY-CLI-TOB-TET	*blaZ*, *mecA*, *dfrA*, *ermC*, *tet*(K), *tet*(M), *ant4'*, *aac6'-aph2''*	t011/CC398	Negative
	Pig 6	3	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN	*blaZ*, *mecA*, *dfrA*, *ermB*, *ermC*, *tet*(M), *aac6'-aph2''*	t011/CC398	Negative
	Pig 6	1	PEN-FOX-SXT-ERY-CLI-TET-TOB	*blaZ*, *mecA*, *dfrA*, *ermB*, *ermC*, *tet*(K), *tet*(M), *ant4'*, *aac6'-aph2''*	t011/CC398	Negative
	Pig 8	2	PEN-FOX-SXT-ERY-CLI-TET	*blaZ*, *mecA*, *dfrG*, *ermA*, *tet*(M)	t4571/CC398	Negative
	Pig-farmer 2	2	PEN-FOX-SXT-ERY-CLI-TET-TOB	*blaZ*, *mecA*, *dfrK*, *ermC*, *ermT*, *tet*(K), *tet*(L), *tet*(M)	t011/CC398	Negative
	Pig-farmer 2	3	PEN-FOX-SXT-ERY-CLI-TET-CIP-GEN-TOB	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(M), *aac6'-aph2''*	t011/CC398	Negative
B	Pig 1	2	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN	*blaZ*, *mecA*, *ermC*, *tet*(K), *tet*(M), *aac6'-aph2''*	t011/CC398	Negative
	Pig 1	1	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL	*blaZ*, *mecA*, *dfrG*, *ermB*, *ermC*, *tet*(M), *aac6'-aph2''*, *fexA*	t011/CC398	Negative
	Pig 3	6	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN	*blaZ*, *mecA*, *ermC*, *tet*(K), *tet*(M), *aac6'-aph2''*	t011/CC398	Negative
	Pig 4	3	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN	*blaZ*, *mecA*, *dfrA*, *ermC*, *tet*(M), *aac6'-aph2''*	t011/CC398	Negative
	Pig 5	3	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN	*blaZ*, *mecA*, *ermC*, *tet*(K), *tet*(M), *aac6'-aph2''*	t011/CC398	Negative
	Pig 7	1	PEN-FOX-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *mecA*, *tet(K)*, *tet*(M), *aac6'-aph2''*, *fexA*	t011/CC398	Negative
	Pig 7	1	PEN-FOX-CLI-TET-CIP	*blaZ*, *mecA*, *tet*(K), *tet*(M)	t011/CC398	Negative
	Pig 7	3	PEN-FOX-CLI-TET-TOB-GEN-CIP	*blaZ*, *mecA*, *tet*(M), *aac6'-aph2''*	t011/CC398	Negative
	Pig 8	4	PEN-FOX-SXT-ERY- CLI-TET-TOB-GEN	*blaZ*, *mecA*, *dfrA*, *ermB*, *ermC*, *tet*(M), *aac6'-aph2''*	t011/CC398	Negative
	Pig 9	7	PEN-FOX-SXT-ERY- CLI-TET-TOB-GEN	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(M), *aac6'-aph2''*	t011/CC398	Negative
	Pig farmer 1	4	PEN-FOX-ERY-CLI-TET-TOB-CHL-CIP	*blaZ*, *mecA*, *dfrG*, *ermC*, *lnuB*, *tet*(K), *tet*(M), *ant4'*, *fexA*	t034/CC398	Negative
	Pig farmer 1	1	PEN-FOX-CLI-TET-TOB-CHL-CIP	*blaZ*, *mecA*, *dfrG*, *lnuB*, *tet*(K), *tet*(M), *ant4'*, *fexA*	t034/CC398	Negative
	Pig farmer 2	2	PEN-FOX-TET-CIP	*blaZ*, *mecA*, *dfrA*, *tet*(M)	t011/CC398	Negative
	Pig farmer 2	2	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(M), *aac6'-aph2''*	t011/CC398	Negative
	Pig farmer 3	5	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(M), *aac6'-aph2''*	t011/CC398	Negative
	Pig farmer 3	1	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), *aac6'-aph2''*, *ant4'*, *fexA*	t011/CC398	Negative

Table 61. Continuation

Farm	Host/ ID	N. strains	AMR Phenotypes	AMR genes detected	*spa*/CC [a]	IEC
C	Pig 1	1	PEN-SXT-CLI-TET-TOB-GEN-CIP	*blaZ*, *dfrA*, *lnuB*, *tet*(K), *tet*(M), *aac6'-aph2''*	t191/CC9	Negative
	Pig 1	2	PEN-SXT-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *ermB*, *lnuB*, *tet*(L), *tet*(M), *aac6'-aph2''*, *fexA*	t1430/CC9	Negative
	Pig 3	5	PEN-SXT-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *dfrA*, *lnuB*, *ermB*, *ermC*, *tet*(K), *tet*(M), *aac6'-aph2''*, *fexA*	t1430/CC9	Negative
	Pig 5	3	PEN-FOX-SXT-ERY-CLI-TET-CIP	*blaZ*, *mecA*, *dfrK*, *ermB*, *tet*(K), *tet*(M)	t011/CC398	Negative
	Pig 5	2	PEN-FOX-ERY-CLI-TET-CIP	*blaZ*, *mecA*, *ermB*, *tet*(K), *tet*(M)	t011/CC398	Negative
	Pig 5	1	PEN-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *lnuB*, *tet*(K), *tet*(L), *aac6'-aph2''*, *fexA*	t1430/CC9	Negative
	Pig farmer 1	2	PEN-FOX-CLI-TET-CIP	*blaZ*, *mecA*, *dfrA*, *ermC*, *lnuB*, *tet*(K), *tet*(M)	t1451/CC398	Negative
	Pig farmer 1	1	PEN-SXT- CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *dfrA*, *dfrG*, *tet*(K), *tet*(M), *aac6'-aph2''*, *fexA*	t1430/CC9	Negative
	Pig farmer 2	1	PEN	*blaZ*	t065/CC45	C
D	Pig 1	5	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), cat_{PC221}	t011/CC398	Negative
	Pig 2	5	PEN-FOX-SXT-ERY-CLI-TET-CHL-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), *fexA*	t011/CC398	Negative
	Pig 2	1	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), cat_{PC221}	t011/CC398	Negative
	Pig 3	3	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), cat_{PC221}	t011/CC398	Negative
	Pig 4	3	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), cat_{PC221}	t011/CC398	Negative
	Pig 4	2	PEN-FOX-SXT-ERY-CLI-TET-CLO-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), *fexA*	t011/CC398	Negative
	Pig 5	3	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), cat_{PC221}	t011/CC398	Negative
	Pig 6	4	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), cat_{PC221}	t011/CC398	Negative
	Pig 7	3	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), cat_{PC221}	t011/CC398	Negative
	Pig 7	2	PEN-FOX-SXT-ERY-CLI-TET-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M)	t011/CC398	Negative
	Pig 8	6	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), cat_{PC221}	t011/CC398	Negative
	Pig 9	1	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), cat_{PC221}	t011/CC398	Negative
	Pig 10	5	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *mecA*, *dfrK*, *ermC tet*(K), *tet*(M), cat_{PC221}	t011/CC398	Negative
	Pig farmer 1	5	PEN-FOX-SXT-ERY-CLI-TET-TOB-GEN-CHL-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M), cat_{PC221}	t011/CC398	Negative
	Pig farmer 1	2	PEN-FOX-SXT-ERY-CLI-TET-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M)	t011/CC398	Negative
	Pig farmer 3	5	PEN-FOX-SXT-ERY-CLI-TET-CIP	*blaZ*, *mecA*, *dfrK*, *ermC*, *tet*(K), *tet*(M)	t011/CC398	Negative

Abbreviation: CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin

Note: All strains were *luk-S/F-PV* and *tst* negative

[a] CC assigned according to the *spa*-type, except for CC398 (determined by specific PCR)

NT: Not tested

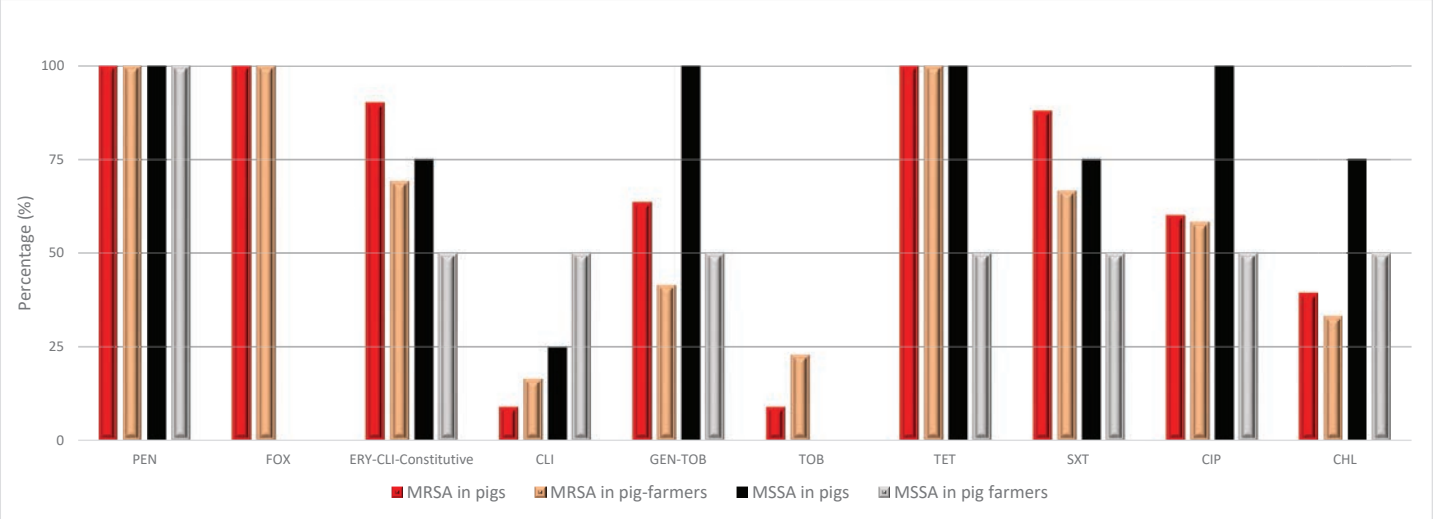


Figure 40. Antimicrobial resistance rates in *S. aureus* strains from farms A to D (both pigs and farmers)

Percentages were based on the collection of *S. aureus* obtained of different samples or those of the same sample but with different AMR phenotype (10, 16, 9, 16 from farms A to D, respectively)

Note: There were 33 and 12 distinct MRSA strains from pigs and pig-farmers respectively. Conversely, 4 and 2 distinct MSSA strains from pigs and pig-farmers, respectively.

Abbreviation: CHL: chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin

3.1.4.4 Genetic typing of the *S. aureus* strains from healthy pig and pig-farmers

All MRSA from pigs and pig-farmers were of the CC398 lineage. The prevalence of MRSA-CC398 lineage among the pigs studied was 60%, while 70% of all the pig-farmers were MRSA-CC398 carriers **(Figure 41).** Also, all MRSA strains from farms A, B and D belonged to the CC398 lineage, however, only 20% of the pigs from farm C carried MRSA-CC398 **(Figure 41).** Based on the *spa*-types of the MRSA-CC398 strains of pigs, all were t011, except one (which was t4571) **(Table 61).** However, of the 12 MRSA from the pig-farmers, MRSA-CC398-t011 (75%) was the predominant, followed by MRSA-CC398-t034 (16.7%), and then MRSA-CC398-t1451 (8.3%). MSSA strains were only detected from pigs and pig-farmers in farm C (66.7% of all strains). The majority of the MSSA were of the CC9 lineage and *spa*-types t191 (n = 1) and t1430 (n = 7). Specifically, all the MSSA strains from the pigs were MSSA-CC9, whereas MSSA-CC45-t065 and MSSA-CC9-t1430 were identified from two pig-farmers **(Table 61).** All the *S. aureus* strains were negative for *luk-S/F-PV* and *tst* genes. All the *S. aureus* were *scn*-negative except one MSSA strain from farm C that was *scn*-positive (IEC-type C) **(Table 61).**

3.1.4.5 Within-host variation of genetic lineages and/or AMR in pigs and pig-famers

Of the 26 pigs with nasal *S. aureus* carriage, 9 (34.6%) harboured strains with varied within-host *spa*-types or resistomes **(Table 61).** Of these, 2 to 3 genetically distinct *S. aureus* strains were detected **(Table 61).** In one of the pigs, one MSSA-CC9 and two MRSA-CC398 strains were detected (pig No. 5 in farm-C). The strains also had different AMR phenotypes/genes, viz.: (PEN-FOX-SXT-ERY-CLI-TET-CIP/ *mecA, dfrK, ermB, tet*(K), *tet*(M); PEN-FOX-ERY-CLI-TET-CIP/ *mecA, ermB, tet*(K), *tet*(M); and PEN-CLI-TET-TOB-GEN-CLO-CIP/ *lnuB, tet*(K), *tet*(L), *aac6'-aph2", fexA*). Also, worth mentioning is the detection in a single pig of an MSSA-CC9-t191 strain carrying *dfrA, lnuB, tet*(K), *tet*(M), *aac6'-aph2"* genes and an MSSA-CC9-t1430 strain carrying *ermB, lnuB, tet*(L), *tet*(M), *aac6'-aph2", fexA* genes **(Table 61).**

Moreover, 6 (75%) of the pig-farmers had *S. aureus* strains with varied within-host *spa*-types or AMR genes **(Table 61)**. Of special relevance is the detection of an MSSA-CC9-t1430 with *dfrA, dfrG, tet(K), tet(M), aac6'-aph2", fexA* resistance genes and an MRSA-CC398-t1451 with *mecA, dfrA, ermC, lnuB, tet(K), tet(M)* genes **(Table 61).**

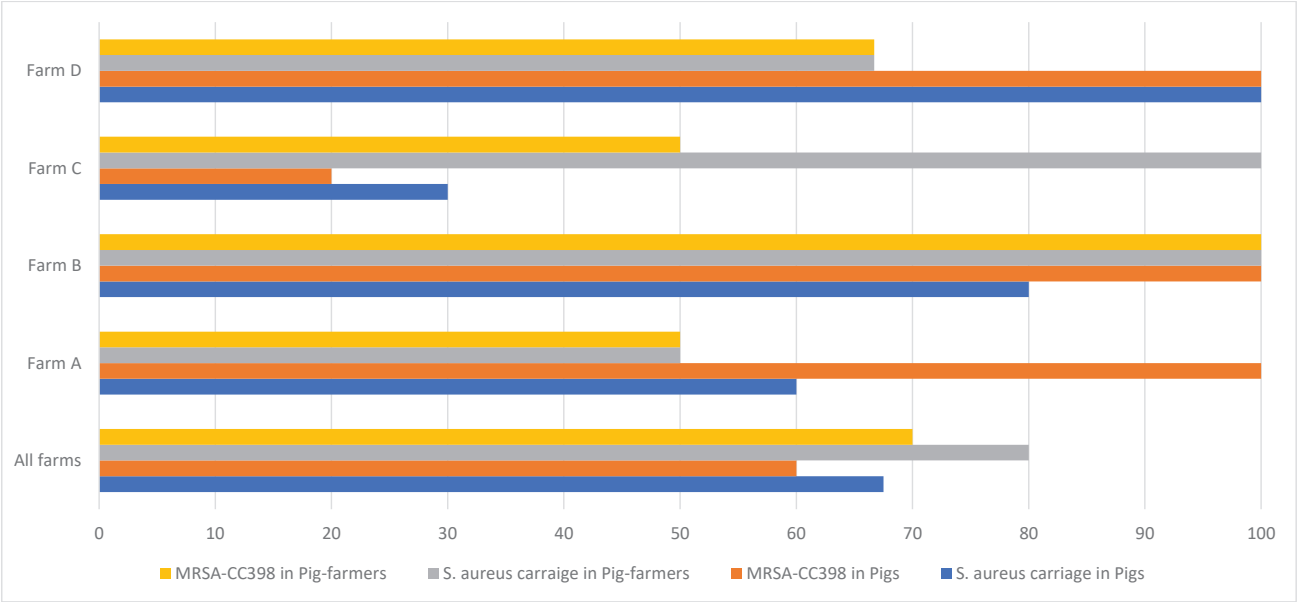


Figure 41. Frequency of *S. aureus* and MRSA-CC398 nasal carriage in pigs and pig-farmers

3.1.4.6 Nasal co-carriage of CoNS and *S. aureus* in pigs and pig-farmers

The majority of the hosts with co-carriage of single CoNS species with *S. aureus* were due to *S. chromogenes* and *S. haemolyticus* **(Table 62).** Nevertheless, most of the hosts with only *S. sciuri* carriage had no *S. aureus* co-carriage (especially in farm C) **(Table 60).** About 40% of pigs and pig-farmers with MRSACC398 had no other CoNS nasal co-carriage, whereas 36.7% had one CoNS co-carriage and 23.3% had ≥2 CoNS carriage **(Table 63).** Conversely, 16.7% of MSSA carriers had no CoNS co-carriage, whereas 33.3% had one CoNS co-carriage and 50% had ≥ 2 CoNS carriages **(Table 63).** About 41.1% who were not *S. aureus* carriers had ≥2 CoNS carriage **(Table 63).** However, there was no significant association between the presence of MRSA, MSSA and the number of CoNS species in pigs and pig-farmers (p>0.05) **(Table 63).**

Table 62. Nasal staphylococci co-carriage in all pigs and pig farmers in the four analysed farms (A-D)

Farm	Host[a]/ Nº carriers	CoNS present	Presence *S. aureus*	Methicillin Susceptibility /*spa*/CC [b]
A	P1	*S. hyicus, S. simulans, S. epidermidis*	Yes	MRSA/t011/CC398
	P2	*S. sciuri, S. haemolyticus, S. epidermidis*	Yes	MRSA/t011/CC398
	P3	*S. chromogenes, S. sciuri*	No	NT
	P4	*S. chromogenes*	Yes	MRSA/t011/CC398
	P5	*S. hyicus*	Yes	MRSA/t011/CC398
	P6	*S. hyicus*	Yes	MRSA/t011/CC398
	P7	*S. haemolyticus, S. epidermidis,*	No	NT
		S. chromogenes, S. saprophyticus	Yes	MRSA/ t4571/CC398
	P8	*S. chromogenes, S. haemolyticus, S. pasteuri*	No	NT
	P9	*S. haemolyticus, S. sciuri*	No	NT
	P10	*S. pasteuri, S. chromogenes, S. saprophyticus*	No	NT
	PF1	*S. epidermidis*	Yes	MRSA/t011/CC398
	PF2	None		
B	P1	*S. epidermidis, S. haemolyticus, S, hyicus*	Yes	MRSA/t011/CC398
	P2	*S. hyicus*	No	NT
	P3	*S. hyicus*	Yes	MRSA/t011/CC398
	P4	*S. haemolyticus*	Yes	MRSA/t011/CC398
	P5	*S. haemolyticus*	Yes	MRSA/t011/CC398
	P6	None	No	NT
	P7	None	Yes	MRSA/t011/CC398
	P8	None	Yes	MRSA/t011/CC398
	P9	*S. chromogenes*	Yes	MRSA/t011/CC398
	P10	None	No	NT
	PF1	*S. hyicus, S. epidermidis, S. saprophyticus*	Yes	MRSA/t034/CC398
	PF2	*S. epidermidis*	Yes	MRSA/t034/CC398
	PF3	None	No	NT

Table 62. Continuation

Farm	Host[a]/ Nº carriers	CoNS present	Presence *S. aureus*	Methicillin Susceptibility /*spa*/CC [b]
C	P1	*S. sciuri, S. chromogenes, S. hyicus*	Yes	MSSA/t191/CC9; MSSA/t1430/CC9
	P2	*S. sciuri*	No	NT
	P3	*S. sciuri*	Yes	MSSA/t1430/CC9
	P4	*S. sciuri*	No	NT
	P5	*S. chromogenes*	Yes	MRSA/t011/CC398; MSSA/t1430/CC9
	P6	*S. sciuri*	No	NT
	P7	*S. sciuri*	No	NT
	P8	*S. sciuri*	No	NT
	P9	*S. hyicus. S. xylosus*	No	NT
	P10	*S. xylosus, S. sciuri*	No	NT
	PF1	*S. epidermidis, S. simulans*	Yes	MRSA/t1451/CC398;MSSA/t1430/CC9
	PF2	None	Yes	MSSA/t065/CC45
D	P1	*S. chromogenes*	Yes	MRSA/t011/CC398
	P2	None	Yes	MRSA/t011/CC398
	P3	None	Yes	MRSA/t011/CC398
	P4	None	Yes	MRSA/t011/CC398
	P5	*S. haemolyticus*	Yes	MRSA/t011/CC398
	P6	None	Yes	MRSA/t011/CC398
	P7	None	Yes	MRSA/t011/CC398
	P8	None	Yes	MRSA/t011/CC398
	P9	None	Yes	MRSA/t011/CC398
	P10	None	Yes	MRSA/t011/CC398
	PF1	None	Yes	MRSA/t011/CC398
	PF2	*S. simulans, S. haemolyticus*	No	NT
	PF3	*S. chromogenes*	Yes	MRSA/t011/CC398

NT: Not tested

Note: [a] P= pig; PF= pig farmer; [b] CC assigned according to the *spa*-type, except for CC398 (determined by specific PCR)

Table 63. Comparison matrix of the presence of MRSA, MSSA and the number of CoNS species in pigs and pig-farmers

	MRSA-CC398 (n=30)	No. (%) with MSSA (n=6)	No. (%) without *S. aureus* (n= 17)
No. (%) with no CoNS	12 (40.0)	1 (16.7)	3 (17.6)
OR (95% CI)	3.11 (0.73-13.2)	0.93 (0.08-11.2)	Referent
p value	0.124	0.956	Referent
No. (%) with 1 CoNS species	11 (36.7)	2 (33.3)	7 (41.1)
OR (95% CI)	0.83 (0.24-2.79)	0.71 (0.10-5.04)	Referent
p value	0.760	0.736	Referent
No. (%) with ≥2 CoNS species	7 (23.3)	3 (50.0)	7 (41.1)
OR (95% CI)	0.43 (0.12-1.57)	1.43 (0.22-9.26)	Referent
p value	0.204	0.708	Referent

Significant association determined by bivariate regression at 95% Confidence interval (CI)
Note*:* A pig and pig-farmer each had both MRSA-CC398 and MSSA-CC9 co-carriage. Also, 1 pig farmer had two MSSA-CC9 with different *spa* types.

3.1.4.7 Antimicrobial Resistance Phenotypes and Genotypes of non-repetitive CoNS

From these 101 CoNS, 75 non-repetitive strains were selected after determining their phenotypes/genotypes of AMR. Of the 75 non-repetitive strains (62 from pigs and 13 from pig-farmers), 92% showed a multidrug resistance (MDR) phenotype **(Table 64 and Figure 42).** Specifically, 83.6% and 100% of the non-repetitive CoNS
from pigs and pig-farmers presented an MDR phenotype, respectively **(Table 64)**. All strains were *lukS-PV/lukF-PV*, *tst*, *eta* and *etb* negative.

All *S. sciuri* strains carried the intrinsic *salA* gene. The following AMR phenotypes were detected among the non-repetitive CoNS (percentage of strains/ genes detected): tetracycline (94.7/*tet*(K), *tet*(L), *tet*(M), and *tet*(O)), penicillin (77.3/*blaZ*), erythromycin-clindamycin-constitutive (77.3/*ermA, ermC, ermT,* and *erm43*), sulfamethoxazole-trimethoprim (66.7/*dfrA, dfrD, dfrG,* and *dfrK*), ciprofloxacin (52), tobramycin (50.7/*ant4'*), chloramphenicol (21.3/*fexA* and cat_{PC221}), clindamycin (16/*lnuA, lnuB,* and *salA*), gentamicin-tobramycin (12/*aac6'-aph2"*), linezolid (2.7/*cfr*), mupirocin (2.7/*mupA*) and erythromycin (1.3/ *msrA*) **(Figure 42, Tables 65 and 66).** About 52% of CoNS were *mecA*-positive (i.e., MRCoNS) and they were associated with SCC*mec* types V (46.2%), IVb (20.5%) and IVc (5.1%). However, 23.1% of MRCoNS were SCC*mec* non-typeable **(Figure 42).**

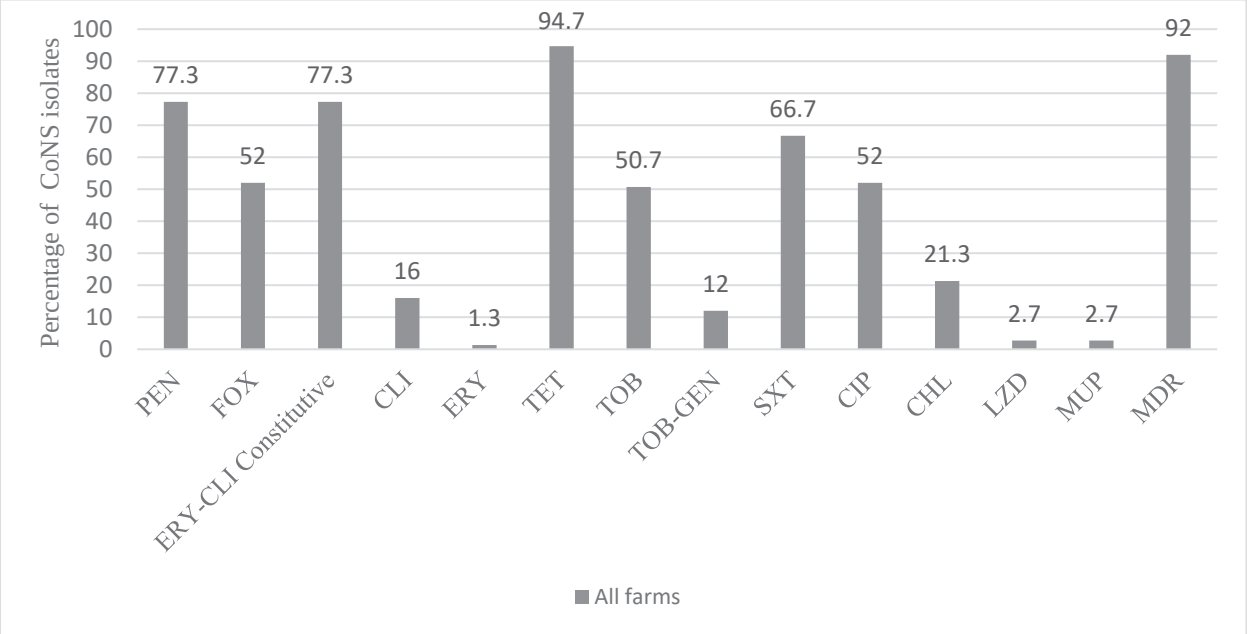


Figure 42. Frequency of antimicrobial resistance profiles in the CoNS strains recovered from nasal cavities of healthy pigs and pig-farmers.

CHL: Chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; LZD: linezolid; MUP: mupirocin; MDR: multi-drug resistance; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin.

Table 64. Coagulase-negative staphylococci from healthy pigs and pig-farmers and those with MDR phenotype from the four farms (A-D).

CoNS species	Total strains	Non-repetitive strains[a]												
		Pigs	Pig-farmers	Strains with MDR phenotype[b]	Strains with MDR phenotype in pigs					Strains with MDR phenotype in pig-farmers				
				Pigs and pig-farmers	All farms	Farm A	Farm B	Farm C	Farm D	All farms	Farm A	Farm B	Farm C	Farm D
S. sciuri	29	17	0	17	17	4	0	13	0	0	0	0	0	0
S. haemolyticus	5	3	1	4	3	0	3	0	0	1	0	0	0	1
S. borealis	12	10	0	10	10	5	4	0	1	0	0	0	0	0
S. chromogenes	15	11	2	9	7	5	1	0	1	2	0	0	0	2
S. epidermidis	13	5	5	10	5	4	1	0	0	5	2	2	1	0
S. hyicus	11	8	1	9	8	3	3	2	0	1	0	1	0	0
S. saprophyticus	7	3	1	4	3	2	1	0	0	1	0	1	0	0
S. simulans	4	1	3	4	1	1	0	0	0	3	0	0	1	2
S. xylosus	3	2	0	0	0	0	0	0	0	0	0	0	0	0
S. pasteuri	2	2	0	2	2	2	0	0	0	0	0	0	0	0
Total (%)	101	62	13	69 (92)	56 (83.6)	26	13	15	2	13 (100)	2	4	2	5

[a]Non-repetitive strains: one of each species per sample, or more than one if they presented a different AMR phenotype. All data presented in this study were obtained with the collection of non-repetitive CoNS strains.
[a]MDR: resistance to at least 3 families of antibiotics. In *S. sciuri*, clindamycin resistance was not considered for MDR analyses (this species has an intrinsic mechanism of lincomycin resistance).

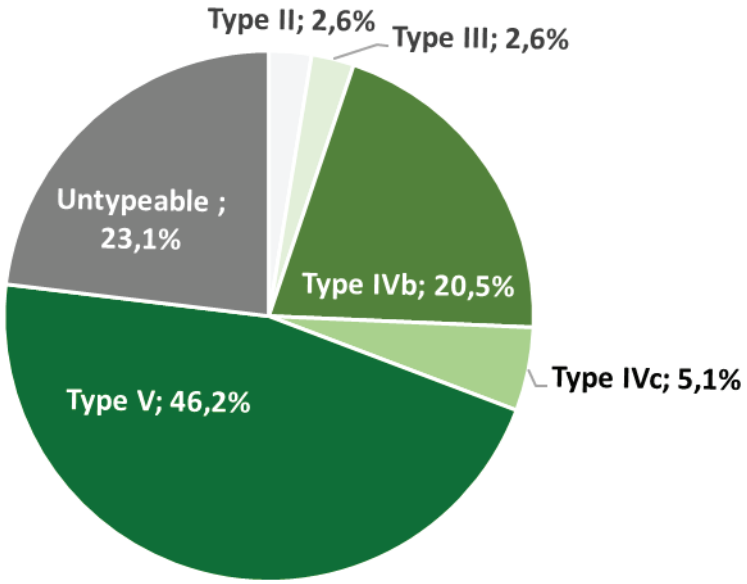


Figure 43. Frequency of the types of SCC*mec* mobile elements identified in the MRCoNS nasal carriers.

3.1.4.8 Comparison of AMR phenotype frequencies by pig farms

To compare the AMR frequencies of non-duplicate CoNS strains from pigs and pig farmers of the four pig farms (A-D), individual chi-squared tests against every antimicrobial agent were computed. Erythromycin-clindamycin constitutive resistance was significantly higher among CoNS strains from pigs and pig farmers in farm A than strains from the other farms (p = 0.018)**.** CoNS strains from pigs and pig farmers in Farm B had significantly higher tobramycin and ciprofloxacin resistances than strains from other farms (p<0.05). **(Table 65).** For the other antibiotics' resistances and the MDR phenotype, no significant associations between the farms were detected (p > 0.05) **(Table 65).**

3.1.4.9 Unusual Antimicrobial Resistance Genes

Interestingly, the linezolid-resistance *cfr* gene (identified in *S. saprophyticus* and *S. epidermidis*-ST16) was detected among two chloramphenicol-resistant CoNS strains of pig and pig-farmers **(Table 66);** one of these strains expressed phenotypic resistance to linezolid (MIC: 12 μg/ml), but the other was susceptible to linezolid (MIC: 1.5 μg/ml) **(Table 66)**. The *ermT* gene was detected in strains of five CoNS species (*S. chromogenes, S. epidermidis, S. borealis, S. sciuri* and *S. hyicus*) **(Tables 65 and 66).** Moreover, the *erm43* gene was detected in 8 CoNS of 4 different species (*S. epidermidis, S. chromogenes, S. haemolyticus* and *S. borealis*) and the gene *mupA* in two strains of the species *S. epidermidis* and *S. sciuri.* (**Tables 66 and 67**).

Table 65. Comparison of the frequencies of antimicrobial resistance phenotypes among CoNS strains from healthy pigs and pig-farmers in farms A to D.

Antimicrobial resistance phenotype	Farm A (%)	Farm B (%)	Farm C (%)	Farm D (%)	χ^2	*p* value
PEN	23 (76.7)	15 (88.2)	17 (80.9)	3 (42.9)	5.078	0.166
FOX	16 (53.3)	10 (58.8)	10 (47.6)	3 (42.9)	0.734	0.865
ERY-CLI constitutive	28 (93.3)	12 (40)	12 (57.1)	6 (85.7)	9.987	0.018*
CLI	0	4 (13.3)	7 (33.3)	1 (14.3)	11.141	0.011*
ERY	1 (3.3)	0	0	0	1.520	0.677
TET	30 (100)	15 (88.2)	20 (95.2)	6 (85.7)	4.208	0.239
TOB	17 (56.7)	14 (82.3)	5 (23.8)	2 (28.6)	14.688	0.002*
TOB-GEN	2 (6.7)	2 (11.7)	3 (14.3)	2 (28.6)	2.733	0.434
SXT	24 (80)	12 (70.6)	8 (38.1)	6 (85.7)	11.375	0.009*
CIP	12 (40)	15 (88.2)	9 (42.9)	3 (42.9)	11.611	0.008*
CHL	9 (30)	4 (23.5)	2 (9.5)	1 (14.3)	3.344	0.341
LZD	1 (3.3)	1 (5.9)	0	0	1.496	0.683
MUP	0	0	2 (9.5)	0	5.284	0.152
MDR	28 (93.3)	17 (100)	17 (80.9)	7 (100)	5.642	0.130

The number of CoNS strains from the farms are Farm A=30, Farm B=17, Farm C=21 and Farm D =7
*Significant association determined by two-tailed chi-squared test at 95% Confidence interval (CI)
Abbreviation: CHL: Chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; LZD: linezolid; MUP: mupirocin; MDR: multi-drug resistance; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin.

Table 66. Intra-host species and intra-species AMR diversity of coagulase-negative staphylococci from healthy pigs and pig-farmers

Host[a]	Staphylococcal species	AMR phenotype	AMR genes detected	LZD		ST	SCC-*mec*
				genes	MIC (µg/ml)[b]		
Farm A							
P1	*S. epidermidis*	PEN-FOX-TET-ERY-CLI-SXT-TOB	*blaZ, mecA, tet*(L), *tet*(M), *ermB, dfrD, aac6'-aph2", ant4'*	-	-	ST25	IVc
	S. hyicus	PEN-TET-ERY- CLI	*blaZ, tet*(L), *ermC*	-	-	-	-
	S. simulans	TET-ERY-CLI-SXT-GEN	*tet*(K), *ermA, dfrG, aac6'-aph6"*	-	-	-	-
P2	*S. sciuri*	PEN-FOX-TET-ERY-CLI-SXT-CHL-CN-TOB-CIP	*mecA, tet*(L), *tet*(M), *ermA, ermB, ermC, lnuA, salA, dfrD, fexA, aac6'-aph2", ant4'*	ND	-	-	IVb
	S. epidermidis	PEN-FOX-TET-ERY-CLI-SXT-TOB	*blaZ, mecA, tet*(M), *erm43, ermC, dfrG, dfrK, ant4'*	-	-	ST25	V
	S. borealis	PEN-FOX-TET-ERY-CLI-SXT-CHL- TOB-CIP	*blaZ, mecA, tet*(L), *tet*(M),*ermC, ermT, lnuB, dfrK, cat*$_{PC221}$, *fexA, ant4'*	ND	-	-	V
P3	*S. sciuri*	PEN-FOX-TET-ERY-CLI-SXT-CHL-TOB-CIP	*blaZ, mecA, tet*(L), *tet*(M), *ermC, ermT, lnuB, dfrK, cat*$_{PC221}$, *fexA, ant4'*	ND	-	-	IVb
	S. sciuri	PEN-FOX-TET-ERY-CLI-SXT-CHL-TOB-CIP	*mecA, tet*(L), *tet*(M), *ermC, msrA, dfrK, cat*$_{PC221}$, *ant4'*	ND	-	-	IVb
	S. chromogenes	PEN-TET-ERY-CLI-TOB-SXT	*blaZ, tet*(L),*erm43, ermT, dfrA, dfrG, dfrK, ant4'*	-	-	-	-
P4	*S. chromogenes*	TET-ERY-CLI-SXT-TOB	*tet*(L), *tet*(M), *ermA, dfrA, ant4'*	-	-	-	-
	S. chromogenes	PEN-FOX-TET-ERY-CLI-SXT	*blaZ, mecA, tet*(L), *erm43, ermA, ermT, dfrA, dfrG, dfrK*	-	-	-	NT
P7	*S. chromogenes*	PEN-TET-ERY-CLI-SXT-TOB	*blaZ, tet*(L), *erm43, ermT, dfrA, dfrG, dfrK, ant4'*	-	-	-	-
	S. epidermidis	PEN-FOX-TET-ERY-CLI-SXT-TOB	*blaZ, mecA, tet*(M), *ermC, dfrK, aac6'-aph2", ant4'*	-	-	ST25	IVc
	S. saprophyticus	PEN-FOX-TET-ERY-CLI-SXT	*blaZ, mecA, tet*(L), *tet*(M), *ermC, ermA, dfrK*	-	-	-	III
	S. borealis	PEN-FOX-TET-ERY-CLI-SXT-CHL-TOB-CIP	*blaZ, mecA, tet*(L), *tet*(M), *ermC, ermT, lnuB, cat*$_{PC221}$, *fexA, dfrK, ant4'*	ND	-		V
P8	*S. chromogenes*	TET-ERY-CLI-TOB	*tet*(L), *tet*(M), *ermC, ant4'*	-	-	-	-
	S. chromogenes	TET-ERY-CLI	*tet*(L), *ermC*	-	-	-	-
	S. epidermidis	TET-ERY-CLI-SXT	*blaZ, tet*(O), *tet*(L), *tet*(M), *ermC, dfrK*	-	-	ST977	-
	S. borealis	PEN-FOX-TET-ERY-CLI-TOB-CIP	*blaZ, mecA, tet*(L), *tet*(M), *erm43, dfrA, dfrG, dfrK, ant4'*	-	-	-	V
	S. borealis	PEN-FOX-TET-ERY-CLI-SXT-CHL-TOB-CIP	*blaZ, mecA, tet*(L), *tet*(M), *ermC, ermT, lnuB, dfrK, fexA, ant4'*	ND	-	-	V
	S. pastueri	PEN-FOX-TET-ERY-CLI-SXT- TOB-CIP	*blaZ, mecA, tet*(K), *tet*(L), *tet*(M), *ermC, dfrK, ant4'*	-	-	-	V
P9	*S. sciuri*	PEN-TET-ERY-CLI-SXT-CHL-TOB	*mecA, tet*(L), *tet*(M), *ermC, lnuA, fexA, dfrK, ant4', aac6'-aph2"*	ND	-	-	IVb
	S. borealis	PEN-FOX-TET-ERY-CLI-SXT-CHL-TOB-CIP	*blaZ, mecA, tet*(L), *tet*(M), *ermC, ermT, lnuB, cat*$_{PC221}$, *fexA, dfrK, ant4'*	ND	-	-	V
P10	*S. chromogenes*	TET-ERY-CLI	*tet*(L), *ermC*	-	-	-	-
	S. saprophyticus	FOX-TET-ERY-CHL-CLI-TOB-SXT	*mecA, tet*(L), *tet*(M), *ermC, dfrK, fexA, ant4'*	*cfr*	12	-	V
	S. pasteuri	PEN-FOX-TET-ERY-CLI-SXT-TOB-CIP	*blaZ, mecA, tet*(L), *tet*(M), *ermC, dfrG, dfrK, ant4'*	-	-	-	V
F1	*S. epidermidis*	PEN-FOX-TET-ERY- SXT-CIP	*blaZ, mecA, tet*(O), *msrA, dfrA, dfrG*	-	-	ST59	V
	S. epidermidis	PEN-FOX-TET- SXT-CIP	*blaZ, mecA, tet*(L), *dfrA, dfrG*	-	-	ST59	V

