UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIA BIOLÓGICAS
DEPARTAMENTO DE GENÉTICA, ECOLOGIA E EVOLUÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

ALESSANDRA LIMA DA SILVA

Genômica comparativa de *Staphylococcus aureus* isolados de mastite em ruminantes

Belo Horizonte
2022

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Tese apresentada ao Programa de Pós-graduação em Genética da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Genética.

Orientador: Prof. Dr. Vasco A. C. Azevedo

Coorientador: Dr. Thiago Sousa e Dr. Marcus Viana

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UNIVERSIDADE FEDERAL DE MINAS GERAIS
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Instituto de Ciências Biológicas

ATA DE DEFESA DE TESE

ATA DA DEFESA DE TESE	**168/2022** **entrada**
ALESSANDRA LIMA DA SILVA	**1°/2018** **CPF: 051.816.555-80**

Às **09:00hs (nove) horas** do dia **12 de dezembro de 2022**, reuniu-se a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"Genômica comparativa de Staphylococcus aureus isolados de mastite em ruminantes"**, requisito para obtenção do grau de Doutora em **Genética.** Abrindo a sessão, o Presidente da Comissão, **Vasco Ariston de Carvalho Azevedo**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	**Instituição**	**CPF**	**Indicação**
Vasco Ariston de Carvalho Azevedo	UFMG	283.171.225-49	Aprovada
Marcus Vinicius Canário Viana	LACEN-ES	074.734.266-05	Aprovada
Thiago de Jesus Sousa	UFMG	041.698.435-50	Aprovada
Renato Santana de Aguiar	UFMG	000.086.336-06	Aprovada
Rodrigo Dias de Oliveira Carvalho	UFBA	080.173.536-00	Aprovada
Túlio Marcos Santos	PUC Minas	675.862.016-68	Aprovada
Siomar de Castro Soares	UFTM	056.951.826-11	Aprovada
Wanderson Marques da Silva	INTA-Argentina	014.183.526-59	Aprovada

Pelas indicações, a candidata foi considerada: **APROVADA**.

O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 12 de dezembro de 2022.

Vasco Ariston de Carvalho Azevedo - Orientador

Marcus Vinicius Canário Viana - Coorientador

Thiago de Jesus Sousa - Coorientador

Renato Santana de Aguiar

Rodrigo Dias de Oliveira Carvalho

Siomar de Castro Soares

Túlio Marcos Santos

Wanderson Marques da Silva

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UNIVERSIDADE FEDERAL DE MINAS GERAIS
Programa de Pós-Graduação em Genética
Instituto de Ciências Biológicas

FOLHA DE APROVAÇÃO

"Genômica comparativa de Staphylococcus aureus isolados de mastite em ruminantes"

ALESSANDRA LIMA DA SILVA

Tese aprovada pela banca examinadora constituída pelos Professores:

Vasco Ariston de Carvalho Azevedo - Orientador
UFMG

Marcus Vinicius Canário Viana - Coorientador
UFMG

Thiago de Jesus Sousa - Coorientador
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Túlio Marcos Santos
PUC Minas

Siomar de Castro Soares
UFTM

Wanderson Marques da Silva
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Dedico esta, bem como todas as minhas demais conquistas, aos meus amados pais Givaldete e José Cícero, pelo incentivo, pela força e principalmente pelo carinho. Valeu a pena toda distância, todo sofrimento e todas as renúncias. Também ao meu fiel companheiro de longas datas Lucas Emmanuel, meu esposo, sempre servindo como um alicerce e não me deixando desistir em dias ruins. E ao nosso filho Cícero, que já encheu nosso lar de esperança e alegria.

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May the force be with you!

“O passado pode doer, mas do jeito que eu vejo você pode fugir dele ou aprender com ele.” (Rafiki, O Rei Leão)

RESUMO

Mastite é uma infecção na glândula mamária que acomete ruminantes, podendo afetar a quantidade e qualidade do leite do animal acometido, que quando ingerido pode causar intoxicação alimentar em humanos. Os principais patógenos responsáveis pelos casos de mastite em animais de produção são os *Staphylococcus* spp. O gênero é dividido entre dois grupos, baseado na capacidade de coagulação do plasma ou sangue. *Staphylococcus* coagulase positivas (CoPS) ocorre majoritariamente na espécie de *S. aureus. Staphylococcus* coagulase negativas (CoNS) são as demais espécies de estafilococos. As CoPS são as que causam maiores prejuízos para o rebanho e os CoNS são considerados patógenos emergentes e vêm ocasionando graves prejuízos sobre a qualidade do leite. A identificação de *S. aureus* coagulase negativa é considerado um evento atípico no diagnóstico clínico, acarretando a caracterização errônea da espécie, como *S.* não *aureus*, caso o resultado do teste de aglutinação seja negativo. *S. aureus* é uma bactéria tanto comensal quanto patogênica, sendo considerada o principal agente etiológico da mastite em ruminantes, apresentando uma difícil cura, possível reincidência e comprometendo a economia e saúde pública mundialmente. Esta tese foi feita com o propósito de sequenciar e analisar genomas de *S. aureus* isolados de mastite em ovinos e bovinos oriundos da França e Brasil. A tese é composta por dois artigos, o primeiro analisa a especificidade, a relação evolutiva e características que podem estar associadas à patogenia de *S. aureus* isoladas de mastite em ovinos. Em geral, os genomas de ovinos apresentam uma alta similaridade nicho-hospedeiro, podendo ser classificados a nível de tipagem molecular e filogenia, ao contrário de sequencias oriundas de bovinos e humanos. O segundo artigo, analisa a nível genômico amostras isoladas de mastite bovina no Brasil, identificadas *in vitro* por meio do teste de coagulase e MALDI-TOF MS como *S. aureus* coagulase-negativas, com o objetivo de identificar particularidades envolvendo estas amostras e sequencias de *S. aureus* coagulase positivas. duas amostras anteriormente identificadas como *S. aureus* coagulase negativas foram classificadas como *S. capiti*. O resultado que mais se destacou foi o comportamento do gene responsável pela expressão da proteína *von Willebrand factor-binding protein* (*vWbp*) que, assim como a enzima coagulase, também é importante no processo de coagulação. No alinhamento de *vWbp* foi identificado uma fragmentação apenas nas sequencias referentes às amostras de *S. aureus* coagulase negativa, podendo estar relacionado à ausência de coagulação nestas amostras. Os resultados obtidos geram novos conhecimentos sobre a arquitetura genômica associada ao risco zoonótico de *S. aureus*. Além disso, este trabalho pode ser considerado um dos primeiros estudos genômicos de *S. aureus* coagulase negativa isoladas de mastite em bovinos, abrindo caminhos para novos estudos filogenômicos do gênero *Staphylococcus*.

Palavras-chave: Genômica comparativa, mastite, ruminante, *Staphylococcus aureus* coagulase-negativa, estafilocoagulase.