Table 66. Continuation

Host[a]	Staphylococcal species	AMR phenotype	AMR genes detected	LZD genes	LZD MIC (µg/ml)[b]	ST	SCC-*mec*
Farm B							
P1	*S. haemolyticus*	PEN-TET-ERY-CLI-SXT-GEN-TOB	*blaZ, mecA, tet*(L), *tet*(M), *erm43, ermC, dfrA, aac6'-aph2", ant4'*	-	-	-	V
	S. haemolyticus	PEN-FOX-TET-ERY-CLI-GEN-TOB-CIP	*mecA, tet*(L), *ermA, ermT, dfrA, dfrG, aac6'-aph2", ant4'*	-	-	-	V
	S. epidermidis	PEN-TET-ERY-CLI-TOB	*blaZ, tet*(K), *tet*(L), *tet*(M), *ermC, ant4'*	-	-	ST100	-
	S. hyicus	PEN-TET-ERY-CLI-TOB-GEN-CIP	*blaZ, tet*(L), *ermT, aac6'-aph2"*	-	-	-	-
P4	*S. borealis*	PEN-FOX-TET-ERY-CLI-SXT-TOB-CIP	*mecA, tet*(K), *tet*(L), *ermA, ermC, dfrK, ant4'*	-	-	-	V
	S. borealis	PEN-FOX-TET-ERY-CLI-CHL-SXT-GEN-TOB-CIP	*blaZ, mecA, tet*(L), *tet*(M), *ermT, fexA, dfrK, aac6'-aph2", ant4'*	ND	-	-	V
	S. haemolyticus	PEN-TET-CLI-GEN-TOB-CIP	*tet*(L), *ermC, lnuA, aac6'-aph2", ant4'*	-	-	-	-
P5	*S. borealis*	PEN-FOX-TET-ERY-CLI-CHL-SXT-GEN-TOB-CIP	*blaZ, mecA, tet*(L), *tet*(M), *ermA, ermT, cat*$_{PC221}$, *fexA, dfrK, aac6'-aph2", ant4'*	ND	-	-	V
	S. borealis	PEN-FOX-TET-ERY-CLI-SXT-TOB-CIP	*mecA, tet*(K), *tet*(L), *ermA, ermC, dfrK, ant4'*	-	-	-	V
F1	*S. epidermidis*	PEN-FOX-TET-CLI-CHL-SXT-TOB-CIP	*blaZ, mecA, tet*(K), *tet*(L), *fexA, dfrK, ant4'*	*cfr*	1.5	ST16	V
	S. hyicus	PEN-FOX-TET-CIP-SXT	*blaZ, mecA, tet*(K),, *tet*(O), *dfrA, dfrG*	-	-	-	NT
	S. saprophyticus	PEN-FOX-TET-ERY-CLI-SXT-TOB-GEN-SXT-CIP	*blaZ, mecA, tet*(K), *tet*(M), *ermC, dfrG, ant4', aac6'-aph2"*	-	-	-	V
Farm C							
P1	*S. sciuri*	PEN-FOX-TET-ERY-CLI-SXT-CIP	*mecA, tet*(L), *tet*(M), *ermB, erm43, dfrK*	-	-	-	NT
	S. chromogenes	TET-ERY-CLI	*tet*(M), *ermC, lnuB*	-	-	-	-
	S. hyicus	PEN-TET-ERY-CLI-SXT-CIP	*blaZ, tet*(L), *ermT, dfrK*	-	-	-	-
P4	*S. sciuri*	PEN-FOX-TET-ERY-CLI-TOB	*mecA, tet*(L), *tet*(M), *ermB, dfrK, ant4'*	-	-	-	NT
	S. sciuri	PEN-FOX-TET-ERY-CLI-CIP-TOB-GEN	*mecA, tet*(L), *ermC, aac6'-aph2"*	-	-	-	V
P6	*S. sciuri*	PEN-TET-ERY-CLI-SXT-CIP	*mecA, tet*(L), *tet*(M), *ermT, dfrG, dfrK*	-	-	-	NT
	S. sciuri	PEN-FOX-TET-CLI-TOB	*mecA, tet*(L), *tet*(M), *lnuA, ant4'*	-	-	-	IVb
P8	*S. sciuri*	TET-CLI-PEN-TOB	*tet*(L), *lnuA, ant4'*	-	-	-	-
	S. sciuri	PEN-FOX-TET-ERY-CLI	*mecA, tet*(M),, *ermB*	-	-	-	IVb
	S. sciuri	PEN-FOX-TET-ERY-CLI-SXT	*mecA, tet*(L), *tet*(M), *ermB, dfrK*	-	-	-	-
P9	*S. hyicus*	PEN- FOX-TET-CLI-SXT-TOB-GEN-CIP	*blaZ, mecA, tet*(M), *lnuA, lnuB, dfrD, aac6'-aph2"*	-	-	-	V
	S. xylosus	PEN-TET	*blaZ, tet*(K)	-	-	-	-
P10	*S. sciuri*	PEN-FOX-TET-ERY-CLI-SXT-CIP	*mecA, tet*(L), *tet*(M), *ermB, dfrK*	-	-	-	NT
	S. sciuri	TET-ERY-CLI-CHL-SXT-CIP	*tet*(L), *tet*(M), *ermA, lnuA, cat*$_{PC221}$, *dfrK*	ND	-	-	-
	S. xylosus	PEN-TET	*blaZ, tet*(K)	-	-	-	-
F1	*S. epidermidis*	PEN-TET-ERY-CLI-TOB-MUP	*blaZ, tet*(K), *tet*(L), *tet*(M), *erm43, dfrA, dfrK, ant4', mupA*	-	-	ST100	-
	S. simulans	TET-CLI-CHL	*tet*(K), *lnuA, fexA*	ND	-	-	-

Table 66. Continuation

Host[a]	Staphylococcal species	AMR phenotype	AMR genes detected	LZD genes	LZD MIC (µg/ml)[b]	ST	SCC-*mec*
Farm D							
F2	*S. simulans*	PEN-FOX-TET-ERY-CLI-TOB-GEN	*blaZ, mecA, tet*(L), *ermA, aac6'-aph2"*	-	-	-	NT
	S. simulans	TET-ERY-CLI-SXT	*tet*(M), *ermC, dfrG*	-	-	-	-
	S. haemolyticus	PEN-FOX-TET-CLI-SXT-TOB-GEN-CIP	*blaZ, mecA, tet*(K), *lnuA, dfrG, aac6'-aph2"*	-	-	-	II
F3	*S. chromogenes*	TET-ERY-CLI-SXT	*tet*(L), *ermT, dfrA, dfrG*	-	-	-	-
	S. chromogenes	ERY- CLI-CHL-SXT	*mecA, tet*(L), *tet*(M), *ermC, dfrK, fexA*	ND	-	-	IVb

[a] P, pig; PF: pig farmer; [b]Linezolid MIC was tested in the strains that carried linezolid resistance genes. Abbreviation: CHL: Chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; LZD: linezolid; MUP: mupirocin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin. ST: Sequence type; NT: Non-typeable; - : Not tested; ND: Not detected. **Note:** All strains were *lukS-PV/lukF-PV, tst, eta* and *etb* negative;

Table 67. CoNS with single antimicrobial resistance profile from healthy pigs and pig-farmers

Host/ Farm	Species	AMR phenotype	AMR genes detected	ST	SCC*mec*
P5/A	*S. hyicus*	PEN-TET-ERY-CLI-SXT	*blaZ, tet*(M), *ermC, dfrA, dfrG*	-	-
P6/A	*S. hyicus*	PEN-TET-ERY-CLI	*blaZ, tet*(L), *ermC*	-	-
P2/B	*S. hycius*	CLI-SXT-GEN-TOB-CIP	*lnuA, lnuB, dfrK, aac6'-aph2", ant4'*	-	-
P3/B	*S. hyicus*	CLI-SXT-GEN-TOB-CIP	*lnuA, lnuB, dfrK, aac6'-aph2", ant4'*	-	-
P6/B	*S. saprophyticus*	PEN-FOX-TET-ERY-CLI-SXT-TOB-GEN-SXT-CIP	*blaZ, mecA, tet*(M), *ermC, dfrA, dfrG, ant4', aac6'-aph2"*	-	V
P9/B	*S. chromogenes*	PEN-TET-ERY-CLI-GEN-TOB-CIP	*blaZ, tet*(L), *ermT, aac6'-aph2", ant4'*	-	-
F2/B	*S. epidermidis*	PEN-FOX-TET-ERY-CLI-CHL-SXT-TOB-GEN-CIP	*blaZ, mecA, tet*(L), *tet*(M), *ermT, lnuB, cat*$_{PC221}$, *fexA, dfrA, dfrK, aac6'-aph2", ant4'*	ST59	V
P2/C	*S. sciuri*	PEN-FOX-TET-CLI-CIP-TOB-GEN-MUP	*mecA, tet*(L), *tet*(M), *lnuA, ant4', mupA*	-	IVb
P3/C	*S. sciuri*	PEN-FOX-TET-ERY-CLI-SXT-CIP	*mecA, tet*(L), *tet*(M), *ermB, dfrK,*	-	NT
P5/C	*S. chromogenes*	CLI	*lnuB*	-	-
P7/C	*S. sciuri*	TET-CLI-PEN-TOB	*tet*(L), *lnuA, ant4'*	-	-
P1/D	*S. chromogenes*	TET-ERY-CLI-SXT-TOB-CIP	*tet*(L), *tet*(M), *tet*(K), *ermC, dfrK, ant4'*	-	-
P5/D	*S. borealis*	PEN-FOX-TET-ERY-CLI-SXT-TOB-CIP	*blaZ, mecA, tet*(L), *ermT, dfrA, dfrK, ant4', aac6'-aph2"*	-	NT

[a] P, pig; PF: pig farmer; CHL: Chloramphenicol; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; FOX: cefoxitin; GEN: gentamicin; LZD: linezolid; MUP: mupirocin; PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline, TOB: tobramycin. ST: Sequence type ; NT: Non-typeable; - : Not tested
Note: All strains were *lukS-PV/lukF-PV, tst, eta* and *etb* negative
[a]Linezolid MIC was tested in the strains that carried linezolid resistance genes.

3.1.4.10 Antimicrobial resistome dynamics and transmission across pigs and pig-farmers

About 28% of the pigs and pig-farmers had intra-host species diversity (> 1 CoNS species in a host) while 26% had intra-species AMR diversity (same species with >1 AMR profile) **(Figure 44 and Table 66).** Pig-to-pig nasal transmission of CoNS with similar MDR genes and SCC*mec* types was detected in 35% of pigs **(Figure 42 and Table 66).** In farm A, *S. sciuri* strains carrying the same resistome and SCC*mec* type were found in pigs 2, 3, and 9; *S. borealis* in pigs 2, 7, 8, and 9; *S. chromogenes* in pigs 3, 7, 8, and 10; *S. epidermidis*-ST25 in pigs 2 and 7; *S. hyicus* in pigs 1 and 6*;* and *S. pasteuri* in pigs 8 and 10 **(Table 66).** In farm B, similar *S. hycius* strains were found in pigs 2 and 3; and *S. borealis* in pigs 4 and 5 **(Table 66).** Whereas in Farm C, similar *S. sciuri* strains were found in pigs 3, 7, 8 and 10; and *S. xylosus* in pigs 9 and 10 **(Tables 66 and 67).** None was detected in farm D.

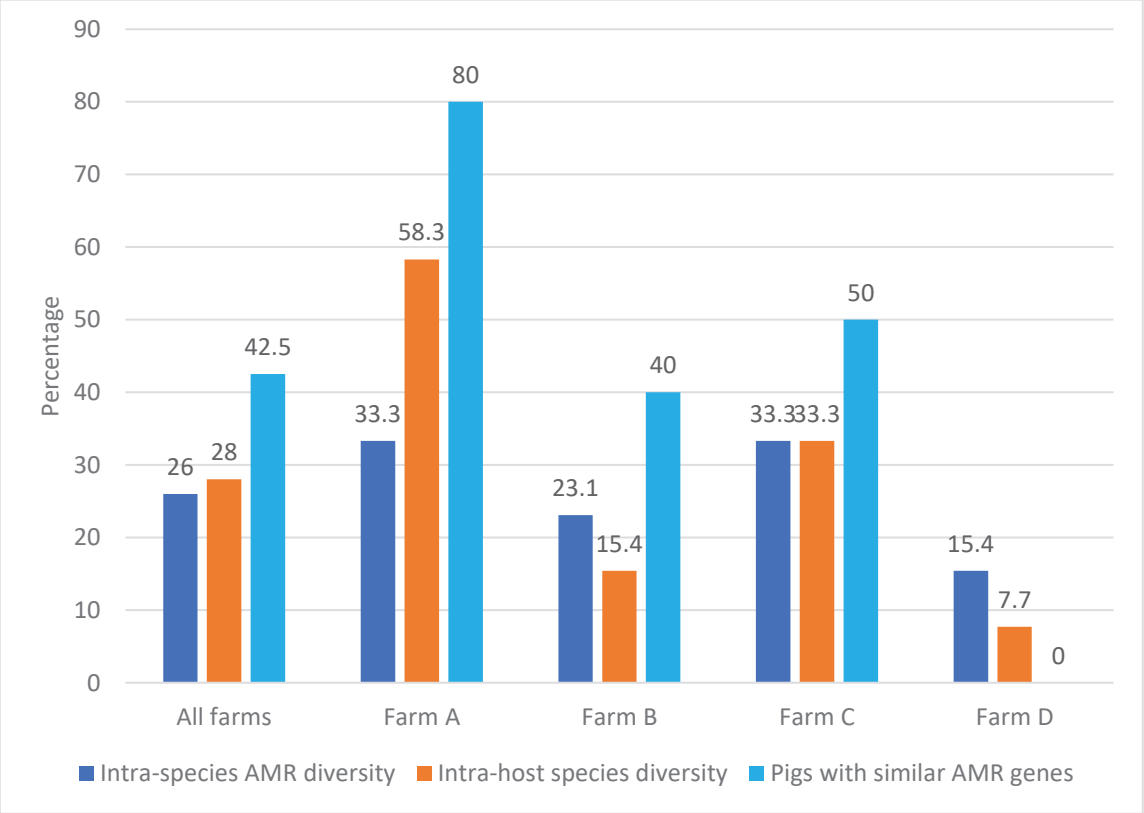


Figure 44. Frequency of intra-species AMR and intra-host species diversity of CoNS among healthy pigs and pig-farmers

Note: The number of individuals included 10 pigs from each farm (a total of 40 pigs) and 10 workers from the pig-farms (2, 3, 2 and 3 humans in farms A, B, C and D, respectively).

3.1.5 NASOTRACHEAL ENTEROCOCCAL CARRIAGE AND RESISTOMES IN NESTLING STORKS, PIGS, PETS, AND IN-CONTACT HUMANS

3.1.5.1 Nasal enterococcal carriage rate in healthy pigs and pig-farmers

Enterococci nasal carriage was found in all the farms. In total, 51 enterococci strains (43 from pigs, and 8 from pig-farmers) were recovered. Of the pigs' strains, 34, 4, 2, 2, and 1 were *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae* and *E. casseliflavus*, respectively. However, among the enterococci strains from the pig-farmers, they were only 4 *E. faecium* and 4 *E. faecalis* strains **(Figure 45).** Of the 40 pigs studied, 29 (72.5%) were enterococci nasal carriers. Of these, 4 (40%), 9 (90%), 8 (80%) and 8 (80%) were obtained in farms A to D, respectively **(Figure 45).**

Specifically, nasal carriage of *E. faecalis* (*n* = 2), *E. casseliflavus* (*n* = 1) and *E. hirae* strains (*n* = 1) were identified in pigs of farm-A; *E. faecalis* (*n* = 9) from pigs of farm-B; *E. faecalis* (*n* = 3), *E. faecium* (*n* = 2), *E. hirae* (*n* = 1), and *E. gallinarum* (*n* = 2) from pigs of farm C; and *E. faecalis* (*n* = 6), and *E. faecalis/E. faecium* co-carriage (*n* = 2) from pigs of farm D **(Figure 45).** In addition, all three farmers (100%) in farm-B were enterococci nasal carriers (66.7% *E. faecalis* and 33.3% *E. faecium*); 1 of the 2 farmers (50%) in farm-C was a nasal carrier (*E. faecium*); 2 of the 3 farmers of farm-D (66.7%) were nasal carriers (50% *E. faecalis* and 50.0% *E. faecium*), but none of the farmers in farm-A were enterococci nasal carriers **(Figure 45).** Only two healthy humans who had no contact with animals were enterococcal nasal carriers (3.5%; *E. faecalis*) **(Table 45)**, and all were susceptible to the tested antibiotics except erythromycin (*ermB* positive).

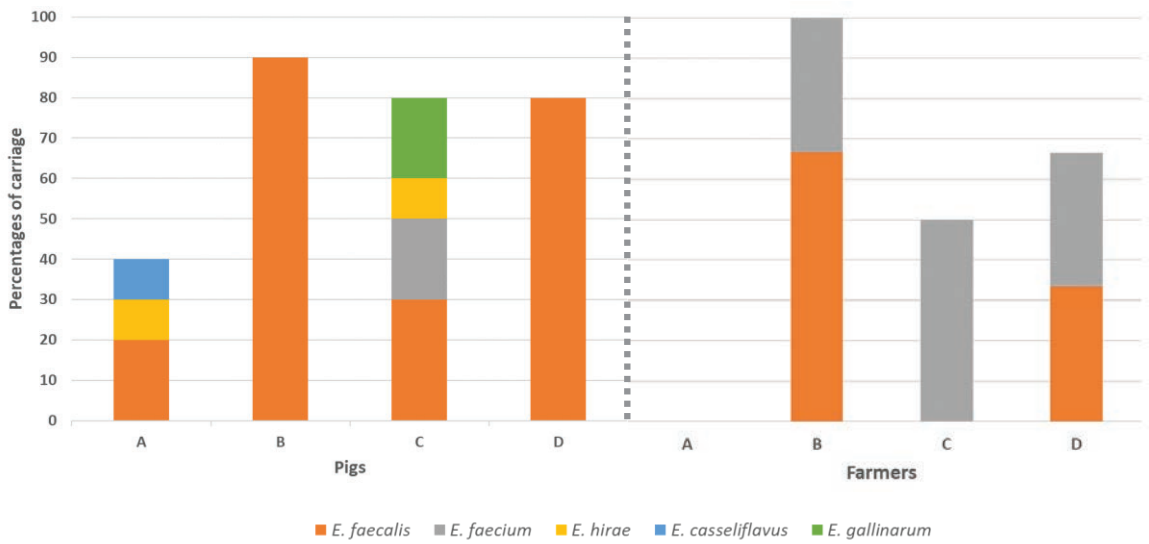


Figure 45. Nasal enterococci carriage in pigs and farmers in the four pig-farms (A, B, C and D).
Note: The number of individuals sampled from pigs and farmers was 40 and 10, respectively

3.1.5.2 Antimicrobial resistomes of *Enterococcus* spp Strains from pigs farms

In farms-A to -D, all the *E. faecalis* strains from pigs and farmers were MDR **(Table 68).** Multiresistance was also detected in other species such as *E. casseliflavus* (carrying *fexA*, *optrA*, *cfrD*, *tet*(K), *tet*(L), and *ermB* genes), *E. hirae* and *E. gallinarum* (carrying *ermB*, *tet*(M), and *ant6* genes). None of the enterococci showed resistance to linezolid by disk diffusion; however, most of the chloramphenicol-resistant strains carried some acquired linezolid resistance genes (*optrA,* and in some cases *cfrD*). Of special relevance was the detection of linezolid-resistance genes in enterococci of pigs: (a) in 33.3% of pigs tested (*E. faecalis* with *optrA* and/or *cfrD*; *E. casseliflavus* with *optrA* and *cfrD*); and (b) in 10% of pig farmers (*E. faecalis* with *optrA*). The MLST of three *E. faecalis* strains carrying the linezolid resistance genes from farms-A (pig) and B (pig and farmer) was performed and found to be of the genetic lineage ST330 in all cases. However, the MLST of three of the linezolid-resistant *E. faecalis* strains from farm-D obtained from two pigs were ST330, ST474 and ST59 **(Table 68).** The strains that carried linezolid resistance genes showed an MIC for linezolid in the range of 8–16 μg/ml **(Table 68).**

3.1.5.3 Nasal enterococcal carriage rate in healthy dogs and human household members

Six out of the 27 households (22.2%) had nasal enterococci carriers**.** In total, 31 enterococci strains (27 from dogs, 4 from human household members) were recovered. Of the dogs' strains, 18, 7 and 2 were *E. faecium*, *E. faecalis*, and *E. raffinosus*, respectively. However, all the strains of humans were *E. faecalis* (*n* = 4) **(Figure 46).** The nasal carriage rate of enterococci among healthy dogs and dog-owning humans were 29.4% and 4.9%, respectively.

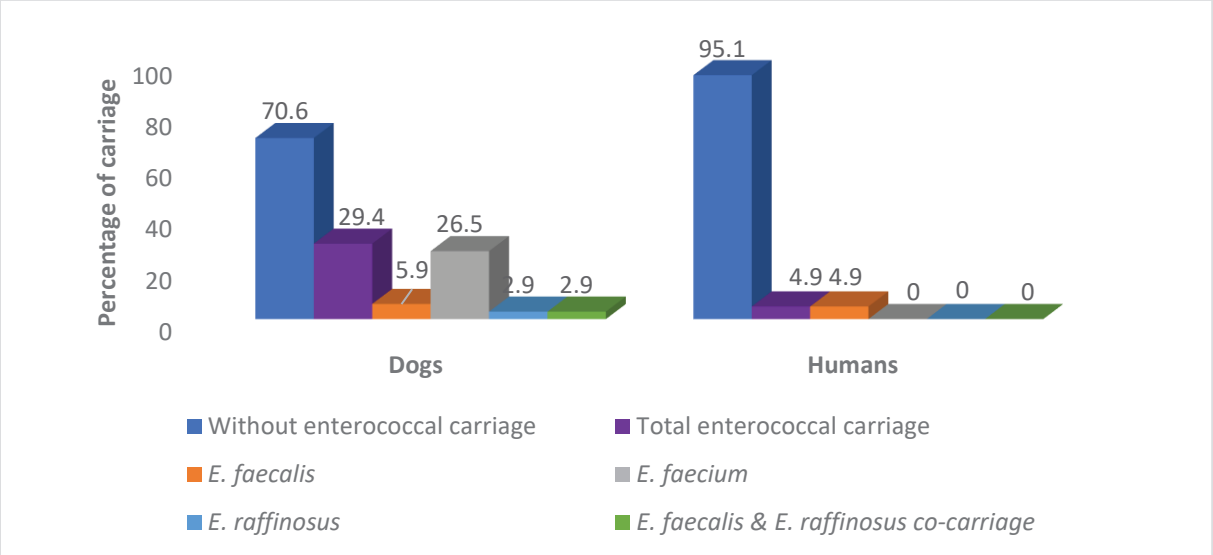


Figure 46. Nasal enterococcal carriage in healthy dogs and dog-owning human household members.
Note: The number of individuals sampled from dogs and dog owners were 34 and 41 respectively, from 27 households.

Table 68. Intra-sample and intra-species variation of resistomes of *Enterococcus spp* strains from all pigs and pig-farmers

Farm	Host/ Nº carriers	*Enterococcus spp*	N. strains	AMR Phenotypes[a,b]	LZD MIC (µg/ml)[c]	AMR genes detected[a]	Sequence Type
Farm-A	Pig/2	*E. faecalis*	2	CHL^2-TET^2-ERY^2-CIP^2 STR^2	10, 12	*fexA*^{2}, *optrA*^{2}, *tet*(M)^2, *ermA*^{2}, *ermB*^{2}, *ant6'*^{2}	ST330
	Pig/1	*E. hirae*	1	PEN^1-TET^1-ERY^1 -STR^1	NT	*ermB*^{1}	NT
	Pig/1	*E. casseliflavus*	1	STR^1-TET^1-ERY^1-CLO^1	8	*fexA*^{1}, *cfrD*^{1}, *optrA*^{1}, *tet*(K)^1, *tet*(L)^1, *ermB*^{1}	NT
Farm-B	Pig/7	*E. faecalis*	7	CLO^7-TET^7-ERY^7-CIP^6-GEN^7-STR^7	12-16	*fexA*^{7}, *optrA*^{7}, *tet*(M)^7, *ermB*^{7}, *aac6'-aph2''*^{7}, *ant6'*^{7}	ST330
	Pig/2	*E. faecalis*	2	TET^2-ERY^2-CIP^2-STR^2	NT	*ermB*^{2}, *ant6'*^{2}	NT
	2nd Pig-farmer	*E. faecalis*	2	CHL^2-TET^2-ERY^2-CIP^2-GEN^2-STR^2	8, 10	*fexA*^{2}, *optrA*^{2}, *tet*(M)^2, *ermB*^{2}, *aac6'-aph2''*^{2}, *ant6'*^{2}	ST330
	1st Pig-farmer	*E. faecalis*	1	CHL^1-TET^1-ERY^1-CIP^1-GEN^1-STR^1	NT	*tet*(M)^1, *ermB*^{1}, *aac6'-aph2''*^{1}, *ant6'*^{1}	NT
	3rd Pig-farmer	*E. faecium*	1	PEN^1- TET^1-ERY^1-CIP^1	NT	*tet*(M)^1, *ermB*^{1}	NT
Farm-C	Pig/1	*E. faecalis*	1	TET^1-ERY^1-CIP^1-STR^1	NT	*tet*(M)^1, *ermA*^{1}, *ant6'*^{1}	NT
	Pig/1	*E. faecalis*	1	PEN^1--TET^1-CIP^1-STR^1	NT	*tet*(M)^1, *ant6'*^{1}	NT
	Pig/1	*E. faecalis*	1	PEN^1-TET^1-CIP^1	NT	*tet*(M)^1	NT
	Pig/1	*E. faecium*	1	TET^1-ERY^1--CIP^1-GEN^1-STR^1	NT	*tet*(M)^1, *ermB*^{1}, *aac6'-aph2''*^{1}, *ant6'*^{1},	NT
	Pig/1	*E. faecium*	1	PEN^1 -TET^1-CIP^1	NT	*tet*(M)^1	NT
	Pig/1	*E. gallinarum*	1	PEN^1 -TET^1-STR^1	NT	*tet*(M)^1, *ant6'*^{1}	NT
	Pig/1	*E. gallinarum*	1	TET^1-ERY^1-CIP^1-STR^1	NT	*tet*(M)^1, *ermB*^{1}, *ant6*^{1}',	NT
	Pig/1	*E. hirae*	1	PEN^1-TET^1-ERY^1-CIP^1	NT	*tet*(M)^1, *ermA*^{1}, *ermB*^{1}	NT
	2nd Pig-farmer	*E. faecium*	1	PEN^1-TET^1	NT	*tet*(M)^1	NT
Farm-D	Pig/4	*E. faecalis*	6	CHL^6-TET^6-ERY^6-CIP^6-GEN^6-STR^6	2-3	*fexA*^{6}, *catA*^{6}, *cfrD*^{6}, *optrA*^{6}, *ermB*^{6}, *tet*(M)^6, *aac6'-aph2''*^{6}, *ant6'*^{6}	ST59
	Pig/1	*E. faecalis*	2	CHL^2-TET^2-ERY^2-CIP^2-GEN^2-STR^2	NT	*catA*^{2}, *tet*(M)^2, *ermB*^{2}, *aac6'-aph2''*^{2}, *ant6'*^{2}	NT
	Pig/1	*E. faecalis*	1	CHL^1-TET^1-ERY^1-GEN^1-STR^1	NT	*catA*^{1}, *tet*(M)^1, *ermB*^{1}, *aac6'-aph2''*^{1}, *ant6'*^{1}	NT
	Pig/1	*E. faecalis*	1	CHL^1-TET^1-ERY^1-GEN^1-STR^1	12	*fexA*^{1}, *optrA*^{1}, *tet*(M)^1, *ermB*^{1} *aac6'-aph2''*^{1}, *ant6'*^{1}	ST330
	Pig/3	*E. faecalis*	4	TET^4-ERY^4-GEN^4-STR^4	NT	*tet*(M)^4, *ermB*^{4}, *aac6'-aph2''*^{4}	NT
	Pig/1	*E. faecalis*	2	CHL^2-TET^2-ERY^2	NT	*tet*(K)^2, *tet*(M)^2, *ermB*^{2}	NT
	Pig/1	*E. faecalis*	2	CHL^2-TET^2-ERY^2-STR^2	NT	*catA*^{2}, *ermB*^{2}, *ant6'*^{2}	NT
	Pig/1	*E. faecalis*	2	CHL^2-TET^2-ERY^2- GEN^2-STR^2	8, 10	*fexA*^{2}, *optrA*^{2}, *aac6'-aph2''*^{2}	ST474
	Pig/1	*E. faecium*	2	CHL^2-TET^2-ERY^2-STR^2	NT	*catA*^{2}, *ermB*^{2}, *ant6'*^{2}	NT
	1st Pig-farmer	*E. faecium*	2	PEN^2 -TET^2-ERY^2-STR^2	NT	*ermB*^{2}, *ant6'*^{2}	NT
	2nd Pig-farmer	*E. faecalis*	1	TET^1- CIP^1-STR^1	NT	*tet*(M)^1, *ant6'*^{1}	NT

[a]In superscript is the number of strains that present the specific phenotype/genotype of AMR; ST; Sequence Type; NT: Not tested

[b]CHL: chloramphenicol; CIP: ciprofloxacin; ERY: erythromycin; GEN; gentamicin; LZD: linezolid; PEN: penicillin; STR: streptomycin; TET: tetracycline

[c]Linezolid MIC was tested in the strains that carried linezolid resistance genes.

Note: Some pigs and pig farmers were carriers of >1 species and/ or the same species with varied AMR phenotypes

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3.1.5.4 Antimicrobial resistomes of *Enterococcus* spp strains from healthy dogs and dog-owning human household members

In one of the 6 households with enterococci carriage (household ID number 10), both humans and dogs were *E. faecalis* carriers and the strains presented a similar AMR phenotype and genotype (*tet*(M) positive) **(Table 69).** Moreover, in another household (household ID number 18), enterococci were detected in both dog owners and dogs but belonged to different species (*E. faecalis* in humans and *E. faecium* in dogs). One of the dogs' samples analyzed in this study (household ID number 18) carried linezolid-resistant *E. faecalis* strains that contained the *fexA*, *optrA*, *tet*(L), *tet*(M), *ermA*, *ermB*, *str*, *aac6'-aph2''*, and *ant6'* genes, and were typed as ST585.

3.1.5.5 Antimicrobial resistomes in the *Enterococcus* strains from white stork nestlings

More than 70% of the 144 *Enterococcus* spp studied were susceptible to all antibiotics tested **(Table 70).** However, 13.2% of enterococci showed tetracycline resistance, all of them of the species *E. faecalis* and *E. faecium*, and they carried the *tet*(M) and/or *tet*(K) genes (except one *E. faecium* strain); moreover, between 4-5% of enterococci showed erythromycin resistance (*ermB* and *ermA* genes) and high-level aminoglycoside resistance (*aac6'-aph2''* or *str* genes) **(Table 70).** In addition, and for the first time in this animal species, an *E. faecium* strain carried an acquired linezolid resistance gene (*poxtA*), in addition to *fexB* gene (associated with chloramphenicol resistance); this strain belonged to the lineage ST1736 and presented an MIC for linezolid of 8 μg/ml. None of the *E. casseliflavus*, *E. hirae*, *E. durans*, and *E. gallinarum* strains of stork origin showed resistance to the antibiotics tested **(Table 70).**

Table 69. Intra-sample and intra-species variation of resistomes of *Enterococcus* spp strains from healthy dogs' household

Host/ ID Nº	Household ID Nº/ population	Species	Nº of strains	AMR Phenotypes[a,b]	AMR genes detected[a]	LZD MIC (µg/ml)[c]	Sequence type
Dog/ 28	11/ 2 humans, 2 dogs	*E. faecium*	1	PEN[1]-TET[1]-CIP[1]	*tet*(M)[1]	NT	NT
Dog/ 29	11/ 2 humans, 2 dogs	*E. faecium*	3	PEN[3]-ERY[3]-CIP[3]	ND[3]	NT	NT
Human/ 23	10/ 2 humans, 2 dogs	*E. faecalis*	1	PEN[1]-TET[1]	*tet*(M)[1]	NT	NT
		E. faecalis	1	PEN[1]-CIP[1]	ND	NT	NT
Dog/ 25	10/ 2 humans, 2 dogs	*E. faecalis*	1	PEN[1]-TET[1]-CIP[1] -GEN[1]	*aac6'-aph2''*[1], *tet*(M)[1]	NT	NT
		E. faecalis	2	PEN[2]-TET[2]-CIP[2]	*tet*(M)[2]	NT	NT
		E. raffinosus	2	PEN[2]	ND[2]	NT	NT
Human/ 47	18/ 2 humans, 2 dogs	*E. faecalis*	2	TET[2]-ERY[2]-CIP[2]-GEN[2]	*aac6'-aph2''*[2], *ant6'*[2], *tet*(M)[2]	NT	NT
Dog/ 48	18/ 2 humans, 2 dogs	*E. faecium*	1	TET[1]	*tet*(M)[1]	NT	NT
		E. faecium	1	CHL[1]-TET[1]	*catA*[1], *tet*(M)[1]	NT	NT
		E. faecium	1	TET[1]-CIP[1]	*tet*(M)[1]	NT	NT
		E. faecium	1	TET[1]-CIP[1]	*tet*(K)[1], *tet*(L)[1]	NT	NT
Dog/ 49	18/ 2 humans, 2 dogs	*E. faecium*	1	TET[1]-CIP[1]	*tet*(M)[1]	NT	NT
		E. faecium	1	TET[1]	*tet*(M)[1]	NT	NT
		E. faecium	1	TET[1]-CIP[1]-STR[1]	*tet*(M)[1]	NT	NT
		E. faecium	1	TET[1]-CIP[1]	*tet*(M)[1]	NT	NT
		E. faecium	1	TET[1]	*tet*(M)[1]	NT	NT
Dog/ 53	19/ 2 humans, 2 dogs	*E. faecium*	1	PEN[1]-LZD[1]-ERY[1]-TET[1]-STR[1]	*tet*(M)[1], *ermB*[1], *ant6'*[1]	NT	NT
		E. faecium	1	PEN[1]-TET[1]- ERY[1]-STR[1]	*ant6*[1]*''*, *tet*(M)[1]	NT	NT
		E. faecium	1	PEN[1]-TET[1]- ERY[1]-STR[1]	*ant6''*[1], *tet*(M)[1]	NT	NT
Dog/ 56	20/ 2 humans, 2 dogs	*E. faecalis*	4	CHL[4]-LZD[4]-TET[4]-ERY[4]-CIP[4]-STR-GEN[4]	*fexA*[4], *optrA*[4], *tet*(L)[4], *tet*(M)[4], *ermA*[4], *ermB*[4], *str*[4], *aac6'-aph2''*[4], *ant6''*[4]	10-12	ST585
Dog/ 73	27/ 3 humans, 1 dog	*E. faecium*	2	Suceptible[2]	NT	NT	NT

[a]In superscript is the number of strains that present the specific phenotype/genotype of AMR; ST; Sequence Type; NT: Not tested; ND: Not detected

[b]CHL: chloramphenicol; CIP: ciprofloxacin; ERY: erythromycin; GEN; gentamicin; LZD: linezolid; PEN: penicillin; STR: streptomycin; TET: tetracycline; VAN: vancomycin.

[b]CHL: chloramphenicol; CIP: ciprofloxacin; ERY: erythromycin; GEN; gentamicin; LZD: linezolid; PEN: penicillin; STR: streptomycin; TET: tetracycline

[c]Linezolid MIC was tested in the strains that carried linezolid resistance genes.

Note: Some dogs and dog owners were carriers of >1 species and/ or the same species with varied AMR phenotype

Table 70. Antimicrobial resistomes of enterococci from nestlings based on forging habitat of their parents

Sample[a]	No.Storks	Species	AMR phenotype[b]	AMR genes	LZD[c]	Foraging habitat	ST
T	30	*E. faecalis*	Susceptible	NT[c]	NT	Landfill	NT
T	6	*E. faecalis*	Susceptible	NT	NT	Natural	NT
T	2	*E. faecalis*	TET	*tet*(M)	NT	Landfill	NT
T	1	*E. faecalis*	TET	*tet*(M)	NT	Natural	NT
T	1	*E. faecalis*	TET-ERY	*tet*(K), *tet*(M), *ermB*	NT	Landfill	NT
T	1	*E. faecalis*	TET-ERY-STR	*tet*(M), *ermB, str*	NT	Landfill	NT
T	1	*E. faecalis*	TET-ERY	*tet*(M), *ermA, ermB*	NT	Landfill	NT
T	1	*E. faecalis*	ERY-GEN	*ermB, aac6'-aph2''*	NT	Landfill	NT
T	1	*E. faecalis*	TET-STR	*tet(K), str*	NT	Landfill	NT
T	1	*E. hirae*	Susceptible	NT	NT	Natural	NT
T	1	*E. gallinarum*	Susceptible	NT	NT	Landfill	NT
T	1	*E. cecorum*	Susceptible	NT	NT	Natural	NT
T	6	*E. cecorum*	Susceptible	NT	NT	Landfill	NT
T	3	*E. faecium*	Susceptible	NT	NT	Natural	NT
T	2	*E. faecium*	Susceptible	NT	NT	Landfill	NT
T	1	*E. faecium*	CHL	*fexB, poxtA*	8	Landfill	ST1736
T	1	*E. faecium*	TET-CIP	*tet(M)*	NT	Landfill	NT
T	1	*E. faecium*	TET-CIP	ND	NT	Landfill	NT
T	3	*E. faecium*	CIP	ND	NT	Landfill	NT
N	3	*E. faecium*	CIP	ND	NT	Landfill	NT
N	7	*E. faecium*	Susceptible	NT	NT	Landfill	NT
N	1	*E. faecium*	TET	*tet*(M)	NT	Landfill	NT
N	1	*E. faecium*	TET	*tet*(M)	NT	Landfill	NT
N	1	*E. canis*	Susceptible	NT	NT	Natural	NT
N	3	*E. faecalis*	TET	*tet*(M)	NT	Landfill	NT
N	3	*E. faecalis*	TET-STR	*tet*(K), *tet*(M), *str*	NT	Landfill	NT
N	1	*E. faecalis*	TET-ERY	*tet*(K), *tet*(M), *ermB*	NT	Landfill	NT
N	1	*E. faecalis*	TET-ERY	*tet*(K), *tet*(M), *ermB*	NT	Landfill	NT
N	23	*E. faecalis*	Susceptible	NT	NT	Landfill	NT
N	3	*E. faecalis*	Susceptible	NT	NT	Natural	NT
N	4	*E. casseliflavus*	Susceptible	NT	NT	Natural	NT
N	1	*E. gallinarum*	Susceptible	NT	NT	Landfill	NT
N	1	*E. durans*	Susceptible	NT	NT	Natural	NT

[a]T, tracheal, N nasal; [b]CHL, chloramphenicol; CIP: ciprofloxacin; ERY: erythromycin; GEN; gentamicin; LZD: linezolid; STR: streptomycin; TET: tetracycline; [c]Linezolid MIC (μg/ml) was tested in the strains that carried linezolid resistance genes. NT: Not tested

3.1.5.6 Comparison of AMR phenotype frequencies among *E. faecalis and E. faecium*

To compare the AMR frequencies of distinct *E. faecalis* and *E. faecium* strains from dogs, pigs and storks' nasal samples, individual chi-squared tests against every antimicrobial agent were computed. The prevalence of tetracycline, erythromycin, chloramphenicol, gentamicin, linezolid, and streptomycin resistances was significantly higher among *E. faecalis* of pigs than in the other two groups ($p < 0.0001$) **(Table 71).** All the enterococci carrying linezolid resistance genes were phenotypically susceptible by disc diffusion tests; however, upon LZD Etest for their MIC, all were found resistant (range: 8 to 16 μg/ml) except six strains (all of the same animal) with MIC range of 2–3 μg/ml **(Tables 68, 69 and 70).**

Concerning *E. faecium* strains, penicillin resistance was significantly present among strains of dogs with respect to the other two groups ($p < 0.05$) **(Table 71).** However, gentamicin, erythromycin, ciprofloxacin, and streptomycin resistances were significantly higher among *E. faecium* of pigs than in the other two groups **(Table 68).** In all cases, storks' nasal *E. faecalis* and *E. faecium* strains had the lowest AMR rates compared to the dogs' and pigs' strains. Among the chloramphenicol-resistant enterococci, strains harbouring linezolid resistance genes (*optrA*, *poxtA*, and *cfrD*) were detected in 16 pigs (33.3%), 1 dog (2.9%), 1 stork (1.1%) and 1 pig-farmer (10.0%) **(Figure 47).**

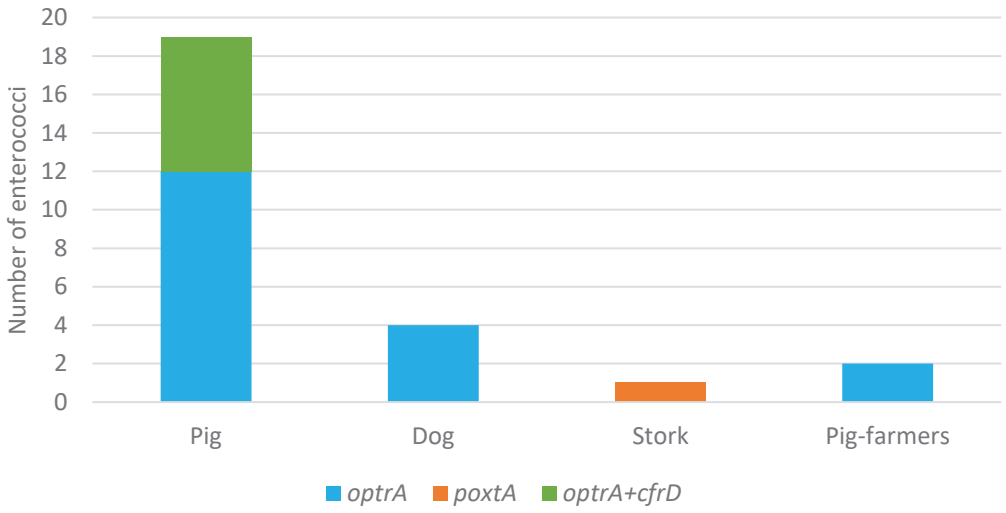

Figure 47. Distribution of acquired linezolid resistance genes among the four hosts

3.1.5.7 Risk factors associated with nasal enterococcal carriage and MDR phenotypes

After bivariate logistic analysis, nasal carriage (OR = 6.33, 95% CI: 2.29–17.42, $p = 0.004$) and occurrence rate of MDR phenotype (OR = 8.57, 95% CI: 2.99–24.56, $p = 0.0001$) were significantly associated with the species of animal **(Table 72).** In these regards, pigs had the enterococci that presented the highest rate of MDR phenotype. Nevertheless, the nasal enterococcal carriage in storks was double but not significantly different from that of dogs (OR = 2.4, 95% CI: 0.93–6.17, $p = 0.069$). Also, nasal enterococci carriage in humans was significantly associated with the species of animal contact (OR = 29.25, 95% CI: 4.36–196.07, $p = 0.0005$). Dog-owning households with > 1 dog & 1 human had relatively higher odds of nasal enterococci carriage than those with only 1 dog & 1 human; however, this was not statistically significant (OR = 3.75, 9% CI: 0.37–37.94, $p = 0.268$) **(Table 72).**

Table 71. Comparison of the AMR phenotype frequencies among distinct *E. faecalis* and *E. faecium* strains from dogs, pigs and nestling storks

Antimicrobial agent	*E. faecalis*			χ^2	*p* value	*E. faecium*			χ^2	*p* value
	Nº (%) of resistant strains from dogs (n=3)	Nº (%) of resistant strains from pigs (n=30)	Nº (%) of resistant strains from storks' nasal (n=34)			Nº (%) of resistant strains from dogs (n=15)	Nº (%) of resistant strains from pigs (n=4)	Nº (%) of resistant strains from storks' nasal (n=25)		
Penicillin	2 (66.7)	0 (0.0)	0 (0.0)	43.979	<0.0001*	3 (20.0)	0 (0.0)	0 (0.0)	6.224	0.0445*
Tetracycline	3 (100.0)	30 (100.0)	8 (23.5)	41.238	<0.0001*	14 (93.3)	4 (100.0)	2 (8.0)	32.814	<0.0001*
Erythromycin	1 (33.3)	30 (100.0)	2 (5.9)	56.802	<0.0001*	3 (20.0)	3 (75.0)	0 (0.0)	17.253	<0.0001*
Ciprofloxacin	3 (100.0)	28 (93.3)	0 (0.0)	59.492	<0.0001*	7 (46.7)	2 (50.0)	3 (12.0)	6.826	0.03294*
Gentamicin	2 (66.7)	24 (80.0)	0 (0.0)	43.979	<0.0001*	0 (0.0)	1 (25.0)	0 (0.0)	10.233	0.00599*
Chloramphenicol	1 (33.3)	28 (93.3)	0 (0.0)	56.68	<0.0001*	0 (0.0)	0 (0.0)	0 (0.0)	NA	NA
Linezolid	1 (33.3)	20 (66.7)	0 (0.0)	32.922	<0.0001*	0 (0.0)	0 (0.0)	0 (0.0)	NA	NA
Streptomycin	1 (33.3)	26 (86.7)	3 (8.8)	39.222	<0.0001*	4 (26.7)	3 (75.0)	0 (0.0)	16.467	0.00026[a]
Vancomycin	0 (0.0)	0 (0.0)	0 (0.0)	NA	NA	0 (0.0)	0 (0.0)	0 (0.0)	NA	NA

[a]Significant association determined by two-tailed chi-squared test at 95% Confidence interval (CI)
Percentages were based on the total *E. faecalis* and *E. faecium* strains with distinct AMR profiles

Table 72. Risk factors associated with nasal enterococcal carriage and MDR phenotypes

Variable	Nº of Enterococci present (%)	Nº of Enterococci absent (%)	OR (95% CI)	*p* value
Nasal carriage by animal type				
Pigs (n=40)	29 (72.5)	11 (27.5)	1.17 (0.47-2.91)	0.7329
Dogs (n=34)	10 (29.4)	24 (70.6)	0.19 (0.07-0.48)	0.0005[a]
Storks (n=52)	36 (69.2)	16 (30.7)	Referent	Referent
MDR phenotype by animal type				
Pigs (n=40)	29 (72.5)	11 (27.5)	43.06 (11.09-167.2)	0.0001
Dogs (n=34)	8 (23.5)	26 (76.5)	5.02 (1.23-20.6)	0.025
Storks (n=52)	3 (5.8)	49 (94.2)	Referent	Referent
Nasal carriage by animal contact				
Pig farm personnel (n=10)	6 (60.0)	4 (40.0)	29.25 (4.36-196.07)	0.0005[a]
Pet ownership (n=41)	2 (4.9)	39 (95.1)	Referent	Referent
Nasal carriage by household density				
>1 dogs &1 humans	5 (29.4)	12 (70.6)	3.75 (0.37-37.94)	0.268
(n=17)	1 (10.0)	9 (90.0)	Referent	Referent
1 dog &1 human (n=10)				

[a]Significant association determined by bivariate regression at 95% Confidence interval (CI)
MDR: Multidrug resistance

3.2 MOBILOME AND VIRULOME OF STAPHYLOCOCCI BY WHOLE GENOME SEQUENCING

3.2.1 *S. aureus* from nestling storks, pigs and pig farmers

Two subclades have been described within the *S. aureus* CC398 lineage. As these *S. aureus*-CC398 subclades are important livestock (MRSA) and human (MSSA) colonizers, their common interaction in both hosts and potential spillover to wild animals requires attention. Moreover, few comparative genomic studies have determined the impact of hosts, habitat, ecology, and human occupation on antibiotic selection pressure and associated mobile genetic elements. The hosts from the two ecological niches (wildlife and pig farms) were selected in these analyses due to their distinct differences in relation to antimicrobial pressures (high and low).

3.2.2 Effects of strains' origin and habitat on antibiotic pressure and mobilome levels

S. aureus strains with diverse CCs from nestling storks compared to those with only two CCs from pigs and pig farmers were analyzed **(Table 73).** Bivariate logistic analysis showed that the frequencies of multidrug-resistant (MDR) phenotype and transposons were significantly lower in nestling storks than in pigs (OR=525, 95%CI: 20.1-13690.5, *p*=0.0002) and pig farmers (OR=375, 95% CI: 14.2-9911.2, *p*=0.0004) **(Table 74)**. However, the presence of IEC genes in *S. aureus* strains from nestling storks was significantly higher than in those of

pigs (OR= 0.006, 95% CI: 0.0003-0.1314, *p*=0.0001) and pig farmers (OR=0.009, 95% CI: 0.0005-0.187, *p*=0.0023). However, the frequencies of plasmids and phages found in the *S. aureus* strains were not significantly associated with the types of hosts (p>0.05) **(Table 74).**

Table 73. Characteristics of *S. aureus* strains from the nestling storks, pigs and pig farmers

Variables	**Nestling storks from parents foraging in landfills (n=18)**	**Nestling storks from parents foraging in natural areas (n=5)**	**Pigs (n =17)**	**Pig farmers (n =12)**
Nº. strains with methicillin resistance.	0	0	14	12
Nº. strains with MDR.	1	0	17	12
spa types	t015, t127, t209, t223, t227, t521, t335, t571, t774, t1094, t1451, t1654, t3380, t7778, t18009	t571, t1451, t2313, t3380 t6220	t011, t034, t1430, t1451	t011, t034, t1451
ST	ST1, ST5, ST15, ST22, ST25, ST26, ST30, ST45, ST97, ST109, ST398	ST97, ST291, ST130, ST398	CC9, CC398	ST398
CC	CC1, CC5, CC9, CC15, CC22, CC25, CC30, CC45, CC97, CC398	CC97, CC130, CC398	ST9, ST398	CC398

Table 74. Comparison of the level of antibiotic pressure and mobile genetic elements among distinct *S. aureus* strains from nestling storks, pigs and pig farmers

Variables	**Stork nestlings (n=23) Low antimicrobial pressure**	**Pigs (n =17) High antimicrobial pressure**	**Pig farmers (n =12) High antimicrobial pressure**
MDR phenotype			
Yes	1	17	12
No	22	0	0
OR (95% CI)	Reference	525 (20.1-13690.5)	375 (14.2-9911.2)
p value	Reference	0.0002*	0.0004*
Presence of plasmids			
Yes	16	17	12
No	7	0	0
OR (95% CI)	Reference	15.9 (0.84-301.1)	11.4 (0.59-218.3)
p value	Reference	0.065	0.1070
Presence of transposons			
Yes	1	17	12
No	22	0	0
OR (95% CI)	Reference	525 (20.1-13690.5)	375 (14.2-9911.2)
p value	Reference	0.0002*	0.0004*
Presence of phages			
Yes	21	15	12
No	2	2	0
OR (95% CI)	Reference	0.71 (0.09-5.65)	2.9 (0.1-65.5)
p value	Reference	0.749	0.5020
Presence of IEC			
Yes	19	0	0
No	4	17	12
OR (95% CI)	Reference	0.006 (0.0003-0.1314)	0.009 (0.0005-0.187)
p value	Reference	0.0001*	0.0023*

*Significant association determined by bivariate regression at 95% confidence interval (CI)

3.2.3 Antimicrobial and metal resistance and associated mobile genetic elements

Generally, most of the *S. aureus* strains from nestling storks were either resistant to only one class of antibiotics (in most cases beta-lactam or macrolides) or entirely susceptible to all antibiotics. In this sense, the resistome profile of the strains was mainly *blaZ, ermT, lnuA,* and *tet*(K) as previously detected by PCR. In addition, we here further identified the *ant9'* gene in strain X3799 which was co-localized with *ermA* in a Tn*554* transposon **(Table 75)**.

Table 76 shows the complete resistome of the 35 *S. aureus* strains from pigs and pig farmers. All the *S. aureus* strains presented an MDR phenotype mediated by a large repertoire of resistance markers (often >5 different genes). In this sense, the resistome of these strains was mainly comprised of genes that mediate resistance to beta-lactams, macrolide-lincomycin-streptogramin B (MLS_b), tetracyclines, aminoglycosides, sulfamethoxazole-trimethoprim, or fluoroquinolones as previously detected by PCR. Aside from these genes, the aminoglycoside gene *str* that mediates resistance to streptomycin was detected in twelve strains. Also, *ant9'* another aminoglycoside gene, was detected co-located with the *ermT* gene on plasmid *repUS18* in a pig (X4905) and a pig farmer (X5473) strain **(Table 76).**

All the *S. aureus* strains from pigs (both MRSA and MSSA) presented an MDR phenotype, contrary to those from the nestling storks where only one strain presented an MDR phenotype/genotype (X3799). All the strains from pigs and pig farmers presented the characteristic tetracycline resistance where 20 *S. aureus* strains from pigs and pig farmers carried *tet*(M), 12 carried *tet*(K) and eight carried *tet*(L) gene. It is noteworthy that *tet*(M) and *tet*(L) genes were located in plasmid *rep22* and *repUS43*, respectively, in all the MRSA-CC398 strains carrying these tetracycline resistance genes. But *tet*(M)- and *tet*(L)-carrying MSSA-CC9 strains from the two pigs and a pig farmer did not harbour these plasmid replicons. Perhaps, these plasmids are emerging mobilomes for *tet*(M)- and *tet*(L) genes in the MRSA-CC398 strains. The *tet*(K) gene in most of the *S. aureus* strains (including the one from a stork nestling (X4139)) was located in *rep7a* while in only one strain (X5751) was not associated with this plasmid replicon. The *tet*(M) gene was localized in various transposons such as those detected in this study (*i.e.,* Tn*6009*, Tn*925* and Tn*916*). Moreover, the *tet*(L) is almost always physically linked with the *dfrK* gene in small plasmids with varied sizes and shapes.

Concerning all the strains carrying *tet*(L) and *dfrK* genes, the plasmid *rep22* was always co-located with these genes and is 99% similar to the plasmid pKKS2187 in an MRSA-CC398 strain from a pig (GenBank accession number: FM207105). For the nestling storks' strains, two of them (X4135 and X4139) presented the *tet*(K) gene carried in circular plasmid *rep7a* while the *tet*(M) and *tet*(L) genes were absent from all nestling stork strains.

Table 75. Antimicrobial resistomes, virulence, mobilomes and genetic lineages of 23 *S. aureus* strains from nestlings of parent storks foraging in landfills and natural areas

Stork №/ Strain ID	*spa*-types/ ST/CC	Resistome	Transposons (associated genes)	Metal/biocide resistance genes	Insertion sequence	Virulome profile	Prophage integrase	Plasmid replicons
Nestlings of parent storks foraging in landfills								
546/ X4139	t127/ST1/CC1	*tet*(K), *lnuA*, *vga(A)V*	ND	*arsB*	ND	*icaA-D, icaR, lukF-PV, lukD, lukE, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	none	*rep7a, rep7c*
489/ X4659	t1094/ST5/CC5	*blaZ*	ND	*cadD, arsB*	ND	*fnbA, icaA-D, lukD, lukE, seo, seu, sen, seg, sei, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	Sa7int, phiJBint	*rep20, rep21*
554/ X3799	t209/ST109/CC9	*blaZ, ermA, ant9'*	Tn*554* (*ermA, ant9'*)	*arsB*	ISSau5	*icaA-D, icaR, lukF-PV, eta, sem, seo, seu, sen, sei, clfA, clfB, clfP, sdrC, sdrD, vWbp, coa, adsA*	Sa1int, Sa3int	none
536/ X4573	t335/ST15/CC15	*blaZ*	ND	*cadD, arsB, lmrS, norA*	ISSep3 ISSau5	*icaA-D, icaR, lukF-PV, lukD, lukE, seb, cna, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	SebogoInt, Sa3int	*rep5a, rep16*
546/ X4135	t774/ST15/CC15	*blaZ, tet*(K)	ND	*cadD, arsB*	ISSep3 ISSau5	*icaA-D, icaR, lukF-PV, lukD, lukE, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	Sa3int	*rep5a, rep16*
490/ X3844	t774/ST15/CC15	*blaZ*	ND	*cadD, arsB*	ISSep3	*icaA-D, icaR, lukF-PV, lukD, lukE, clfA, clfB, clfP, sdrC, sdrD, vWbp, coa, adsA*	Sa3int	*rep5a, rep16*
489/ X4610	t7778/ST15/CC15	*blaZ*	ND	*cadD, arsB*	ISSep3	*icaA-D, icaR, lukF-PV, lukD, lukE, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	Sa3int	*rep5a, rep16*
510/ X4541	t223/ST22/CC22	*blaZ*	ND	*arsB*	ISSau2 ISSau5	*icaA-D, icaR, lukF-PV, lukD, lukE, tst, sen, seg, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	Sa9int, Sa3int	none
545/ X3777	t227/ST25/CC25	*blaZ*	ND	*cadD, arsB*	ND	*icaA-D, icaR, lukF-PV, seb, seo, seu, sen, sei, seg, lukD, lukE, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	Sa3int	*rep7c, rep 16, rep19*
530/ X3723	t18009/ST26/CC25	*blaZ*	ND	*cadD, arsB*	ND	*icaA-D, icaR, lukF-PV, lukD, lukE, sen, sem, sei, seg, seu, seo, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	Sa2int, Sa3int	*rep7c, rep16, rep19*
507/ X4654	t1654/ST30/CC30	*blaZ*	ND	*arsB, copB*	ISSau6	*icaA-D, icaR, lukF-PV, lukD, lukE, sem, sen, seu, sei, seo, tst, clfA, clfB, clfP, sdrC, sdrE, vWbp, coa, adsA*	Sa2int, Sa5int	*rep5a, rep16*
512/ X4548	t015/ST45/CC45	*blaZ*	ND	*cadD, arsB*	ISSau3	*icaA-D, icaR, lukF-PV, lukD, lukE, seg, sem, seu, sei, sen, seo, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa*	Sa7int, Sa3int	*rep5a, rep15, rep16*

Table 75. Continuation

Stork №/ Strain ID	*spa*-types/ ST/CC	Resistome	Transposons (associated genes)	Metal/biocide resistance genes	Insertion sequence	Virulome profile	Prophage integrase	Plasmid replicons
Nestlings of parent storks foraging in landfills								
552/ X4675	t521/ST97/CC97	ND	ND	*arsB*	ND	*fnbA, icaA-D, icaR, lukD, lukE, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	SebogoInt, Sa3int	none
546/ X4138	t3380/ST97/CC97	None	ND	*arsB*	ND	*icaA-D, icaR, lukF-PV, lukD, lukE, she, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	SebogoInt, Sa3int	none
544/ X3775	t2313/ST291	*blaZ*	ND	*cadD, arsB*	ND	*icaA-D, icaR, lukF-PV, lukD, lukE, clfA, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	Sa1int, Sa3int	*rep5a, rep16*
505/ X4630	t571/ST398/CC398	*blaZ, ermT*	ND	*cadD, cadR, arsB*	ND	*icaA-D, icaR, lukD, lukE, clfA, clfP, sdrE, vWbp, adsA*	Sa9int, Sa3int	*rep13*
505/ X4603	t1451/ST398/CC398	*blaZ, ermT*	ND	*cadD, cadR, arsB*	ND	*icaA-D, icaR, fnbA, lukD, lukE, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	Sa3int	none
556/ X4706	t1451/ST398/CC398	*ermT*	ND	*cadD, cadR, arsB*	ND	*fnbB, icaA-D, icaR, lukD, lukE, clfA, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	SebogoInt. Sa3int	*rep13*
Nestlings of parent Storks foraging in natural areas								
433/ X4422	t3380/ST97/CC97	None	ND	*arsB*	ND	*fnbA, fnbB, icaA-D, icaR, lukF-PV, lukD, lukE, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	SebogoInt, Sa3int	none
436/ X4036	t6220/ST130/CC130	None	ND	*arsB*	ND	*fnbB, icaA-D, icaR, lukD, lukE, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	none	none
426/ X4406	t2313/ST291	*blaZ*	ND	*cadD, arsB*	ND	*fnbA, fnbB, icaA-D, icaR, lukF-PV, lukD, lukE, clfA, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	Sa1int, Sa3int	*rep5a, rep16*
474/ X3913	t571/ST398/CC398	*blaZ, ermT*	ND	*cadD, cadR, arsB*	ND	*fnbB, icaA-D, icaR, lukD, lukE clfA, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	Sa3int, Sa9int	
470/ X3906	t1451/ST398/CC398	*blaZ, ermT*	ND	*cadD, cadR, arsB*	ISSau30	*fnbB, icaA-D, icaR, isdA-G, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, coa, adsA*	Sa3int	*rep13*

Table 76. Antimicrobial resistomes, virulence genes, mobilomes and genetic lineages of 35 *S. aureus* strains from pigs and pig-farmers

Strain ID	Host Nº / Farm*	*spa*-types/ ST/CC	Resistomes	Transposon (associated genes)	Metal/biocide resistance genes	Mutations	SCC*mec*	Insertion sequence	Virulome profile	Prophage integrase	Plasmid replicons
Pigs											
X5153 **X5078**	P1/ C P3/ C	t1430/ST9/ CC9	*blaZ, dfrK, lsaE, lnuB, fexA, tet*(L), *tet*(M), *aac6'-aph2", ant6'*	Tn*6009* (*tet*(M)) Tn558 (*fexA*)	*arsB, copB, qacG*	GyrA (S84L), GrlA (S80F)	ND	ND	*fnbA, fnbB, icaA-D, icaR, sem, seo, seu, sei, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	None	*rep21*
X5082	P3/ C	t1430/ST9/ CC9	*blaZ, dfrK, lsaE, lnuB, fexA, tet*(L), *tet*(M), *aac6'-aph2", ant6'*	Tn*6009* (*tet*(M)) Tn558 (*fexA*)	*arsB, copB, qacG*	GyrA (S84L), GrlA (S80F)	ND	ISSau3 IS256	*fnbA, fnbB, icaA-D, icaR, sem, seo, seu, sei, adsA*	None	*rep21*
X4889 **X5408** **X5441** **X4915** **X5411** **X5401**	P2/ A P4/ B P8/ B P6/ A P5/ B P3/ B	t011/ST398/ CC398	*blaZ, mecA, dfrK, ermC, vga(A)LC, tet*(*L*), *tet*(M), *str, aac6'-aph2", ant4'*	Tn*6009* (*tet*(M))	*arsB, copA, czrC, qacG*	ND	Vc	IS256	*fnbA, icaA-D, icaR, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	Sa2int	*rep7b, rep10, rep16, rep21, rep22, repUS43, repUS76*
X5451	P9/ B	t011/ST398/ CC398	*blaZ, mecA, dfrK, ermC, tet*(L), *tet*(M), *str, aac6'-aph2", ant4'*	Tn*6009* (*tet*(M))	*arsB, copB, qacG*	ND	Vc	IS256 ISSau8	*fnbA, icaA-D, icaR, clfA, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	Sa2int	*rep10, rep16, rep21, rep22, repUS43, repUS76*
X4905	P4/ A	t011/ST398/ CC398	*blaZ, mecA, dfrK, ermC, ermT, tet*(*K*), *tet*(L), *tet*(M), *str, ant9'*	Tn*925* (*tet*(M))	*arsB, copA, czrC, qacG*	GyrA (S84L), GrlA (S80F)	Vc	ND	*fnbA, icaA-D, icaR, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	Sa2int, Sa9int, SebagoInt	*rep7a, rep10, rep21, rep22, repUS18, repUS43*
X5711	P2/ D	t011/ST398/ CC398	*blaZ, mecA, dfrK, ermC, cat*$_{pC221}$, *tet*(K), *tet*(M)	Tn*6009* (*tet*(M)) Tn*559* (*dfrK*)	*arsB, copB*	GyrA (S84L), GrlA (S80F)	Vc	ISSau8	*fnbA, icaA-D, icaR, clfA, clfP, sdrC, sdrD, sdrE, vWbp*	phiJBint Sa2int, Sa9int	*rep7a, rep10, rep21, repUS43*
X5393	P3/ B	t011/ST398/ CC398	*blaZ, mecA, dfrK, ermC, vga(A)LC, tet*(L), *tet*(M), *aac6'-aph2", ant4'*	Tn*6009* (*tet*(M))	*arsB, copB, qacG*	ND	Vc	IS256	*fnbA, icaA-D, icaR, adsA*	Sa1int Sa2int	*rep7b, rep10, rep16, rep21, rep22, repUS43, repUS76*

Table 76. Continuation

Strain ID	Host № / Farm*	*spa*-types/ ST/CC	Resistomes	Transposon (associated genes)	Metal/biocide resistance genes	Mutations	SCC*mec*	Insersion sequence	Virulome profile	Prophage integrase	Plasmid replicons
Pigs											
X5492	P6/A	t011/ST398/CC398	*blaZ, mecA, ant4', lnuA, dfrK, tet(L), ermC, tet*(M), *vgaA(LC), aac6'aph2"*	Tn*6009* (*tet*(M))	*arsB, copA, czrC, qacG*	ND	Vc	IS256	*fnbA, icaA-D, icaR, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	Sa2int	*rep7b, rep10, rep16, rep21, rep22, repUS43, repUS76*
X5751 **X5702**	P9/ D P2/ D	t011/ST398/CC398	*blaZ, mecA, dfrK, ermC, cat*$_{pC221}$, *tet*(K), *tet*(M)	Tn*6009* (*tet*(M)) Tn559 (*dfrK*)	*arsB, copB*	GyrA (S84L), GrlA (S80F)	Vc	ISSau8	*fnbA, icaA-D, icaR, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	Sa2int, Sa6int Sa9int	*rep7a, rep10, rep21, repUS43*
X5720	P4/D	t11451/ST398/CC398	*blaZ, mecA, tet*(M), *tet*(K), *lnuB, lsaE, aph3'*	Tn*6009* (*tet*(M))	*arsB, copA, czrC, qacG*	GrlA (S80F), GyrA (S84L)	Vc	ND	*fnbA, icaA-D, icaR, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	Sa2int, Sa6int Sa9int	*repUS22, repUS43*
Pig-farmers											
X5484 **X5471** **X5792**	F2/ A F2/ B F3/ B	t011/ST398/CC398	*blaZ, mecA, dfrK, ermC, vga(A)LC, tet*(L), *tet*(M), *str, aac6'-aph2", ant4'*	Tn*6009* (*tet*(M))	*arsB, copB, qacG*	ND	Vc	IS256	*fnbA, icaA-D, icaR, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	Sa2int	*rep7b, rep10, rep16, rep21, rep22, repUS43, repUS76*
X5764	F1/D	t011/ST398/CC398	*blaZ, mecA, dfrK, ermC, cat*$_{pC221}$, *tet(K), tet*(M)	Tn*6009* (*tet*(M)) Tn*559* (*dfrK*)	*arsB, copB*	GyrA (S84L), GrlA (S80F)	Vc	ND	*fnbA, icaA-D, icaR, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	Sa2int, Sa6int, Sa9int	*rep7a, rep10, rep21, repUS43*
X5786	F3/ D	t011/ST398/CC398	*blaZ, mecA, dfrK, ermC, tet(K), tet*(M)	Tn*6009* (*tet*(M)) Tn559 (*dfrK*)	*arsB, copB*	GyrA (S84L), GrlA (S80F)	Vc	ND	*fnbA, icaA-D, icaR, clfA, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	phiJBint Sa2int, Sa9int	*rep7a, rep10, repUS43*

Table 76. Continuation

Strain ID	Host Nº / Farm*	*spa*-types/ ST/CC	Resistomes	Transposon (associated genes)	Metal/biocide resistance genes	Mutations	SCC*mec*	Insersion sequence	Virulome profile	Prophage integrase	Plasmid replicons
Pig-farmers											
X5771	F1/ D	t011/ST398/CC398	*blaZ, mecA, dfrK, tet*(M), *tet(K), ermC*	Tn*6009* (*tet*(M)) Tn*559* (*dfrK*)	*arsB, copA, copB*	ND	Vc	ISSau8	*fnbA, icaA-D, icaR, adsA*	Sa2int, Sa9int	*rep7a, rep10, repUSA43*
X5466	F2/ B	t011/ST398/CC398	*blaZ, mecA, lnuB, lsaE, tet*(M), *ant6', aph3'*	Tn*916* (*tet*(M))	*arsB, copB, qacG*	GyrA (S84L), GrlA (S80F)	Vc	ND	*fnbA, icaA-D, icaR, adsA*	Sa2int	*rep21, repUS43*
X5473	F2/ A	t011/ST398/CC398	*blaZ, mecA, dfrK, ermC, ermT, tet(K), tet(L), tet*(M), *str, ant9'*	Tn*916* (*tet*(M))	*arsB, copB, qacG*	GyrA (S84L), GrlA (S80F)	Vc	ISsau8	*fnbA, icaA-D, icaR, adsA*	Sa1int, Sa2int, SebagoInt	*rep7a, rep10, rep21, rep22, repUS18, repUS43*
X5457	F1/ B	t034/ST398/CC398	*blaZ, dfrG, ermC, lsaE, lnuB, fexA, tet(K), tet*(M), *ant6', ant4'*	Tn*6009* (*tet*(M)) Tn*558* (*fexA*)	*arsB, copA, copB*	GyrA (S84L), GrlA (S80Y)	Vc	ND	*fnbA, icaA-D, icaR, adsA*	Sa1int, Sa2int, Sa6int, Sa9int	*rep7a, rep10, rep19b, rep22, repUS43*
X5458	F1/ B	t034/ST398/CC398	*blaZ, mecA, dfrG, lsaE, lnuB, fexA, tet(K), tet*(M), *ant4', ant6'*	Tn*6009* (*tet*(M)) Tn*558* (*fexA*)	*arsB, copA, copB*	GyrA (S84L), GrlA (S80Y)	Vc	ISSau8	*fnbA, icaA-D, icaR, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	Sa2int	*rep7a, rep19b, rep22, repUS43*
X5460	F1/ B	t034/ST398/CC398	*blaZ, mecA, dfrG, lsaE, lnuB, fexA, tet(K), tet*(M), *ant4', ant6'*	Tn*6009* (*tet*(M)) Tn*558* (*fexA*)	*arsB, copA, copB*	GyrA (S84L), GrlA (S80Y)	Vc	ND	*fnbA, icaA-D, icaR, clfA, clfB, clfP, sdrC, sdrD, sdrE, vWbp, adsA*	Sa2int	*rep7a, rep19b, rep22, repUS43*
X5124	F1/ C	t1451/ST398/CC398	*blaZ, mecA, lnuB, lsaE, tet(K), tet*(M), *ant4', ant6', aph3'*	Tn*6009* (*tet*(M))	*arsB, copB, qacG*	GyrA (S84L), GrlA (S80F)	Vc	ND	*fnbA, icaA-D, icaR, adsA*	phiJBint Sa2int, SebagoInt	*rep7a, repUS22, repUS43*

The pigs (10 per farm) are named P1-P10 in each farm (A-D). In the case of humans working in the farm, they are designated as F1, F2, F3 and the farm (A-D)

Aside from tetracycline, resistance to other clinical antibiotics was also detected, such as to MLS_a antibiotics. AMR to this class of antibiotics was specifically mediated by *lnuB* and *lsaE* in nine *S. aureus* strains from the pigs and pig farmers. However, MLS_a resistance was mediated by *ermA* and *vga(A)V* among the two non-CC398 strains from nestling storks (X3799 and X4139). Also, the *vga*(A)*LC* gene is located on a *rep7b* in ten strains **(Table 75).**

A striking AMR difference between the strains from the two ecological niches was the ciprofloxacin resistance mediated by DNA topoisomerase IV point mutations at GyrA (S84L) and DNA gyrase at GrlA (S80F or S80Y). These common mutations were found in 16 (55.2%) of the *S. aureus* strains from pigs and pig farmers but none among those from the nestling storks **(Tables 75 and 76).**

Concerning the heavy metal resistance genes, certain categorical differences in the genes encoding copper and zinc were observed and widespread among the *S. aureus* strains from the pigs and pig farmers, whereas these were absent in the nestling storks' strains. Interestingly, only the *czrC* gene was detected among the MSSA-CC9 strains. Moreover, the *copA* gene was exclusively associated with MRSA strains. Apart from the *cadX* gene that was only found on the MSSA-CC398 subclade with nestling stork strains, the *arsB* gene that encodes for arsenic pump membrane protein was only found in an MSSA-CC130 strain **(Table 74).** Concerning biocide resistances, only the *qacG* gene was detected in 20 (68.9%) *S. aureus* strains (MRSA-CC398 and MSSA-CC9), all from pigs and pig farmers **(Table 76).**

3.2.4 Genetic environment of the *ermT* gene in MSSA and MRSA strains

The *in-silico* analysis of the *ermT* sequences of two MRSA strains from a pig (X4905) and a pig farmer (X5473) and five MSSA strains from nestling storks (X3906, X3913, X4603, X4630, X4703) revealed striking differences in their genetic environment **(Figure 48).** First, the five *ermT*-carrying MSSA strains were all associated with cadmium-resistance genes, *cadR* and *cadD*, which were absent from the two *ermT*-carrying MRSA strains. The IS*257* was flanked upstream of the *ermT* gene in MSSA strains, except in one strain in which it was flanked downstream. Interestingly these markers were absent in the *ermT*-carrying MRSA strains. The *ermT*-gene of the MSSA strains was associated with plasmid *rep13*.

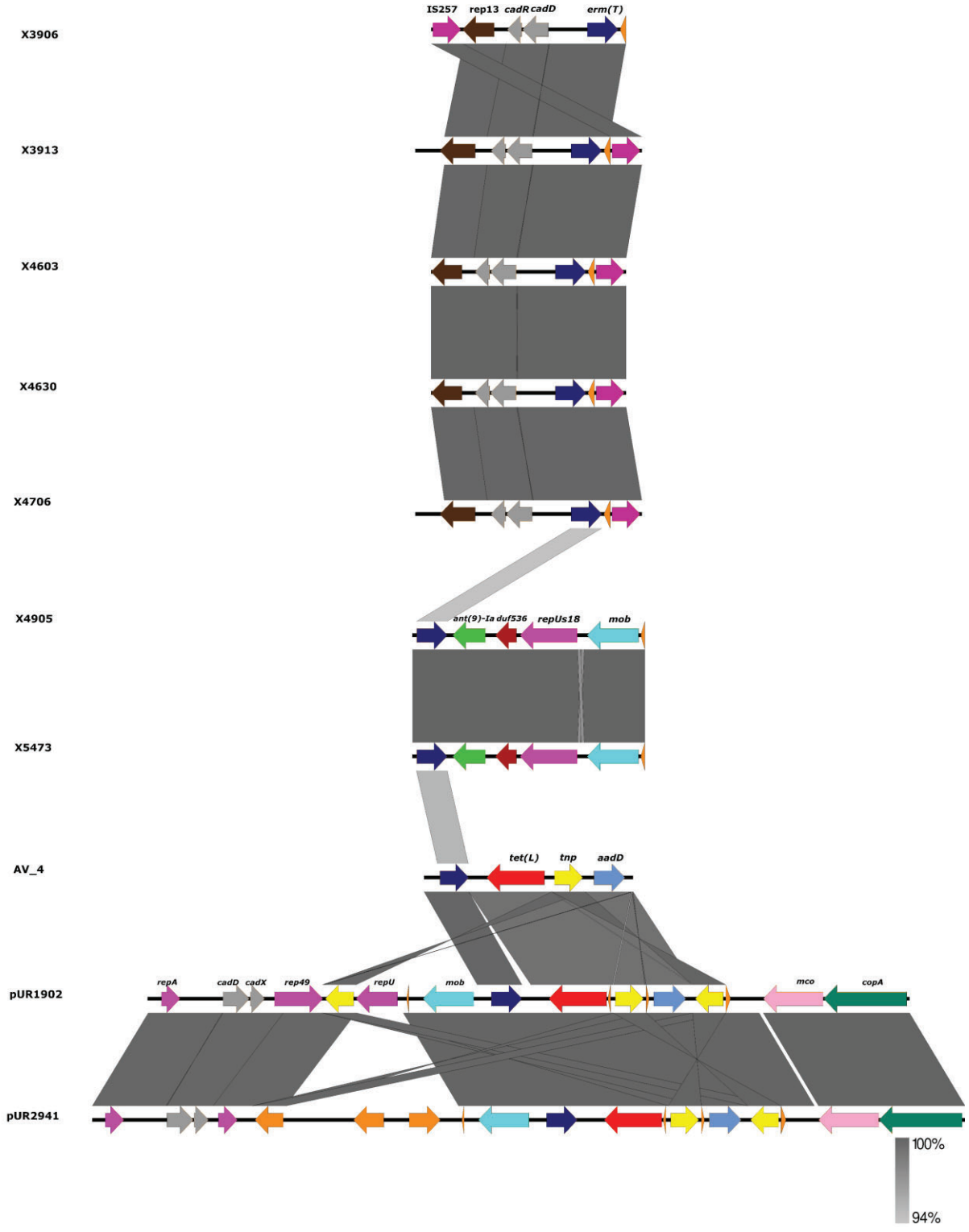

Figure 48. Schematic comparison between the environment of the *ermT* gene in the five MSSA-CC398 and two MRSA-CC398 strains with the reference strain MRSA-pURX2941 (HF583290) and pURX1902 (Gene bank accession number: HF583291) and MRSA-AV_4 (Gene bank accession number: SAMN00828682). Colors and arrows indicate the represented genes and their orientation.

3.2.5 Virulome profile of the *S. aureus* strains from nestling storks and pig farm hosts

The MRSA-CC398 strains were entirely negative for *tst, lukS/F-PV, eta, etb, etc, etd* and all genes encoding enterotoxins. However, the *sem, seo, seu,* and *sei* genes were identified in the three MSSA-CC9 strains from pigs. Moreover, *sen, sem, sei, seg, seu,* and *seo* genes were also identified in four strains of the nestling storks belonging to the lineages MSSA-CC5, -CC9, -CC25 and -CC45 **(Table 75).** From our findings, it appears that virulence genes are more predominant in MSSA than in MRSA strains.

Aside from the toxins, some *S. aureus* enzymes were commonly present in all the strains, such as the *adsA* that encodes adenosine synthase A. Furthermore, the *icaABCD* operon and its *icaR* were present in all the strains **(Tables 75 and 76).**

3.2.6 Host adaptation markers of *S. aureus*

Nineteen (82.6%) strains from the nestling storks carried the *scn* gene mediated by the prophage φ3 **(Table 75).** Whereas all *S. aureus* (MRSA-CC398 and MSSA-CC9) from pigs and pig farmers lacked the prophage φ3 marker (i.e., *scn*); they can be considered either as animal-adapted strains or originating from non-human hosts **(Table 76).** After a phylogenetic analysis of our CC398 strains with a collection of previously deposited strains, two different clusters of human- and animal-adapted subclades were observed **(Figure 49).** Aside from the prophage φ3, other prophages such as φ2, φ6, and φ9 were also frequently identified in *S. aureus* strains of the nestling storks, pigs and pig farmers regardless of the methicillin susceptibility and genetic lineages **(Tables 75 and 76).** Importantly, the presence of β-converting φ3-prophage variants carrying an IEC characterizes the MSSA-CC398 subclade. However, this subclade also harbours other prophages carrying integrase genes SebogoInt and Sa9int **(Table 75).**

3.2.7 Mobilomes of MSSA-CC398 strains from Healthy humans

About 77.8% of the MSSA-CC398 strains had the *ermT* gene that was in plasmid *rep*13 flanked by IS257 which were upstream of *cadR* and *cadD* genes **(Figure 50)**. However, one of the MSSA-CC398 strains carried the *ermC* gene in plasmid *rep*10 **(Figure 51).** Interestingly these markers were absent in one of the MSSA-CC398 strains (X6610) **(Figures 50 and 51).** Only the MSSA-CC398-*ermT*-positive strains were highly related (SNPs <50) and carried the φ*Sa3 prophage* (IEC type-C) **(Figure 52).** Analyses with other publicly available *S. aureus* CC398 genomes revealed relatedness of the X6417 and X6379 with SRR15903565 in France (SNP<200) **(Figure 52)**.

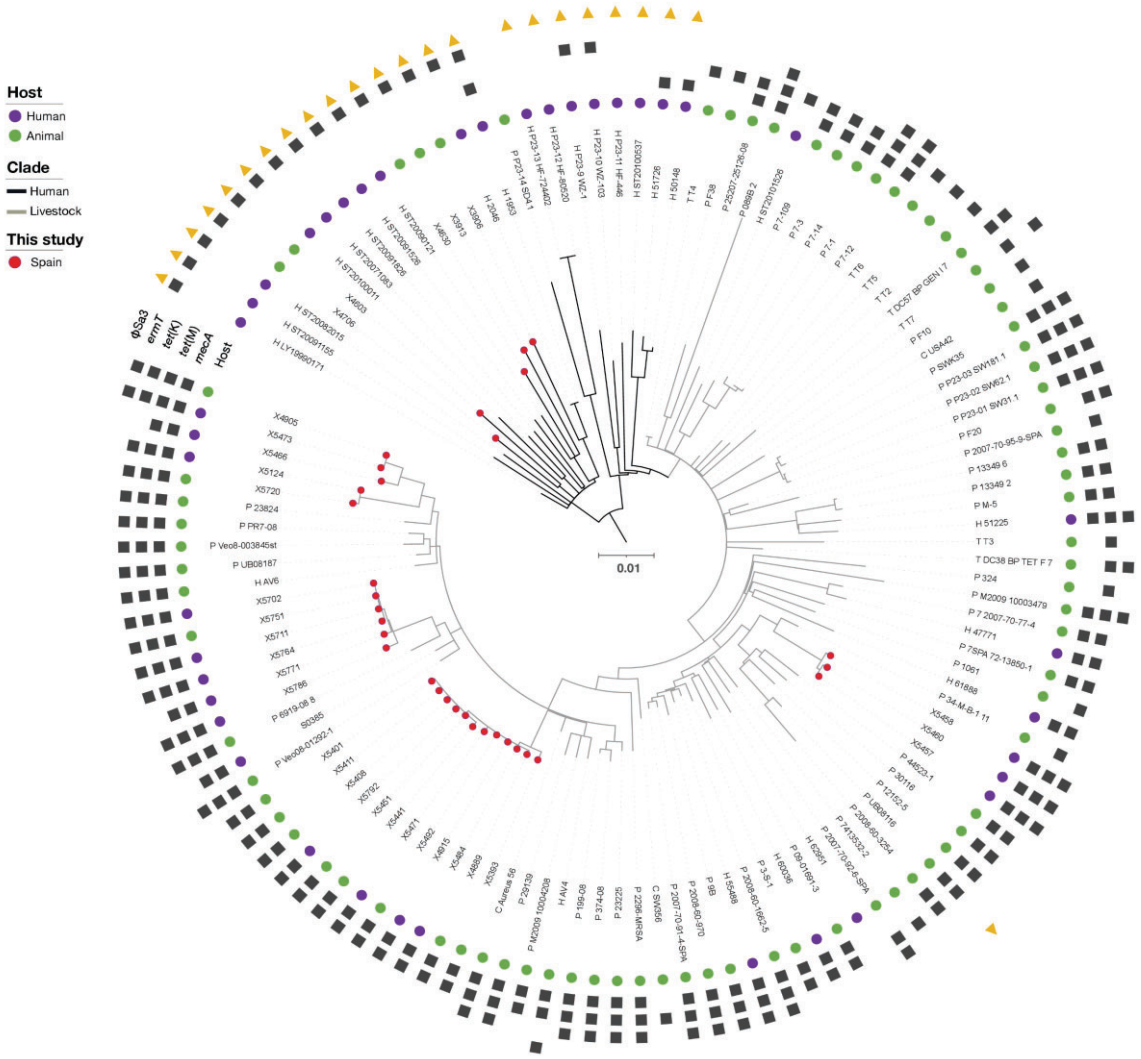

Figure 49. Circular phylogenetic tree to show the human- and animal-adapted subclades based on SNP analyses of CC398 strains obtained from this study in comparison with publicly available genomes of CC398 lineages from Price *et al* (2012)

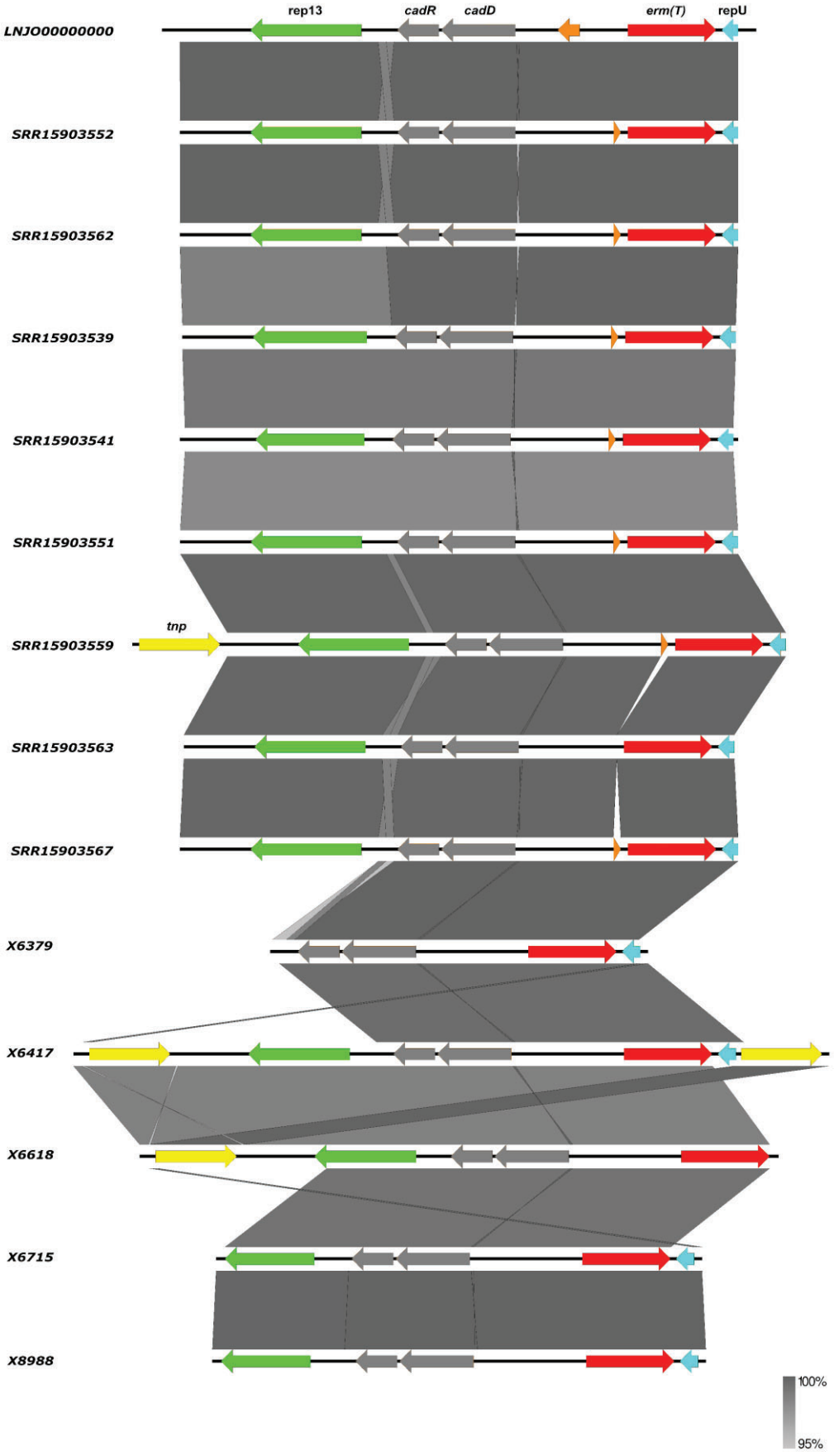

Figure 50. Genetic environment of the *ermT* gene of the five MSSA-CC398 strains in healthy humans. Shown in the figure are *ermT* gene located in the same contigs and frames with their corresponding mobile genetic elements. The percentage of identity and scale bar legends are presented on the right side of the image.