ABSTRACT

Mastitis is an infection of the mammary gland that affects ruminants and can affect the quantity and quality of milk from the affected animal, which when ingested can cause food poisoning in humans. The primary pathogen responsible for cases of mastitis in production animals are Staphylococcus spp. The genus is divided into two groups, based on the ability to coagulate plasma or blood. Coagulase positive Staphylococcus (CoPS) occurs mostly in the S. aureus species. Coagulase negative Staphylococcus (CoNS) are the other species of staphylococci. CoPS are the most damaging for the herd and CoNS are considered emerging pathogens and have been causing serious damage to the quality of milk. Identifying coagulase-negative S. aureus is considered an atypical event in clinical diagnosis. The erroneous characterization of the species as S. non-aureus if coagulation does not occur. S. aureus is both a commensal and pathogenic bacterium; it is considered the primary aetiology of mastitis in ruminants, presenting a problematic cure, possible recurrence, and compromising the economy and public health worldwide. This thesis was done with the purpose of sequencing and analyzing genomes of S. aureus isolated from mastitis in ovine and bovine from France and Brazil. The thesis is composed of two papers, the first analyzes the specificity, evolutionary relationship and characteristics that may be associated with the pathogenicity of S. aureus isolated from mastitis in ovine. In general, ovine genomes show a high niche-host similarity and can be classified at the molecular typing and phylogeny level, unlike sequences from cattle and humans. The second article analyzes at genomic level samples isolated from bovine mastitis in Brazil, identified in vitro by coagulase test and MALDI-TOF MS as coagulase-negative S. aureus, aiming to identify particularities involving these samples and sequences of coagulase-positive S. aureus. Two samples previously identified as coagulase-negative S. aureus were classified as S. capiti. The result that stood out the most was the behaviour of the gene responsible for the expression of the von Willebrand factor-binding protein (vWbp), besides the enzyme coagulase, which is also essential in the coagulation process. In the vWbp alignment, fragmentation was identified only in the sequences referring to the coagulase-negative S. aureus samples; this may be related to the absence of coagulation in these samples. The results generate new knowledge about the genomic architecture associated with the zoonotic risk of S. aureus. Furthermore, this work can be considered one of the first genomic studies for coagulase-negative S. aureus isolated from mastitis in bovine, opening the way for new studies to classify the genus of Staphylococcus.

Keywords: *Comparative genomics, mastitis, ruminant, coagulase-negative Staphylococcus aureus, staphylocoagulase.*

PREFÁCIO

I.1 COLABORADORES

Este trabalho foi realizado no Laboratório de Genética Celular e Molecular (LGCM) do instituto de Ciências Biológicas (ICB) da Universidade Federal de Minas Gerais (UFMG) coordenado pelo Prof. Vasco Ariston de Carvalho Azevedo, com colaboração entre os seguintes pesquisadores em ordem alfabética:

Prof. Dr. Brenig Bertram – pesquisador e professor na *Georg August University Göttingen*, Alemanha.

Profª Drª Elaine Dorneles – pesquisadora e professora no Departamento de Medicina Veterinária da Escola de Veterinária da UFLA (Universidade Federal de Lavras), Brasil.

Profª Drª Flávia Aburjaile – pesquisadora e professora no Departamento de Medicina Veterinária da Escola de Veterinária da UFMG.

Prof. Dr. Geraldo Costa - pesquisador e professor no Departamento de Medicina Veterinária da UFLA (Universidade Federal de Lavras), Brasil

Prof. Dr. Henrique César Pereira Figueiredo, professor da Escola de Veterinária, Departamento de Medicina Veterinária Preventiva e coordenador do Laboratório (AQUACEN) pela UFMG.

Prof. Dr. Mateus Matiuzzi – pesquisador e professor no Departamento de Medicina Veterinária Preventiva da Escola de Veterinária da UNIVASF (Universidade Federal do Vale do São Francisco), Brasil.

Dr. Yves Le Loir – pesquisador no INRAE **(***French National Research Institute for Agriculture, Food and Environment*), França.

O trabalho foi apoiado pela: Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

I.2 PESQUISA GENÔMICA DE *STAPHYLOCOCCUS AUREUS*

Em 2018, o Laboratório de Genética e Biologia Molecular (LGCM), recebeu doze amostras de *S. aureus* isoladas de diferentes estágios de mastite ovina, anos e regiões geográficas da França, fornecidos pelo pesquisador Dr. Yves Le Loir. Esses isolados foram encaminhados para o Dr. Brenig Bertram a fim de realizar o sequenciamento genômico, utilizando a plataforma de sequenciamento Illumina Hiseq. Em 2018, esses genomas foram montados, anotados e depositados pela equipe com o intuito de explorar as características genômicas desses isolados, sendo um trabalho pioneiro em estudos envolvendo genomas de *S. aureus* isolados de mastite em ovinos. As análises que necessitaram de uma capacidade alta de processadores foram realizadas utilizando servidores de alto desempenho, com o colaborador Prof. Dr. Henrique Figueiredo. Participei nas etapas de curadoria da montagem, depósito na plataforma de banco de dados público de genomas o NCBI (*National Center for Biotechnology Information*) e todas as análises comparativas geradas no trabalho. Esse material foi utilizado no desenvolvimento do primeiro artigo da tese, intitulado "*Genome host-specificity in Staphylococcus aureus isolated from ovine mastitis*", onde busca analisar a especificidade pelo

hospedeiro, a relação evolutiva entre os isolados e características que podem estar associadas à virulência, a fim de identificar e compreender traços envolvidos na especialização por hospedeiro, utilizando dados genômicos e contribuindo assim para o desenvolvimento de novas estratégias de controle e tratamento dessa infecção.

Durante a qualificação da Tese em 2020, diante resultados como a identificação de *Staphyloferrin B synthase* e *Staphyloferrin B transporter* e a predição de genes ligados a virulência e resistência apresentados *in silico*, foram sugeridas algumas análises fenotípicas. Por exemplo, teste de susceptibilidade antimicrobiana à penicilina, fosfomicina, norfloxacina, lincomicina e tetraciclina; identificação *in vitro* da produção de biofilme, testes fenotípicos de sideróforo e alfa e beta hemolisinas. Através da colaboração com o Prof. Dr. Mateus Matiuzzi foi possível realizar essas etapas, e enviamos o material e reagentes para os testes serem realizados. Infelizmente, devido a pandemia COVID-19, alguns resultados não foram gerados até a presente data de escrita da tese. Todos os 12 isolados produziram biofilme, além disso foram testadas 12 concentrações de 512 a 0,250 microgramas/ml de penicilina, tetraciclina e norfloxacina. Todas inibiram o crescimento bacteriano, confirmando a susceptibilidade. Posteriormente foi reduzida a concentração do estoque para testar abaixo de 0,250 micrograma/ml. Apenas fosfomicina apresentou concentrações mais altas. Neste mesmo ano, a EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) enviou para o LGCM três amostras identificadas como *Staphylococcus aureus* isoladas de leite de cabra diagnosticadas com mastite. Essas amostras foram isoladas e enviadas para sequenciamento. Apenas uma amostra retornou do sequenciamento, pois as outras duas apresentaram baixa qualidade. O isolado retornado foi submetido ao pipeline de montagem de genomas de *S. aureus.* Durante as etapas de análise de qualidade do sequenciamento e identificação de espécie, o genoma obteve maiores valores de identidade com genomas representantes de *Enterococcus faecalis* e o conteúdo GC (conteúdo de guanina-citosina) não era coerente com os valores esperados para *S. aureus*.