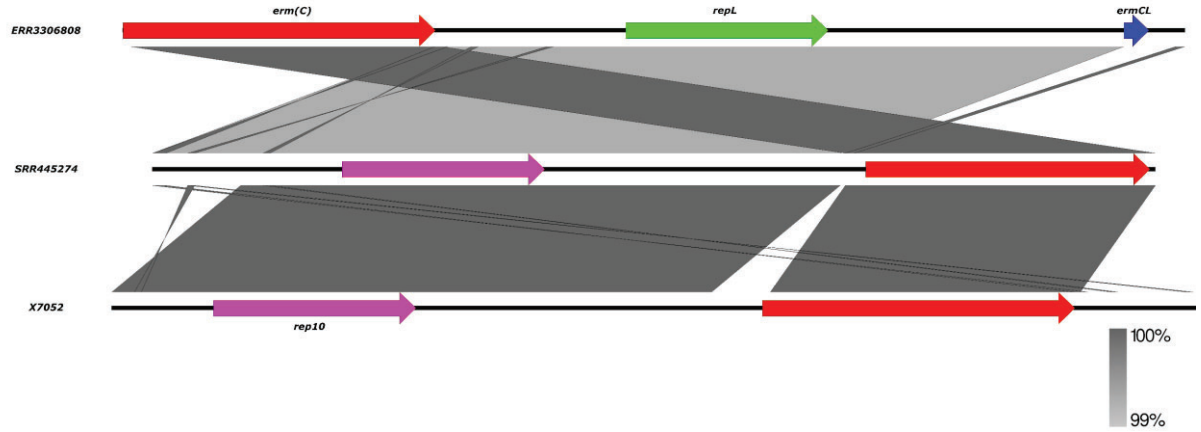

Figure 51. Genetic environment of the *ermC* gene of one MSSA-CC398 (X7052).
Shown in the figure are *ermC* gene located in the same contigs and frames with their corresponding plasmid replicon. The percentage of identity and scale bar legends are presented on the right side of the image.

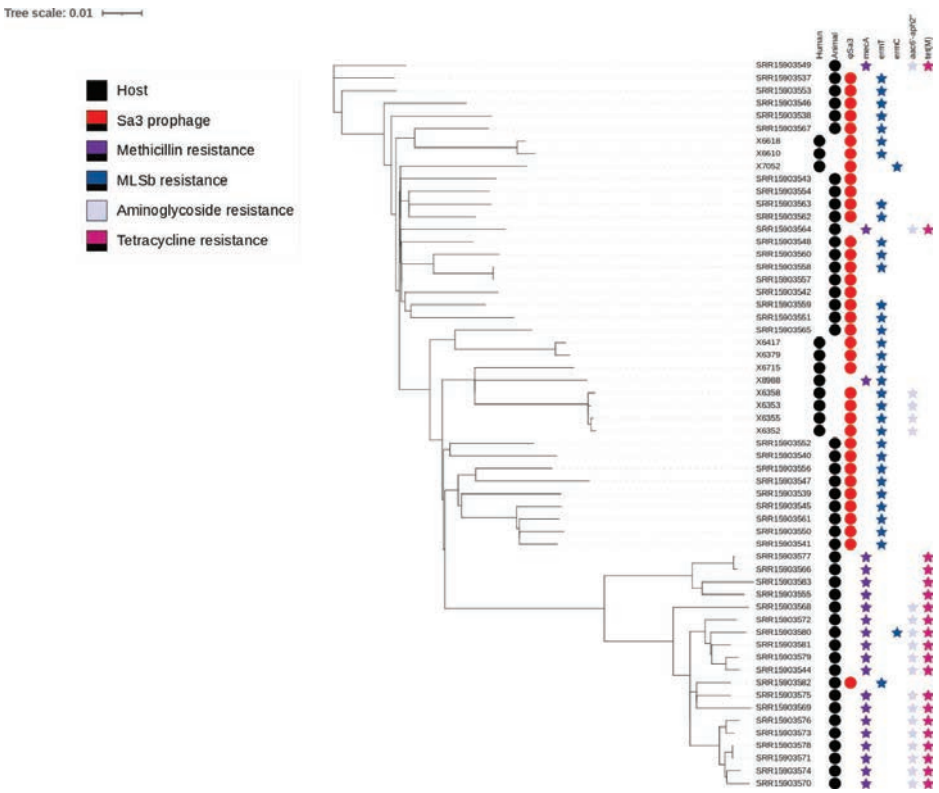

Figure 52. Phylogenetic tree based on core genome SNP analysis of seven MSSA-CC398 strains from healthy humans with 51 publicly available MSSA-CC398 and MRSA-CC398 genomes. Colors (in circles) of the hosts and φSa3 prophage, while those in stars are the AMR genes.

3.2.8 Dogs and Dog owners

Upon WGS, very few SNPs difference (<15) were detected among *S. aureus* strains from human carriers within the same household (Nº 10 and 21) **(Figure 53),** and these all shared the same repertoire of AMR genes, IEC types, virulence genes and plasmid replicons **(Table 77; Figure 53)**. Concerning the MSSA-CC398 strains, one strain from a single person without a co-carrier in their household (Nº 17) had >250 SNP differences with those from another household (Nº 21) with two human MSSA-CC398 carriers **(Figure 53).** The major difference between these MSSA-CC398 strains was the absence of the *blaZ, aac6'-aph2"*, and *fnbA* genes in the strain from household number 17 **(Figure 53).** Concerning the two *S. pseudintermedius*, each from a dog and human from the same household (Nº 10), they were both ST1115 NS identical (zero SNP differences) and shared identical virulence genes **(Table 77).**

Clonally related *S. aureus* or *S. pseudintermedius* strains were found in humans or dogs among 11.1% of households (*n* = 3). Two of the 16 households (household Nºs 11 and 21) positive for nasal *S. aureus* had human carriers with similar clonal complexes (CCs), *spa*-types and IEC types **(Table 77).** In one of these households (Nº 11), MSSA-CC30-*spa*-type t1070 strains (*scn*-negative) were identified in two humans, however, a different lineage, MSSA-CC8 of the *spa*-type t121 (IEC type-D), was identified in their dog **(Table 77).** In the second household (Nº 21), two humans carried MSSA-CC398 strains of different *spa*-types (t1451 and t571), although the dog was not *S. aureus* carrier. Moreover, in another household (Nº 10), a dog and a human were carriers of the same genetic lineage of *S. pseudintermedius* (MSSP-ST1115); in this household, the human also carried MSSA-CC97-t267 and a dog MSSA-t2013-CC15 **(Table 77).**

The household in our study with both human and dog *S. pseudintermedius* carriers strongly suggested intrahousehold transmission, as the strains had no SNP (zero) difference and were confirmed as clones by our genomic analyses. To our knowledge, this lineage (ST1115) has not been reported so far for MSSP from dogs.

Table 77. Genetic lineages, virulence and AMR genes of CoPS strains among households with both dog and human carriers

Household ID/ population[a]	**Strain** ID/ **host** ID[a]	**AMR**	**AMR genes**	**Virulence genes**	**IEC**	**Plasmid replicons**	***spa*-types/ ST/CC**
S. aureus							
11/2H&2Ds	X6019/H26	PEN	*blaZ*	*aur, cap8A-J, clfA, clfB, coa, cbp, fnbA, fnbB, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, lukF-PV, vWbp, seg, sei, sem, sen, seo, seu*	Negative	*rep5, rep16, rep19*	t1070/ST30/CC30
	X6040/H27	PEN	*blaZ*	*aur, cap8A-J, clfA, clfB, coa, cbp, fnbA, fnbB, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, lukF-PV, vWbp, seg, sei, sem, sen, seo, seu*	Negative	*rep5, rep16, rep19*	t1070/ST30/CC30
	X6036/D29	PEN-CIP	*blaZ, grlA* (S80F)	*aur, cap8A-G, cap8L-P, clfA, clfB, coa, cbp, fnbA, fnbB, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, lukF-PV, vWbp*	D	*rep7, rep20*	t121/ST8/CC8
10/2H&2Ds	X6061/H23	Susceptible	NT	*aur, splA, splB, splE, hlgA, hlgB, hlgC, lukD-PV, lukE-PV*	E	None	t267/ST97/CC97
	X6065/D24	PEN	*blaZ*	*aur, cap8A-P, clfA, clfB, coa, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, lukF-PV, vWbp*	C	*rep5, rep16*	t2013/ST15/CC15
21/2H&1Ds	X6352/H57	PEN-ERY-CLI^{Ind}-GEN-TOB	*blaZ, ermT, aac6'-aph2''*	*aur, cap8A-G, cap8L-P, clfA, clfB, coa, cbp, fnbA, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, vWbp*	C	*rep13*	t1451/ST398/CC398
	X6355/H57	PEN-ERY-CLI^{Ind}-GEN-TOB	*blaZ, ermT, aac6'-aph2''*	*aur, cap8A-G, cap8L-P, clfA, clfB, coa, cbp, fnbA, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, vWbp*	C	*rep13*	t571/ST398/CC398
	X6353/H58	PEN-ERY-CLI^{Ind}-GEN-TOB	*blaZ, ermT, aac6'-aph2''*	*aur, cap8A-G, cap8L-P, clfA, clfB, coa, cbp, fnbA, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, vWbp*	C	*rep13*	t1451/ST398/CC398
	X6358/H58	PEN-ERY-CLI^{Ind}-GEN-TOB	*blaZ, ermT, aac6'-aph2''*	*aur, cap8A-G, cap8L-P, clfA, clfB, coa, cbp, fnbA, hlB, hlD, icaA-D, icaR, isdA-G, hlgA, hlgB, hlgC, vWbp*	C	*rep13*	t571/ST398/CC398
S.pseudintermedius							
10/2H&2Ds	X6050/D25	Susceptible	NT	*lukS/F-I, siet, sient, clpP, hlgB,*	Negative	*rep7*	ST1115
	X6059/H23	Susceptible	NT	*lukS/F-I, siet, sient, clpP, hlgB,*	Negative	*rep7*	ST1115

[a]H, humans; D, dogs; CIP: ciprofloxacin; ERY-CLI^{ind}: erythromycin-clindamycin inducible; GEN; gentamicin; PEN: penicillin; TOB: tobramycin; NT: Not tested.

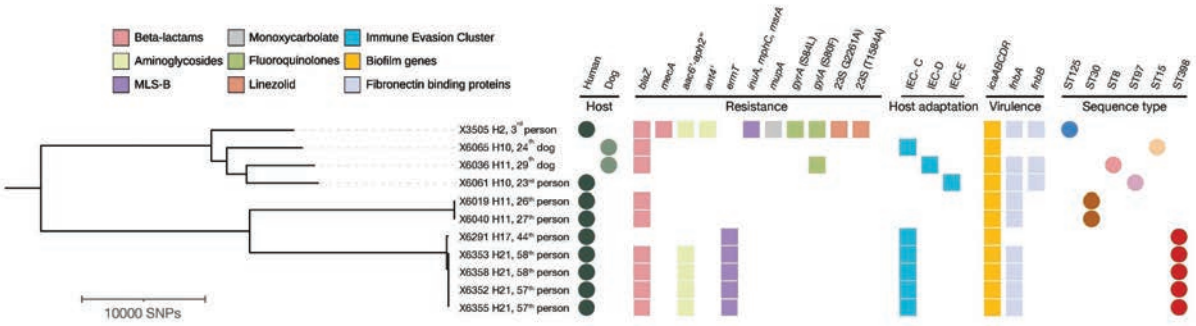

Figure 53. Phylogenetic tree based on core genome SNP analysis of 11 MRSA, MSSA-CC398, and other MSSA strains from households with ≥2 humans and/or dogs' carriers. The presence of antimicrobial resistance genes, immune evasion cluster (IEC), *icaABCDR* operon, fibronectin binding proteins, host type and sequence types (ST) are indicated. The filled circles indicate hosts and sequence types while the filled and squares indicate the confirmed antimicrobial resistance, host adaptation markers and virulence determinants extracted from MLST 2.0, ResFinder, PointFinder and VirulenceFinder. Visuals were obtained using iTOL v6.6. MLS-B, Macrolide-Lincosamide-Streptogramin-B. H, Household.

3.2.9 Mobilome-bound antimicrobial resistance in coagulase-negative staphylococci

Most of the CoNS strains from healthy pigs and pig farmers in this study presented an MDR phenotype to six or more classes of antibiotics **(Table 78).** Concerning genes that encode MLSb resistance, particularly the erythromycin-clindamycin-constitutive, the *ermC* gene was localized in three different *rep* plasmids (*rep10, repUS12, rep24c*) in eight of the 11 CoNS strains **(Table 78),** but predominantly by repUS12. The *repUS12* carrying *ermC* is almost identical (99.8%) with two small plasmids (7.1kb and 4.1kb) in an *S. aureus* pMSA16 (GenBank accession number: JQ246438.1) and *S. saprophyticus* plasmid pSES22 (GenBank accession number: AM159501.1). The *ermB* and *erm45* genes were detected in only one *S. scuiri* strain from a pig (X4892), of which the *ermB* gene was located in two plasmids *repUS76* and *rep16*. Moreover, it is important to remark on the detection of the unusual *ermT* gene in two staphylococcal species: *S. borealis* (carried by plasmid *repUS18*) and *S. hyicus* (with no associated plasmid). Usually, the *ermB, ermT,* and *erm45* genes are not common for MLSb resistance in CoNS. On the other hand, clindamycin resistance genes such as *vgaA(LC), vga(E),* and *vga(A)* detected in ten strains were not associated with any mobile genetic element.

Tetracycline resistance was found in all the pig and pig farmer strains but mediated by different combinations of genes. In this regard, *tet*(K), *tet*(L), *tet*(M), and *tet*(45) were found in 6, 10, 7, and 8 pigs/pig farmer strains, respectively. Also, the *tet*(L) gene was found in one *S. epidermidis* (X6293) strain from a dog-owner. It is important to mention that the *tet*(L) gene was located in plasmid *rep22* in all the pig and pig farmer strains, except in an *S. hyicus* strain (X5069) from a pig. Moreover, all the *tet*(M) and *tet*(45) carrying strains were not associated with any mobile genetic element **(Table 78).**

The *tet*(K) gene in most of the CoNS strains was located in *rep7a,* while only in one strain (X6049b) was located in plasmid rep*20,* three others from pigs were not associated with this plasmid replicon **(Table 77).** Concerning *tet*(M) gene, it was previously found to be localized in various types of transposons in MRSA-CC398 stains. It is important to highlight that all the plasmid-bound *tet*(L) gene were linked with the *dfrK* gene in similar plasmid repUS12. However, Tn559 bound *dfrK* in an *S. hyicus* strain from a pig (X5069) which had *tet*(L) not bound to any plasmid.

Aside from these plasmid-bound AMR genes, other genes that mediate resistance to aminoglycosides (such as *ant4'* and *bleO*) were located in plasmid *repUS12,* clindamycin (*eg., lnuA*) in *rep22* and sulfamethoxazole-trimethoprim (*e.g., dfrK*) in *repUS12* and *rep22.* In some instances, these AMR genes were not associated with any plasmid.

Aside from these mobilome-bound AMR genes, the aminoglycoside and MLSb resistance genes *ant9'* and *ermA* were also carried by Tn*554* in an *S. epidermidis* strain from a dog-owner (X3617) **(Table 78).** In this study, only *fexA* which was carried by Tn*554* and *Tn558* was identified in four pigs' strains **(Table 78).** In this regard, two *cfr*-carrying *S. epidermidis* and *S. saprophyticus* strains from a pig farmer and a pig previously identified were characterized. The *cfr* gene in *S. saprophyticus* strain was located in a plasmid *rep15*, while in *S. epidermidis* was not associated with any plasmid but was flanked by ISSau9 **(Table 78).**

3.2.10 Antimicrobial resistance mediated by chromosomal point mutations

As high as 46.2% (n=12) of CoNS strains carried one or more mechanisms of ciprofloxacin resistance mediated by DNA topoisomerase IV point mutations at GyrA (S84L) and DNA gyrase at GrlA (S80F). Strikingly was the detection of 21 unknown mutations on the GyrA on one *S. simulans* strain from a healthy pig (X5777) **(Table 78)**. A major difference in the ciprofloxacin resistance rate was observed between the strains from the pigs and pig farmers and those of the other hosts: 7 (50%) of the CoNS strains from pigs and pig farmers expressed one or more mutations on quinolone-resistance-determining region, whereas three CoNS strains from healthy humans (*S. epidermidis* and *S. haemolyticus*) and one from a nestling stork (*S. arlettae*) exhibit this mutation.

3.2.11 Plasmid-bound biocide and metal resistance among the coagulase-negative staphylococci strains

Concerning biocide resistances, various plasmid bound-biocide resistance genes (such as *qacA* [*rep20, rep22*], *qacC* [*rep13*], *qacG* [*rep21*], *qacJ* [*rep21*]) were detected in 34.6% of the MDR-CoNS **(Table 78).** In addition, *smr* gene that encodes resistance against cationic antiseptic compounds was identified in four strains **(Table 78).** Two genes that encode for cadmium and zinc resistance (*cadD, czrC*) were identified, of which *czrC* predominated (42.3%) **(Table 78).**

Table 78. Antimicrobial resistome, metal/biocide resistance determinants in 26 multi-drug-resistant CoNS and their associated mobile genetic elements.

Strain ID	Species	Source / ID	ST/CC	SCC *mec*	Resistome (plasmid replicons)	No. of antibiotic classes with resistance	Metal/biocide resistance (plasmid replicons)	Other plasmid replicons	Chromosomal point mutations	Transposon (AMR genes)	IS (AMR genes)
X4922	*S. borealis*	HP/A-8	NT	Vc	*blaZ*, *mecA*, *ermC* (*repUS12*), *lnuB*, *lsaE*, *vgaA*(*LC*), *tet*(L) (*repUS12*), *tet*(M), *tet*(45), *dfrK* (*repUS12*), *ant4'*(*repUS12*), *ant6'*, *bleO* (*repUS12*), *fexA*	7	*czrC*	*rep5b*, *rep13*, *rep19b*, *rep39*	GyrA (E84G)	Tn*554* (*fexA*)	ISSep3 (none)
X5417	*S. borealis*	HP/B-4	NT	Vc	*mecA*, *ermA*, *ermC* (*repUS12*, *rep24c*), *vga*(*E*), *tet*(K), *tet*(L) (*repUS12*, *rep24c*), *tet*(45), *dfrK* (*repUS12*, *rep24c*), *ant4'* (*repUS12*, *rep24c*), *ant6'*, *ant9'*, *aph3'*, *bleO* (*repUS12*, *rep24c*)	6	*qacG* (*rep21*), *czrC*	None	GyrA (E84G)	None	IS256 (none),ISSep3 (none)
X5418	*S. borealis*	HP/B-5	NT	Vc	*mecA*, *ermA*, *ermC* (*repUS12*), *vga*(*E*), *dfrK* (*repUS12*), *tet*(K), *tet*(L) (*repUS12*), *ant4'* (*repUS12*), *ant6'*, *ant9'*, *aph3'*, *bleO* (*repUS12*)	6	*qacG* (*rep21*), *czrC*	*repUS24c*	GyrA (E84G)	None	IS256, ISSep3, ISSha1
X5409	*S. borealis*	HP/B-4	NT	Vc	*blaZ*, *mecA*, *ermT* (*repUS18*), *vga*(*A*)*LC*, *tet*(L), *tet*(M), *dfrK*, *aac2'-aph2"*, *ant4'*, *ant6'*, *ant9'* (*repUS18*), *aph3'*, *bleO*, *fexA*, *sat4*	8	*qacJ*, *smr*, *czrC*	*rep5e*, *rep15*, *rep19b*, *rep20*, *rep24c*, *rep39*, *repUS76*	GyrA (E84G)	Tn*558* (*fexA*)	ISsep3, ISSha1
X4944	*S. saprophyticus*	HP/A-P10	NT	Vc	*mecA*, *ermC*, *lsaB* (*rep15*), *tet*(L) (*rep22*), *tet*(M), *tet*(45), *dfrK* (*rep22*), *ant4'* (*rep22*), *str* (*rep7a*), *fexA*, *cfr* (*rep15*), *fusD*, (*rep10*)	7	*qacJ* (*rep21*), *czrC*	*rep19c*, *rep20*, *rep21*	None	Tn*554* (*fexA*)	ISSau9 (*cfr*, *lsaB*)
X5435	*S. saprophyticus*	HP/ B-P6	NT	IV (2B)	*blaZ*, *mecA*, *ermC*, *lnuB*, *lsaE*, *tet*(K) (*rep7a*), *tet*(M), *dfrC*, *dfrG*, *aac6'-aph2"*, *ant4'* (*rep22*), *ant6'*, *aph3'*, *fusD*	6	*qacJ* (*rep21*), *czrC*	*rep19c*, *rep20*, *rep21*, *rep24c*	None	None	IS256
X5462	*S. saprophyticus*	HPF/ B-F1	NT	IV (2B)	*blaZ*, *mecA*, *ermC* (*rep10*), *lnuB*, *lsaE*, *vga*(*A*)*V*, *tet*(K) (*rep7a*), *tet*(M), *dfrC*, *dfrG*, *aac6'-aph2"*, *ant4'* (*rep22*), *ant6'*, *aph3'*, *str*, *fusD*	6	*qacJ* (*rep21*), *czrC*	*rep20*, *rep24c*	None	None	IS256
X5776	*S. haemolyticus*	HPF/D-F2	ST30	Vc	*blaZ*, *mecA*, *vga*(*A*)*LC* (*rep5b*), *tet*(K) (*rep7a*), *dfrG*, *aac6'-aph2"*	6	*czrC*	*rep39*, *repUS70*	GyrA (S84L)	None	None
X7059	*S. haemolyticus*	HH-/34	ST30	Vc	*blaZ*, *mecA*, *msrA*, *mphC*, *tet*(K) (*rep7a*), *dfrG*, *aac6'-aph2"*, *ant4'* (*repUS12*), *bleO* (*repUS12*)	7	*czrC*	None	GyrA (S84L)	None	IS256
*X3784	*S. haemolyticus*	NS/546	ST68	V	*blaZ*, *mecA*, *lnuA* (*rep22*), *tet*(K) (*rep7a*), *dfrG*, *aac6'-aph2"*, *ant4'* (*rep22*)	5	*qacJ*, *smr*, *czrC*	*rep20*, *repUS22*	None	None	IS256
X4892	*S. sciuri*	HP/A-P2	ST212	VIII	*mecA*, *mecA1*, *ermA* (*repUS18*), *ermC* (*rep10*), *ermB* (*repUS76*, *rep16*), *erm45*, *lnuA*, *salA*, *tet*(M) (*repUS43*), *tet*(L) (*repUS12*), *tet*(45), *dfrD* (*rep22*), *aac6'-aph2"*, *ant4'* (*repUS12*), *ant9'* (*repUS18*), *bleO* (*repUS12*), *str* (*rep7a*, *repUS18*), *fexA*	6	*qacG* (*rep21*), *czrC*	*rep19a*	None	Tn*558* (*fexA*, *salA*) Tn*6006* (none)	None
X5485	*S. epidermidis*	HPF/B-F1	ST16/ CC5	IV (2B)	*blaZ*, *mecA*, *lsaB*, *vga*(*A*)*LC* (*rep5b*), *tet*(K) (*rep7a*), *tet*(L) (*rep22*), *tet*(45), *dfrK* (*rep22*), *ant4'* (*rep22*), *str* (*rep7a*), *fexA*, *cfr*, *fosB*	9	None	None	GyrA (S80F), GyrL (E84G)	None	ISSau9 (*cfr*, *lsaB*), ISSau4 (none)

Table 78. Continuation

Strain ID	Species	Source / ID	ST/CC	SCC *mec*	Resistome (plasmid replicons)	No. of antibiotic classes with resistance	Metal/biocide resistance (plasmid replicons)	Other plasmid replicons	Chromosomal point mutations	Transposon (AMR genes)	IS (AMR genes)
X6590	*S. epidermidis*	HH/19	ST89/ CC2	None	*blaZ, ermC* (*repUS12*), *fosB, mupA*	4	None	*rep7a*	None	None	None
X6628a	*S. epidermidis*	HH/22	ST210	None	*blaZ, ermC, lnuA, dfrC, dfrG, tet*(K) (*rep7a*), *ant4'* (*rep22*), *fosB*	6	None	*rep13, rep20, repUS22*	GyrA (S80F)	None	ISSau4
X9066	*S. epidermidis*	HH/46	ST173	V	*blaZ, mecA, vgaA (rep5), lnuA, vga*(*A*)*LC* (*rep5*), *tet*(K) (*rep7a*), *dfrC, aac6'-aph2", ant4'* (*rep22, rep20*), *fosB*	7	*qacA* (*rep22, rep20*)	None	GyrA (S80F)	None	ISSau4
X3617	*S. epidermidis*	HDO/19	ST59/ CC2	None	*blaZ, ermA, ant9', fosB, mupA*	5	None	*rep7a*	None	Tn*554* (*ant9', ermA*)	None
X6049b	*S. epidermidis*	HDO/26	ST35/ CC5	V	*blaZ, mecA msrA, mphC, tet*(K) (*rep20*), *fosB, fusB, mupA*	7	None	None	L3 (I188V, G218V, N219I, L220D) and L4 (N158S)	none	None
X6293	*S. epidermidis*	HDO/44	ST297	II	*blaZ, mecA, ermC, tet*(L), *tet*(45), *dfrC, ant4', bleO, fosB*	6	*qacC, qacJ, smr*	repUS22	None	None	ISSep3
X4430	*S. epidermidis*	NS/487	ST595	None	*blaZ* (*rep20, repUS70*), *msrA (rep20, repUS70), fosB, mupA*	4	*qacC* (*rep13*), *smr*	*rep7a, rep39, rep40*	None	None	None
X4638	*S. lentus*	NS/507	NTs	Hybrid VII	*blaZ, mecA, mecC, mphC, tet*(K) (*rep7a*), *fosD*	5	None	None	None	None	None
X3574	*S. hominis*	HD/8	ST33	VI (4B)	*blaZ, mecA, msrA, mphC, ant4'* (*rep22*), *bleO* (*rep22*), *fusC*	4	*qacA*	*rep20, rep21, rep39*	None	None	ISSau4
X4592	*S. arlettae*	NS/535	NT	None	bla_{ARL}, *lnuA, mphC, msrA, tet*(K) (*rep7a*), *aph2'*	5	*qacG* (*rep21*)	*rep16*	GyrA (S84L)	None	None
X4956	*S. pastueri*	HP/A-P8	NT	Vc	*blaZ, mecA, ermC* (*rep10*), *vga*(*A*)*LC, tet*(K), *tet*(L) (*rep22*), *tet*(M), *tet*(45), *dfrK* (*rep22*), *ant4'* (*rep22*), *str* (*rep7a*)	5	*czrC*	*rep21, rep39*	None	None	None
X5069	*S. hyicus*	HP/C-P1	NT	None	*blaZ, ermT, lnuB, lsaE, tet*(L), *tet*(45), *dfrK, ant6'*	6	None	None	None	Tn*559* (*dfrK*)	None
X5447	*S. hyicus*	HP/B-P9	NT	None	*blaZ, ermT, lnuB, lsaE, tet*(L), *tet*(45), *aac6'-aph2", ant4', ant6'*	6	None	*rep22*	None	None	IS256
X5777	*S. simulans*	HP/C-P2	NT	None	*blaZ, ermA, ant9'*	4	None	*rep7a, rep21*	GyrA (E214T, V248E, S63P, Q6E, Y366R, A367T, S173A, C377H, S376V, I368V, L191V, A169V, K364R, S16N, A32S, K200H, L188M, N153T, S158E, S245A, L4Y)	Tn*559* (*ermA, ant9'*)	None

Unusual AMR determinants in bold; NS: Nestling stork; HP: Healthy pig; Healthy pig farmer: HPF; Healthy dog: HD; Healthy dog owner: HDO; Healthy human without animal contact: HH

The pigs (10 per farm) are named P1-P10 in each farm (A-D). In the case of humans working on the farm, they are designated as F1, F2, F3 and the farm (A-D)
*All strains were of nasal origin, except *S. haemolyticus* X3784 of nestling stork which was from tracheal sample.

3.2.12 Genetic environment of the unusual antimicrobial resistance gene *ermT* in CoNS strains

The *in-silico* analysis of the *ermT* sequences of three CoNS strains (*S. borealis* and *S. hyicus*) from healthy pigs revealed striking differences in their genetic environment **(Figure 54).** The *ermT* gene is in the opposite direction with a*nt9'* and both are located in plasmid *repUS18* in *S. borealis* strain. However, the *ermT* gene in the other two *S. hyicus* strains (X5447 and X5069) is not associated with any plasmid, perhaps it is chromosomally located **(Figure 54).**

The *in-silico* analysis of *S. lentus* strain (X4638) showed that it carried a hybrid SCC*mec*-*mecC*, which is over 95% similar to an *S. sciuri* strain **(Figure 55)**. Specifically, the hybrid SCC*mec* consist of a class E *mec* complex (*mecI-mecR1-mecC1-blaZ*) located immediately downstream of a SCC*mec* type VII element. Most of the cassette comprise of *mecA*/*mecI*/*mecR2* and *cadD*/*cadA*/*cadC* **(Figure 55).** The *mecC* gene of the *S. lentus* (X4638) was quite different from conserved SCC*mec* type XI, a classical type that was first *in S. aureus* LGA251 (accession number FR821779)**.**

Linezolid resistance mechanisms mediated by *cfr* gene which was located in a 41.5kb plasmid (pURX4944) in an *S. saprophyticus* strain from a pig **(Figure 56)**. The *cfr* gene was upstream of *lsaB* of the *S. saprophyticus* **(Figure 57)** and 96% identical to the plasmid of a clinical *S. epidermidis* strain from Italy (GenBank Accession number: KR230047.1), whereas the *cfr* gene of the *S. epidermidis*-ST16 strain from a pig farmer was not associated with a plasmid **(Table 78)**.

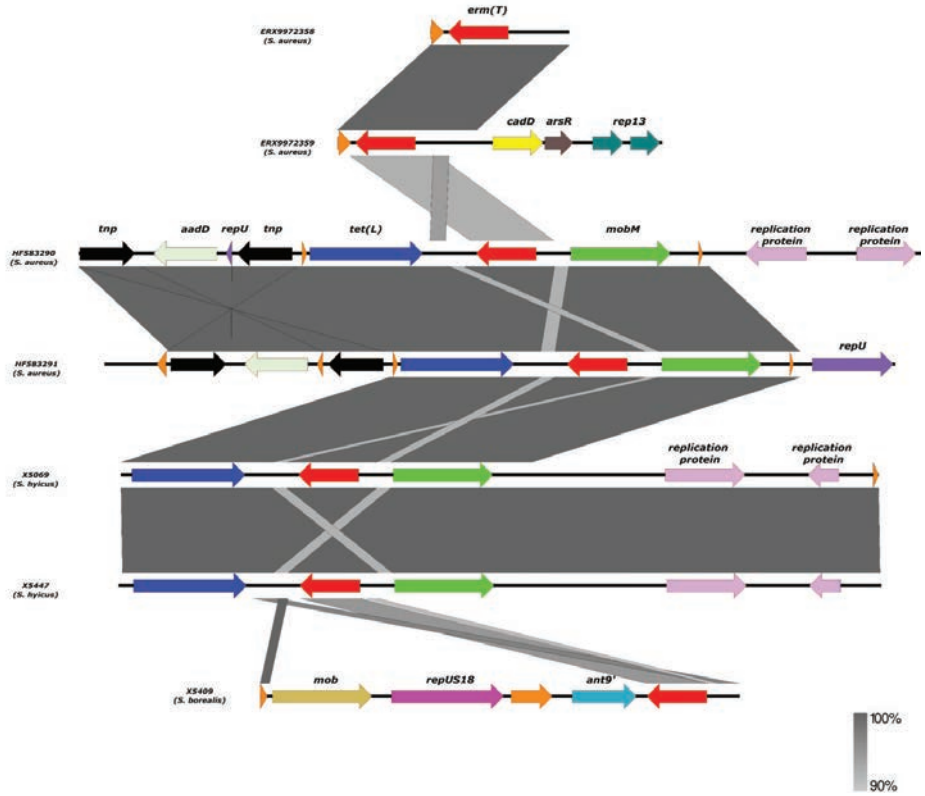

Figure 54. Genetic environment of *ermT* gene in coagulase-negative staphylococci in comparison with four reference strains.

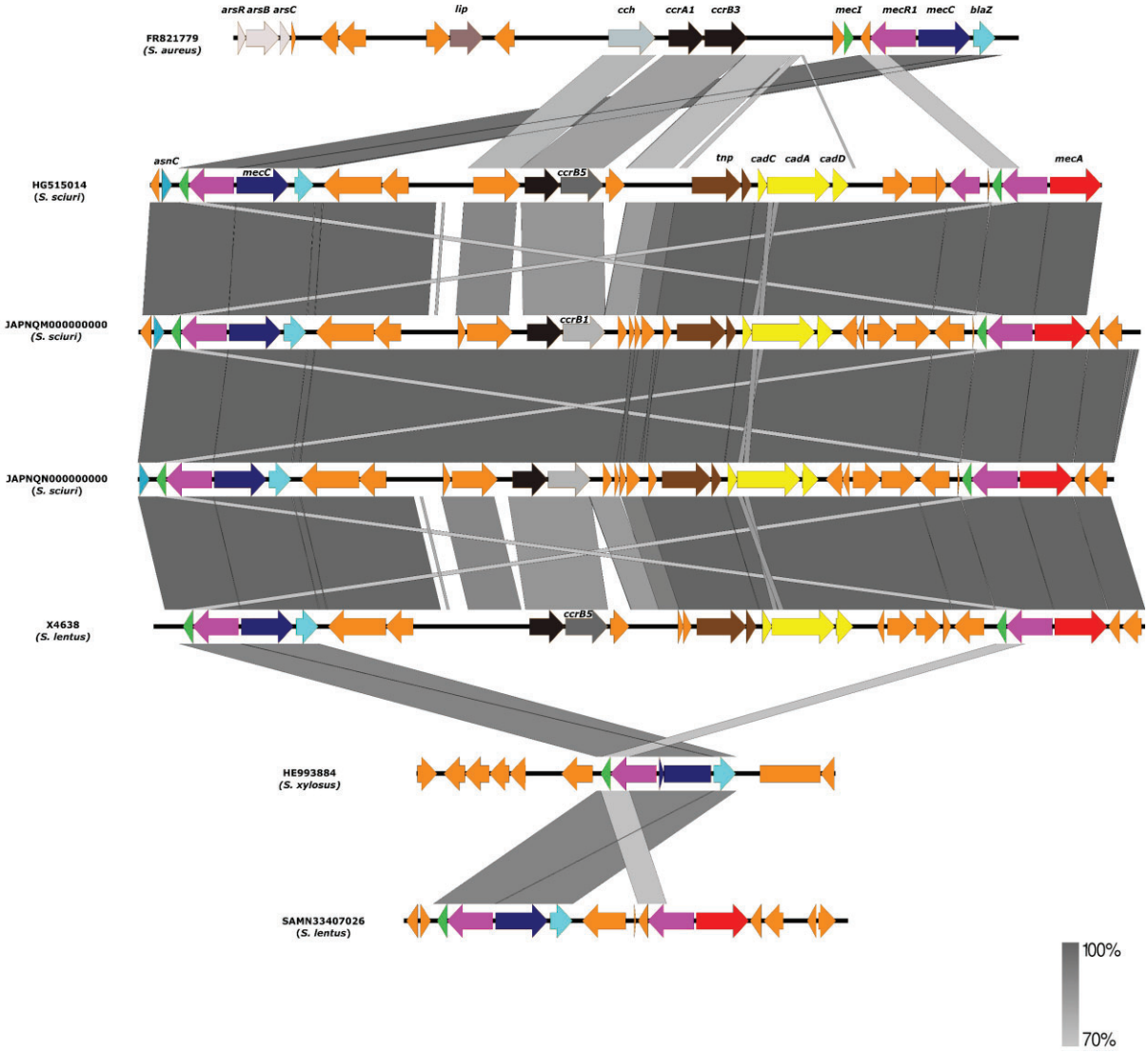

Figure 55. The environment of the *mecC* gene of *S. lentus* (X4638) in comparison with previously described *mecC*-carrying coagulase-negative staphylococci and the *S. aureus*$_{LGA251}$ strain

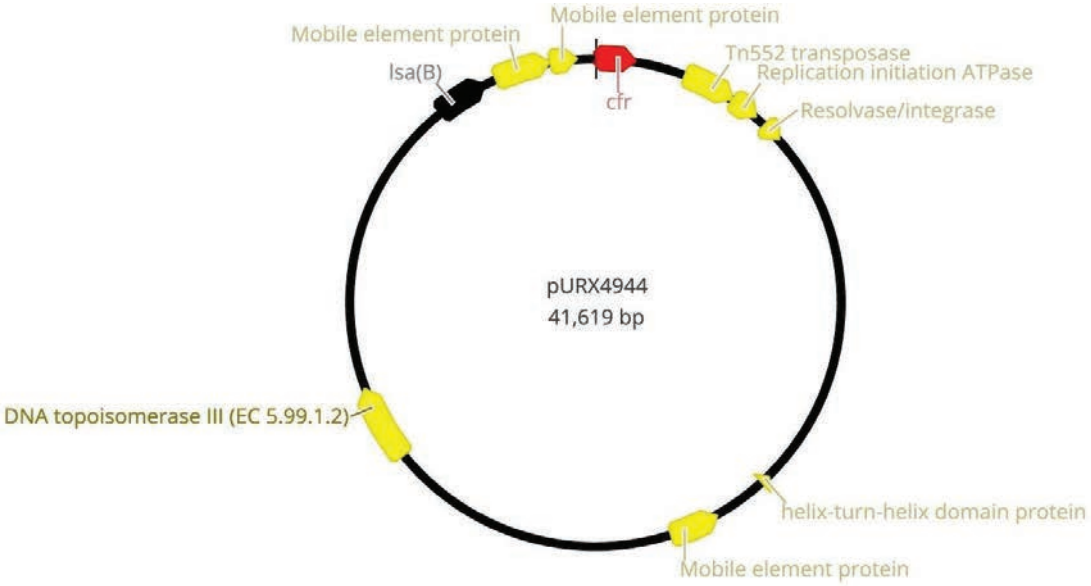

Figure 56. Circular representation of the plasmid-carrying the *cfr* gene in *S. saprophyticus.*

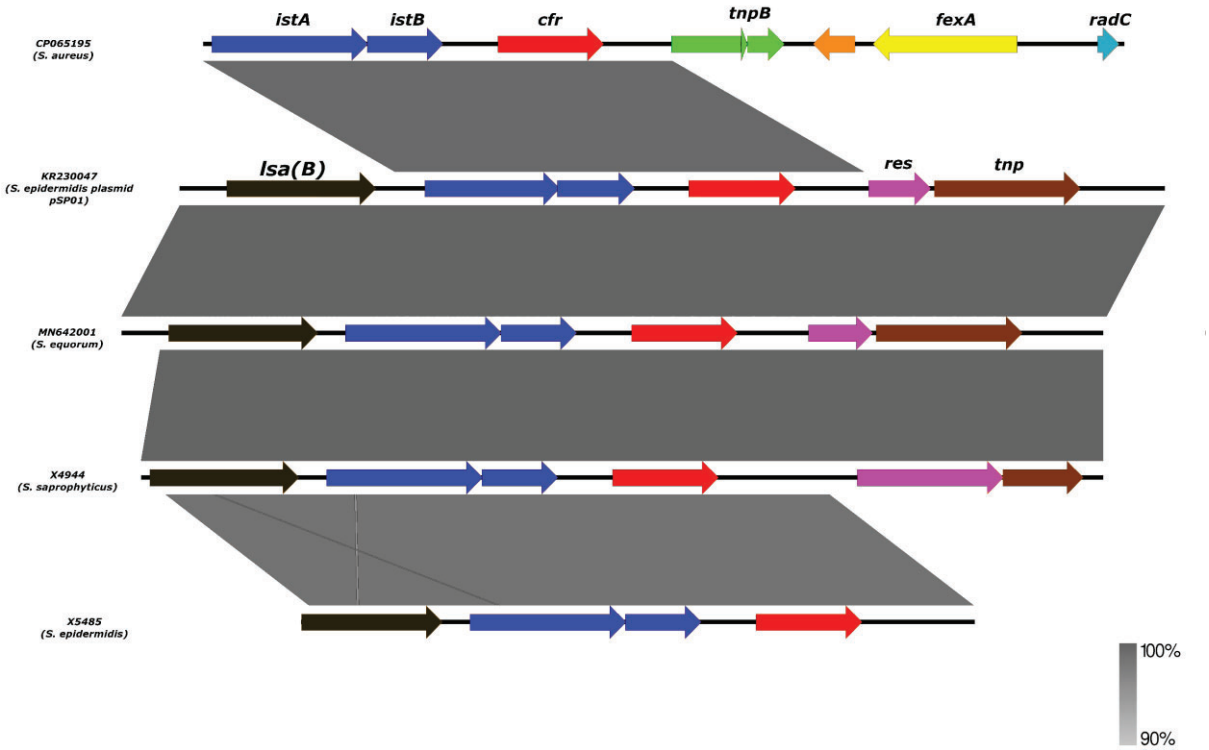

Figure 57. Environment of the *cfr* gene of *S. epidermidis* (X5485) and *S. saprophyticus* (X4944) in comparison with previously described *cfr*-carrying coagulase-negative staphylococci and *S. aureus.*

3.2.13 Virulome profile of the coagulase-negative staphylococci strains

The frequency and distribution of virulence genes among the different CoNS strains from the four hosts were investigated. About 96.1% of the MDR-CoNS strains harboured one or more of the diverse adherence, exoenzyme, haemolysin, or immune evasion genes **(Table 79).** It is important to highlight the detection of *sec*- and *sel*-carrying *S. epidermidis* strain of the lineage ST595 **(Table 79)**.

Aside from the toxins, many CoNS harboured genes such as the *capB* and *capC* (encode capsules) and *adsA, galE, wbtE, wbtP* genes that facilitate immune evasion by CoNS. Furthermore, the *icaABC* operon and its *icaR* were present in five strains (19.2%) **(Tabe 79).** Other very relevant adhesin genes such as *fnbA* and *fnbB* (encode fibronectin-binding proteins) and *cna* (encodes collagen-binding adhesin) were identified in an *S. hyicus* strain from a healthy pig (X5447) **(Table 79).**

3.2.14 CRISPR-Cas system distribution among the staphylococci

All the *S. aureus* strains carried no CRISPR-Cas system. However, complete CRISPR-Cas systems were detected in 19.2% of the CoNS strains, of which cas-1, -2 and -9 predominated in *S. borealis* (75%) **(Table 80)**. In other species, Cas 3 type I CRISPR was identified in *two S. epidermidis* strains (X6590 and X6049b) from humans. Furthermore, the *mecC*-carrying *S. lentus* strain harboured the Cas-2 type I and Cas-9 type II systems **(Table 80).**

Table 79. Virulence determinants and prophages in the 26 MDR-CoNS

Strain ID	Species	Source/ID	Intact staphylococcal phages identified (GenBank Accession Number)	Classes of virulence factors						
				Adherence	Exoenzymes	Haemolysin	Immune evasion	Mobile genetic element	Metal uptake	others
X4922	*S. borealis*	HP/A-P8	IME_SA4 (NC_029025)	*atl, ebp,*	*lip*	None	*adsA*	None	None	None
X5417	*S. borealis*	HP/B-P4	vB_SepiS-phiIPLA5 (NC_018281)	*atl, epb,*	*lip*	None	*adsA, wbtP*	None	None	*uge*
X5418	*S. borealis*	HP/B-P5	vB_SepiS-phiIPLA5 (NC_018281)	*atl, epb,*	*lip*	None	*adsA, wbtP*	None	None	*uge*
X5409	*S. borealis*	HP/B-P4	IME_SA4 (NC_029025)	*atl, epb, sdrE,*	*lip*	None	*adsA, capB, wbtP*	None	None	None
X4944	*S. saprophyticus*	HP/A-P10	47 (NC_007054)	*atl, sdrC,*	*lip, sspA*	None	None	None	None	None
X5435	*S. saprophyticus*	HP/B-P6	phiRS7 (NC_022914)	*atl, ebp*	*lip, geh*	None	*capB, galE*	None	*vctC*	None
X5462	*S. saprophyticus*	HPF/B-F1	phiRS7 (NC_022914)	*atl*	*lip, geh, sspA*	None	*capB, galE,*	None	*vctC*	None
X5776	*S. haemolyticus*	HPF/D-F2	stB12 (NC_020490)	None	*sspB, geh*	None	*adsA, capB, galE, wbtE, wbtP*	None	None	*cylR2*
X7059	*S. haemolyticus*	HH$^-$/34	stB12 (NC_020490)	*atl, ebp*	*lip*	None	None	None	None	None
*X3784	*S. haemolyticus*	NS/546	None	*atl, epb*	*lip*	None	*adsA, wbtP*	None	None	None
X4892	*S. sciuri*	HP/A-P2	None	*icaA, icaB, icaC, clpP, lgt*	*sspA*	None	None	None	*vctC*	*lisR*
X5485	*S. epidermidis*	HPF/B-F1	StB20 (NC_019915)	*icaA, icaB, icaC, icaR, sdrH, sdrG, atl, ebh, ebp*	*sspA, sspB, lip, geh*	*hlb*	None	ACME	None	None
X6590	*S. epidermidis*	HH$^-$/19	None	*atl, ebh, ebp, sdrH, geh*	*sspA, sspB, lip*	*hlb*	None	None	None	None
X6628a	*S. epidermidis*	HH$^-$/22	None	*sdrH, sdrG, atl, ebh, ebp*	*sspA, sspB, lip, geh*		None	None	None	None

Table 79. Continuation

Strain ID	Species	Source/ID	Intact staphylococcal phages identified (GenBank Accession Number)	Classes of virulence factors						
				Adherence	**Exoenzymes**	**Haemolysin**	**Immune evasion**	**Mobile genetic element**	**Metal uptake**	**others**
X9066	*S. epidermidis*	HH^-/46	None	*atl, ebp, ebh, clfA, icaA, icaB, icaC, icaR, sdrD, sdrG*	*sspB, geh, lip, sspA*	*hlb*	*capB*	ACME	None	None
X3617	*S. epidermidis*	HDO/19	None	*sdrH, sdrG, atl, ebh, ebp,*	*sspA, sspB, lip, geh*	*hlb*	None	ACME	None	None
X6049b	*S. epidermidis*	HDO/26	stB12 (NC_020490)	*icaA, icaB, icaC, icaR, sdrH, sdrG, atl, ebh, ebp, eno*	*sspA, sspB, lip, geh*	*hlb*	None	ACME	None	None
X6293	*S. epidermidis*	HDO/44	None	*icaA, icaB, icaC, icaR, sdrF, sdrG, sdrH, atl, ebh, ebp*	*sspA, sspB, lip, geh*	*hlb*	None	ACME	None	None
X4430	*S. epidermidis*	NS/487	None	*alt, ebh, epb, sdrG*	*lip, sspB, geh, esa, esaD, esaE, esaG, essB, essC, esxB, esxC, esxD*	*hlb*	None	None	None	*sec, sel*
X4638	*S. lentus*	NS/507	None	*clfB, lgt*	*sspA, ndk, lplA1*	None	*gtaB, wbtP*	None	*vctC, ctpV,*	*lisR*
X3574	*S. hominis*	HD/8	stB_27 (NC_019914)	*atl, ebp*	*lip*	None	*capB*	None	None	None
X4592	*S. arlettae*	NS/535	vB_SauS_phi2 (NC_028862)	None	*lip, sspA*	None	*gale, wbtP*	None	None	None
X4956	*S. pastueri*	HP/A-P8	vB_SepiS-phiIPLA7 (NC_018284)	*atl, ebh, ebp, icaA, icaB, icaC*	*lip, sspB*	None	*capB, manA*	None	None	None
X5069	*S. hyicus*	HP/C-P1	EW (NC_007056)	*clfA, clfB, cna, fnbA, fnbB*	*sspB, hysA, geh, esaB, essB, essC, esxA*	*hlb*	*capB, capC*	None	None	*set26, cvtC, lisR, lgt*
X5447	*S. hyicus*	HP/B-P9	None	*clfA, cna, fnbA, fnbB, lgt*	*sspB, hysA, geh, esaB, essB, essC, esxA*	*hlb*	*adsA,capB, capC*	None	*vctC*	*set15, lisR*
X5777	*S. simulans*	HPC-P2	37 (NC_007055)	*atl, ebp*	None	None	*capB*	None	None	None

uge: antiphagocytic capsule *; lisR*: signal transduction system; Unusual virulence genes in bold

NS: Nestling stork; HP: Healthy pig; Healthy pig farmer: HPF; Healthy dog: HD; Healthy dog owner: HDO; Healthy human without animal contact: HH^-

The pigs (10 per farm) are named P1-P10 in each farm (A-D). In the case of humans working on the farm, they are designated as F1, F2, F3 and the farm (A-D)

*All strains were of nasal origin, except *S. haemolyticus* X3784 of nestling stork which was from tracheal sample.

Table 80. CRISPR-Cas system distribution among the CoNS

Strain ID	Species	Source/ID	Nº of Standalone CRISPR/ Nº of with Cas protein	CRISPR-Cas class	Cas type (orientation)	Total Number of Spacers/ Spacers with Cas
X4922	*S. borealis*	HP/A-P8	14/0	None	None	14
X5417	*S. borealis*	HP/B-P4	12/3	Class 2 type II	Cas1-type II (+); Cas2-type-I, II, III (+); Cas9-type II (+)	18/3
X5418	*S. borealis*	HP/B-P5	13/3	Class 2 type II	Cas1-type II (-); Cas2-type-I, II, III (-); Cas9-type II (-)	19/3
X5409	*S. borealis*	HP/B-P4	18/3	Class 2 type II	Cas1-type II (-); Cas2-type-I, II, III (-); Cas9-type II (-)	26/ 3
X4944	*S. saprophyticus*	HP/A-P10	1/0	None	None	1
X5435	*S. saprophyticus*	HP/B-P6	2/0	None	None	2
X5462	*S. saprophyticus*	HPF/B-F1	1/0	None	None	1
X5776	*S. haemolyticus*	HPFD-F2	2/2	None	None	2
X7059	*S. haemolyticus*	HH-/34	3/0	None	None	3
*X3784	*S. haemolyticus*	NS/546	3/0	None	None	3
X4892	*S. sciuri*	HP/A-P2	6/0	None	None	6
X5485	*S. epidermidis*	HPF/B-F1	4/0	None	None	4
X6590	*S. epidermidis*	HH-/19	5/1	None	Cas3-type I (+)	5/1
X6628a	*S. epidermidis*	HH-/22	1/0	None	None	1
X9066	*S. epidermidis*	HH-/46	4/0	None	None	4
X3617	*S. epidermidis*	HDO/19	1/0	None	None	1
X6049b	*S. epidermidis*	HDO/26	4/1	None	Cas3-type I (-)	1
X6293	*S. epidermidis*	HDO/44	2/0	None	None	2
X4430	*S. epidermidis*	NS/487	4/0	None	None	4
X4638	*S. lentus*	NS/507	8/2	Class 2 type II	Cas2-type I, II, III (+); Cas9-type II (+)	15/3
X3574	*S. hominis*	HD/8	3/0	None	None	3
X4592	*S. arlettae*	NS/535	None	None	None	None
X4956	*S. pastueri*	HP/A-P8	1/0	None	None	1
X5069	*S. hyicus*	HP/C-P1	2/3	Class 2 type II	Cas1-type II (-); Cas2-type-I, II, III (-); Cas9-type II (-)	26/ 3
X5447	*S. hyicus*	HP/B-P9	2/0	None	None	20
X5777	*S. simulans*	HPC-P2	1/0	None	None	1

NS: Nestling stork; HP: Healthy pig; Healthy pig farmer : HPF; Healthy dog: HD; Healthy dog owner: HDO; Healthy human without animal contact: HH
The pigs (10 per farm) are named P1-P10 in each farm (A-D). In the case of humans working on the farm, they are designated as F1, F2, F3 and the farm (A-D)
*All strains were of nasal origin, except *S. haemolyticus* X3784 of nestling stork which was from tracheal sample

3.2.15 Mobilomes of antimicrobial resistance genes and metal resistance determinants in the linezolid-resistant enterococci

The genomes of the seven LZD^R *E. faecalis* strains had sizes in the range of 2.8-3.1 Mb and contigs range from 100-164 **(Table 81).** The LZD^R *E. faecium* strain had a genome size of 2.7 Mb and 136 contigs, whereas the LZD^R *E. casseliflavus* strain had a genome size of 3.6 Mb and 347 contigs **(Table 81).**

The *in silico* analysis of the *optrA* sequences revealed that two LRE strains harboured the wild-type *optrA*, while the remaining five carried three *optrA* variants (types 5, 7 and 15) **(Table 81).** The *optrA* type 5 variant was only found in one *E. faecalis*-ST59 strain from a pig. The *optrA* type 7 variant was detected in two *E. faecalis* strains from a pig and a dog (ST330 and ST585), respectively. Whereas the *optrA* variant 15 was identified in an *E. faecalis*-ST330 strains from a pig. Other *E. faecalis* strains from a pig-farmer (ST330) and a pig (ST474) carried the wild type *optrA* variant (**Table 81**). In addition, the linezolid resistant-*E. faecium* strain carried a *poxtA* type 1 **(Table 81).**

All the enterococci carried at least one *rep* gene (1–5 *rep* genes). The three *optrA*-positive *E. faecalis*-ST330 strains carried different numbers of plasmid replicons **(Table 81).** The *repUS52* in X5386 was found co-located with the aminoglycoside resistance *ant4'* gene. Three resistance genes (*tet*(L), *tet*(M), *a*nd *cat*) were co-located on *rep*US43 of all *E. faecalis* strains from the pigs and pig farmer **(Table 81).** Moreover, the *agg* virulence gene was also co-located on the *rep*9a contig of *E. faecalis*-ST330 (X5463) and *E. faecalis*-ST32 (X5445). Three replicons were identified in the *E. faecium* strain (*rep*29, *rep*1, *rep*US15) and none was co-located with any other AMR genes. Moreover, five replicons (*rep*14b, *rep*US41, *rep*US1, *rep*1, and *rep*40) were identified in the *optrA*-positive *E. casseliflavus* X4962 strain, of which *rep*US40 was associated with the *optrA*-*fexA* genes **(Figure 58)** and was 99.83% identical with plasmid pE3954 of *E. faecalis* (GenBank accession no: KP399637).

Two different transposons were identified, *viz*: Tn*6260* and Tn*554* associated with *lnuG* and *fexA* genes of *E. faecalis*-ST330 and -ST59 strains, respectively **(Table 81).** In this regard, only one *E. faecalis* strain carried Tn*554* linked with *fexA* **(Figure 58)**. However, the Tn*6260* linked with *lnuG* was identified in three of the *optrA*-positive *E. faecalis* strains **(Table 81).** Different insertion sequences were detected among our LZD^R strains: *E. faecalis*-ST59 (ISS1N and ISEnfa4), *E. faecalis*-ST330 (ISEnfa1), *E. faecium* (ISSsu5) and *E. casseliflavus* (ISEnfa1) **(Table 81)**. The *optrA* gene was chromosomally located in all our *E. faecalis* strains. Nevertheless, the *optrA* gene of *E. casseliflavus* strain X4962 was located in a plasmid (37.9kb,

pURX4962) **(Figure 59)**, that showed 99.98% similarity with the one of an *E. faecalis* strain from China (GenBank Accession number: KP399637.1). All our LZD^R *E. faecalis* and *E. faecium* strains carried at least one prophage, of which *E. faecalis* -ST330 (X4957) from a pig carried the highest variety (n=5), *viz*: phiEf11, phiFL4A, phiFL2A, EFC_1 and LP_101 **(Table 81)**.

Aside from the AMR genes, chromosomal point mutations leading to ciprofloxacin resistance were detected in both ParC (S80I) and GyrA (E87G) of *E. faecalis*-ST330 from pigs of farms B and D. In the dog *E. faecalis*-ST475 strain, only mutation in ParC (S80I) was detected. In addition, 17 different point mutations on the penicillin binding protein 5 (S27G, A68T, A216S, T172A, V24A, 885D, K144Q, A499T, L177I, N496K, G66E, E100Q, D204G, P667S, E525D, T324A, R34Q) were detected in the *E. faecium* strain from stork nestling (*poxt*A-positive) **(Table 80).**

Chromosomal point mutations putatively conferring resistance to linezolid (in 23S rRNA and ribosomal proteins L3/L4/L22) were not detected in all of our strains. However, *E. faecalis*-ST330 from healthy pigs and pig farmers presented ParC (S80I) and GyrA (E87G) point mutations associated to fluoroquinolone resistance **(Table 81).**

All strains except *E. casseliflavus* X4962 carried ≥2 metal resistance genes (MRGs), of which *E. faecium*-ST1739 carried most of them (*arsA, copA, fief, ziaA, znuA, zosA, zupT, zur)* **(Table 81).** Moreover, *E. faecalis* X5386 carried only two of the MRGs (*cutC* and *znuA*). Other strains carried three MRGs, *cutC, tcrB,* and *znuA* **(Table 81)**.

3.2.16 Virulence determinants in the linezolid-resistant enterococci

Many virulence genes that have been associated with surface adherence, biofilm formation and cytolysis were detected in the *optrA*-carrying *E. faecalis* strains, most frequently being the *ebpA, tpx, elrA, hylA, srtA, gelE, fsrB, ace, cOB1, cCF10, dad, agg, camE, efaAfs, hylB,* and *cylA* genes. In the *E. faecium* X3877 strain, only *acm* and *efaAfm* genes were detected. However, none of these genes was found in the *E. casseliflavus* X4962 strain **(Table 81).** Regarding virulence factors of the *poxtA*-positive *E. faecium* X3877 strain, it carried the *acm* and *efaA* genes that encode collagen-binding **(Table 81).**

Table 81. Genomic characteristics of 9 linezolid-resistant *Enterococcus* spp investigated in this study*

Strain ID	Source/ ID	ST/ CC[a]	No. contigs/ Genome size (Mb)	LZD[R] genes		AMR genes	Metal resistance	Point mutation	Pro-phage	Tn	Plasmid	IS	Virulence
				Chromo -some	Plasmid								
E. casseli-flavus													
X4962	Pig/Farm A/ P3	NT	247/ 36.8	*cfrD*	*optrA* [wild type] (*rep*US40)	*fexA, lnuB, lsaE, aph3', dfrG, vanC3XY*	none	none	none	none	*rep*1, *rep*14b, *rep*US1, *rep*US41	ISEnfa1	none
E. faecium													
X3877	Stork/ Landfill/	ST-1739	136/2.6	*poxtA* type 1	none	*fexB*	*arsA, copA, fief, ziaA, zosA, zupT, zur, znuA*	[b]17 mutations in pbp5	vB_IME 197 BCJA1c	none	*rep*1, *rep*29, *rep*US15	ISSsu5	*acm, efaAfm*
E. faecalis													
X4957	Pig/ farm A/ P8	ST-330	164/3.1	*optrA* type 15	none	*cat, fexA, lnuG, lsaA, ermA, tet(L), tet(M), aac6'-aph2", aph3', dfrG*	*cutC, tcrB, znuA*	ParC (S80I), GyrA (E87G)	phiEf11 phiFL4A phiFL2A EFC _1 LP_101	Tn*6260* (*lnuG*)	*rep*1, *rep*9a *rep*US1, *rep*US43	ISEnfa1	*ace, agg, cad, camE, cOB1, cCF10, ebpA, ebpC, elrA, efaAfs, fsrB, gelE, hylA, hylB, srtA, tpx*
X5386	Pig/ farm B/ P1	ST-330	137/3.0	*optrA* type 7	none	*cat, fexA, lnuG, lsaA, ermA, tet(L), tet(M), aac6'-aph2", ant4', aph3', dfrG*	*cutC, znuA*	ParC (S80I), GyrA (E87G)	phiEf11 BCJA1c phiFL4A	Tn*6260* (*lnuG*)	*rep*9a, *rep*9b, *rep*9c, *rep*US43, *rep*US52	none	*ace, agg, camE, cOB1, cCF10, dad, ebpA, ebpC, elrA, efaAfs, fsrB, gelE, hylA, hylB, SrtA, tpx*
X5463	Pig-farmer/ farm B/ F2	ST-330	116/2.9	*optrA* type 7	none	*cat, fexA, lnuG, lsaA, ermA, tet(L), tet(M), aac6'-aph2", ant6', aph3', dfrG*	*cutC, tcrB, znuA*	ParC (S80I), GyrA (E87G)	phiFL4A Lj928 BCJA1c	Tn*6260* (*lnuG*)	*rep*9a, *rep*9b, *rep*US43	ISS1N	*ace, agg, cad, camE, cOB1, cCF10, dad, ebpA, ebpC, elrA, efaAfs, fsrB, gelE, hylA, hylB, srtA, tpx*

Table 81. Continuation

Strain ID	Source/ ID	ST/ CC[a]	No. contigs/ Genome size (Mb)	LZD[R] genes		AMR genes	Metal resistance	Point mutation	Pro-phage	Tn	Plasmid	IS	Virulence
				Chromo-some	Plasmid								
E. faecalis													
X5799	Pig/ Farm D/ P5	ST59	139/ 2.9	*optrA* type 5	none	*cat, fexA, lnuB, lsaA, lsaE, ermA, tet(L), tet(M), aac6'-aph2", aph3', dfrG*	*cutC, tcrB, znuA*	none	phiFL4A	Tn*554* (*fexA, optrA* type 5)	*rep*9a, *rep*9c, *rep*US43	ISS1N, ISEnfa4	*ace, agg, camE, cOB1, cCF10, cylA, dad, ebpA, ebpC, elrA, efaAfs, fsrB, gelE, hylA, hylB, srtA, tpx*
X5809	Pig/ Farm D/ P10	ST-474	100/2.8	*optrA* (wild type)	none	*cat, fexA, lnuB, lsaA, lsaE, ermA, tet(L), tet(M), aac6'-aph2", aph3', dfrG*	*cutC, tcrB, znuA*	ParC (S80I), GyrA (E87G)	phiFL3A	none	*rep*9a, *rep*9c, *rep*US43	ISS1N	*ace, agg, camE, cOB1, cCF10, cylA, dad, ebpA, ebpC, elrA, efaAfs, fsrB, gelE, hylA, hylB, srtA, tpx*
X5445	Pig/ farm B/ P8	ST32	122/2.9	*optrA* (wild type)	none	*cat, fexA, lnuG, lsaA, ermA, ermB, tet(L), tet(M), aac6'-aph2", ant9', aph3', dfrG*	*cutC, tcrB, znuA*	none	phiFL3A EFC_1	none	*rep*9a, *rep*9c, *rep*US43	ISS1N	*ace, agg, cad, camE, cOB1, cCF10, cylA, dad, ebpA, ebpC, elrA, efaAfs, fsrB, gelE, hylA, hylB, srtA, tpx*
X6347	Dog/ household 56	ST585/ CC5	148/2.8	*optrA* type 7	none	*fexA, lnuB, lsaA, ermA, ermB, tet(L), tet(M), str*	*cutC, tcrB, znuA*	ParC (S80I)	PHBC6A5A	none	*rep*7a, *rep*9a, *rep*9b, *rep*US43	none	*ace, agg, cad, camE, cOB1, cCF10, cylA, cylL dad, ebpA, ebpC, elrA, efaAfs, fsrB, gelE, hylA, hylB, srtA, tpx*

[a]ST, Sequence Type; CC, Clonal Complex

[b]17 mutations in pbp5 = S27G, A68T, A216S, T172A, V24A, 885D, K144Q, A499T, L177I, N496K, G66E, E100Q, D204G, P667S, E525D, T324A, R34Q

Abbreviations: AMR= Antimicrobial Resistance; F= Pig-farmer; P= Pig

*All strains were of nasal origin, except *E. faecium* X3877 which was from tracheal sample.

3.2.17 Genetic Environment of the Linezolid Resistance genes

The *fexA* gene, which confers resistance to phenicols, was detected upstream of the *optrA* gene in all 7 strains **(Figure 58)**. Moreover, an *ermA*-like gene was located downstream of the *optrA* gene in all the strains except X5809 and X4962 **(Figure 58)**. This *ermA*-like gene was identical to the one detected in a *Streptococcus suis strain* (GenBank accession number: EU348758). Regarding the *cfrD* gene, we could identify the presence of a *guaA* gene encoding a glutamine-hydrolyzing guanosine monophosphate synthase in the downstream region. Upstream of the *cfrD* gene, we detected the *ermB* gene flanked by IS1216 and ISNCY **(Figure 60)**. The genetic environment of the *cfrD* gene (1,074 bp) revealed 100% nucleotide similarity with that of an *E. faecium* strain in France (GenBank accession number: NG_067192).

Sequence analysis revealed that *E. faecium*-X3877 harboured the wild-type *poxtA* gene with 100% nucleotide sequence identity to that of *E. faecium* plasmid pGZ8 (GenBank accession number: CP038162) **(Figure 61).**

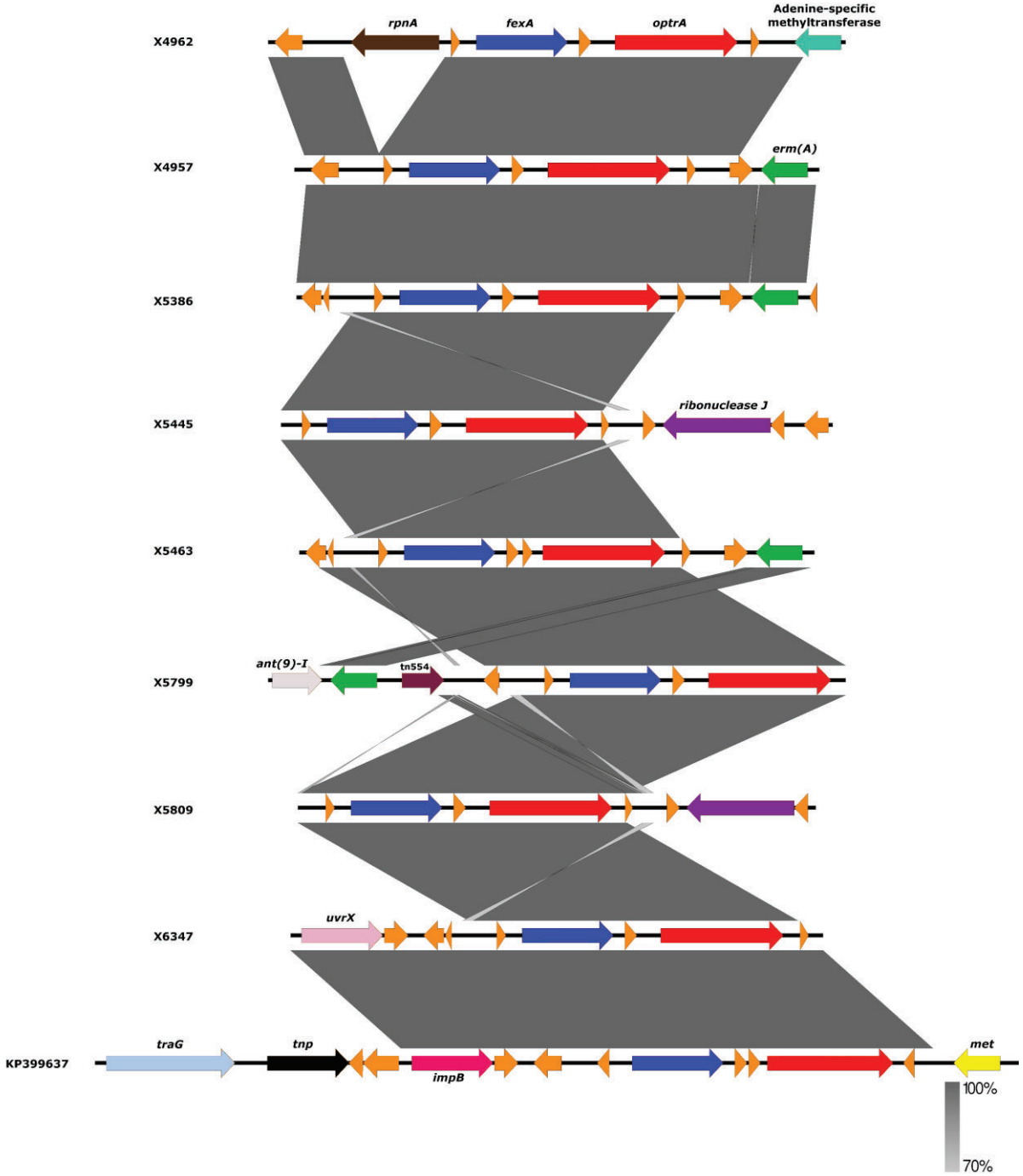

Figure 58. Genetic environment of the *optrA* gene in the eight *E. faecalis* and *E. casseliflavus* from healthy pigs, pig farmer, and a dog.

Shown in the figure are AMR genes located in the same contigs and frames with their corresponding mobile genetic elements. The percentage of identity and scale bar legends are presented on the right side of the image. The comparison was made with a reference *E. faecalis* strain *trG* (GenBank Accession number: KP399637)

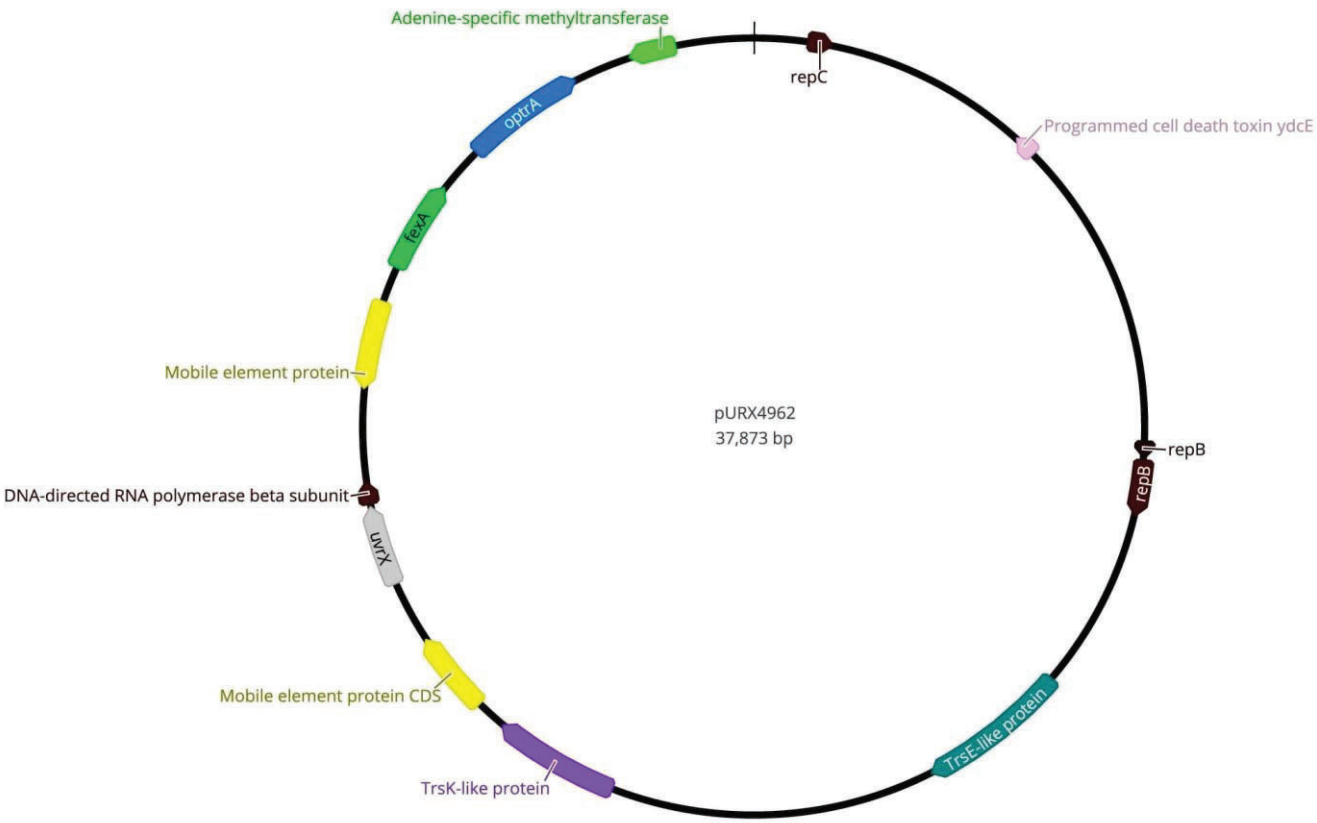

Figure 59. Circular representation of the plasmid carrying the *optrA* gene in *E. casseliflavus*
Colors and arrows indicate the represented genes and their orientation.

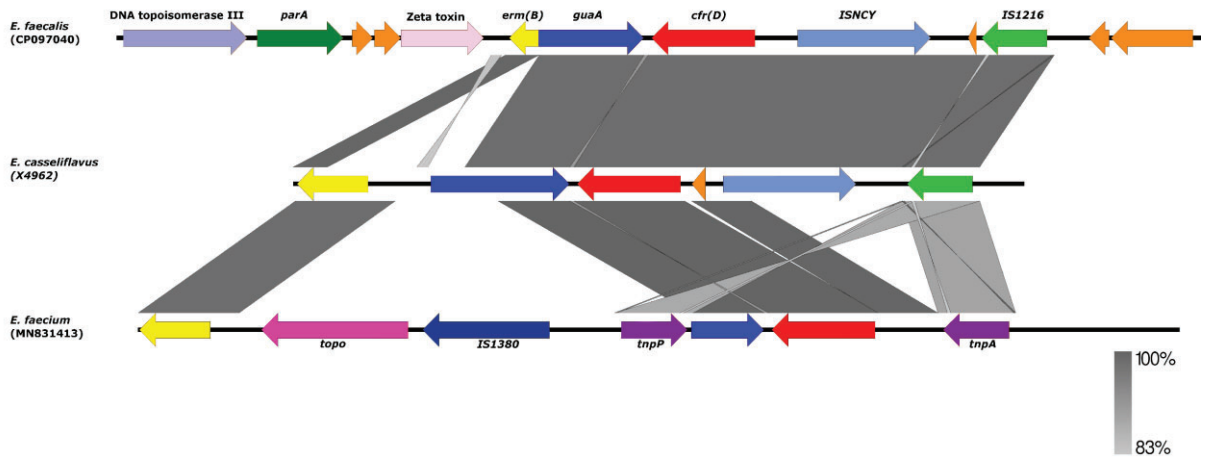

Figure 60. Schematic comparison between the environment of the *cfrD* gene in the strain *E. casseliflavus* (X4962) with *E. faecalis* (GenBank Accession number: CP097040) and *E. faecium* (GenBank Accession number: MN831413).

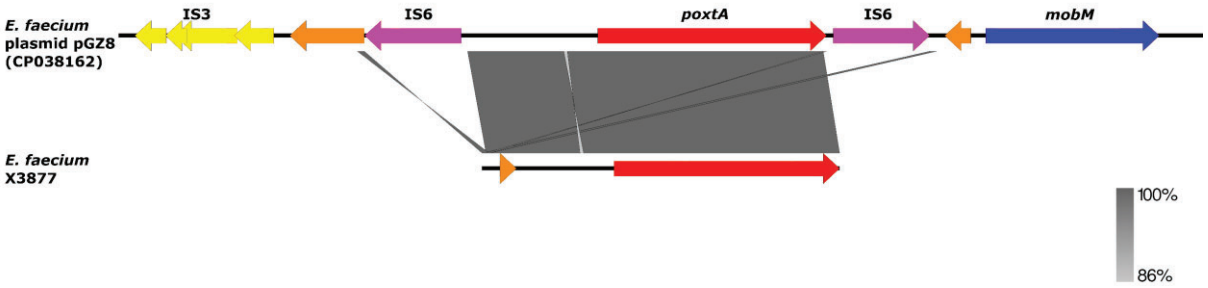

Figure 61. Genetic environment of the *poxtA* gene in the strains *E. faecium* (X3877) in comparison with the previously described plasmid-bound *poxtA* gene in an *E. faecium* strain pGZ8 (GenBank Accession number: CP038162)

3.3 Genetic relatedness of Staphylococci and enterococci strains

3.3.1 *S. aureus* strains from nestling storks, pigs and pig farmers

First, core-genome SNP analyses identified a high relatedness and clustering of the five MSSA-CC398 strains (<10 SNPs) from nestling storks **(Figure 62)**. Second, all the MSSA-CC9 from pigs clustered into the same cluster but different from the MSSA-CC9 of the nestling storks **(Figure 62).** Furthermore, closely related MRSA-CC398 strains carrying the same AMR profile were detected between pigs and pig farmers from the same farm (SNP <10) **(Figure 61),** indicating within-farm transmission of these strains. Stork strains from the two habitats are mixed, indicating no clear difference in spillover patterns between the two groups.

3.3.2 Relatedness of the coagulase-negative staphylococci strains

The phylogenetic analysis identified clusters of related strains of various CoNS species with other countries. Specifically, the *cfr*-carrying *S. epidermidis*-ST16 (X5485) was related to an *S. epidermidis*-ST16 strain from a human blood sample (SNP =70) from Canada (id-41749). The *S. epidermidis*-ST35 from a dog owner is related to an strain from a human strain from Portugal (id-43340) (SNP=90). Moreover, the *S. epidermidis*-ST297 from a healthy human in our study is related to three human strains from Germany, UK and Switzerland (SNP<80) **(Figure 63).** Also, the *S. epidermidis*-ST173 (X9066) was related to an animal strain in Thailand (id-44496) (SNP= 76) **(Figure 63).** Furthermore, the *S. epidermidis*-ST595 is related to strains from Portugal and Italy (ID-43921, Id-43401). It is important to remark that despite the relatedness of some strains from Portugal (id-43340) and Canada (id-41749) with our two linezolid-resistant strains (X5485 and X6049b), none of them from the two countries were linezolid-resistant. This suggests that our strains might have acquired the gene and mutation flowing antibiotic pressure. These findings highlight the international circulation of related *S. epidermidis* strains between various humans and animals as confirmed by the phylogenetic analysis (SNP <100) **(Figure 63).**

Aside from the *S. epidermidis* strains, we found closely related *S. borealis* strains (SNP <10) between pigs (**Figure 64)**. However, the *S. saprophyticus* strains from a pig and pig farmer from the same farm were not related (SNP=346) **(Table 82, Figure 65).**

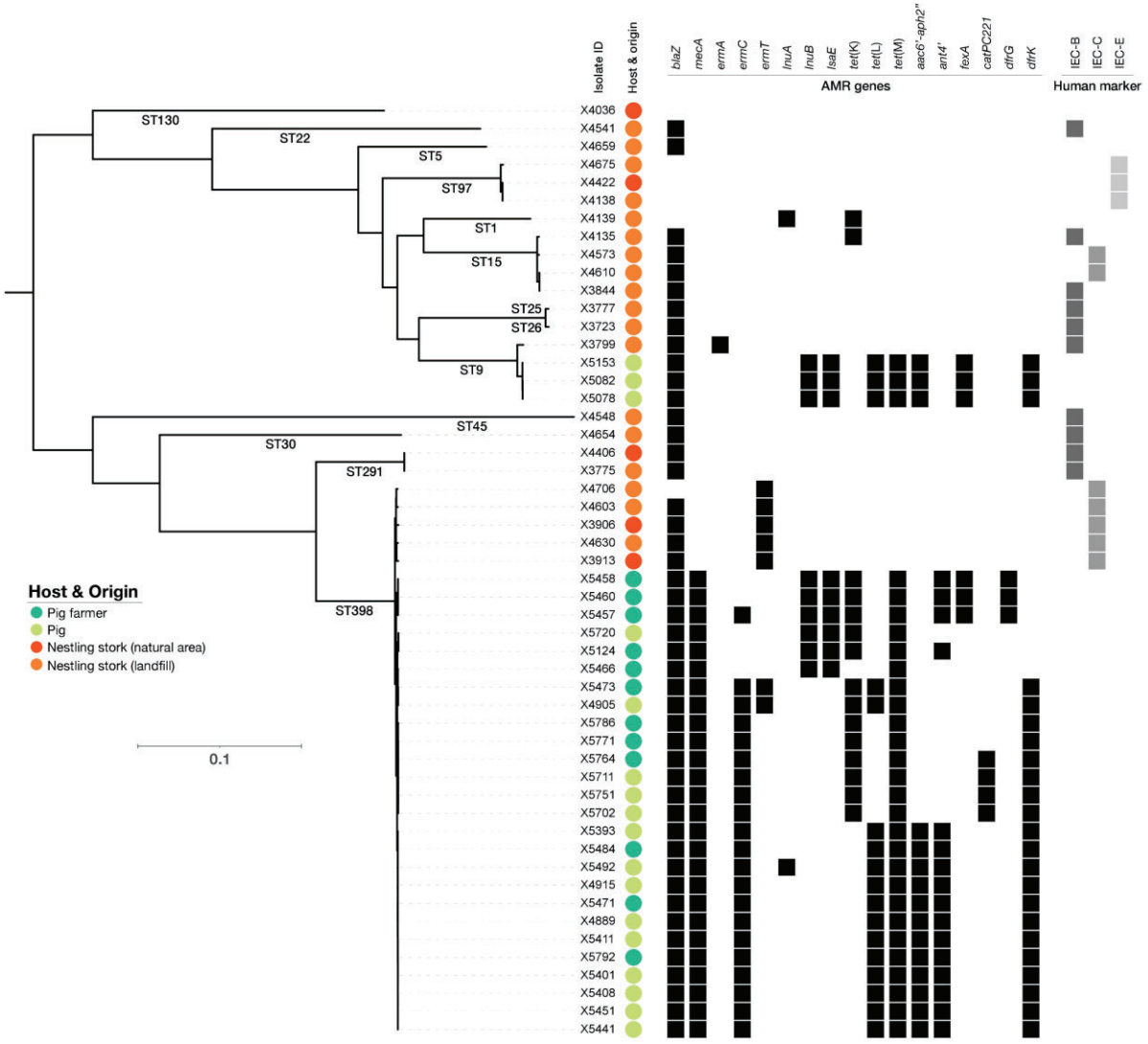

Figure 62. Phylogenetic tree based on core genome SNP analysis of 52 *S. aureus* strains to demonstrate the influence of antibiotic pressure based on the host.