Em 2021, os nossos colaboradores, Profª. Drª Elaine Dorneles e o Prof. Dr. Geraldo Costa, forneceram um banco de dados contendo mais de 400 amostras identificadas como *S. aureus* isolados de mastite de bovino de diferentes regiões do Sudeste do Brasil. Todas as amostras foram testadas fenotipicamente através dos testes de coagulase, catalase, produção de biofilme, susceptibilidade antimicrobiana, presença de enterotoxinas, hemolisinas e outros. Dessas, foram selecionadas 102 amostras de *S. aureus* isoladas de mastite em bovino para sequenciamento, 90 das quais fazem parte da dissertação "*Detection of virulence and antimicrobial resistance genes in Staphylococcus aureus isolated from bovine mastitis in Brazil*" (Pérez, 2019, disponível em http://repositorio.ufla.br/jspui/handle/1/34712). Além dessas amostras, a Profª. Drª Elaine e o Prof. Dr. Geraldo tinham em seu banco de *S. aureus* no Laboratório de Epidemiologia Molecular seis amostras caracterizadas como *S. aureus* Coagulase-negativa, exploradas epidemiologicamente no trabalho de dissertação "Caracterização molecular e fenotípica de *Staphylococcus* coagulase negativos isolados de mastite bovina" (Custódio, 2019, disponível em http://repositorio.ufla.br/handle/1/34242). As outras seis amostras fornecidas, foram isoladas de amostras clínicas. Todo o material foi enviado para o Prof. Dr. Brenig Bertram, para a realização do sequenciamento genômico, utilizando a plataforma de sequenciamento Illumina Hiseq. Em dezembro de 2021, as amostras retornaram do sequenciamento. Dentre as 102 amostras sequenciadas, três isolados apresentaram baixa qualidade, não sendo possível realizar o sequenciamento delas. Um desafio

de tese foi lançado e em janeiro de 2022 e todos os 99 isolados que apresentaram boa qualidade foram submetidos ao pipeline de montagem de genomas referente a *S. aureus*. As amostras caracterizadas como *S. aureus* Coagulase-negativa foram utilizadas para o desenvolvimento do artigo 2 da tese, intitulado "*Genome-Wide analysis of coagulase-negative Staphylococcus spp.".* Utilizando sequenciamento e ferramentas de bioinformática foi possível realizar um uma análise comparativa entre genomas de *S. aureus* coagulase negativa e positiva, sendo abordado suas especificidades e possíveis genes que podem ser utilizados para um diagnóstico mais preciso de *S. aureus*, sobretudo para casos atípicos de coagulase-negativa. Na literatura, não foi identificado nenhum trabalho genômico referente a *S. aureus* coagulase-negativa advindos de mastite bovina.

I.3 ESTRUTURA DA TESE

A presente tese foi redigida no formato de "artigos científicos", sendo dividida em nove seções:

A 1ª seção contém uma introdução geral onde apresentará conceitos gerais sobre o microrganismo de estudo, a patogênese e o uso das ferramentas de bioinformática buscando entender a epidemiologia genômica de *S. aureus*.

A 2ª seção compreende a justificativa dos estudos realizados.

A 3ª seção apresenta o objetivo geral e objetivos específicos do projeto.

As 4ª e **5ª seções** apresentam metodologia e resultados divididos em Capítulos (1 e 2). Cada Capítulo, contará com objetivos específicos, artigo, anexos (material suplementar):

Capítulo I:

a. Objetivos específicos;

i. Obter as sequências genômicas completas e anotadas das linhagens O55, O17, O82, O217, O267, O268, O322, O326, O331, O408, O11 e O46 de *S. aureus* isoladas de ovinos;

ii. Proceder com a tipagem molecular *in silico* das linhagens de *S. aureus* utilizadas no estudo por meio da identificação de *Sequence Types* (STs) e complexos clonais (CCs);

iii. Avaliar como as linhagens de *S. aureus* isoladas de ovinos se relacionam filogeneticamente entre si e com as demais linhagens selecionadas de outros hospedeiros;

iv. Definir um padrão de segregação filogenômica capaz de distinguir grupos de linhagens oriundos de ovinos de grupos de linhagens encontradas em outros hospedeiros;

v. Avaliar os elementos genômicos, incluindo genes, ilhas genômicas, fatores de virulência, resistência e profagos, presentes apenas nas linhagens ovinas de *S. aureus* em comparação com as linhagens de outros hospedeiros;

b. Artigo I: "*Genome host-specificity in Staphylococcus aureus isolated from ovine mastitis*". Esse artigo caracteriza genomas de *S. aureus* isolados de mastite em ovinos, com o foco em analisar a especificidade pelo hospedeiro, a relação evolutiva entre os isolados e características que podem estar

associadas à virulência, a fim de contribuir para novas estratégias de controle e tratamento dessa infecção.

c. Anexos

Capítulo II:

a. Objetivos específicos

i. Sequenciar, montar, anotar e depositar, no banco de dados do NCBI, os genomas das seis amostras 23S, 42S, 48S, 56S, 62S, 73S de *S. aureus* isoladas de bovinos;
ii. Identificar a presença de plasmídeos;
iii. Verificar a taxonomia das amostras por meio da Identidade Média de Nucleotídeos (ANI);
iv. Avaliar como as linhagens identificadas como *Staphylococcus* coagulase negativa se relacionam filogeneticamente com as *S. aureus* positivas e outras espécies;
v. Identificar genes ortólogos;
vi. Avaliar por meio de alinhamento como os genes relacionados a coagulação, as estafilocoagulases, estão agrupados;
vii. Avaliar o uso das técnicas de MALDI-TOF, teste de coagulase e sequenciamento na identificação de *S. aureus*.
viii. Predizer fatores de virulência, patogenicidade e resistência nos genomas das linhagens de *S. aureus* isoladas de casos de mastite em ovinos e bovinos.
ix. Observar a presença e ausência de fatores de virulência, patogenicidade e resistência entre os grupos de *S. aureus* coagulase positiva e negativa;

b. Artigo II: "*Genome-Wide analysis of coagulase-negative Staphylococcus spp.". Staphylococcus* coagulase negativa são identificados apenas em espécies que se diferenciam de *S. aureus.* Nesse artigo são apresentados genomas de *S. aureus* coagulase negativa advindos de mastite bovina. Utilizando sequenciamento e ferramentas de bioinformática, foi possível realizar um uma análise comparativa entre genomas de *S.* coagulase positiva (CoPS) e *S.* coagulase negativa (CoNS), sendo abordado suas especificidades e possíveis genes que podem ser utilizados para um diagnóstico mais preciso de CoNS em diferentes espécies.

c. Anexos

A 6ª seção apresenta uma conclusão geral da tese

A 7ª seção aborda as perspectivas dos trabalhos desenvolvidos durante a tese.

A 8ª seção é um anexo que retrata toda a produção científica gerada durante a tese, como artigos, livro, capítulo de livro, resumos publicados. Atuação profissional, como disciplinas ministradas, cursos complementares, cursos ministrados e organização de eventos. Participação na realização de análises em colaboração com outras instituições de ensino e pesquisa.