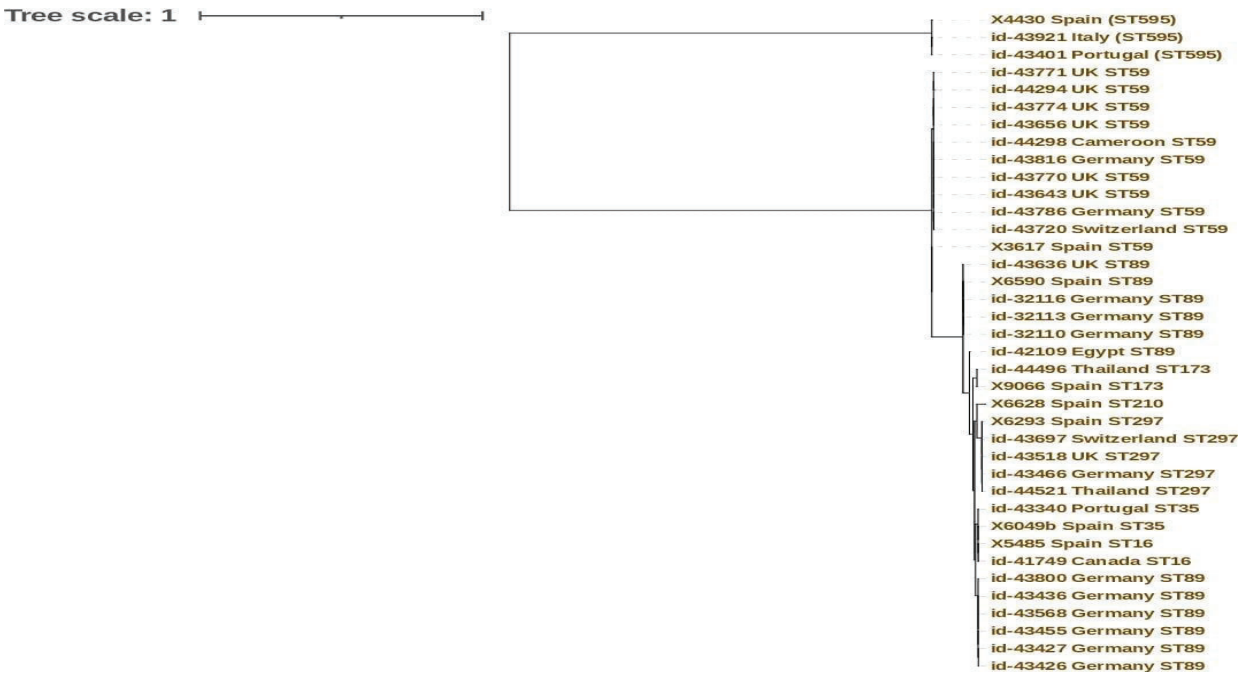

Figure 63. Phylogenetic tree based on core genome SNP analysis of nine *S. epidermidis* strains to with 38 publicly available genomes with similar genetic lineages.

Figure 64. SNIP-based phylogenic tree of the four *S. borealis* strains mapped with all the 13 publicly genomes available from four countries.

Table 82. Single nucleotide polymorphism matrix of the core genome of the three *S. saprophyticus* strains with reference ATCC 15305

	Reference	**X4944**	**X5435**	**X5462**
Reference	0	5185	5185	16888
X4944	5185	0	17552	17552
X5435	16888	17552	0	346
X5462	17120	17549	17549	0

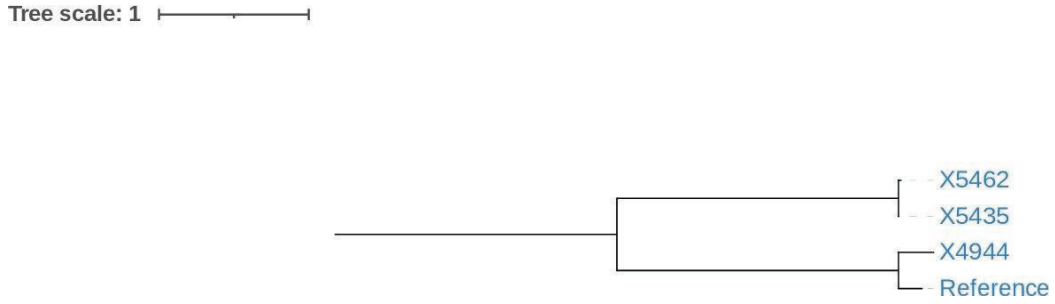

Figure 65. SNIP-based phylogenic tree of the three *S. saprophyticus* strains mapped with genome of reference ATCC 15305 (Accession no. AP008934.1)

3.3.3 Relatedness of the *E. faecalis* strains

The phylogenetic tree of the *E. faecalis* included 12 publicly available genomes (GenBank accession numbers: SRR17662732, ERR2008110, ERR2008112, ERR1599987, ERR1599986, ERR2008113, ERR2008114, SRS7549315, SRS7549355, SRS7549357, SRS7549371, SRS7549400). First, SNPs analyses identified high relatedness (SNP= 4) of pig strain (X5386) with that of the pig farmer (X5463) from the same farm **(Figure 66).** Then, analyses with other publicly available genomes revealed the relatedness of an *E. faecali*s-ST32 (X5445) from a pig with another similar strain (SRR17662732) from a healthy human (SNP=86) in Switzerland. Moreover, the dog strain (X6547) was related to two strains from hospitalized patients (SNP=152 and 156) in Spain (ERR2008110 and ERR2008112) **(Figure 66).** These findings illustrate the potential flow and transfer of linezolid-resistant-*E. faecalis* strains from multiple sources and countries.

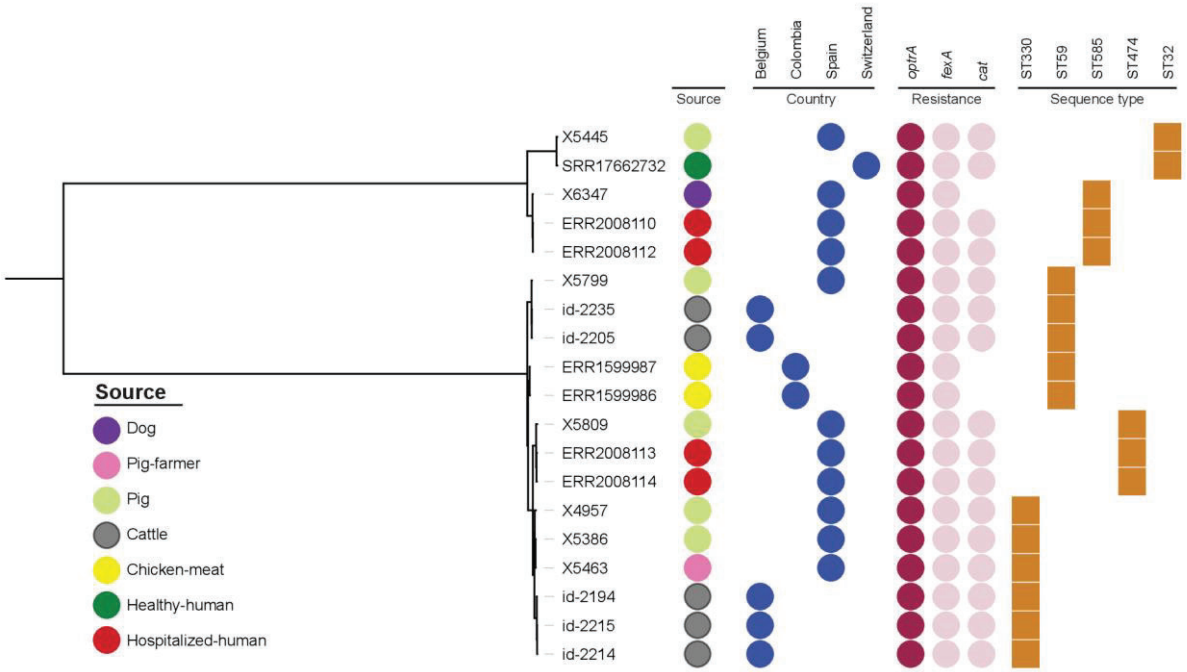

Figure 66. Phylogenetic tree based on core genome SNPs analysis of seven *E. faecalis* strains from healthy hosts in this study with twelve publicly available *E. faecalis* genomes with similar STs and linezolid resistance genes. Colors (in circles) of the AMR genes are as follows: dark purple for *optrA* while and light purple for *fexA* and *cat.*

CHAPTER FOUR

DISCUSSIONS

4.0 DISCUSSIONS

4.1 Species diversity of staphylococci that colonize nasotracheal cavities of nestling storks, nose of healthy humans (with/without contact with animals), dogs and pigs

4.1.1 *Staphylococcus* species from the nasal and tracheal cavities of nestling storks

Migratory birds (such as storks) have been suggested to play a vital role in the spread of bacteria of public health concern across habitats and regions of the world. Key factors for a vector role are exposure to point sources of such bacteria, colonization, persistence and excretion. The former is closely related to the ecology of the species and the behaviour of individuals. In this regard, the acquisition of pathogenic bacteria through the diet (i.e. foraging) is more evident and has been reported for digestive tract samples (Wilharm *et al*. 2016; Höfle *et al*., 2020; Jarma *et al*., 2021). In contrast, there is a paucity of evidence for the respiratory tract to constitute a reservoir of *Staphylococcus* spp (Gómez *et al*., 2016). In particular, detailed bacterial diversity data on the respiratory tract and their association with the foraging habits of storks remain very scarce. Such data from nestling white storks could also reflect the behaviour of their parents, as during the breeding season they are spatially bound to their nest, foraging primarily close to the location of the colony (Pineda-Pampliega *et al*., 2021).

Gram-positive cocci were the most frequently detected bacteria from the nasal and tracheal cavities of nestling storks**,** followed by Gram-positive bacilli, while *Enterobacterales* and Gram-negative non-fermenters were relatively less frequent. Anatomically, Gram-positive cocci are often aerobic and could have a higher affinity to colonize the upper respiratory tissues (nasal and tracheal) (Yildiz *et al*., 2020), and it is expected for them to be more prevalent than Gram-negative bacilli which are facultative anaerobes (such as *Enterobacterales*) and have more affinity to the intestinal lumen and tissues. This is because the gut contains low levels of oxygen due to oxygen consumption by facultative anaerobes (Franzin *et al*., 2021).

Some bacterial species were recovered in high frequencies from nestlings of parent storks foraging in landfills. The exception was *S. sciuri*, which was identified in higher frequency from the trachea of storks foraging in natural habitats. The high CoNS carriage rate detected in the nasal and tracheal samples in the nestling storks (> 80%), is similar to the high prevalence rate previously detected in different types of wild birds in Spain (60%) (Ruiz-Ripa *et al*., 2020b) and Portugal (75%) (Sousa *et al*., 2016), but much higher than the prevalence reported in wild birds in Italy (11.4%) (Gambino *et al*., 2021). These differences could reflect variations in nasal and tracheal staphylococci colonization rates in the wild animal species, and

could also be due to differences in methodologies used by the studies. Behavioural traits that could also influence this high prevalence could be the sharing of pastures with livestock such as cattle and small ruminants and the consumption of dung beetles by the storks, as well as the habit of storks to use cattle manure in the nest presumably to aid in the thermoregulation of newly hatched chicks (Ferreira *et al*., 2019; Tortosa and Villafuerte, 1999).

Highly diverse *Staphylococcus* spp were detected, of which *S. sciuri* and *S. aureus* accounted for over 85% of strains of the entire genus detected. A possible explanation for the abundance of *S. sciuri* could be that this species is largely adapted to wildlife, especially wild birds (Ruiz-Ripa *et al*., 2020b), whereas *S. aureus* has a very broad host range of adaptation across various ecosystems (Guinane *et al*., 2010). *S. aureus* nasotracheal carriage rate of 31% was found in the white stork nestlings analysed in this study, with a higher prevalence in nasal samples (36.5%) than in tracheal samples (11.9%) of the nestlings. Other studies analysed the tracheal *S. aureus* carriage rate in wild birds with various frequencies (4.6–34.8%) (Ruiz-Ripa *et al*., 2019a; Gómez *et al*., 2016; Sousa *et al*., 2014; Wardyn *et al*., 2012).

Concerning the foraging habit of the birds, nestling storks of parents that forage in landfills had a relatively higher *S. aureus* carriage rate than those with parents that forage in natural habitats. This could be because storks foraging in natural habitats might also have relatively lesser chances of *S. aureus* contamination than those foraging in landfills (Ruiz-Ripa *et al*., 2019a). In the study of Gómez *et al* (2016), the high *S. aureus* carriage could be attributed to the stork nestling's exposure to human residues. Thus, the exploitation of human household residues as a major food source seems to be a risk factor for the acquisition of *S. aureus* by wild birds (Abdullahi *et al*., 2021). Especially in the case of migratory birds (such as storks), these could then act as vectors between habitats, regions and even continents (Wilharm *et al*., 2014).

4.1.1 *Staphylococcus* species from the nasal cavities of healthy humans who had no contact with animals

The detection of *S. epidermidis* at a very high frequency in healthy humans is not surprising, as it is the major colonizer of the skin and nasal cavity of people. This is in line with a German study that also reported a very high recovery rate of *S. epidermidis* in healthy volunteers (Marincola *et al*., 2021). Most of the studies focused on CoNS are related on clinical samples or on humans who had contact with animals perhaps because these studied intended to determine the influence of ill-health, healthcare facilities or animals in the acquisition of antibiotic-resistant strains (Asante *et al*., 2021; Khanal *et al*., 2021; Gizaw *et al*., 2020).

Moreover, a high nasal carriage rate of *S. aureus* (36.8%) was detected in healthy individuals in this study. The frequency of nasal *S. aureus* in healthy human populations without any risk of prior colonization varies, depending on the region and/or country. However, lower carriage rates (19.1% and 22.2%) were reported in Spain by Falomir *et al* (2014) and Lozano *et al* (2011a), respectively. The variation in the frequencies of *S. aureus* carriers could be due to difference in detection methods with or without enrichment before cultural plating for bacterial isolation. Moreover, other factors such as the study methodologies, differences in timescale (i.e., the year of study), specific biodata of the participants or level of previous antimicrobial use could be responsible for these variations (Abdullahi *et al*., 2021a). Following these two *Staphylococcus* species, *S. warneri* and *S. haemolyticus* were also detected but in low frequency (6.8% and 5.3%, respectively). Concerning *S. warneri*, a closely similar frequency (11.1%) was previously reported in Valencia city of Spain (Falomir *et al.,* 2019).

4.1.3 *Staphylococcus* species from the nasal cavities of dogs and dog owners

The potential influence of dog-ownership on the nasal staphylococcal community (especially *S. aureus* and *S. pseudintermedius* colonization) of dogs and in-contact humans needs continuous surveillance. The *S. aureus* household carriage rate (at least one dog and/or one human) in this study (44.4%) was lower than the 51.2% detected in a previous study performed a decade ago in Spain (Gómez-Sanz *et al*., 2013a), and the *S. aureus* carriage rate in dog owners in this study was also lower (34.1% *vs* 41.8%) (Gómez-Sanz *et al*., 2013a). Concerning studies from other European countries, the *S. aureus* prevalence obtained in healthy dog owners was higher than those reported in Germany (22%) and Hungary (23.8%) (Holtfreter *et al*., 2016; Sahin-Tóth *et al*., 2021).

Data on *S. aureus* nasal carriage rate among healthy dogs in community settings are sparsely available, varying between 2–8% according to different sources (Fazakerley *et al*., 2010; Rubin and Chirino-Trejo, 2010; Walther *et al*., 2012; Sahin-Tóth *et al*., 2021). The majority of previous studies focused on sick or staphylococcal-infected dogs, such as a recent one conducted in Spain (Ruiz-Ripa *et al.,* 2021a). In this present study, 5.9% of the healthy dogs had nasal *S. aureus* carriage. However, in some African and Asian countries, high *S. aureus* nasal carriage has been reported in healthy dogs, such as in Nigeria (36.9%), Indonesia (48.0%), Bangladesh (25.0%) and India (35.0%) (Mustapha *et al*., 2016; Sekhar *et al*., 2017; Rahman *et al*., 2018; Decline *et al*., 2020). The wide variation in nasal *S. aureus* carriage in dogs across the continents could be influenced by the local epidemiology of the *S. aureus*, differences in methodologies, dogs' hygiene, environmental sanitation, antibiotic use in

animals, and/or the health status of owners (Collignon and Voss, 2015; Fletcher, 2015; Valiakos *et al*., 2020). As was observed in this study, the *S. aureus* nasal carriage rate in healthy dog owners was much higher than in dog carriers. No significant association was obtained between the household density and carriage of *S. aureus* between dogs and dog owners. This could indicate less transmission rate between humans and dogs.

Concerning *S. pseudintermedius*, 2.4% and 32.4% nasal carriage rates were detected in the studied humans and dogs, respectively. The *S. pseudintermedius* carriage rate among dogs in this study was higher than previously reported for dogs from Spain (22.7%) (Gómez-Sanz *et al*., 2013a), but lower than those reported in Canada (46%) or Germany (34.3-37.4%) (Hanselman *et al*., 2009; Han *et al*., 2016; Sahin-Tóth *et al*., 2021; Cuny *et al*., 2022). Moreover, there was a significant association between the household densities and nasal carriage of *S. pseudintermedius* in households with > than 1 dog and >than 1 human, pointing out the possibility that a relatively higher household population is related to a higher detection rate of *S. pseudintermedius*. This suggests that the transfer between nasal *S. pseudintermedius* among healthy dogs could depend on the number of dogs in the household. This corroborates results obtained in previous reports in which diverse MSSP lineages were analyzed from Germany and France (Haenni *et al*., 2020; Cuny *et al*., 2022). On the other hand, nasal *S. pseudintermedius* carriage was found in a person living with dogs in one of the investigated households (*i.e.*, 2.4% of all dog owners). This is lower when compared with previously reported human cases of nasal *S. pseudintermedius* carriage in Korea, Spain, and Canada (Hanselman *et al*., 2009; Gómez-Sanz *et al*., 2013a; Han *et al*., 2016).

It appears that humans are not natural hosts for *S. pseudintermedius*, but adaptation to humans could occur. A recent large study has shown the diversity between the *S. pseudintermedius* strains of human and dog infections with similar pathogenicity islands and virulence gene-containing prophages (Phumthanakorn *et al*., 2021). The households in this study with both human and dog *S. pseudintermedius* carriers strongly suggested intrahousehold transmission, as the strains had no SNP (zero) difference and were confirmed as a clone by genomic analyses.

Contrary to the findings of Penna *et al*. (2013), which reported *S. schleiferi* subsp. *schleiferi* carriage among ~32% of healthy dogs in Brazil, only 2 dogs from this study had *S. schleiferi* nasal carriage (5.4%). This finding is similar to the *S. coagulans*-positive dogs (4.9%) from the study of Lee *et al*. (2019) in Korea, but relatively higher than the 1.0% previously reported in Spain (Gómez-Sanz *et al*., 2013a). Since its first identification in humans in 1988

(Freney *et al*., 1988), several *S. coagulans* infections have been reported in humans and pets (May *et al*., 2005; Abraham *et al*., 2007; Yarbrough *et al*., 2017).

In the present study, several CoNS species were isolated from the two hosts, but more diverse in healthy dogs than in their owners. This is similar to the report from a study in Trinidad where significantly more diverse species of CoNS were identified from healthy dogs than the owners (Suepaul *et al*., 2021). However, the CoNS species detected widely differed between this study and that of Suepaul *et al* (2021), perhaps due to the type of bred dog or the geographical/ hygienic status of the hosts. Most of the CoNS isolated in the present study belonged to the species *S. epidermidis* in both dogs and their owners. *S. epidermidis* is the most reported among CoNS in humans (Burke *et al*., 2023) and has often been detected in healthy pets (Han *et al*., 2016). Being the major nasal commensal in humans, the predominance of *S. epidermidis* is not surprising in the dog-owners. Although at lower rates, it is also a predominant species of healthy dogs (Gomez-Sanz *et al*., 2019; Han *et al*., 2016), perhaps due to the influence of human-pet direct or indirect contact in their household.

Aside from *S. epidermidis, S. hominis* was identified at a moderate rate from both hosts. Reports on the carriage of *S. hominis* in healthy dogs and humans are scarce. The same for other species identified with low rates (*S. haemolyticus*, *S. pastueri* and *S. warneri*). A recent similar study reported the same trend, but in nasal skin samples of healthy dogs (Štempelová *et al*., 2022). Remarkably, *S. ludgunensis* was only identified in four dog-owners but not in dogs. This is a relevant CoNS species in humans causing diverse clinical infections (Fernandez-Fernandez *et al*., 2022). None of the dogs was colonized by this species, however, colonized dog-owners could place their dogs at risk of anthroponotic infections, as it has previously been implicated in canine infections (Rook *et al*., 2012).

4.1.4 *Staphylococcus* species from the nasal cavities of healthy pigs and pig farmers

The findings from this study showed that the most prevalent staphylococcal species in healthy pigs was *S. aureus*. This is not unexpected as it is consistent with previous findings from similar designs in Spain which reported up to 89.6% carriage *S. aureus* rate by Abreu *et al* (2019), 85.6% by Morcillo *et al* (2012) and other European countries such as 96.1% in Portugal by Lopes *et al* (2019) or 65.5% in Belgium by Peeters *et al* (2015). Also, similar nasal *S. aureus* carriage rates in healthy pigs (75.2%) were reported in Australia (Sahibzada *et al*., 2021), the USA (67.7%) (Linhares *et al*., 2015), India (71.4%) (Zehra *et al*., 2017) and China (47.9%) (Wang *et al*., 2012). However, lower frequencies were reported in a Spanish study, 12.7% (Moreno-Flores *et al*., 2020) and in other countries in Africa and middle east Asia

(Egyir *et al*., 2020; Khalid *et al*., 2009). The varied frequencies of *S. aureus* detection rate reported by these studies could be due to the age of the pigs studied or variations in studied methodologies/protocols and the level of intensive pig-farming in the study areas (Van *et al*., 2020).

S. aureus is a multi-host bacterium and is generally a major component of the nasal and skin microbiota of pigs. *S. aureus* colonization could transform to cause a variety of infections in humans, but *S. aureus* is an asymptomatic colonizer in pigs (Rossi *et al*., 2019). Hence, the similarly high nasal carriage of *S. aureus* in pig farmers beyond the usual 30-40% threshold rate is a clear indication of the influence of occupational exposure (Chen and Wu, 2020).
Conversely, other CoNS detected in high frequencies among the pigs, such as *S. chromogenes* and *S. haemolyticus* corroborated with previous reports on the nasal CoNS carriage rate in livestock (Ménard *et al*., 2020: Egyir *et al*., 2020). Although *S. sciuri* was reported in low rates from the pigs, a much higher prevalence of 80% was detected in healthy pigs in Ghana (Egyir *et al*., 2020). The low detection rate of *S. sciuri* from the pigs in this study and its absence in the pig-farmers could be due to the displacement of this species from the nasal cavity by *S. aureus*, as the individuals were heavily colonized by *S. aureus (*Ménard *et al*., 2020). However, this observation needs to be further elucidated.

Concerning the co-carriage of *S. aureus* and CoNS, there was no significant association between *S. aureus* carriage rate and other CoNS. Instead, the colonization of the pigs' and pig farmers' noses with *S. aureus* could be associated with other non-staphylococcal species (Verstappen *et al*., 2017).

4.2 Antimicrobial resistance, virulence genes and genetic lineages of staphylococci of the four hosts

4.2.1 Nestling storks

Most of the *S. aureus* strains from nestling storks presented with a low-level AMR or were entirely susceptible. Specifically, very few of the strains carried the tetracycline resistance gene, *tet*(K), while only one showed a MDR phenotype.
Generally, very diverse CCs were identified especially among the *S. aureus* strains from nestling of parent storks that foraged in landfill areas. Concerning the MSSA CCs, the CC130 was identified in two strains and could be related to nasal carriage by the nestlings from parent storks that had foraged in an area previously contaminated by excretions from small ruminants (such as sheep) (Achek *et al*., 2020). It has been proposed that this lineage evolved from humans to ruminants (Achek *et al*., 2020). None of the CC130 strains contained IEC genes.

This finding is consistent with a previous study that indicated that MSSA-CC130 appears to be a common lineage in sheep causing several infections (Agabou *et al*., 2017). Concerning the MSSA-CC130 from this study, the two strains were from nestlings of parent storks foraging in natural habitats, that consist of open woodland employed for extensive livestock grazing that involves mainly ruminants (sheep and cattle).

Findings from the AMR profile of the *S. aureus* strains from nestling storks showed that 79.1% of the strains were resistant to penicillin. This denoted that about 21% were penicillin-susceptible (PEN^S), a recent trend that could open more therapeutic options against invasive *S. aureus* strains (Mama *et al*., 2021a). Aside from the MSSA-CC398-PEN^S clone with a high potential for invasive infections, other MSSA-PEN^S clones, such as the CC5, were often reported to cause bloodstream infections (BSIs) in a large-scale study in Spain (Mama *et al*., 2021a). The lineage CC5 has also been associated with avian colonization or infections and it was previously reported that a subtype of human MSSA-CC5 was identified in a human-to-poultry 'host switch' (Murray *et al*., 2017). Interestingly, 6 out of 7 of the MSSA-CC5 strains in this study harboured IEC genes (IEC type B).

Besides the MSSA-CC398 lineage, MSSA-CC15 and MSSA-ST291 were detected in high frequency from both nasal and tracheal samples. The MSSA-ST291 lineage has been reported by previous studies and is globally distributed (Kechrid *et al*., 2011; Stegger *et al*., 2013; Bouchiat *et al*., 2015; Havaei *et al*., 2013; Mediavill *et al*., 2012; Mehraj *et al*., 2014). It is important to remark that ST291 is a double-locus variant of ST398 and has previously been misassigned to CC398 (Havaei *et al*., 2013; Stegger *et al*., 2013). Although scarcely described in recent times, MSSA-ST291-*spa*-type t2313 has been responsible for invasive infections with low-level AMR (often carrying only *blaZ* gene) (Mediavilla *et al*., 2012). However, it was previously detected in healthy people in Germany (Mehraj *et al*., 2014).

The pathogenicity of MSSA is largely determined by the presence of certain important virulence factors (especially Panton-Valentine leucocidin, toxic shock syndrome toxin and exfoliative toxins). This is the first time that *tst*-carrying MSSA strains of the genetic lineage CC22 has been found in storks. Nevertheless, *tst*-carrying MSSA-CC30-t012 strains were previously reported by Gomez *et al*. (2016) in storks. Also, *tst*-positive MSSA-CC522 strains were previously reported in other wild animals (such as wild boar) in Spain as well as in healthy ewe in Tunisia (Ben Said *et al*., 2017; Ruiz-Ripa *et al*., 2019a). It is worth mentioning that *tst*-carrying MSSA-CC22 strains (*spa*-type t790) have been associated with wound infections in Iran (Navidinia *et al*., 2021). A possible link between the detection of MSSA strains containing virulence genes in wild birds and livestock could be that the storks foraged on pastures or in

farm areas contaminated with livestock droppings. Also, the toxigenic MSSA from storks in the present study originated all of them from nestlings fed food foraged in landfills which might contain visceral tissues including intestines. In addition to the presence of *tst* gene, the detection of MSSA-*eta*- and *etb*-positive strains in the two storks from this study is also relevant and can pose clinical and public health implications. To my knowledge, there is no previous report of *etb*-positive MSSA in wild birds.

In this study, two new *spa* types were identified. One of them, t7778, belonged to the ST15/CC15 while the other t18009 belonged to the ST26/CC25 lineage. Concerning the MSSA-CC15, this lineage has previously been described with diverse *spa* types circulating in some European countries and Asia (Udo *et al*., 2020; Grundmann *et al*., 2010). Concerning the MSSA-ST26, this lineage has rarely been reported in previous studies. However, worthy to mention is the detection of MSSA-ST26-*blaZ*-positive strains from human oral samples in Japan (Hirose *et al*., 2021). Hence, these CCs need to be monitored to fully understand their evolution in the One Health ecosystems.

Being wild animals, the AMR rates detected among the strains in the present study are worrisome, considering that white storks are not expected to be under antibiotic selective pressure. In the present study, the highest frequency of antimicrobial-resistant *S. sciuri* and other CoNS was to penicillin (with a combined frequency of 26.1%). This finding is similar to the 28.7% resistance to penicillin among CoNS from free-living birds in Poland (Sulikowska *et al*., 2022). However, other studies have shown a varying percentage of resistance to other antimicrobial agents by CoNS from wild birds in Europe and Asia (Sousa *et al*., 2016; Ruiz-Ripa *et al*., 2020b; Elsohaby *et al*., 2021; Sulikowska *et al*., 2022). These variations could be due to the differences in the animal types, contact level with the anthroponotic environment and methodologies of sample collection and processing used for the studies. About 8.2% of the CoNS strains exhibited MDR phenotype. This is relatively lower than the 34% MDR rate reported in healthy free-ranging birds in Spain (Ruiz-Ripa *et al*., 2020b). However, this represents an important source of transmission of pathogens and their antimicrobial resistance mechanisms to other species of animals. The occurrence of MDR-CoNS in our white storks may be related to their foraging habitat. As expected, it was relatively higher in storks foraging in landfills than in natural areas.

Antimicrobial residues are common in solid waste plants and perhaps areas with hospital wastes which are the main compositions of landfills. Conversely, storks foraging in natural areas that had previously been contaminated by livestock manure could also be a source of MDR-CoNS as observed in some of the *S. sciuri* strains in our study. The *mec*A gene, a

major determinant of methicillin resistance in staphylococci was found in some of the CoNS and in most of them, the SCC*mec* mobile genetic element was detected and typed. It is worth mentioning that some CoNS that carry SCC*mec* elements are potential reservoirs of the *mec*A gene and could transmit the same to *S. aureus* (Maree *et al*., 2022).

Remarkably was the detection of an MDR-*S. lentus* carrying *mecA* and *mecC* genes, as well as the SCC*mec* type-XI mobile genetic element. To our knowledge, this is the first case of dual *mecA* and *mecC*-mediated methicillin resistance in *S. lentus* from storks in Spain. This could be because most previous *mecC*-carrying staphylococci studies focused on MRSA in wild animals. Thus, the *mecC*-carrying MDR-*S. lentus* strain in the present study highlights their potential role in the evolutionary origin, genetic transfer, and dissemination of the *mecC* gene to MRSA in the stork population, as the *mecC* gene in our *S. lentus* strain was carried by the classical SCC*mec* type XI. In a recent related study, five *S. lentus* strains carrying *mecC* hybrid in SCC*mec*-VII were identified but from dromedary camels in Algeria (Belhout *et al*., 2023). Also, *mecC*-mediated methicillin resistance (SCC*mec*-*mecC* hybrid) was first identified in two *S. sciuri* strains about ten years ago (Harrison *et al*., 2014). Subsequently, sporadic cases of *mecC* gene in CoNS have been reported. For instance, in a large collection of CoNS from wild and domestic animals, several MR-CoNS (*S. caprae, S. xylosus, S. stepanovicii, S. warneri* and *S. sciuri*) have been demonstrated to carry the *mecC* gene (Lancoric *et al.,* 2019). Similarly, two *S. sciuri*-ST71 strains carrying both *mecA* and *mecC* hybrid were detected from livestock (ewe) in Brazil (de Moura *et al*., 2023). Put together, our findings and previous reports on *mecC*-carrying CoNS suggest the potential expansion of the ecological niches of the *mecC* gene in non-*aureus* staphylococci in some countries. It is important to mention that three MR-CoNS strains were non-typeable. This shows the diverse nature and potentially novel SCC*mec* elements in CoNS as compared to those available in *S. aureus.*

The species *S. sciuri* has previously been shown to exhibit intrinsic resistance to clindamycin (mediated by *salA*), however, low-level resistance was detected in other CoNS identified (mediated by *lnuA* and *vgaA*) that could have contributed to its reduced effectiveness for lincosamide chemotherapy (Lozano *et al*., 2012a). The *salA* gene detected in the *S. sciuri* strains in our study conferred a diverse degree of resistance to clindamycin, as some were completely susceptible while others had intermediate resistance or were fully resistant. This gene had previously been considered to confer low-level intrinsic resistance to other lincosamides, pleuromutilins and streptogramin A (Hot *et al*., 2014). Moreover, the *salA* gene could also confer clindamycin resistance in *S. haemolyticus, S. epidermidis*, and *S. xylosus* strains (Deng *et al*., 2017). In this regard, none of the non-*sciuri*-CoNS in our study carried the

salA gene. Aside from the *salA* gene, *lnuA* was also detected in very few of the *S. sciuri* strains. This gene could have been acquired through plasmids from other non-*sciuri* species within the same microenvironment, such as *lnuA*-mediated plasmids in *S. aureus* (Lozano *et al*., 2012b).

Worth mentioning is the detection of the *ermT* gene in an *S. epidermidis* causing erythromycin-clindamycin constitutive resistance. The *ermT* gene was previously considered unusual in CoNS. This is contrary to the erythromycin-clindamycin-inducible resistance it confers in *S. aureus* and that serves as a good biomarker for the MSSA-CC398 subclade in humans and animals (Mama *et al*., 2021b). The detection of some plasmid-mediated AMR genes such as *dfrG, fexA, tet*(L) and transposon-encoded-*tet*(M) and -*dfrK* suggests that these white storks could have acquired these resistance genes during foraging in areas contaminated with livestock faeces that harboured *S. aureus* containing these genes as in these areas they share part of the pastures with extensively farmed sheep and/or cattle. Importantly, most of the *tet*(M), *dfrK* and *tet*(L) carrying CoNS were *S. lentus*. Similar tetracycline and trimethoprim/sulfamethoxazole resistance genes were previously reported in *S. lentus* strains from wild birds in Spain (Ruiz-Ripa *et al*., 2020b). In this study, most of the *S. lentus* strains showed an MDR phenotype which highlights the need to monitor the potential of this species to spread high-level AMR in the future. Moreover, the five *S. sciuri* strains carrying *fexA, fexB* and cat_{PC221} further highlight the impact of livestock wastes on the acquisition of AMR genes in white storks foraging in habitats concurrently with livestock or previously contaminated with livestock dungs, as chloramphenicol resistance is often associated with environments with high pig-farming (Martins-Silva *et al*., 2023).

Significantly more MDR-*S. sciuri* were detected in nestlings of parent storks foraging in natural areas. This further highlights the potential influence of livestock contact/wastes in natural areas on the acquisition of MDR-*S. sciuri* as this species appears to be related to this habitat (Abdullahi *et al*., 2021). Overall, the differential distribution of *S sciuri* and CoNS resistance genotypes in storks in association with foraging habits suggests that MDR in *S. sciuri* and CoNS of storks is not intrinsic but externally acquired.

4.2.2 Healthy humans

No MRSA carriage was detected in our study, as this could be because our participants did not have any contact with high-risk locations (such as hospitals and livestock farms) or animals that could predispose to MRSA colonization in humans.

The majority of our MSSA strains were susceptible to most of the antibiotics tested except penicillin. Aside from penicillin, resistance to macrolides-lincomycin-streptogramins B

(MLS_b) predominates and this could be explained by the wide use of this class of antibiotics in the treatment of Gram-positive bacterial infections (Bamigbola *et al.,* 2023). Moreover, three strains were ciprofloxacin-resistant and this resistance was mediated by mutations at the common quinolone resistance-determining *regions* of GrlA and GyrA (Afzal *et al.,* 2022). Also, the detection of mupirocin-resistant MSSA strains could render the use of this nasal de-colonizer or topical treatment of superficial skin infections less useful (Nong *et al.,* 2023).

In this study, highly diverse *spa* types were identified among the recovered MSSA strains. This depicts that most of the *S. aureus* carriers were sparsely related. However, it is important to remark on the predominance of the MSSA-CC398 and MSSA-CC121. Among the MSSA-CC398 was the *spa* type t571, a classical lineage that has been associated with invasive human infections (Mama *et al*., 2021b; Davies *et al*., 2011). Nevertheless, in this study, all the MSSA-CC398 strains were recovered from healthy people without prior risk factors of colonization or infection. In addition, most of them harboured the *ermT* gene which appears to be a very useful biomarker for MSSA-CC398 subclade. However, it is important to remark on the first detection of MSSA-CC398 with erythromycin-clindamycin-inducible resistance mediated by *ermC* in Spain. Consequently, the environment and the associated plasmid replicons of *ermT* and *ermC* genes in MSSA-CC398 strains were illustrated for the first time in healthy people. Collectively, it appears that the *ermT*-positive MSSA-CC398 subclade is fast expanding across some European countries and many ecological niches and hosts (Laumay *et al.,* 2021). Whereas the *ermC*-carrying MSSA-CC398 is silently evolving in healthy people, as has rarely been reported (Tegegne *et al.,* 2022).

Although all the MSSA-CC398 strains carry very few or no enterotoxin genes, more studies should be conducted in the future to unravel the mechanism(s) by which MSSA-CC398 acquires virulence and causes clinical infections in humans. Conversely, MSSA-CC121 has now been evolving as a hypervirulent etiological agent of staphylococcal scalded skin syndrome mediated by the exfoliative toxin genes (*eta* and *etb*). Conversely, *etd*-carrying *S. aureus* is primarily associated with mild cutaneous infections, particularly the MSSA-CC5 lineage has been implicated in paediatric skin abscesses since many years (Conceição *et al.*, 2011; Bukowski *et al.,* 2010; Yamasaki *et al.,* 2006).

The MSSA-CC152 lineage is often associated with PVL, especially in Africa and Europe causing community-acquired pneumonia (Azarian *et al*., 2021; Shen *et al*., 2019; Lawal *et al*., 2022; Baig *et al*., 2020). Concerning the *tst*-carrying MSSA, all were of the CC22 lineage that was previously reported in Iran (Goudarzi *et al.,* 2020; Tayebi *et al.,* 2020; Shahini Shams-Abadi *et al*., 2018). The detection of the high frequency of enterotoxin, re-emerging exfoliative

and other virulent MSSA strains underscores the potential roles of healthy humans in the transmission of *S. aureus* infection to vulnerable people such as children, the critically ill and immunosuppressed persons who can have personal contact with these carriers via kissing or from their nasal discharge (Raineri *et al*., 2022).

Findings from this study showed that MR- and MDR-CoNS are carried in the nasal samples of healthy people in the community. Methicillin resistance trait in staphylococci is a major cause for concern especially when they are carried by the SCC*mec* mobile genetic elements. In this study, the prevalence of MRCoNS strains was high (26.7%), 30.2% of the CoNS presented the MDR phenotype, of which 17 (48.6%) of the MDR-CoNS strains was methicillin-resistant. This high MR-CoNS rate is in agreement with previous reports on CoNS in healthy people (He *et al.,* 2020; Lebeaux *et al.,* 2012; Barbier *et al.,* 2010). However, the frequency may vary considerably, as seen in a study that reported as high as 50% (Kateete *et al*., 2020). Relatively high erythromycin, mupirocin and tetracycline resistance rates were also found among the CoNS strains. For the macrolide resistance, it was not surprising, as it is among the top classes of antibiotics that are frequently prescribed (Myers and Clark, 2021; Fan *et al*., 2020), whereas mupirocin has been used to decolonize nasal MRSA (Williamson *et al*., 2017). Thus, resistance to these categories of antibiotics might be associated with high selective pressure due to their frequent use (Marincola *et al*., 2021). Fortunately, linezolid resistance was not detected in any of the *Staphylococcus* strains. However, very low (only one) resistance to chloramphenicol was recorded. These strongly indicate that our study participants do have no contact with livestock as resistance to these antibiotics is relatively more common in livestock farmers than the healthy people with no previous contact with animals (Uddin *et al*., 2021).

MDR was high in our CoNS carriers, as even some strains presented resistance against five or six classes of antibiotics (MAR index > 5). Therefore, MDR-CoNS may limit the available chemotherapeutic options against staphylococcal and many other Gram-positive bacterial infections (Lord *et al*., 2022). Another important phenomenon to remark on is the high intra-host species and intra-species AMR diversity. To our knowledge, this is one of the few studies on healthy people to determine this phenomenon. Being heterogeneous, many *S. epidermidis* carriers had genetically diverse strains with varied AMR genes and/or lineages. In future, this may pose a difficulty in eradicating *S. epidermidis* when they cause infections such as prosthetic joint infections and sepsis (Joubert *et al*., 2022; Widerström *et al*., 2022). The detection of methicillin-resistant *S. epidermidis* (MRSE)-ST59, a known community-associated lineage highlights its versatility and ease of transmission in the human population

(Liu *et al*., 2022; Xu *et al*., 2018). Also, MRSE-ST2, a well-established hospital-associated genetic lineage (Ruiz-Ripa *et al*., 2021b), was identified from one of the participants.