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Artigo 2:

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Artigo 1:

Artigo 2:

LISTA DE ABREVIAÇÕES

ACT	*Artemis Comparison Tool* (Ferramenta de Comparação Artemis)
AMR	*Antimicrobial resistance* (Resistência antimicrobiana)
ANI	*Average Nucleotide Identity* (Identidade Média de Nucleotídeos)
ARG-ANNOT	*Antibiotic Resistance Gene-ANNOTation* (Gene-ANOTação de Antibióticos Resistentes)
BLAST	*Basic Local Alignment Search Tool* (Ferramenta Básica de Pesquisa de Alinhamento Local)
BPGA	*Bacterial Pan Genome Analysis Tool* (Ferramenta de Análise do Genoma Bacteriano Pan)
BRIG	*Blast Ring Generator* (Gerador de Blast Ring)
CARD	*Comprehensive Antibiotic Resistance Database* (Banco de Dados Abrangente de Resistência a Antibióticos)
CC	*Clonal Complex* (Complexo clonal)
CCS	Contagem de Células Somáticas
CHIPS	*Chemotaxis inhibitory protein of Staphylococcus* (Proteína inibidora de quimiotaxia de *Staphylococcus*)
CLF	*Clumping Factor* (Fator de aglutinação)
CMT	*California Mastitis Test* (Teste de Mastite da Califórnia)
COG	*Cluster of Orthologous Groups of proteins* (Aglomerado de Grupos Ortólogos de proteínas)
CoPS	*Staphylococcus* coagulase-positivos
CoNS	*Staphylococcus* coagulase-negativos
DMT	*Drug/metabolite transporter* (Transportador de droga / metabólito)
DNA	*Deoxyribonucleic acid* (Ácido desoxirribonucleico)
Embrapa	Empresa Brasileira de Pesquisa Agropecuária
Fig.	*Figure* (Figura)
FnB	*Fibrinogen-binding function* (Função de ligação de fibrinogênio)
FnBPs	*Fibronectin binding proteins* (Proteínas de ligação à fibronectina)
GEIs	*Genomic Islands* (Ilhas Genômicas)
GYPSy	*Genomic Island Prediction Software* (Software de Predição da Ilha Genômica)
IBGE	Instituto Brasileiro de Geografia e Estatística
KEGG	*Kyoto Encyclopedia of Genes and Genomes* (Enciclopédia de Genes e Genomas de Quioto)
LB	Luria Bertani
MALDI-TOF	*Matrix Associated Laser Desorption-Ionization - Time of Flight* (Dessorção-Ionização a Laser Associada à Matriz - Tempo de Voo)
MLEE	*Multilocus Enzyme Electrophoresis* (Eletroforese Enzimática Multilocus)
MLSA	*Multilocus Sequence Analysis* (Análise de Sequência Multilocus)
MLST	*Multilocus Sequence Typing* (Tipagem de Sequência Multilocus)

MRSA	*Methicillin resistant Staphylococcus aureus* (*Staphylococcus aureus* resistente à meticilina)
MSSA	*Methicillin susceptible Staphylococcus aureus* (*Staphylococcus aureus* suscetível à meticilina)
MSCRAMM	*The microbial surface components recognizing adhesive matrix molecules* (Proteínas de adesão)
NaAc	Acetato de sódio
NaCl	Cloreto de sódio
NCBI	*National Center for Biotechnology Information (*Centro Nacional para Informação Biotecnológica)
NGS	*Next Generation Sequence* (Sequenciamento de nova geração)
OMS	Organização Mundial da Saúde
PAIs	*Pathogenicity Islands* (Ilhas de Patogenicidade)
PATRIC	*Pathosystems Resource Integration Center* (Centro de Integração de Recursos Patossistemas)
PB	Pares de base
PCR	*Polymerase Chain Reaction* (Reação em cadeia da polimerase)
PROKKA	*Rapid prokaryotic genome annotation* (Anotação rápida do genoma procariótico)
PVL	*Panton-Valentine leukocidin* (Leucocidina Panton-Valentine)
RAST	*Rapid Annotations using Subsystems Technology* (Anotações Rápidas usando Tecnologia de Subsistemas)
RPM	Rotações por minuto
SCC	*Staphylococcal Chromosome Cassete* (Cassete Cromossômico *Staphylococcal*)
SCIN	*Staphylococcal complement inhibitor* (Inibidor do complemento *Staphylococcal*)
SI	*Symbiosis Islands* (Ilhas de Simbiose)
SNP	*Single Nucleotide Polymorphism* (Polimorfismo de nucleotídeo único)
ST	*Sequence Type* (Tipo de sequência)
TM	*Trademark* (Marca registrada)
Tris-HCl	Tris-hidrocloreto
tRNA	*transfer Ribonucleic Acid* (Ácido ribonucleico transportador)
VFDB	*Virulence Factors of Pathogenic Bacteria Database* (Database de Fatores de Virulência de Bactérias Patogênicas)

LISTA DE SÍMBOLOS

°C	Graus Celsius
EDTA	*Ethylenediamine tetraacetic acid* (Ácido etilenodiamino tetra-acético)
GC	Guanina e Citosina
GLPT	Glycerol-3-phosphate (Glicerol-3-fosfato)
GMP	*Glutamine-hydrolyzing* (Hidrólise de glutamina)
h	Horas
H_2O	Água
µl	Microlitro
µm	Micrômetro
M	Molar
Mb	Megabase
mg	Miligramas
min.	Minutos
mL	Mililitro
mm	Milímetro
PfoR	*Pyruvate-ferredoxin oxidoreductase* (Piruvato-ferredoxina oxidoredutase)
pH	Potencial hidrogeniônico

SUMÁRIO

1. INTRODUÇÃO

1.1. Características de *Staphylococcus aureus*

1.1.1. Taxonomia

A espécie *Staphylococcus aureus* pertence ao gênero *Staphylococcus* (do grego *staphyle* - cacho de uvas e *kokkos* - baga), identificado em 1880 por Alexandre Ogston em um abcesso cirúrgico. Em 1884 já se observava variações na coloração de espécies do gênero pelo médico Friedrich Julius Rosenbach , *S. aureus* (do latim *aurum*, ouro) e *S. albus* (do latim *albus*, branco). A espécie *S. albus* foi posteriormente renomeada para *S. epidermidis* devido à sua onipresença na pele humana [60, 103]. O gênero faz parte do grupo *Bacillus-Lactobacillus-Streptococcus* de bactérias Gram-positivas com baixo conteúdo GC (30% a 39%) [10, 65]. Atualmente, o gênero[1] reúne 85 espécies e 30 subespécies. São consideradas bactérias comensais (vivem no organismo do hospedeiro) ou patógenos oportunistas colonizando locais como pele, nasofaringe e diversas membranas mucosas de seres humanos e de outros animais [45]. A maioria possui importância clínica.

1.1.2. Aspectos microbiológicos e bioquímicos

Staphylococcus aureus é uma bactéria comensal Gram-positiva. Suas células possuem formato esférico (1µm de diâmetro), podendo ser encontradas isoladas ou em arranjos (cacho de uva), aos pares ou em aglomerados [37]. Metabolicamente, são anaeróbicos facultativos, imóveis (não possuem flagelos) e não formam esporos. Em termos de crescimento, apresentam crescimento rápido e em diferentes meios bacteriológicos, sendo observado colônias de tamanho entre 1,0 a 2,0 mm de diâmetro após 24h de incubação em condições aeróbias (presença de oxigênio). Também são capazes de realizar fermentação em anaerobiose (sem a presença de oxigênio) [112, 19]. São consideradas relativamente resistentes a cloreto de sódio, tolerando uma concentração de até 10% de NaCl no meio de cultura e crescem em uma larga faixa de temperatura de 7°C a 48,5°C [45, 76]. Em geral, *S. aureus* forma colônias que podem produzir uma pigmentação amarelo-dourado ou amarelo-alaranjado, sendo opacas e convexas [67].

1.1.3. Identificação de Staphylococcus aureus

Uma das características principais de diferenciação entre *S. aureus* e outros estafilococos Gram-positivos é a sua capacidade de produzir a enzima extracelular coagulase, sendo considerado coagulase e catalase positiva e oxidase negativa [19, 9, 43]. No fluxograma da Figura 1, encontra-se descrito o processo utilizado para identificação das espécies de estafilococos. Através de testes bioquímicos rápidos é possível identificar e classificar espécies

[1]Genus *Staphylococcus*, novembro de 2022: https://lpsn.dsmz.de/genus/staphylococcus

a partir da produção de enzimas consideradas fatores de diferenciação e por isso são considerados como testes confirmatórios. O teste de catalase é utilizado para distinguir estafilococos dos estreptococos. Ao identificar a formação de bolhas após a amostra entrar em contato com o peróxido de hidrogênio afirma-se que há presença da enzima catalase. A presença dessa enzima permite distinguir microrganismos do gênero *Streptococcus* catalase negativa de outros cocos Gram-positivos produtores de catalase, por exemplo, *Staphylococcus* spp. Para fazer a distinção entre espécies de estafilococos, o teste de coagulase é utilizado como padrão ouro. A enzima coagulase é considerada chave para diferenciação entre as espécies do gênero de *Staphylococcus* entre *S. aureus* versus *S. não aureus* [38]. No entanto, outras espécies como *S. intermedius* e algumas linhagens de *S. lugdunensis* também possuem atividade de coagulase [95, 68, 101]. Desta forma, o teste bioquímico da coagulase é considerado um teste rotineiro na identificação de *S. aureus,* sendo utilizado por Loeb desde 1903 [61]. Este teste verifica a capacidade do microrganismo reagir com o plasma sanguíneo e formar coágulo (Figura 2). Geralmente, a ausência de coagulação sugere a presença de outras espécies de *Staphylococcus*, embora a identificação de cepas de *Staphylococcus aureus* coagulase-negativas seja um fenômeno raro, mas possível, que pode levar a um diagnostico incorreto. Portanto, a confirmação da identificação de *S. aureus* deve ser feita por meio de testes adicionais, como a detecção do gene *nuc* ou a pesquisa de outros fatores de virulência.

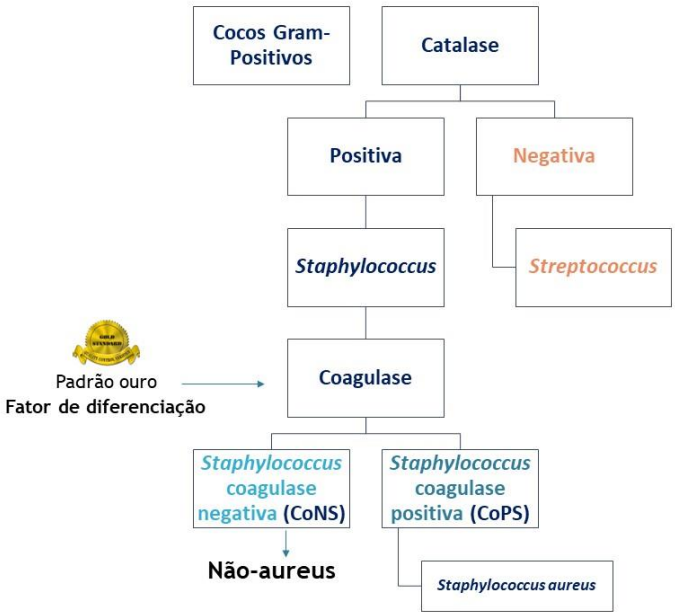

Figura 1. Fluxograma de identificação simplificada de *Staphylococcus aureus*.

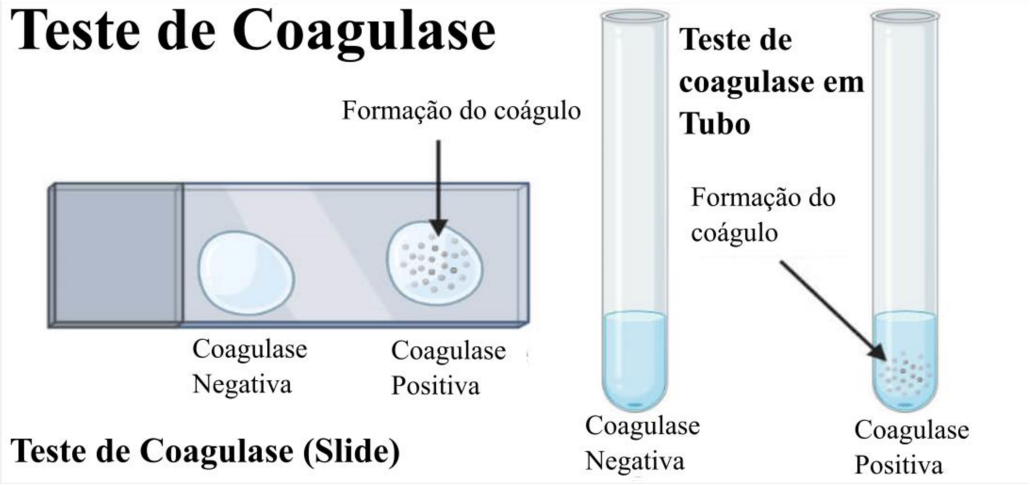

Figura 2. Representação esquemática de um teste de coagulase, na forma de lâmina (slide) e em tubo. Ambas são representadas pela formação de coágulo, a não identificação desses grumos é caracterizado como coagulase negativa. Fonte: Adaptado de https://microbenotes.com/wp-content/uploads/2020/10/Coagulase-Test.jpeg

Alguns trabalhos observaram diferenças no tempo de coagulação entre plasmas de diferentes espécies de mamíferos, demonstrando que a eficiência do teste de coagulase depende da origem da amostra [14, 83, 102]. A atividade da coagulase pode variar significativamente dependendo da origem do sangue utilizado no teste. Por exemplo, uma cepa de estafilococos isolada de bovino pode apresentar um tempo de formação de coágulo mais longo em plasma/sangue humano em comparação com plasma/sangue bovino. Geralmente, esse teste ´e realizado em plasma/sangue de coelho, sendo necessária cautela na interpretação dos resultados, já que a origem do plasma/sangue pode influenciar a sensibilidade e especificidade do teste de coagulase [16].