It is important to remark on the detection of multi-resistant *S. haemolyticus* (MAR index = 0.58) that carried multiple genes that mediate MDR phenotype (*blaZ, mecA, msrA, mphC, tet(K), aac6'-aph2'', ant4', dfrG*). In recent times, *S. haemolyticus* has been evolving as a major cause of neonatal sepsis (Westberg *et al*., 2022), consequently, this species deserves genome-base surveillance to track its relevance in clinical infectious diseases.

4.2.3 Healthy dogs and dog owners

Several studies have reported the transmission of CoPS between pets and their owners (Gómez-Sanz *et al*., 2013a; Han *et al*., 2016; Sahin-Tóth *et al*., 2021; Cuny *et al*., 2022). However, the present study is among the few that have studied intra-species and within-host genetic diversities of CoPS in these hosts. Such information can better illustrate the complexity of challenges in the control of AMR in healthy dog-owning households.

In this study, no MRSP was detected among healthy dogs and similar results were obtained in a study in Sweden (Börjesson *et al*., 2015). However, other studies reported low rates of MRSP nasal carriage in dogs, as 0.9% in Germany, 4.5% in Canada and 2.6% in Norway (Hanselman *et al*., 2009; Kjellman *et al*., 2015; Cuny *et al*., 2022). Moreover, a pooled 4.6% MRSP was reported among healthy dogs in Spain (Gómez-Sanz *et al*., 2011); of the nine MRSP nasal carriers, one was from a household dog, while the remaining eight were from stray dogs (Gómez-Sanz *et al*., 2011). The absence of MRSP in this study (healthy animals and household members) and the previously low MRSP in healthy dog studies are remarkably different to the high prevalence in dogs receiving treatment in veterinary clinics in France (16.9%) (Haenni *et al*., 2014). MRSP seems to be associated with animal-hospital-lineages (Ruiz-Ripa *et al*., 2021b), whereas strains that are susceptible or have low AMR levels may represent natural colonizers of dogs.

The MSSA-CC398 was a predominant lineage in this study, although detected only in humans. In many cases, the MSSA-CC398 clade is associated with the predominant *spa* type t571 and the macrolide resistance gene *ermT* (Mama *et al*., 2021b). Worryingly, this MSSA-CC398 human clade has been recently considered an emergent lineage in invasive human infections in Spain and other countries (Laumay *et al*., 2021; Mama *et al*., 2021b). Concerning MSSA-CC398 in dog-owning households, a previous study by Gómez-Sanz *et al*. (2013a) also reported MSSA-CC398 of the *spa* type t571. An important difference between the previous study and the current one is that here, MSSA-CC398 strains were only detected in humans and

all were IEC type C of the *spa* type t571 and t1451. In another study by Gómez-Sanz *et al*. (2013b), about 7.1% of 98 kennel dogs also carried MSSA-CC398-*scn*-negative strains but of different *spa* types (t034, t5883 and t108), and all were pan-susceptible.

The MSSA CC398 strains from the present study were resistant at least to one antibiotic and worryingly 50% of the strains presented an MDR phenotype. A major difference between the MSSA-CC398 strains reported by Gómez-Sanz *et al*. (2013b) and this study was that here, human strains were *scn*-positive (IEC type C). Recently in France, the MSSA-CC398 lineage (t571, t1451 and t18379) was also reported in 14.6 and 27.3% of dogs and cats, respectively (Tegegne *et al*., 2022). Worthy mentioning is the detection of MSSA-CC398-t571-*scn*-negative in a cat (Tegegne *et al*., 2022). The reason for this variation is not fully understood, however, it could be attributed to the *spa* type associated with the MSSA CC398 strains or due to the carriage status of the *Sa3* prophage (Gómez *et al*., 2020). The loss of φ*Sa3* in the *scn*-negative strains is a major determinant of the human-to-animal transmission of MSSA-CC398 (Price *et al*., 2012; Matuszewska *et al*., 2022). The findings of Gómez-Sanz *et al*. (2013a, 2013b) and the ones from the present study suggest the persistence of MSSA-CC398 in humans and dogs.

Another finding of special epidemiological relevance is the dual MRSA/MSSA carriage detected in this study as both MSSA-CC30 and MRSA-CC5 were identified in a human household member. In a previous Spanish study, simultaneous carriage of both MRSA and MSSA of the CC398 lineage was reported in a farm worker with occupational exposure (Gómez *et al*., 2020). In another study among healthcare students in Portugal, concurrent detection of MRSA and MSSA in a single person was also reported (Coelho *et al*., 2021).

Though all the MRSA strains had an MDR phenotype, more than 20% of the MSSA and MSSP strains were also MDR. Generally, the AMR rate was moderate, but the most common AMR in *S. aureus* strains were to penicillin, aminoglycosides, and erythromycin-clindamycin. Conversely, similarly to previous studies in *S. pseudintermedius*, the predominant AMR phenotype was to sulfamethoxazole-trimethoprim, erythromycin, and tetracycline (Rynhoud *et al*., 2021). Novel mutations (G2261A & T1584A) in the domain V region of the 23S rDNA of one MRSA strain were observed, and although the predicted *in silico* resistance did not reveal a currently known AMR phenotype attributable to this mutation, the strain was phenotypically linezolid resistant. The inability to detect the linezolid phenotype from the genome database could be due to that this mutation has not been fully characterized (not previously reported and deposited in the genome database), as opposed to the most frequently detected 23S rDNA point mutation in staphylococci (G2576T) (Gostev *et al*., 2021; Ruiz-Ripa

et al., 2021b). However, recently, novel point mutations in 23S rRNA associated with linezolid resistance in staphylococci have been reported in *S. epidermidis* in Austria (Huber *et al*., 2021) and in *S. capitis* in China (Han *et al*., 2022). It could be those novel mutations in domain V of 23S rRNA are silently emerging and mandate the need for close surveillance.

Regarding *S. aureus* virulence factors, all were negative for TSST-1, PVL, ETA and ETB encoding genes. All except one of the nasal *S. pseudintermedius* strains of dog and human origins carried the *lukS/F-I, siet,* and *expA* genes. These leucocidins and exfoliatins are responsible for host-specific clinical infections in dogs (Gharsa *et al*., 2013; Gómez-Sanz *et al*., 2013a)

Concerning the AMR profile of the CoNS in this study, *mecA*-positive CoNS was found in 23.2% of the strains of healthy dog-owners and is slightly higher than those detected in former studies among clinically healthy people (less than 20%) (Suepaul *et al*., 2021; Abadi *et al*., 2015; Xu *et al*., 2018). However, a slightly higher rate of 27.9% was reported from dog-owners in Spain (Gomez-Sanz *et al*., 2019). Conversely, the carriage rate of MR-CoNS obtained in this study was closely similar to a multinational study on hospital workers (21.4%) (Morgenstern *et al*., 2016). Put together, these data indicate the variation of nasal carriage of MR-CoNS based on occupation, age, geographical region and contact with animals. However, it is important to remark that studies on MRCoNS from dog owners are particularly sparsely available. Most of the MRCoNS from this study were of the species *S. epidermidis*, others include *S. hominis* in both dogs and owners, but *S. cohnii* only in dogs. The MR-*S. cohnii* and MR-*S. hominis* are *rarely isolated* from *healthy dogs. If one considers the* comparably similar types of SCC*mec* elements in some of these MRCoNS strains, one could suggest the potential transfer of the *mecA* gene to non-*epidermidis*-MRCoNS strains.

Half of the CoNS from dogs and their owners were penicillin-resistant mediated by the *blaZ* gene that produces a beta-lactamase. A similar rate was reported by Seupaul *et al* (2019). This is not surprising as penicillin is one of the first-line antibiotics and its frequent use may contribute to selection pressures in the *Staphylococcus* spp. Thereafter, various forms of macrolide-lincosamide-streptogramin (MLS) resistance phenotypes/genotypes such as the erythromycin-resistant-clindamycin-susceptible (by *msrA, mphC*), erythromycin-clindamycin-constitutive (by *ermA* and *ermT*), erythromycin-clindamycin-inducible (by *ermC*) and erythromycin susceptible-clindamycin-resistant (by *vgaA, lsaB*) were exhibited by over 50% of the CoNS. These classes of antibiotics are relevant treatment options in most clinical staphylococcal infections (Mahfouz *et al*., 2023). Hence, the AMR to this category of drugs is of serious concern. Of note, is the detection of the *ermT* gene in *S. epidermidis* and *S. hominis*.

This is an unusual mechanism of erythromycin-clindamycin-constitutive resistance that appears to be evolving in humans and animals.

Resistance to aminoglycosides and chloramphenicol was reported at very low rates. The aminoglycosides are used extensively in clinical settings (Han *et al*., 2016; Conner *et al*., 2018) and chloramphenicol is not in use in humans or pets. Perhaps, this was why the *fexA* and *fexB* genes were not detected among the chloramphenicol-resistant strains, rather *catPC221* which appears to be one of the common mechanisms of resistance in non-*aureus* staphylococci. Most important is the detection of three linezolid-resistant *S. epidermidis* strains mediated by multiple point mutations on L3 and one on L4 ribosomal proteins. This is the first report on this mechanism of linezolid resistance in *S. epidiermidis*-ST35 in the literature. These mechanisms of resistance are not transferable to other species or bacteria but confirm the silent and slow emergence of high-level resistance in CoNS in dog owners.

Tetracycline resistance was generally detected at a moderate level but relatively more in dog owners than in dogs. Tetracycline is widely used in both humans and animals (di Cerbo *et al*., 2019). Most studies on mupirocin resistance focused on *S. aureus* strains and very few data are available on the CoNS species (Gómez-Sanz *et al*., 2019), especially from healthy pets and their owners. In this study, the mupirocin resistance rate was high among the CoNS from both hosts and this is a cause for concern as mupirocin has long been used in the nasal decolonization of *S. aureus* (Allport *et al*., 2022).

MDR was high in both dogs and dog-owners, but slightly lesser in dogs than their owners. MDR from healthy pets and their owners may limit the available treatment options for staphylococcal infections in humans and animal medicine (Lord *et al*., 2022). Another important phenomenon to remark on is the high intra-host species and intra-species AMR diversity. This is one of the few studies on healthy pets and owners to determine this phenomenon. Being heterogeneous, more *S. epidermidis* exhibited intra-species diversity with varied AMR genes. This may pose a difficulty in eradicating *S. epidermidis*, especially in old people suffering from prosthetic joint infections (Widerström *et al*., 2022). There are few studies like the one carried out in this thesis in which the potential transmission of *S. epidermidis* between owners and their pets has been detected. This was detected in five households where the dogs and dog owners were colonized by similar strains with the same genetic lineages. Similar findings were previously reported in Spain but on MR-*S. epidermidis* (Gomez-Sanz *et al*., 2019). Put together, this highlights the transmission of *Staphylococcus* species other than *S. aureus* and *S. pseudintermedius* in dog-owning households.

4.2.4 Healthy pigs and pig farmers

Several studies have reported the nasal carriage rates and transmission patterns of *S. aureus* between pigs and pig farmers. Worth mentioning is the first detection and continued persistence the MRSA-CC398 for the past decade in pigs, humans in contact with pigs, pig-derived foods, pig-farm environmental samples and human residents close to pig farms as well as patients in hospitals located in areas with high pig density in Spain (Mama *et al*., 2021b; Lozano *et al*., 2011a, 2011b; Gómez-Sanz *et al*., 2010; Benito *et al*., 2014; Ceballos *et al*., 2019; Ruiz-Ripa *et al*., 2020c). These put together highlight the endemic status of MRSA-CC398 in Spain. However, the present study further elucidated the within-host variability of AMR of *S. aureus* of the same or different genetic lineages and their potential association with CoNS species in the same nasal niche. This information can better explain the complex existence of varied *spa*-types and AMR within the same CCs and their potential implication in the control of AMR in pig herds and zoonotic/occupational transmission.

Concerning the MRSA recovery rate in pigs, the majority of the *S. aureus* strains (all in farms A, B, and D and few in farm C) were MRSA (>90%). This finding is similar to the previous report from another Spanish region (Catalonia) where all the *S. aureus* (100%) were methicillin-resistant (Reynaga *et al*., 2016). Similarly, about 80% of the *S. aureus* from the pig farmers were MRSA. However, this observation is different from another Spanish study in the Canary Islands, where a relatively low prevalence (15%) of nasal MRSA was reported in pig farmers (Morcillo *et al*., 2020).

It has been shown that exposure to high amounts of MRSA in the environment (such as the air) of pig farms and time spent on the farm are major determinants for MRSA nasal carriage in pig farmers (Bos *et al*., 2016). Also, a higher pig density of farms could contribute to the nasal carriage rate of MRSA-CC398 in pig farmers (Reynaga *et al*., 2016). This could be the reason why MRSA-CC398 was relatively less in farm-C which had the least population of pigs.

The prevalence of MRSA found in pigs (62.5%) was similar to those reported in Germany (52%) and the Netherlands (56%) (Bos *et al*., Alt *et al*., 2011). But much higher than the report from La Rioja (Spain), where a 21% MRSA nasal carriage rate was reported among fattening in a slaughterhouse (Gómez-Sanz *et al*., 2010). These differences reflect the physical conditions and the age of the pigs during sampling collection.

A very interesting finding related to the MRSA-CC398 detected in the pig-farmers is the *spa*-type t034 and t1451 which was not detected in any of the pigs. Also, all the *S. aureus* strains were IEC-negative (i.e., lacked the human-adaptation marker), except one

MSSA-CC45-t065 from a pig-farmer which was IEC-type C. These put together suggest that the MRSA-CC398-t034 and -t1451 lineage and MSSA-CC9 from pig-farmers were animal-adapted subclades (Broens *et al*., 2010). However, none of the pigs tested had MRSA-CC398 with these *spa*-types. This raises a question of the source of these MRSA-CC398-*spa*-types t034 and -t1451 strains in the pig-farmers. Nevertheless, their absence, even in very low frequency cannot be categorically exonerated from the pig population.

Concerning the AMR phenotypes of the *S. aureus* strains, all the MRSA-CC398 strains presented tetracycline resistance. It has been demonstrated that tetracycline resistance is a good phenotypic marker of MRSA-CC398 (Lozano *et al*., 2011a; Lozano *et al*., 2011b; Camoez *et al*., 2013) and the *tet(M)* gene is classically integrated into the SCC*mec* of MRSA-CC398 (Ceballos *et al*., 2019). The MRSA strains from this study showed high-level resistance to erythromycin and clindamycin. In 90.1% of the MRSA strains from pigs, erythromycin-clindamycin constitutive resistance was detected (mediated mainly by *ermB* and *ermC*), while a small proportion showed solely clindamycin-resistance (with erythromycin susceptibility) mediated by the *lnuB* gene, which is often enriched among MRSA-CC398 strains (Benito *et al*., 2014). Importantly, the presence of *lnuA* or *lnuB* genes seems to be related to *S. aureus* animal-dependent lineages (Lozano *et al*., 2012b). Regarding the MLS_B resistance genes, *ermT* was also detected in two strains from a pig and pig farmer with similar AMR profiles. The *ermT* gene is very unusual in MRSA-CC398 strains, in most cases, this gene (*ermT*) is associated with plasmids and metal resistance genes such as *cadD, cadX* and *copA* (Gómez-Sanz *et al*., 2011).

Of note, some of the pigs and pig farmers had within-host diversity of genetic lineages and methicillin resistance profiles (i.e., carriers of both MRSA-CC398 and MSSA-CC9). Also, heterogeneity in the AMR phenotypes and genes of within-host MRSA strains was recorded in a significant number of pigs and pig-farmers with each host harbouring 2 or 3 distinct AMR phenotypes. These phenomena highlight the importance of selecting multiple colonies from all *S. aureus* nasal carriers to obtain complete epidemiological data.

The AMR profiles detected in the CoNS greatly varied, with high levels of resistance to tetracycline, chloramphenicol and erythromycin. It is understandable for the very high level of tetracycline resistance presented with the predominance of *tet(M)* and *tet(L)* genes, as this is a common antibiotic in animal husbandry (Soundararajan *et al*., 2022). Florfenicol is also frequently used in livestock which could contribute to the persistence of chloramphenicol and the emergence of linezolid resistance (Yang *et al*., 2022). More worrisome is the linezolid resistance genes detected in two strains. Linezolid has never been licenced for use in livestock

(Ruiz-Ripa *et al*., 2020c). However, other classes of antibiotics could have contributed to the increase of cross-resistance, especially to the *cfr* gene (Pholwat *et al*., 2020), which confers resistance to florfenicol and contributes to the emergence of linezolid-resistance in Gram-positive cocci (Brenciani *et al*., 201). Aside from the *cfr*-carrying *S. saprophyticus* and *S. epidermidis*, several MDR-*S. borealis* strains carrying SCC*mec* type-V were detected among pigs from three farms studied. To our knowledge, this is the first report on the molecular characterization of AMR genes of MDR-*S. borealis* strains from healthy pigs in the literature. The *S. borealis* was first described by whole genome sequencing and ascribed to a distinct species due to the significant phylogenetic distance from *S. haemolyticus* (Pain *et al*., 2020). Despite being a relatively new species isolated from human skin and blood samples, it needs to be monitored and fully characterized to determine its potential to spread MDR and critical AMR genes in other ecological niches. The presence of *cfr* genes did not translate to phenotypic LZD resistance by both the disc diffusion test and E-test in the *S. epidermidis* strain. These results confirm the silent emergence of LZD resistance at the molecular level in *S. epidermidis* from a pig-farmer. It appears that the pig-farm environment favours the persistence of linezolid resistance and MDR genes (Martins-Silva *et al*., 2023).

Many of the identified AMR genes in the CoNS strains are commonly found within mobile genetic elements, such as *mecA*. In this sense, the MRSA strains have long been considered to have originated from the acquisition of SCC*mec* from MRCoNS. However, whether the same SCC*mec* types are present in MRSA and MRCoNS that reside in the same nasal niche needs to be elucidated. Even though the high-level AMR genes detected were from CoNS strains (often considered harmless), they can exchange mobile genetic elements with pathogenic species (Rossi *et al*., 2020). Unfortunately, the molecular surveillance of these multiresistant CoNS is underrated (Rossi *et al*., 2020). It is important to acknowledge the frequent detection of *S. epidermidis* ST59, a clone that has very high community transmission potential (Chen *et al*., 2013) and may facilitate the transmission and persistence of AMR genes in various ecological niches. One of the *cfr*-carrying strains is an *S. epidermidis*-ST16. This genetic lineage has previously been reported to cause bloodstream infection but not carrying the *cfr* gene as in the case of this study (Shelburne *et al*., 2020).

The results obtained with the statistical analysis performed indicate that different factors in pig farming could be involved in some AMR rates detected among CoNS, as in the case of significantly high rates of ciprofloxacin and chloramphenicol in farm A compared to others. This difference could be due to the hygienic status of the farm, the population of herds (Reynaga *et al*., 2016), and other potential factors that need to be thoroughly investigated.

Some strains identified had phenotypic resistance (especially to penicillin) but did not harbour the corresponding genes tested. Perhaps, this could be due to certain amino acid changes or polymorphisms in the *blaZ* gene, or the *mecA* gene in the bacteria mediated the penicillin resistance without expression of *blaZ* gene (Miragaia, 2018).

Worth mentioning is the detection of the *ermT* gene in some species of CoNS causing erythromycin-clindamycin constitutive resistance which is an unusual mechanism in CoNS. To our knowledge, this study is the first to report the presence of this gene in CoNS strains of pigs and pig-farmers in Spain. Although, the gene has previously been reported in an *S. haemolyticus* strain in an environmental sample from a pig farm (Ruiz-Ripa *et al*., 2020c), there is a paucity of data on the description of *ermT* gene in CoNS species.

Another point to mention is the detection of similar species of CoNS with different AMR profiles and genes in the same host. This underscores the enormous challenge these strains could pose in the control of AMR at the farm level. More especially, as some of CoNS strains carrying similar AMR profiles were identified ≥ 3 pigs on the same farm. This is a strong indicator of transmission events of similar CoNS strains across the pig herds.

4.3 Host adaption, resistome, virulome, mobile genetic elements, and CRISPR-Cas systems of staphylococci

4.3.1 *S. aureus* from different origins

4.3.1.1 Host adaptation: The case of *S. aureus* CC398 subclades

Two subclades have been described within the *S. aureus* CC398 lineage. As these *S. aureus*-CC398 subclades are important livestock (MRSA) and human (MSSA) colonizers, their common interaction in both hosts and potential spillover to wild animals requires attention.

After a phylogenetic analysis of the CC398 strains from the present study with a collection of previously deposited strains (Price *et al*., 2012), two different clusters of human- and animal-adapted subclades were observed. Originally, the CC398 lineage was divided into a human-adapted MSSA clade (*scn*-positive) and the emerging livestock-associated (LA)-MRSA clade (*scn*-negative) (Mama *et al*., 2021b; Price *et al.*, 2012). However, lately, other variants, (a) of the ancestral human subclade that is *scn*-negative, and (b) of the derived livestock subclade that is human-adapted (*scn*-positive) have been reported but in very rare cases (Laumay *et al*., 2021).

Regarding *S. aureus* CC398 strains detected in this thesis, all stork MSSA-CC398 strains (both from natural and landfill areas) cluster within the human clade, whereas the pig and pig farmer MRSA-CC398 strains cluster in the LA clade. This is indicative of the storks obtaining MSSA-CC398 from human sources (probably via human waste), whereas the pig farmers are exposed to the pigs and therefore obtain LA MRSA-CC398 strains. The phylogenetic analyses of the MRSA-CC398 strains indicated within-farm transmission of these strains. On the other hand, the nestling stork strains from the two analysed habitats are mixed, indicating no clear difference in spillover patterns between the two groups.

Moreover, it is important to remark that prophage φ3 is particularly important in the evolution of the four subclades of CC398. The presence of β-converting φ3-prophage variants carrying an IEC characterizes the MSSA-CC398 subclade. Indeed, human-to-animal transmission is strongly correlated with the loss of φ*Sa3*, but it seems that LA-CC398 MRSA can, in rare cases, readapt to the human host through the regain of an IEC-harbouring φ*Sa3* (Sieber *et al*., 2020). Similar findings in relation to presence of φ*Sa3* in all the MSSA-CC398 strains was observed in the *S. aureus* strains from dog owners and healthy humans without animal contact. However, the clustering and presence of φSa3 in the MSSA-CC398 strains from the nestling storks highlight a 'spillover' event from humans. Collectively, these denote the fast expansion of the MSSA-CC398 subclade across several hosts as recently reported by Tegegne *et al* (2023).

4.3.1.2 Antimicrobial/metal/biocide resistance and associated mobile genetic elements

In the nestling storks' strains, few ARGs were associated with mobile genetic elements. The *ant9'* gene which was co-located with *ermA* in a Tn*554* transposon in an MSSA-CC9 strain (X3799) had never been reported, suggesting the expansion of the ecology of these genes outside methicillin-resistant *S. lugdunensis* which was first described (Chang *et al.,* 2019; Chang *et al.,* 2021). However, the plasmid *rep7a* that was associated with *tet*(K) gene in one of the strains MSSA-CC1 (X4139) has commonly been identified as a major plasmid that disseminates tetracycline resistance among staphylococci (Al-Trad *et al.,* 2023).

Concerning the pigs and pig farmers' strains, most of the ARGs were carried by mobile genetic elements. Most importantly, it is noteworthy that *tet*(M) and *tet*(L) were located in plasmid *rep22* and *repUS43* but not in MSSA-CC9 strains from the two pigs and a pig farmer. Perhaps, these plasmids are emerging mobilomes for *tet*(M)- and *tet*(L) genes in the MRSA-CC398 strains. The *tet*(M) gene was localized in three different types of transposons (*i.e.,* Tn*6009*, Tn*925* and Tn*916*). The Tn*6009* is a member of the Tn*916*–Tn*1545* family and is a

conjugative (non-composite) transposon of Tn*916* (Soge *et al*., 2008). The *tet*(M) has previously been reported to be carried by *repUS43* alongside Tn*6009* in an *E. faecalis* strain (Founou *et al*., 2021), but never in *S. aureus.* This denotes the diversity of transposons associated with *tet*(M)-mediated tetracycline resistance and suggests their essential role in the evolution and horizontal spread of this resistance marker in MRSA-CC398 of pigs and pig farmers. This could particularly occur in other Gram-positive cocci, as Tn*925*-associated *tet*(M) has previously been detected in *Enterococcus* species (Zahid *et al*., 2017). It appears that *tet*(M) gene (in both MRSA and MSSA) was strongly associated with tetracycline resistance in pigs and pig farmers, which could be used as a marker for livestock-associated *S. aureus* (Ceballos *et al*., 2019; Price *et al.,* 2012).

Aside from tetracycline, the *ermT* gene that mediates a peculiar MLS_b resistance phenotype (erythromycin-resistant/clindamycin-inducible-resistance) was found in five MSSA-CC398 strains from nestling storks and two MRSA-CC398 strains from a pig and pig farmer. It is important to highlight that the *ermT* gene is very common in MSSA-CC398 but it is an unusual mechanism of MLS resistance in MRSA-CC398 strains (Gomez-Sanz *et al.* 2012). Worthy to note is that the *ermT* gene was carried by two different types of plasmids in the MSSA-CC398 and MRSA-CC398 strains. Nevertheless, the gene *str* that mediates resistance to the aminoglycoside (streptomycin) was detected in twelve strains. Also, *ant9'*, another aminoglycoside resistance gene was detected co-located with the *ermT* gene on plasmid *repUS18* in a pig (X4905) and a pig farmer (X5473) strains. Perhaps, the dual presence of *ant9'* and *ermT* genes in *repUS18* could be distinguishing markers between *ermT*-positive MRSA-CC398 and MSSA-CC398.

Interestingly, only the *czrC* gene was detected among the MSSA-CC9 strains. The *czrC* gene is part of the SCC*mec* element of MRSA-CC398 strains (Cavaco *et al.*, 2011), so probably the *czrC* in MSSA-CC9 was acquired independently. Moreover, the *copA* gene appears to be exclusively associated with MRSA strains. Apart from the *cadX* gene that was only found on the MSSA-CC398 subclade in nestling stork strains, the *arsB* gene that encodes for arsenic pump membrane protein was only found in an MSSA-CC130 strain as previously described (Monecke *et al*., 2022). However, this gene has also been largely present in MRSA-CC130 strains of wild animals (Gomez *et al*., 2021). Moreover, the *cadD* (cadmium transport protein D) gene was carried by fifteen (57.7%) MSSA strains (including CC398) from nestling storks. The presence of these genes suggests the presence of metal pollution in the environment where these strains were found, which could be linked to human activities (Briffa *et al*., 2020). It is

important to highlight that heavy metal resistance is a matter of public health concern due to its potential hazards in the food chain and the co-selection of AMR (Belloso Daza *et al.*, 2022).

All the MRSA-CC398 strains of pigs and pig farmers carried the SCC*mec* type Vc (5C2&5). The vast majority of MRSA-CC398 strains carry this SCC*mec* type which often contains the *czrC* and *copA* genes that detoxify cadmium and copper (Karampatakis *et al*., 2021), of which the *copA* contributes to MRSA-CC398 survival in the pig farm environment (Schijffelen *et al*., 2010). The acquisition of *qacG* carried on plasmid *rep*21 by 68.9% of the pig and pig farmer strains could facilitate the persistence of these lineages in the pig/pig farmer environment, as they are resistant to quaternary ammonium disinfectants, thus making their eradication very difficult (Seier-Petersen *et al*., 2015).

4.3.1.3 Virulome profile of the *S. aureus* strains

Toxins constitute important virulence determinants of *S. aureus*, with enterotoxins being the most implicated in food safety, especially in meat and dairy products from livestock (Grispoldi *et al.,* 2021). Moreover, other virulence factors could be responsible for a range of *S. aureus*-related infectious diseases (Howden *et al.,* 2023). The MRSA-CC398 strains were entirely negative for *tst, lukS/F-PV, eta, etb, etc, etd* and all genes encoding enterotoxins. This observation has been previously confirmed (Mama *et al*., 2020). However, the *sem, seo, seu,* and *sei* genes were identified in the three MSSA-CC9 strains from pigs. Moreover, *sen, sem, sei, seg, seu,* and *seo* genes were also identified in four strains of the nestling storks belonging to the lineages MSSA-CC5, -CC9, -CC25 and -CC45.

Aside from the toxins, some *S. aureus* enzymes were commonly present in all the strains, such as the *adsA* that encodes adenosine synthase A, a cell wall-anchored enzyme that converts adenosine monophosphate to adenosine and seems to be related to the evasion of host immune responses in *S. aureus* (Darisipudi *et al*., 2021). Furthermore, the presence of *icaABCD* operon and its *icaR* gene in all the strains denote that the *S. aureus* strains easily adhere to the mucosa and serve as a fundamental step in nasal colonization and persistence on environmental surfaces and fomites (Idrees *et al*., 2022).

4.3.2 Coagulase-negative staphylococci from different origins

CoNS have long been considered reservoirs of AMR genes, however, very few genomic studies have elucidated the extent to which different ecological niches and hosts have impacted the level of AMR and the MGEs associated with them in a healthy state. Moreover, genomic insight has been provided on the transmission of certain CoNS species between pigs and pig

farmers within the same pig farm. Moreover, to our knowledge, this is the first study to perform the genomic characterization of *S. borealis* in Spain.

4.3.2.1 Relatedness of the CoNS strains

The phylogenetic analysis suggested that the strains might have acquired the resistance genes and target mutations following antibiotic pressure, especially the CoNS from pigs and pig farmers. Moreover, related *S. epidermidis* strains were found with the mapped genomes obtained from publicly available databases. This could indicate the international circulation of related *S. epidermidis* strains between various humans and animals as confirmed by the phylogenetic analysis (SNP <100).

4.3.2.2 Mobilome-bound antimicrobial/metal/biocide resistance in CoNS

Concerning plasmid bound-AMR genes, all the MRCoNS from pigs and pig farmers had *mecA* genes carried by SCC*mec* type Vc except the two *S. saprophyticus* strains that had *mecA* in SCC*mec* type IVb. The predominance of the SCC*mec* type Vc in these strains reflects the fact that the epidemiological traits of pig-associated MRSA are often similar. Thus, it has been speculated that the SCC*mec* type Vc in LA-MRSA was an evolutionary precursor of SCC*mec* in CoNS carried in the same ecological niche (such as nostrils in this case) (Matuszewska *et al*., 2022). Whereas the SCC*mec* type IV in *S. saprophyticus* from the pig and pig farmer could denote community-associated strains brought to the pig farm. *S. saprophyticus* is often associated with uncomplicated urinary tract infection (Lawal *et al*., 2021a).

In nestling storks, the MDR-*S. arlettae* and *S. epidermidis* were methicillin-susceptible, whereas the MR-*S. haemolyticus* carried *mecA* gene located in SCC*mec* type V, and the MR-*S. lentus* in SCC*mec* type III. In addition, the *S. lentus* carried the *mecC* gene located in SCC*mec* type VII. The *mecA* gene might be an intrinsic gene in *S. lentus* carried in SCC*mec* III (Saber *et al*., 2017). Of the MR-CoNS strains from dogs/owners and healthy humans, both the classical hospital and community-associated SCC*mec* elements were detected. This shows the SCC*mec* type in these hosts aside from pigs/farmers has no categorical predilection.

It is important to remark on the detection of the unusual *ermT* gene in two species of *S. borealis* carried by plasmid *repUS18* and *S. hyicus* with no associated plasmid. Usually, the *ermB, ermT,* and *erm45* genes are not common for MLS_b resistance in CoNS. It appears these genes are silently evolving in CoNS causing a different MLS_b resistance phenotype from that of the MSSA that produces erythromycin-clindamycin-inducible resistance.

The plasmid-bound tetracycline resistance genes were similar to the findings obtained in MRSA-CC398 strains from the same pig farms. It is important to highlight that all the plasmid bound-*tet*(L) were linked with the *dfrK* gene in similar plasmid *repUS12*. A similar observation was reported in an MRSA-CC398 strain from a pig (GenBank accession number: FM207105). However, *tet(L)* was not found to be located in any plasmid in one of the *S. hyicus* strain from a pig (X5069) carrying a Tn*559*-bound *dfrK*. This denotes difference in the pattern of acquisition of *tet*(L) gene and potential of inter-species transfer in CoNS and *S. aureus* in a pig farm setting.

In some instances, some AMR genes (such as *lnuA* and *bleO*) were plasmid-bound in some strains while they were not associated with any plasmid in others. A categorical explanation could not be made for the reason some AMR genes were located in plasmids in some of the CoNS strains while they were not located in any plasmid in other bacteria carrying the same AMR genes. It could be that the bacteria lost the plasmids during horizontal transfer but the recipient bacteria retained the AMR genes (Dimitriu *et al.,* 2022). The similarity in plasmids that carry many AMR genes in all the CoNS strains demonstrates their impact on bacterial fitness for survival and capability to transfer these resistant genes intra-species (the same species), interspecies and between different hosts. Also, some plasmids appeared to carry multiple AMR genes from different classes of antibiotics (such as *repUS12* and *rep22*). The transferability of AMR genes between different *Staphylococcus* spp has been strongly suggested by the sequence similarity of their associated mobilome especially plasmids (Souza-Silva *et al.,* 2022).

Of clinical and public health concern is that other critical AMR genes such as those that mediate transferable linezolid resistance could be co-selected. In this regard, two *cfr*-carrying *S. epidermidis* and *S. saprophyticus* from a pig farmer and a pig previously identified were characterized. Of which, the *cfr*-carrying *S. saprophyticus* was in plasmid *rep15*. It has been suggested that the emergence and dissemination of the *cfr* gene in humans and animals that have never used any of the oxazolidinones might be due to the selective pressure from non-prudent use of closely related antibiotics (phenicols, lincosamides, and pleuromutilins) in treatment, prophylaxis and livestock production (Gostev *et al*., 2021).

The plasmid-bound resistance to quaternary ammonium compounds (such as *qacA, qacC, qacG* and *qacJ*) could facilitate the persistence of MDR-CoNS, as these genes make it very difficult to eradicate these staphylococci (Seier-Petersen *et al*., 2015). Concerning metal resistance, it has previously been hypothesized to co-select for AMR more and often linked to SCC*mec* elements (Lawal *et al*., 2021b) and plasmids in LA-MRSA, *S. epidermidis, S.*

saprophyticus, S. haemolyticus, etc. (Lawal *et al*., 2022; Argudín and Butaye, 2016; Schijffelen *et al*., 2010). Specifically, determinants of copper (*copA*) and zinc (*czrC*) were widespread among the MRCoNS strains of the pigs and pig farmers, but absent or minimal in other hosts. This could denote the potential selection of resistance to these metals due to their persistence in pig farm settings (*e.g.,* in pig feed) especially when plasmid linked (Cavaco, *et al.,* 2011).

4.3.2.3 Virulome profile of the CoNS strains

Diverse virulence genes were identified in the genomes of all the sequenced CoNS. However, of special relevance are the ones that are associated with staphylococcal enterotoxin. Enterotoxins are the most implicated in food-borne gastroenteritis due to staphylococcal infections (Kadariya *et al.,* 2014). Moreover, other virulence factors could be responsible for a range of staphylococcal-related infections that are rarely detected in non-*aureus* staphylococci (Nanoukon *et al*., 2018). However, it is important to highlight the detection of *sec*- and *sel*-carrying *S. epidermidis* of the lineage ST595.

Similar studies have previously reported these virulence genes and their associated pathogenicity islands in *S. epidermidis* (Lin *et al*., 2021; Nasaj *et al*., 2021; Banaszkiewicz *et al*., 2019). Moreover, it has been suggested that only *S. epidermidis* from animals or food but not from humans typically produce *S. aureus*-related SEs (Podkowik *et al*., 2016; Veras *et al*., 2008; Stach *et al*., 2015; Nanoukon *et al*., 2018). However, some *sec* and *sel* genes have been identified in association with plasmids, phages and pathogenicity islands (Alibayov *et al*., 2014; Banaszkiewicz et al., 2019). Thus, they can be horizontally transmitted between any host, including humans. It appears that the *sec* and *sel*-carrying *S. epidermidis* strains from nestling storks are not transferable as they were not associated with a mobile genetic element. Moreover, simultaneous colonization of the nostril by several *Staphylococcus* spp could promote the transfer of enterotoxin genes from *S. aureus* to commensal *S. epidermidis* strains (Nanoukon *et al*., 2018).

4.3.4 CRISPR-Cas system distribution among the staphylococci (CoPS and CoNS)

None of the *S. aureus* carried the CRISPR-Cas system. Usually, a complete CRISPR-Cas system is very rare in *S. aureus*. In this regard, certain genetic lineages, especially MRSA-ST630 are associated with CRISPR-Cas (Mikkelsen *et al*., 2023). The CRISPR-Cas often limits phage multiplication and are offshoot from the SCC *mec* cassette (Mikkelsen *et al*., 2023).

However, complete CRISPR-Cas systems were detected in about 19% of the CoNS. The low frequency of CRISPR-Cas positive strains identified in this study is closely similar to the 12.3% rate by Rossi *et al* (2017) which consisted of mainly class 1 type IIIA and class 2 type IIC systems.

Considering that most CRISPR-Cas reduces or eliminates mobile genetic elements like plasmid, the low frequency of CRISPR-Cas-carrying MDR-CoNS in this study could be because all the strains had ARGs carried by multiple plasmids. Conversely, the data from CRISPR-Cas analyses suggest that the CRISPR-Cas system did not influence AMR and mobilome levels in *S. borealis* strains compared to other species. This is because, despite the demonstration of complete CRISPR-Cas systems in three of the *S. borealis* strains, high repertoires of AMR genes and plasmids were also found in them. Therefore, further studies on large-scale genomes could lend more information about these differences.

4.4 Genetic environments and biomarkers of diagnostic relevance for antimicrobial resistance traits

4.4.1 The *ermT* gene in staphylococcal strains

Despite the involvement of the *ermT* gene as a major mediator of inducible MLS_b resistance in MSSA-CC398 strains, its environment and its associated genes and mobilome have rarely been described First, the five *ermT*-carrying MSSA-CC398 strains were all associated with cadmium-resistance genes, *cadR* and *cadD,* which were absent in the genomes of the two *ermT*-carrying MRSA-CC398 strains. The IS*257* was flanked upstream of the *ermT* gene in MSSA strains, except in one strain in which it was flanked downstream. Interestingly these markers were absent in the *ermT*-carrying MRSA strains. The *ermT* gene of the MSSA strains was associated with plasmid *rep13*. Often, the *ermT* gene in MSSA is carried by a plasmid and has recently been recognized as a biomarker of erythromycin-clindamycin inducible resistance in the MSSA-CC398 lineage (Mama *et al*., 2021b). Contrary to this observation, the *ermT* gene in MRSA strains produces an erythromycin-clindamycin constitutive resistance phenotype, and this phenomenon has previously been reported in the literature (Gomez-Sanz *et al*., 2013c). Moreover, the *ermT* gene in the *S. borealis* strain was in the opposite direction with a*nt9'* and both genes were located in plasmid *repUS18*.

Therefore, the *ermT* gene in the three CoNS and the MRSA-CC398 strains mediated the erythromycin-clindamycin constitutive resistance phenotype and highlights their evolution in MLS_B resistance among CoNS and MRSA-CC398. Whereas the presence of erythromycin-clindamycin-inducible resistance with a characteristic D-shaped zone of inhibition along the

clindamycin and *ermT* gene could serve as a biomarker of MSSA-CC398 in public health laboratories.

4.4.2 The *mecC* gene in CoNS

In relation to the *mecC*-carrying *S. lentus*, it has been previously described that most *mecC*-harbouring CoNS strains carry a hybrid SCC*mec* element comprised of *mecA* encoding SCC*mec* type VII and a *mecC* region consisting of the class E *mec* complex (de Moura *et al*., 2023; Belhout *et al*., 2023; Paterson *et al*., 2020). However, *blaZ*-SCC*mec* XI was initially found to be associated with *mecC* in the *S. lentus* (X4630) by PCR and amplicon sequencing by Sanger (Abdullahi *et al.*, 2023b). Following WGS, the *mecC* gene of the *S. lentus* (X4638) was noted to be quite different from the classical SCC*mec* type XI that was first demonstrated in *S. aureus* LGA251 (accession number FR821779)**.** The reason for this variation is subject of further analysis. But, it could be that a recombination event took place between the SCC*mec* type III (intrinsic for most MR-*S. lentus*) of the *mecA* gene and SCC*mec* type XI of the *mecC* to produce the SCC*mec*-*mecC* hybrid (i.e., the SCC*mec* type VII). In this regard, there is a need for caution in the use of PCR-based assays for the detection of SCC*mec* types in *mecC*-carrying non-*aureus* staphylococci. This report represents the first description of a *mecC* in an *S. lentus from a wild bird. This* suggests the expansion of this mechanism of methicillin resistance in CoNS across various ecological niches including wild animals, which were previously proposed to be the major reservoirs of the *mecC* gene in *S. aureus* (Abdullahi *et al*., 2021).

4.5 Mechanisms of antimicrobial resistance in *Enterococcus* species from the four hosts with special focus on the linezolid resistance and its genetic environment

To our knowledge, there is no previous study that simultaneously investigated the nasal enterococci communities of food-producing animals, pets and wild animals. Perhaps because they are frequent intestinal commensals, most studies focus on gastrointestinal enterococci carriage (Torres *et al*., 2018). However, in all three animal hosts studied, the enterococcal nasal carriage rate was high (especially in pigs and storks).

The high nasal enterococci rate detected in this study highlights the frequent association of *Enterococcus* spp. with the respiratory tracts of the animals. Thus, it is essential to remark that enterococci are not only found at high rates in the gastrointestinal tract but also in nasal samples, as demonstrated in this study. On the other hand, healthy dogs were relatively fewer

carriers of enterococci, and this might be due to host adaption differences to respiratory epithelia.