1.1.4. Epidemiologia de S. aureus

S. aureus é um patógeno zoonótico capaz de colonizar e causar infecções em diversos hospedeiros, representando uma séria ameaça à saúde animal, à segurança alimentar e, consequentemente, à saúde pública. A gravidade das infecções varia, podendo causar desde intoxicação alimentar e infecções cutâneas leves até mastite (inflamação das glândulas mamárias) e síndromes de choque tóxico [78, 88, 51, 48]. A patogenicidade de uma cepa de *S. aureus* reside na combinação de fatores de virulência, como toxinas extracelulares e propriedades invasivas. Uma infecção particularmente preocupante é a síndrome do choque tóxico, observada em animais com mastite causada por *S. aureus*. Essa síndrome está associada à ingestão de enterotoxinas pré-formadas, como a toxina da síndrome do choque tóxico (TSST-1) e enterotoxinas estafilocócicas (*sea*, *seb*, *sed*, *seg*, *seh* e *sei*), que possuem propriedades de superantígenos [21, 64, 96].

Em contraste com outras espécies de estafilococos, *S. aureus* frequentemente exibe um comportamento de especificidade de hospedeiro (*host-specific*). Isso significa que amostras isoladas de bovinos tendem a se agrupar em um grupo filogeneticamente distinto daquelas isoladas de ovinos e outros hospedeiros [73].

1.1.5. Determinantes de virulência

Muitos fatores determinam a virulência bacteriana, ou seja, a capacidade de um microrganismo causar infecção e doença. Quanto mais virulenta a bactéria, maior o dano potencial ao hospedeiro. A ausência de genes de virulência pode influenciar diretamente a sobrevivência e a capacidade de infecção da bactéria. Esses genes codificam moléculas que auxiliam a bactéria em diversas etapas da patogênese, como adesão, invasão e evasão do sistema imune [25, 47, 99].

As coagulases são enzimas que convertem fibrinogênio em fibrina, promovendo a coagulação do plasma ou sangue. Cepas de *Staphylococcus aureus* classificadas como coagulase-positivas são geralmente consideradas mais virulentas do que as coagulase negativas. A capacidade de coagular pode estar relacionada ao aumento da virulência, pois as cepas coagulase-positivas frequentemente expressam um arsenal mais amplo de toxinas, embora ambas as cepas compartilhem a capacidade de aderir, invadir e persistir em tecidos do hospedeiro [39, 74]. A produção de coagulase está correlacionada `a patogenicidade, pois essas cepas produzem diversas enzimas e toxinas extracelulares, que podem causar diferentes manifestações clínicas, como intoxicações alimentares e síndrome do choque tóxico [45, 24]. Os genes associados `a coagulação pertence ao grupo das estafilocoagulases, a coagulase clássica (*coa*) possui o tamanho variando entre 600 700 resíduos de aminoácidos. O *von Willebrand factor-binding protein* (vWbp) com tamanho variando entre 400 482 resíduos de aminoácido, ´e considerada uma enzima zimogênio bifuncional, devido a existência do ligante *von Willebrand* além da função de se ligar a protrombina [52, 14].

A enzima é dividida em seis partes, contendo N-terminal (primeiros 33 aminoácidos), domínio de ligação da protrombina (região D1 e D2), região central (função desconhecida), região repetitiva (27 aminoácidos) e a porção C-terminal (5´ últimos aminoácidos). Existem trabalhos que mostram algumas particularidades de cada uma delas. O gene *coa* apresenta uma região contendo cinco ou oito cópias de regiões repetitivas que agem na ligação do fibrinogênio. O gene *vWbp* pode promover coagulação apenas com os 250 primeiros aminoácidos, porém se houver alguma variação que gere o truncamento (*stop códon*) nessa região, não acontece atividade coagulante [89].

Além da coagulação, a capacidade de aderir às células do hospedeiro é crucial para a sobrevivência e patogenicidade dos estafilococos. *S. aureus* possui diversas proteínas de superfície, classificadas como MSCRAMMs (*Microbial Surface Components Recognizing Adhesive Matrix Molecules*), que medeiam agregação (*clumping*) como a adesão e invasão em tecidos de hospedeiros, evasão do sistema imune e formação de biofilme [38, 33]. A agregação é diferente da coagulação, mas também é importante no processo de patogenicidade. Essas proteínas de superfície celular interagem diretamente com fibrinogênio formando grandes agrupamentos de células (*large clusters of cells*) ou também chamado de micro colônias [90]. Em *S. aureus*, a formação de biofilme é intermediada pela participação de 12 genes, o gene das proteínas de ligação ao fibrinogênio (*fibrinogen-binding proteins - fib*), fibronectina (*fibronectin-binding proteins - fnb*A e *fnb*B), elastina (*elastin binding protein - ebps*), laminina (*laminin binding protein - eno*) os genes da adesão intercelular (*intercellular adhesion - ica*A, B, C e D), o fator de aglutinação (*clumping factor - clf* A e B) e o gene da ligação ao colágeno (*collagen binding protein - cna*) [51]. Os genes *clf* AB e *fnbp*AB participam do processo de coagulação juntamente com as estafilocoagulases, onde os mecanismos de agregação e coagulação estão associados a interação patógeno hospedeiro [24].

A expressão de toxinas está associada diretamente à patogenicidade e virulência bacteriana. As toxinas secretadas mais relevantes são 25 enterotoxinas estafilocócicas (*sea – selz*), hemolisinas (*hla* e *hlb*), leucotoxinas / *Panton-Valentine leukocidin* (*lukF*-PV e *lukS*-PV), toxinas esfoliativas (*eta* e *etb*), e toxinas da síndrome do choque tóxico (*tsst*-1), no entanto novos tipos são frequentemente descobertos [34]. *S. aureus* expressa SEs (*sea*, *seb*, *sec*1, *sec*3, *sed*, *ver*, *seh* e *selk*), dividido em dois grupos baseados na homologia da sequência de aminoácidos, o primeiro grupo (*sea, see, sed* e *seh*) e o segundo grupo (*seb, sec* e *seg*). Estão relacionadas entre as causas mais comuns de intoxicação alimentar, associada a animais de produção. Essas toxinas são termo resistentes (resistentes ao calor), quando secretadas nos derivados e há o consumo de leite, queijo e carne malpassada, desses animais infectados, representa um sério problema de saúde pública [21, 100, 32]. Alguns fatores de virulências são expressos por genes localizados em elementos genéticos m´oveis, como ilhas de patogenicidade (*tsst*-1), bacteriófagos ou profagos (inserido no cromossomo da bactéria) e outros como os inibidores estafilocócicos do complemento (SCIN) [78, 106].

1.1.6. Resistência a antibióticos

Além das infecções, o desenvolvimento de resistência antimicrobiana, impulsionado pelo uso indiscriminado de antibióticos, tem se agravado. Essa resistência ameaça a eficácia da prevenção e do tratamento de um número crescente de infecções por *S. aureus* em humanos e animais. Nos últimos anos, essa evolução tem ocorrido de forma acelerada [26]. A Organização Mundial da Saúde (OMS)[2] considera a resistência antimicrobiana uma das maiores ameaças à saúde global no século XXI, demandando medidas urgentes. Desde 2015, a OMS tem implementado um plano de ação global para combater a resistência antimicrobiana, visando reduzir o uso de antibióticos na saúde humana e animal, incentivando o desenvolvimento econômico sustentável e o investimento em novos medicamentos, ferramentas de diagnostico, vacinas e outras intervenções[3].

Em 1928 o médico bacteriologista Alexander Fleming trabalhava com amostras de *Staphylococcus aureus*, responsável por abscessos na pele de soldados, a fim de identificar algum mecanismo que reduzisse o sofrimento dos combatentes que tinham suas feridas infectadas. Ocasionalmente o bacteriologista deixou que essas amostras sofressem contaminação do ar, gerando a formação de mofo dentro dos recipientes de estudo. No processo de descarte das amostras foi observado que onde tinha se formado bolor não havia *Staphylococcus aureus* em crescimento, havendo uma substância que se movia em torno do fungo *Penicilium notatum* causador do mofo. Alexander Fleming identificou o primeiro antibiótico, marcado por uma das mais vitais descobertas da história, a penicilina, chamada de a "droga milagrosa" (*miracle drug*). O uso terapêutico da penicilina só foi iniciado em 1940 na Inglaterra, a substância inibia o crescimento em diferentes tipos de espécies bacterianas [20, 46]. A utilização de antibióticos na criação de animais foi liberada em 1951, este uso era administrado na dieta animal (promotores de crescimento) e sem receita veterinária [104, 40]. A meticilina, um agente antimicrobiano resistente aos - *lactamases*, foi introduzida em 1959. O primeiro relato de *Staphylococcus aureus* resistente à meticilina (MRSA) foi em Londres em 1961, podendo ser classificado em *Staphylococcus aureus* sensível à meticilina (MSSA) e *Staphylococcus aureus* resistente à meticilina (MRSA), a resistência aos medicamentos de *S. aureus* aumentou gradualmente, onde a taxa de infecção por MRSA aumentou a nível mundial, e o tratamento clínico anti-infeccioso de MRSA tornou-se mais difícil [42, 113]. Além disso, *S. aureus* faz parte do grupo ESKAPE, acrônimo para as seguintes bactérias: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* e *Enterobacter* spp., microrganismos multirresistentes que são

[2]OMS https://www.who.int/pt/
[3]Plano de ação disponível em: https://www.who.int/

considerados um grave problema de saúde pública [31]. A Figura 3 apresenta dados de 30 artigos sobre a resistência a 45 antibióticos em isolados de *S. aureus* de animais de consumo (bovinos, galináceos, suínos e ovinos) no Brasil[4]. A maioria dos estudos relata resistência a antibióticos em isolados de bovinos, incluindo antibióticos de última geração. Em ovinos, essa resistência tem se tornado mais frequente nos últimos anos, como indicado na Figura 3. No entanto, alguns estudos que avaliaram a susceptibilidade de *S. aureus* isolados de ovinos entre 1985 e 2015 indicam que essas cepas geralmente são suscetíveis a diversos antibióticos, sugerindo que a resistência ainda não se alterou significativamente ao longo do tempo nesse hospedeiro [7].

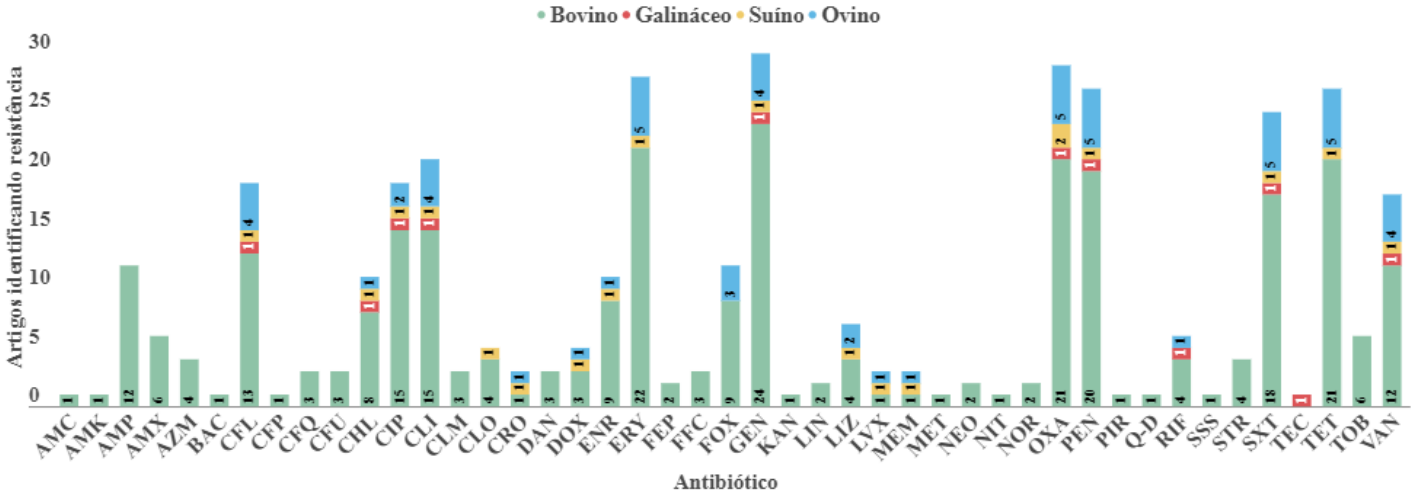

Figura 3. Trinta artigos que envolvem estudos referentes a presença ou ausência de resistência à 45 antibióticos em *S. aureus* isoladas de animais de consumo, como bovino, galináceo, suíno e ovino do Brasil. Figura gerada pelo autor a partir de dados retirados do Resistance Bank (https://resistancebank.org/) em 30/07/2022.

A resistência a um antibiótico presente na natureza não implica necessariamente que a bactéria seja patogênica. No entanto, essa bactéria pode atuar como um reservatório e disseminar genes de resistência por meio de transferência horizontal (plasmídeos, transposons e bacteriófagos). É importante ressaltar que uma bactéria pode ser altamente infecciosa (virulenta) e, ao mesmo tempo, altamente susceptível a antimicrobianos [44, 29].

1.2. Mastite

1.2.1. Manifestação clínica, patogenia, diagnóstico e tratamento

Mastite é uma infecção intramamária, sendo uma das mais frequentes em animais de produção. Segundo Murphy (1947) [75], o desenvolvimento da mastite é fundamentado em três estágios: (1) invasão de um organismo, (2) infecção e (3) inflamação, chamado de complexa tríade (animal, agente etiológico e meio ambiente), como visto na Figura 4 [97].