There is growing evidence that the use of chloramphenicol chemotherapy in animal husbandry can select for enterococci harbouring *optrA* and *poxtA* genes which confer resistance to the critically important antibiotic linezolid, posing a risk to human health via the food chain and contact with livestock. In this study, the majority (over 90%) of the enterococci carrying oxazolidinone resistance genes belonged to *E. faecalis* or *E. faecium*, which are the predominant *Enterococcus* species in humans and animals (including pets and pigs), but also belong to one of the most important Gram-positive nosocomial pathogens worldwide (Zaheer *et al*., 2020).

A relatively higher frequency of LRE was found in pigs from the present study than those reported in other studies on faecal samples of pigs in Switzerland (5%), Belgium (11%) and Italy (21%) (Nüesch-Inderbinen *et al*., 2022; Fioriti *et al*., 2020; Timmermans *et al*., 2021). Notably, comprehensive data for comparative analysis are still scarce. The variations between countries from which data are available should be interpreted with caution due to the differences in study designs, sample types and testing methodologies. Nevertheless, the present study indicates that the occurrence of chloramphenicol-resistant enterococci among the pigs was high. Worryingly, the use of antibiotics in pig farming in the last years has been very high in Spain, highlighting the need to raise awareness within the agricultural sector to mitigate the emergence and spread of linezolid-resistant enterococci in the future. Moreover, most of the enterococci in this study were associated with the presence of tetracycline resistance genes. Tetracycline is the most frequent veterinary antibiotic used for treating many swine bacterial diseases and is likely to promote the spread and persistence of LRE in pigs (Schwarz *et al*., 2021; Swiss Veterinary Society, 2081)

The *optrA*-carrying-*E. faecalis*-ST330, -ST474 and -ST59 circulating in 3 of the 4 studied farms have been previously reported in human and many animal hosts such as macaques, pigs, chickens, poultry meat, and vultures (Fioriti *et al*., 2020; Timmermans *et al*., 2021; Wang *et al*., 2015; McHugh *et al*., 2022; Freitas *et al*., 2022; Woods *et al*., 2017; Cavaco *et al*., 2017; Almeida *et al*., 2020; Roy *et al*., 2020; Càmara *et al*., 2019). These *optrA*-positive lineages appear to be non-host specific. The detection of LRE in pig farmers and a dog indicates a potential risk of transmission to other humans and animals outside the pigs-farm environment and dog-owning households, respectively. These put together with the several *optrA*-positive *E. faecalis* strains reported in dogs fed with raw meat/vegetables in China (Wu *et al*., 2019) underscore the relevance of the ‘One-Health’ approach for investigating LRE, as they can be

shared by animals, humans and environment. However, the direction of transfer is often difficult to prove, especially as none of the humans in contact with the dogs were carriers of LRE. Currently, the knowledge of the LRE prevalence in companion animals is limited and therefore a joint approach to monitor the emergence and dissemination of resistance mechanisms of public health importance is needed.

The MDR *E. faecalis*-ST585 strain detected in a dog in this study was similar to previously reported LZD^{R}-*E. faecalis* strains from Spanish hospitals (Moure *et al*., 2020). Moreover, this is the first description of ST585 carrying the *optrA* gene in dogs from Spain. Plasmid-encoded *optrA* and *poxtA* in *E. durans* and *E. hirae* were previously reported in pigs in Switzerland, as well as *poxtA*-carrying *E. hirae* from China and Italy (He *et al.,* 2016; Schwarz *et al*., 2016; Fioriti *et al*., 2020), and *optrA*-carrying *E. gallinarum* from a fattening pig in Belgium (Timmermans *et al*., 2021) were recently identified. Also, a *cfrD*-carrying *E. casseliflavus* strain has recently been reported from pigs' manure in Italy (Cinthi *et al*., 2022) and *optrA*/*cfr*-carrying *E. casseliflavus* from a faecal swab of a pig in China (Lei *et al*., 2021). The detection of *E. casseliflavus* carrying *optrA* and *cfrD* in a pig from this study is the first report in Spain. This suggests that pigs could be potential reservoirs and vectors of dissemination of LZD^{R}- *E. casseliflavus.* Since *E. casseliflavus* occasionally causes opportunistic infections in humans (Zaheer *et al*., 2020), the presence of linezolid resistance genes in this species from pigs may not pose a direct threat to human health but could play an important role in transferring this resistance mechanism. It is worth mentioning that none of the enterococci strains in this study had a mutation in their 23S rDNA, as well as in the L3, L4 and L22 ribosomal proteins.

Concerning the stork's *E. faecium*-ST1736 carrying *poxtA* in this study, migratory birds may be an important link in the spread of LRE. This strain was obtained from a nestling that was fed food foraged by its parents in the landfills; so, the exposure could be from human household residues, wastewater treatment plant sludge discarded on landfills or animal remains. This is the first time that LR-*E. faecium* ST1736 has been reported in storks. The detection of linezolid resistance genes is highly relevant since these genes could be in plasmids and be transmitted to clinical settings, production animals or the environment.

It is of interest to remark that all the linezolid-resistant enterococci were recovered in the ChromAgar LIN agar plates in which strains were grown as green colonies. Nevertheless, linezolid-susceptible strains were also recovered in this media, as previously indicated by a previous study (Girlich *et al*., 2022).

In storks, a vast majority of the *Enterococcus* species were susceptible to all the antibiotics tested. This difference may reflect the level of selection pressure, particularly due to the extensive use of antibiotics in pig farming as compared to antibiotic chemotherapy in dogs and humans (Benjamin *et al*., 2017). Although vancomycin-resistant enterococci (VRE) are considered high-priority pathogens of great public health concern resistance (European Centre for Disease Prevention and Control, 2017), none of the strains carried the *vanA* and *vanB* genes. Notably, the detected AMR genes in *E. faecium* or *E. faecalis* strains from storks were all from landfill-associated colonies (except one). Most likely, the nestlings were fed landfill-foraged food by their parents. Both acquired and intrinsic resistance properties drastically reduce the options for antimicrobial therapy. Bearing this in mind, the performance of antimicrobial susceptibility tests prior to the start of antimicrobial therapy is of particular significance to guide the application of antimicrobial agents in pigs-farming and canine medicine.

All the enterococci carried at least one plasmid replicon (1–5 *rep*). Generally, the *E. faecalis* strains analyzed carried plasmids belonging to many of the known replicon families in enterococci (Freitas *et al*., 2020: Clewell *et al*., 2014). The variability of plasmid content found illustrates the diverse nature of MGE in enterococci and their potential to facilitate the dissemination of some critical AMR genes, such as the *optrA*, as in the case of the *E. casseliflavus* that was the only strain with plasmid-bound linezolid resistance gene identified in this study.

All the LZD^{R} *E. faecalis* and *E. faecium* strains carried at least one prophage. When detected, most intact prophage-associated sequences are found in clinical enterococci in human and animal populations (Matos *et al*., 2013), but their distribution and involvement in the pathogenesis of enterococcal infection are poorly characterized (Freitas *et al*., 2020).

4.5.1 Relatedness of the LZD^{R}-*E. faecalis* strains

The phylogenetic tree of the LZD^{R}-*E. faecalis* included 12 publicly available genomes and close relatedness was identified between a pig strain with annother strain from a pig farmer in the same farm. This denotes inter-host transmission. Furthermore, the analyses with other publicly available genomes revealed relatedness of the *E. faecalis*-ST330 and -ST59 with three cattle *E. faecalis* strains in Belgium. Moreover, *E. faecalis*-ST32 was closely related to strains from a healthy human in Switzerland. In addition, the dog strain was related to two strains from hospitalized patients in Spain**.** These findings illustrate the potential flow and transfer of LZD^{R}-*E. faecalis* strains across multiple hosts and countries.

CHAPTER FIVE

CONCLUSIONS/CONCLUSIONES

5.0 Conclusions

1. Bacterial communities of the nasal and tracheal cavities of nestling storks were highly diverse being staphylococci predominant, particularly the *S. sciuri* species.
2. One-third of the nestling storks were *S. aureus* nasotracheal carriers, being significantly more frequent in nasal samples. Moreover, up to 13 CoNS species were detected.
3. All *S. aureus* recovered from the nestling storks were MSSA. Moreover, MSSA-CC398 lineage was detected in 8.2% of the storks. Both the *S. aureus* and CoNS strains presented low rates of antibiotic resistance.
4. The detection of unusual AMR genes (such as *mecC* and *ermT*), diversity of SCC*mec* types in CoNS and relevant virulence genes in *S. aureus* from storks highlights the need for meticulous and comprehensive surveillance of wildlife.
5. Healthy humans (who had no animal contact) were frequent *S. aureus* nasal carriers (37%), being the only CoPS species detected. Of the MSSA strains, CC398 was the most frequent lineage (33%), followed by CC121 (18%). Methicillin resistance was frequently detected among CoNS. The detection of toxigenic and virulent *S. aureus* lineages and also of MDR-CoNS strains need to be monitored for their potential human health implications.
6. CoPS were detected in more than one-third of the dog household members, specially *S. aureus* among dog owners (mostly MSSA, including the CC398 lineage) and *S. pseudientermedius* among dogs. Moreover, human-to-human MSSA and dog-to-dog owner MSSP transmissions were identified.
7. Up to nine CoNS species were detected among dogs and six in dog owners, and *S. epidermidis* was predominant in both hosts. Moderate levels of AMR were presented by CoNS from these hosts.
8. Nasal *S. epidermidis* with similar genetic lineages were identified from dogs and dog owners in the same household suggesting the anthroponotic human-to-dog transmission.
9. A very high rate of MRSA-CC398 nasal carriage was found among pigs and pig farmers (60% and 70%, respectively), and it is also of interest the high level of MDR detected among *S. aureus* and CoNS in these hosts. Events of MRSA and MDR-CoNS transmission were confirmed among pigs and pig farmers.
10. The detection of LZD^R-staphylococci (both CoPS and CoNS) and -enterococci in all studied hosts (except healthy humans without animal contact) required special

attention, mainly in the pig farm environment due to the highest frequency of detection and plasmidic location of linezolid resistance genes (*optrA* and *cfr*).

11. Since MSSA-CC398 was detected in three ecosystems studied (healthy humans without animal contact, dog owners and storks), the combination of erythromycin-clindamycin inducible resistance and *ermT* gene could be useful diagnostic markers of MSSA-CC398 subclade.
12. The level of AMR detected in the different hosts analysed in this thesis seems to be correlated with the antibiotic pressure exerted in the different ecosystems; in increasing order from wild birds, healthy humans (without animal contact), dogs –dog owners, and pig-pig farmers.
13. Collectively, this report underscores the need to strengthen the genomic epidemiological approach and inclusion of all *Staphylococcus* species from all hosts (even the healthy ones) to adequately understand the global spread of antimicrobial-resistant strains and track pathogenic ones using the "One Health" model.
14. This thesis showed the influence of ecological niches on AMR levels and the presence and/ or transmission of various epidemic *Staphylococcus* species, lineages and unusual AMR mechanisms across healthy humans and animals and their relatedness with international strains.

5.0 Conclusiones

1. Las comunidades bacterianas de las cavidades nasal y traqueal de los pollos de cigüeña fueron muy diversas, predominando los estafilococos, particularmente la especie *S. sciuri.*

2. Un tercio de los pollos de cigüeña fueron portadores nasotraqueales de *S. aureus,* siendo significativamente más frecuente en muestras nasales. Además, se detectaron hasta 13 especies de SCoN.

3. Todos los *S. aureus* recuperados de las muestras de cigüeña fueron SASM y se detectó la línea genética SASM-CC398 en el 8,2% de las mismas. Tanto las cepas de *S. aureus* como las de SCoN presentaron bajas tasas de resistencia a los antibióticos.

4. La detección de genes inusuales de resistencia a los antimicrobianos (como *mecC* y *ermT*), la diversidad de tipos de SCC*mec* en SCoN y la identificación de genes de virulencia relevantes en *S. aureus* de cigüeñas resaltan la necesidad de una vigilancia meticulosa y exhaustiva de la vida silvestre.

5. Los humanos sanos (que no tuvieron contacto con animales) fueron portadores nasales frecuentes de *S. aureus* (37%), siendo la única especie de SCoP detectada. De las cepas SASM, CC398 fue el linaje más frecuente (33%), seguido de CC121 (18%). La resistencia a la meticilina se detectó con frecuencia entre los SCoN. Es necesario monitorear la detección de linajes toxigénicos y virulentos de *S. aureus* y también de cepas multirresistentes-SCoN por sus posibles implicaciones para la salud humana.

6. Se detectaron SCoP en más de un tercio de los miembros de los hogares con perros, especíalmente *S. aureus* entre los dueños de los perros (principalmente SASM, incluido el linaje CC398) y *S. pseudientermedius* entre los perros. Además, se identifió la transferencia de SASM de persona a persona y de SPSM de perro a dueño de perro.

7. Se detectaron hasta nueve especies de SCoN entre perros y seis entre dueños de perros; en ambos huéspedes, *S. epidermidis* fue predominante. SCoN presentó niveles moderados de RAM en estos huéspedes.

8. Se identificaron cepas de *S. epidermidis* con líneas genéticas similares en muestras nasales de perros y dueños de perros en el mismo hogar, lo que sugiere una transmisión antroponótica de humano a perro.

9. Se encontró una tasa muy alta de portación nasal de SARM-CC398 entre cerdos y trabajadores en contacto con estos animales (60% y 70%, respectivamente), siendo también de interés el alto nivel de multirresistencia detectado en las cepas de *S. aureus* y SCoN en estos huéspedes. Se confirmaron eventos de transmisión de SARM y cepas de SCoN multirresistentes entre cerdos y trabajadores.

10. La detección de estafilococos (tanto SCoP como SCoN) y enterococos resistentes a linezolid (LZD^R) en todos los huéspedes estudiados (excepto en humanos sanos sin contacto con animales) requirió atención especial, principalmente en el entorno de granjas porcinas debido a la mayor frecuencia de detección y ubicación plasmídica de genes de resistencia a este antibiótico (*optrA* y *cfr*).

11. Dado que SASM-CC398 se detectó en tres ecosistemas estudiados (humanos sanos sin contacto con animales, dueños de perros y cigüeñas), la combinación de resistencia a eritromicina y resistencia inducible a clindamicina y la detección del gen *ermT* podrían ser marcadores de diagnóstico útiles del subclado SASM-CC398.

12. El nivel de RAM detectado en los diferentes hospedadores analizados en esta tesis parece estar correlacionado con la presión antibiótica ejercida en los diferentes ecosistemas; en orden creciente desde aves silvestres, humanos sanos (sin contacto con animales), perros-dueños de perros y cerdo-trabajadores de granjas.

13. En conjunto, este trabajo subraya la necesidad de fortalecer el enfoque epidemiológico genómico y la inclusión de todas las especies de *Staphylococcus* de todos los huéspedes (no solo cepas clínicas sino también individuos sanos) para comprender adecuadamente la propagación global de cepas resistentes a los antimicrobianos y rastrear aquellas que puedan resultar patogénicas utilizando el modelo "Una salud".

14. Esta tesis mostró la influencia de diferentes nichos ecológicos en los niveles de RAM y la presencia y/o transmisión de varias especies epidémicas de *Staphylococcus*, sus líneas genéticas y mecanismos inusuales de RAM entre humanos y animales sanos así como su relación con cepas internacionales.

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Antimicrobial resistome of coagulase-negative staphylococci from nasotracheal cavities of nestlings of *Ciconia ciconia* in Southern Spain: Detection of *mecC*-SCC*mec* type-XI-carrying *S. lentus*

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ABSTRACT

The antimicrobial resistance (AMR) genes of 268 non-duplicated coagulase-negative staphylococci (CoNS) previously obtained from nasotracheal cavities of nestling storks were characterized. They included *S. sciuri* isolates (n = 191), and non-*sciuri*-CoNS isolates (NSc-CoNS, n = 77). All *S. sciuri* carried the intrinsic *salA* gene (for clindamycin-resistance) and so, clindamycin was not considered for general analysis in this species. About 71.7%/41.6% of the *S. sciuri*/NSc-CoNS isolates were susceptible to all antibiotics tested; moreover, 14.1%/16.9% and 3.1%/20.8% of *S. sciuri*/NSc-CoNS showed single antibiotic resistance and multidrug resistance (MDR) phenotype, respectively. Of the ten *mecA*-positive CoNS isolates, six were associated with SCC*mec* types-III, -IV or -V elements. Remarkably was the detection of one MDR-*S. lentus* isolate carrying both *mecA* and *mecC* genes, as well as the SCC*mec* type-XI element. MDR-CoNS was relatively higher in nestlings of parent storks foraging in landfills (21.3%) than those in natural areas (9.7%) ($\chi^2 = 3.421$, df=1, $p = 0.064$). AMR phenotypes (and genes detected) include penicillin (*blaZ*, bla_{ARL}), erythromycin-clindamycin-constitutive (*ermA, ermC, ermT*), clindamycin (*lnuA, salA, vgaA*), erythromycin (*msrA, mphC*), tetracycline (*tetK, tetL, tetM*), tobramycin (*ant4′*), tobramycin-gentamicin (*aac6′-aph2″*), sulfamethoxazole-trimethoprim (*dfrA, dfrG, dfrK*), chloramphenicol (*fexA, fexB, catPC221*), and mupirocin (*mupA*). Interestingly, one *S. epidermidis* isolate carried the *ermT* gene. About 29.9% of nestlings harboured more than one non-duplicated CoNS (with varied 2–5 AMR profiles). This study demonstrated that most of the CoNS isolates were susceptible to all the antibiotics tested (63.1%). However, AMR genes of public health importance were found, including the *mecC*-mediated methicillin resistance trait.

1. Introduction

Antimicrobial resistance (AMR) develops from the natural defense of microbes against antimicrobial agents through genetic processes [1]. However, the selective pressure developed from the use of antimicrobial agents in humans, animals (especially livestock and pets), as well as in aquaculture and even in plant production is the major driver of the emergence, re-emergence, and dissemination of AMR in bacteria [2]. The sharing of common habitats, water and food could result in the transfer of antimicrobial-resistant bacteria (ARB) between wildlife, food-producing animals and humans. Several bacterial species with transferable AMR genes have been reported in wildlife [3]. Interestingly, wildlife is considered a well-established source of ARB entering the food chain through meat and green foods [3]. However, the eco-epidemiological relevance of wildlife as reservoirs and routes of AMR transfer in bacterial pathogens need to be fully elucidated.

The dynamic epidemiology of the most relevant *Staphylococcus* spp (i.e., *S. aureus*) has been extensively studied in many ecological niches, however, not so many detailed reports are available on the coagulase-negative staphylococci (CoNS). Even, some CoNS species are emerging pathogens with clinical and public health importance carrying critical AMR and virulence genes [4]. Some CoNS are considered relevant bacterial aetiology of nosocomial as well as community-acquired infections [5–7]. Moreover, potentially pathogenic CoNS carrying

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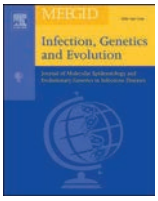

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1. Introduction

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1. Introduction

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Systematic Review

Wild Animals Are Reservoirs and Sentinels of *Staphylococcus aureus* and MRSA Clones: A Problem with "One Health" Concern

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Abstract: *Background:* The availability of comprehensive data on the ecology and molecular epidemiology of *Staphylococcus aureus*/MRSA in wild animals is necessary to understand their relevance in the "One Health" domain. *Objective:* In this study, we determined the pooled prevalence of nasal, tracheal and/or oral (NTO) *Staphylococcus aureus* (*S. aureus*) and methicillin-resistant *S. aureus* (MRSA) carriage in wild animals, with a special focus on *mecA* and *mecC* genes as well as the frequency of MRSA and methicillin susceptible *S. aureus* (MSSA) of the lineages CC398 and CC130 in wild animals. *Methodology:* This systematic review was executed on cross-sectional studies that reported *S. aureus* and MRSA in the NTO cavities of wild animals distributed in four groups: non-human primates (NHP), wild mammals (WM, excluding rodents and NHP), wild birds (WB) and wild rodents (WR). Appropriate and eligible articles published (in English) between 1 January 2011 to 30 August 2021 were searched for from PubMed, Scopus, Google Scholar, SciElo and Web of Science. *Results:* Of the 33 eligible and analysed studies, the pooled prevalence of NTO *S. aureus* and MRSA carriage was 18.5% (range: 0–100%) and 2.1% (range: 0.0–63.9%), respectively. The pooled prevalence of *S. aureus*/MRSA in WM, NHP, WB and WR groups was 15.8/1.6, 32.9/2.0, 10.3/3.4 and 24.2/3.4%, respectively. The prevalence of *mecC*-MRSA among WM/NHP/WB/WR was 1.64/0.0/2.1/0.59%, respectively, representing 89.9/0.0/59.1/25.0% of total MRSA detected in these groups of animals.The MRSA-CC398 and MRSA-CC130 lineages were most prevalent in wild birds (0.64 and 2.07%, respectively); none of these lineages were reported in NHP studies. The MRSA-CC398 (mainly of *spa*-type t011, 53%), MRSA-CC130 (mainly of *spa* types t843 and t1535, 73%), MSSA-CC398 (*spa*-types t571, t1451, t6606 and t034) and MSSA-CC130 (*spa* types t843, t1535, t3625 and t3256) lineages were mostly reported. *Conclusion:* Although the global prevalence of MRSA is low in wild animals, *mecC*-mediated resistance was particularly prevalent among MRSA isolates, especially among WM and WB. Considering the genetic diversity of MRSA in wild animals, they need to be monitored for effective control of the spread of antimicrobial resistance.

Keywords: wild animals; MRSA-CC398; *mecC*-MRSA; livestock-associated MRSA; nasal carriage; bacterial zoonosis

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1. Introduction

Antimicrobial resistance (AMR) constitutes one of the major global health challenges of the twenty-first century. The holistic approach, "One Health", is being considered as an important tool to avoid the emergence and spread of multi-drug resistant bacteria and preserve the efficacy of existing antibiotics. "One Health" is a concept of global health that emphasised the inter-relation or inter-connection of the health of humans to that of animals (pets, livestock and wild) and the environment. Among bacterial pathogens, staphylococci

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Article

Within-Host Diversity of Coagulase-Negative Staphylococci Resistome from Healthy Pigs and Pig Farmers, with the Detection of *cfr*-Carrying Strains and MDR-*S. borealis*

Idris Nasir Abdullahi [1], Carmen Lozano [1], Carmen Simón [2], Myriam Zarazaga [1] and Carmen Torres [1,*]

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Abstract: The ecology and diversity of resistome in coagulase-negative staphylococci (CoNS) from healthy pigs and pig farmers are rarely available as most studies focused on the livestock-associated methicillin-resistant *S. aureus*. This study aims to characterize the antimicrobial resistance (AMR) mechanisms, intra-host species diversity (more than one species in a host), and intra-species AMR diversity (same species with more than one AMR profile) in CoNS recovered from the nasal cavities of healthy pigs and pig farmers. One-hundred-and-one CoNS strains previously recovered from 40 pigs and 10 pig farmers from four Spanish pig farms were tested to determine their AMR profiles. Non-repetitive strains were selected ($n = 75$) and their AMR genes, SCC*mec* types, and genetic lineages were analyzed by PCR/sequencing. Of the non-repetitive strains, 92% showed a multidrug resistance (MDR) phenotype, and 52% were *mecA*-positive, which were associated with SCC*mec* types V (46.2%), IVb (20.5%), and IVc (5.1%). A total of 28% of the pigs and pig farmers had intra-host species diversity, while 26% had intra-species AMR diversity. High repertoires of AMR genes were detected, including unusual ones such as *tetO*, *ermT*, *erm43*, and *cfr*. Most important was the detection of *cfr* (in *S. saprophyticus* and *S. epidermidis*-ST16) in pigs and pig farmers; whereas MDR-*S. borealis* strains were identified in pig farmers. Pig-to-pig transmission of CoNS with similar AMR genes and SCC*mec* types was detected in 42.5% of pigs. The high level of multidrug, within-host, and intra-species resistome diversity in the nasal CoNS highlights their ability to be AMR gene reservoirs in healthy pigs and pig farmers. The detection of MDR-*S. borealis* and linezolid-resistant strains underscore the need for comprehensive and continuous surveillance of MDR-CoNS at the pig farm level.

Keywords: coagulase-negative staphylococci; *Staphylococcus borealis*; multidrug resistance; pig farms; linezolid resistance; *cfr*

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1. Introduction

Antimicrobial resistance (AMR) is one of the greatest global health threats of the late 21st century [1,2]. The global AMR crisis has persisted mainly due to the transfer of antibiotic-resistant bacteria between animals, humans, and the environment through their shared habitats [3]. The emergence and spread of antibiotic-resistant staphylococci are often blamed on the over-prescription of antibiotics for treatment in humans and animals and as growth enhancers in livestock production [4]. The use of antibiotics as growth enhancers is now banned in many countries, but this is still allowed in others.

Coagulase-negative staphylococci (CoNS) are primarily nasal commensals, although some strains can be opportunistic pathogens; they have been implicated in many infections in humans and animals such as catheter-associated, prosthetic joint, and laryngeal infections or sepsis, among others [5,6]. Recently, new CoNS species have been re-classified. In this regard, it is important to mention the reclassification of *S. borealis* nov. sp., which was

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Clonal relatedness of coagulase-positive staphylococci among healthy dogs and dog-owners in Spain. Detection of multidrug-resistant-MSSA-CC398 and novel linezolid-resistant-MRSA-CC5

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Introduction: Nasal carriage of coagulase-positive staphylococci (CoPS) in healthy dogs could indicate increased risks of colonization for in-contact people or vice versa. This study determined the nasal carriage rate of CoPS among healthy dogs and in-contact people, their genotypic characteristics and phylogenetic relatedness.

Methods: Nasal samples were collected from 27 households (34 dogs and 41 humans) in Spain. Staphylococci were identified by MALDI-TOF-MS, their antimicrobial resistance (AMR) genes and *spa*-types were tested by PCR/sequencing. The relatedness of CoPS from the same households was assessed by core genome single nucleotide polymorphisms (SNPs) analyses.

Results: *Staphylococcus aureus* carriage was found in 34.1% of humans (including one methicillin-resistant *S. aureus* MRSA-CC5-t2220-SCC*mec* type-IV2B) and 5.9% of dogs; *Staphylococcus pseudintermedius* in 2.4% of humans and 32.4% of dogs; while *Staphylococcus coagulans* was only detected in dogs (5.4%). Remarkably, one human co-carried *S. aureus*/*S. pseudintermedius*, while a dog co-carried the three CoPS species. Household density was significantly associated with *S. pseudintermedius* carriage in households with > than 1 dog and >than 1 human (OR=18.10, 95% CI: 1.24–260.93, p=0.034). Closely related (<15 SNPs) *S. aureus* or *S. pseudintermedius* were found in humans or dogs in three households. About 56.3% *S. aureus* carriers (dog or human) harboured diverse within-host *spa*-types or AMR genotypes. Ten clonal complexes (CCs) were detected among the *S. aureus*, of which methicillin-susceptible *S. aureus*-CC398-IEC-type C (t1451 and t571) was the most frequent, but exclusive to humans. *S. aureus* and *S. pseudintermedius* isolates harboured resistance genes or mutations associated to 9 classes of antimicrobials including linezolid (G2261A & T1584A point mutations in 23S rDNA). The *S. coagulans* isolates were susceptible to all antimicrobials. Most of the *S. pseudintermedius* carried *lukS/F-I, siet,* and *sient* genes, and all *S. aureus* were negative for *lukS/F*-PV, *tst-1, eta* and *etb* genes.

Discussion: Clonally related human-to-human MSSA and dog-to-human MSSP were found. The detection of the MSSA-CC398 clade highlights the need for its continuous surveillance from One Health perspective.

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REVIEW ARTICLE

Nasal *Staphylococcus aureus* and *S. pseudintermedius* carriage in healthy dogs and cats: a systematic review of their antibiotic resistance, virulence and genetic lineages of zoonotic relevance

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Abstract

The molecular ecology of *Staphylococcus aureus*, *Staphylococcus pseudintermedius* and their methicillin-resistant strains in healthy dogs and cats could serve as good models to understand the concept of bacterial zoonosis due to animal companionship. This study aims to provide insights into pooled prevalence, genetic lineages, virulence and antimicrobial resistance (AMR) among healthy dogs and cats. Original research and brief communication articles published from 2001 to 2021 that reported the nasal detection of *S. aureus* and *S. pseudintermedius* in healthy dogs and cats in the community, homes and outside veterinary clinics were examined and analysed. Forty-nine studies were eligible and included in this systematic review. The pooled prevalence of nasal carriage of *S. aureus*/methicillin-resistant *S. aureus* (MRSA) in healthy dogs and cats were 10.9% (95% CI: 10.1–11.9)/2.8% (95% CI: 2.4–3.2) and 3.2% (95% CI: 1.9–4.8)/0.5% (95% CI: 0.0–1.1), respectively. Conversely, the pooled prevalence of *S. pseudintermedius*/methicillin-resistant *S. pseudintermedius* (MRSP) in healthy dogs and cats were 18.3% (95% CI: 17.1–19.7)/3.1% (95% CI: 2.5–3.7) and 1.3% (95% CI: 0.6–2.4)/1.2% (95% CI: 0.6–2.3), respectively. Although highly diverse genetic lineages of *S. aureus* were detected in healthy dogs and cats, MSSA-CC1/CC5/CC22/CC45/CC121/CC398 and MRSA-CC5/CC93/CC22/CC30 were mostly reported in dogs; and MSSA-CC5/CC8/CC15/CC48 and MRSA-CC22/CC30/CC80 in cats. Of note, MSSA-CC398 isolates (*spa*-types t034 and t5883) were detected in dogs. Genetic lineages often associated with MSSP/MRSP were ST20/ST71, highlighting the frequent detection of the epidemic European MRSP-ST71 clone in dogs. *S. aureus* isolates carrying the *luk-S/F-PV*, *tst*, *eta*, *etb* and *etd* genes were seldomly detected in dogs, and *luk-S/F-PV* was the unique virulence factor reported in isolates of cats. *S. pseudintermedius* isolates harbouring the *luk-S/F-I*, *seint* and *expA* genes were frequently found, especially in dogs. High and diverse rates of AMR were noted, especially among MRSA/MRSP isolates. There is a need for additional studies on

pathogens

MDPI

Review

Ecology and Genetic Lineages of Nasal *Staphylococcus aureus* and MRSA Carriage in Healthy Persons with or without Animal-Related Occupational Risks of Colonization: A Review of Global Reports

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Abstract: In this conceptual review, we thoroughly searched for appropriate English articles on nasal staphylococci carriage among healthy people with no reported risk of colonization (Group A), food handlers (Group B), veterinarians (Group C), and livestock farmers (Group D) published between 2000 and 2021. Random-effects analyses of proportions were performed to determine the pooled prevalence of *S. aureus*, MRSA, MRSA-CC398, and MSSA-CC398, as well as the prevalence of PVL-positive *S. aureus* from all eligible studies. A total of 166 eligible papers were evaluated for Groups A/B/C/D (n = 58/31/26/51). The pooled prevalence of *S. aureus* and MRSA in healthy humans of Groups A to D were 15.9, 7.8, 34.9, and 27.1%, and 0.8, 0.9, 8.6, and 13.5%, respectively. The pooled prevalence of MRSA-CC398 nasal carriage among healthy humans was as follows: Group A/B (<0.05%), Group C (1.4%), Group D (5.4%); and the following among Group D: pig farmers (8.4%) and dairy farmers (4.7%). The pooled prevalence of CC398 lineage among the MSSA and MRSA isolates from studies of the four groups were Group A (2.9 and 6.9%), B (1.5 and 0.0%), C (47.6% in MRSA), and D (11.5 and 58.8%). Moreover, MSSA-CC398 isolates of Groups A and B were mostly of *spa*-t571 (animal-independent clade), while those of Groups C and D were *spa*-t011 and t034. The MRSA-CC398 was predominately of t011 and t034 in all the groups (with few other *spa*-types, livestock-associated clades). The pooled prevalence of MSSA and MRSA isolates carrying the PVL encoding genes were 11.5 and 9.6% (ranges: 0.0–76.9 and 0.0–28.6%), respectively. Moreover, one PVL-positive MSSA-t011-CC398 isolate was detected in Group A. Contact with livestock and veterinary practice seems to increase the risk of carrying MRSA-CC398, but not in food handlers. Thus, this emphasizes the need for integrated molecular epidemiology of zoonotic staphylococci.

Keywords: *Staphylococcus aureus*; MRSA; nasal colonization; genetic lineages; CC398; livestock; *S. pseudintermedius*; CoNS

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1. Introduction

Many coagulase-positive and negative staphylococci are normal microbiota of the nasal cavity. However, some of them are of great public health importance due to their capacity to produce staphylococcal infections and diseases in humans and animals, and being responsible for zoonosis [1,2].

The main reservoir site for *staphylococcal* nasal carriage is the anterior nares and vestibules [3]. *Staphylococcus aureus* (*S. aureus*) is the most important nasal staphylococci and has been found in about 30% of healthy adults [3]. It was estimated that previous nasal colonization in 30% of the cases of bacteremia was due to *S. aureus* [4]. Essentially, *S. aureus* is an important cause of community-acquired (CA) and hospital-acquired (HA)

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RESEARCH

Staphylococcus aureus Carriage in the Nasotracheal Cavities of White Stork Nestlings (*Ciconia ciconia*) in Spain: Genetic Diversity, Resistomes and Virulence Factors

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Abstract
The molecular ecology of *Staphylococcus aureus* in migratory birds (such as white storks) is necessary to understand their relevance in the "One Health" ecosystems. This study determined the nasotracheal carriage rates of *S. aureus* from white storks in Southern Spain and genetically characterized the within-host diversity. A collection of 67 *S. aureus* strains, previously obtained from 87 white stork nestlings (52 nasal and 85 tracheal samples) fed by their parents with food foraged in natural and landfill habitats, were tested for their antimicrobial resistance (AMR) phenotypes. Moreover, the AMR genotypes, immune evasion cluster (IEC), virulence genes and the detection of CC398 lineage were studied by PCR. The *spa* types and multilocus-sequencing-typing (MLST) were also determined by PCR and sequencing. *Staphylococcus aureus* carriage was found in 31% of storks (36.5%/11.9% in nasal/tracheal samples). All isolates were methicillin-susceptible (MSSA) and 8.8% of them were also susceptible to all tested antibiotics. The AMR phenotype/percentage/genes detected were as follows: penicillin/79.1%/*blaZ*; erythromycin-clindamycin-inducible/19.1%/*ermA, ermT*; tetracycline/11.9%/*tetK*; clindamycin/4.5%/*lnuA* and ciprofloxacin/4.5%. Twenty-one different *spa* types, including 2 new ones (t7778-ST15-CC15 and t18009-ST26-CC25), were detected and ascribed to 11 clonal complexes (CCs). MSSA-CC398 (8.2%), MSSA-CC15 (7.1%) and MSSA-ST291 (5.9%) were the most prevalent lineages in storks. Moreover, *tst*-positive (MSSA-CC22-t223 and MSSA-CC30-t1654), *eta*-positive (MSSA-CC9-t209) and *etb*-positive strains (MSSA-CC45-t015) were detected in four storks. The 18.5% of storks harboured distinct MSSA strains (with different lineages and/or AMR genes). Nestlings of storks foraging in landfills (10 CCs) had more diverse *S. aureus* strains than those of parents foraging in natural habitats (3 CCs). Low level of AMR was demonstrated among *S. aureus* strains. The predominance of MSSA-CC398 (an emergent clade) and toxigenic MSSA strains in stork nestlings highlight the need for continuous surveillance of *S. aureus* in wild birds.

Keywords *Staphylococcus aureus* · MSSA-CC398 · Wild birds · MSSA-ST291 · Antimicrobial resistance · White storks

Introduction

Humans are dependent on healthy ecosystems. The "One Health" approach, under which the World Health Organisation (WHO) has based the global strategy to tackle the problem of antimicrobial resistance (AMR), is based on the close link between human, animal and environmental health [1]. The recent focus on the "One Health" framework of research includes wild animals such as migratory birds [2].

Recently, white stork (*Ciconia ciconia*) among other wild birds (such as starlings, cowbirds and gulls) has attracted interest in the study of bacterial ecology and epidemiology as they have been shown to forage in human settlements, farmlands and dumpsites (which may include urban and hospital wastes) [3–5]. Also, during migration, storks can travel long distances across continents such as Africa to Europe and vice versa. These phenomena make storks potential reservoirs and carriers for transcontinental transmission of bacteria of public health concerns [2, 6].

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ORIGINAL ARTICLE

Nasotracheal enterococcal carriage and resistomes: detection of *optrA*-, *poxtA*- and *cfrD*-carrying strains in migratory birds, livestock, pets, and in-contact humans in Spain

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Abstract

This study determined the carriage rates and antimicrobial resistance (AMR) genes of enterococci from nasotracheal samples of three healthy animal species and in-contact humans. Nasal samples were collected from 27 dog-owning households (34 dogs, 41 humans) and 4 pig-farms (40 pigs, 10 pig-farmers), and they were processed for enterococci recovery (MALDI-TOF–MS identification). Also, a collection of 144 enterococci previously recovered of tracheal/nasal samples from 87 white stork nestlings were characterized. The AMR phenotypes were determined in all enterococci and AMR genes were studied by PCR/sequencing. MultiLocus-Sequence-Typing was performed for selected isolates. About 72.5% and 60% of the pigs and pig-farmers, and 29.4% and 4.9%, of healthy dogs and owners were enterococci nasal carriers, respectively. In storks, 43.5% of tracheal and 69.2% of nasal samples had enterococci carriages. Enterococci carrying multidrug-resistance phenotype was identified in 72.5%/40.0%/50.0%/23.5%/1.1% of pigs/pig-farmers/dogs/dogs' owners/storks, respectively. Of special relevance was the detection of linezolid-resistant enterococci (LRE) in (a) 33.3% of pigs (*E. faecalis*-carrying *optrA* and/or *cfrD* of ST59, ST330 or ST474 lineages; *E. casseliflavus*-carrying *optrA* and *cfrD*); (b) 10% of pig farmers (*E. faecalis*-ST330-carrying *optrA*); (c) 2.9% of dogs (*E. faecalis*-ST585-carrying *optrA*); and (d) 1.7% of storks (*E. faecium*-ST1736-carrying *poxtA*). The *fexA* gene was found in all *optrA*-positive *E. faecalis* and *E. casseliflavus* isolates, while *fexB* was detected in the *poxtA*-positive *E. faecium* isolate. The enterococci diversity and AMR rates from the four hosts reflect differences in antimicrobial selection pressure. The detection of LRE carrying acquired and transferable genes in all the hosts emphasizes the need to monitor LRE using a One-Health approach.

Keywords Nasal enterococci · Antimicrobial resistomes · Linezolid resistance · *optrA* · *cfrD* · *poxtA* · Migratory birds · Livestock · Pets

Introduction

Enterococcus spp. are commensals and predominantly found in the intestinal habitat, but they might be translocated to other animal tissues or organs [1]. Among the over 50 different enterococci species, *Enterococcus faecium* (*E. faecium*) and *Enterococcus faecalis* (*E. faecalis*) constitute most of the gastrointestinal tract (GI) enterococci communities in humans [2]. However, in livestock, *E. faecium*, *E. cecorum*, *E. faecalis* and, to some extent, *E. hirae* predominate [3]. In contrast, *E. mundtii* and *E. casseliflavus* are commonly found in plant and environmental samples [2, 4]. Moreover, the ecologic-epidemiology of *E. faecalis* and *E. faecium* has

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EcoHealth

Original Contribution

Nasotracheal Microbiota of Nestlings of Parent White storks with Different Foraging Habits in Spain

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Abstract: Migratory storks could be vectors of transmission of bacteria of public health concern mediated by the colonization, persistence and excretion of such bacteria. This study aims to determine genera/species diversity, prevalence, and co-colonization indices of bacteria obtained from tracheal (T) and nasal (N) samples from storks in relation to exposure to point sources through foraging. One-hundred and thirty-six samples from 87 nestlings of colonies of parent white storks with different foraging habits (natural habitat and landfills) were obtained (84 T-samples and 52 N-samples) and processed. Morphologically distinct colonies (up to 12/sample) were randomly selected and identified by MALDI-TOF-MS. About 87.2% of the total 806 isolates recovered were identified: 398 from T-samples (56.6%) and 305 from N-samples (43.4%). Among identified isolates, 17 genera and 46 species of Gram-positive and Gram-negative bacteria were detected, *Staphylococcus* (58.0%) and *Enterococcus* (20.5%) being the most prevalent genera. *S. sciuri* was the most prevalent species from T (36.7%) and N (34.4%) cavities of total isolates, followed by *E. faecalis* (11.1% each from T and N), and *S. aureus* [T (6.5%), N (13.4%)]. Of N-samples, *E. faecium* was significantly associated with nestlings of parent storks foraging in landfills ($p = 0.018$). *S. sciuri* ($p = 0.0034$) and *M. caseolyticus* ($p = 0.032$) from T-samples were significantly higher among nestlings of parent storks foraging in natural habitats. More than 80% of bacterial species in the T and N cavities showed 1–10% co-colonization indices with one another, but few had $\geq$ 40% indices. *S. sciuri* and *E. faecalis* were the most frequent species identified in the stork nestlings. Moreover, they were highly colonized by other diverse and potentially pathogenic bacteria. Thus, storks could be sentinels of point sources and vehicles of bacterial transmission across the ''One Health'' ecosystems.

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UNIVERSIDAD DE LA RIOJA

Resistome, virulome and mobilome of nasal staphylococci from healthy humans and animals: A One Health approach with public health implications

Resistoma, viruloma y mobiloma de estafilococos nasales de personas y animales sanos: Un enfoque *One Health* con implicaciones en salud Publica

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PhD thesis with International Mention

Idris Nasir Abdullahi

Logroño (Spain), 2023