[4]Lista de artigos disponíveis em: https://resistancebank.org/

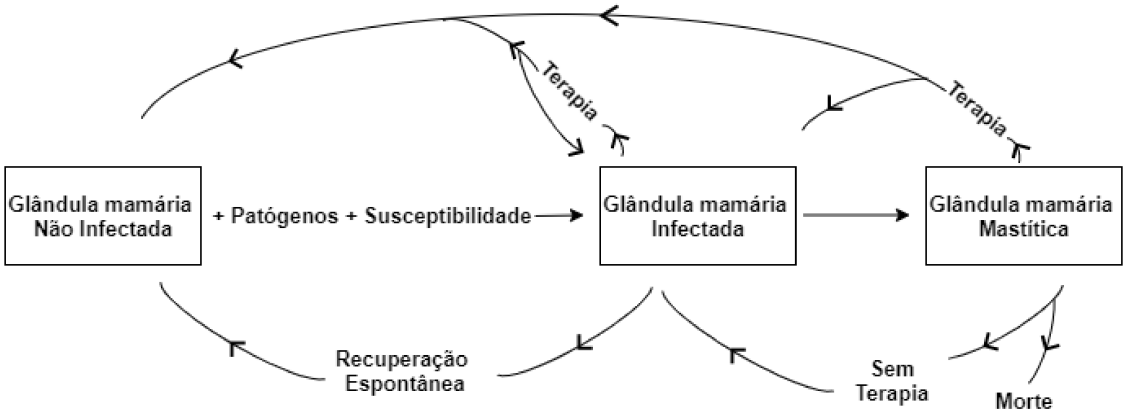

Figura 4 - Possível sequência de eventos no desenvolvimento de infecção e mastite. Fonte: adaptado de Ruegg, 2017.

Existem diversas barreiras físicas que a bactéria precisa superar para estabelecer uma infecção na glândula mamária. Como ilustrado na Figura 5, o músculo esfíncter no canal do teto mantém o orifício externo fechado, isolando o interior da glândula. O canal do teto ´e revestido por queratina, dificultando a penetração de bactérias. A ruptura dessa barreira física é um fator predisponente para o desenvolvimento de mastite. Além disso, a glândula mamária possui mecanismos de defesa imune, como o recrutamento de neutrófilos, a ativação de linfócitos e a produção de citocinas, que atuam no combate a agentes infecciosos [82, 77].

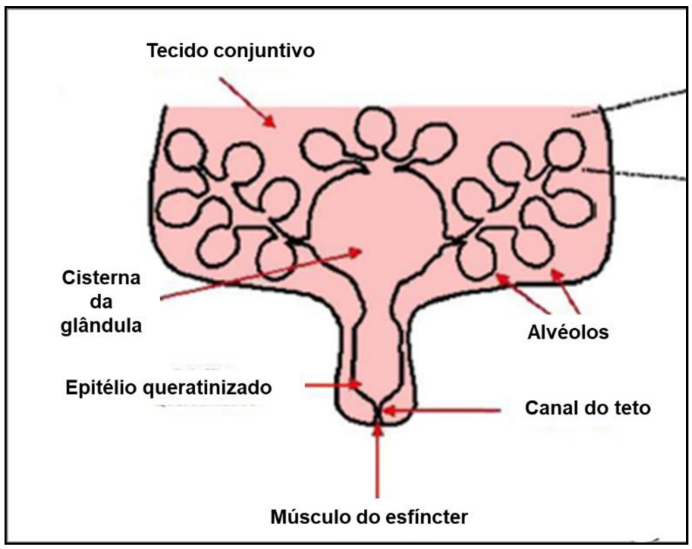

Figura 5: Estrutura da glândula mamária. Fonte: adaptado de Tartaglia, 2016.

A gravidade da inflamação pode variar de subclínica a gangrenosa, podendo evoluir ao estágio crônico ou gerar uma infecção sistêmica [57, 97]. De acordo com uma revisão realizada por Contreras et al. (2007a) [23], a prevalência de mastite subclínica em pequenos ruminantes em 2007 era de 5-30% por ano, porém o estágio clínico é menor que 5%, em 2020 foi observado

a prevalência de 50% [2, 53]. As infecções contagiosas tendem a serem persistentes na glândula mamária, por isso a alta taxa de mastite em sua forma subclínica (assintomática). *S. aureus* é a principal causa de mastite em ruminantes. Outros patógenos como *Streptococcus* spp., *Enterobacteriaceae, Pseudomonas* spp., *Escherichia coli, Klebsiella* spp., *Corynebacterium*, bem como outras bactérias e fungos, podem também contribuir para o desenvolvimento de infecções intramamária em pequenos ruminantes [109, 73, 85].

A epidemiologia da mastite varia de acordo com a espécie, quantidade, patogenicidade e infectividade dos agentes envolvidos. Dentre os agentes causadores, as bactérias são as maiores responsáveis, constituindo cerca de 80 a 90% dos casos de mastite. Esses patógenos são classificados como contagiosos (*Staphylococcus aureus, Streptococcus agalactiae* e *Mycoplasma* spp) e ambientais (*Escherichia coli* e espécies de *Enterobacter, Klebsiella, Proteus, Serratia* e *Pseudomonas*) [22]. *S. aureus* é um dos mais importantes patógenos causadores de mastite, geralmente sua transmissão está envolvida no processo da ordenha, de um quarto mamário para outro ou de um animal lactante para outro. Outras vias preocupantes de transmissão são durante a higienização do teto e dos objetos de ordenha, as mãos do ordenhador, materiais usados para lavar e secar tetos de múltiplas vacas ou equipamento de ordenha com funcionamento inadequado. A mastite subclínica é capaz de sobreviver no ambiente por longos períodos, tende a persistir devido à ausência de sintomas evidentes e, portanto, subestima a produção de animais lactantes infectadas [5, 97].

O tratamento contra a mastite em ruminantes é feito com antibioticoterapia [12]. Para os animais de pequeno porte, como ovinos e caprinos, a variedade de antibióticos regulamentados é inferior à dos bovinos [85, 79, 51]. O uso de medicamentos direcionados para o tratamento de bovinos em pequenos ruminantes pode ser de alto risco, devido à confiança e eficiência destes medicamentos, em geral, serem desconhecidas. Alguns estudos relatam a indução de resistência devido a doença apresentar infecções persistentes Mesmo com o tratamento com antibiótico, a bactéria consegue sobreviver, colonizar e gerar a infecção intramamária, propiciando uma gradual redução de cura [87, 96, 84]. Assim, outros ruminantes do rebanho e outros quartos mamários não-afetados do mesmo animal podem se infectar [58, 1]. A higiene do local de ordenha, dos objetos e do úbere do animal, durante e imediatamente após a ordenha podem contribuir para a diminuição da taxa de infecção por *S. aureus* no rebanho [108]. A utilização de medicamentos homeopáticos e fitoterápicos tem demonstrado resultados satisfatórios durante a terapia dos casos de mastite, porém os estudos são voltados em sua maioria para espécies bovinas. Devido as ações governamentais visando a redução do uso de

antibióticos para o tratamento de infecções bacterianas, hoje são abordadas novas estratégias, como a utilização de bacteriófagos no tratamento de mastite bacteriana [51, 31, 6, 91, 71].

O diagnóstico da mastite é realizado por meio de metodologias diretas e indiretas. As diretas envolvem a identificação do agente etiológico (presença ou ausência do microrganismo) na amostra de leite coletada, enquanto as indiretas se baseiam na forma clínica da doença (subclínica, clínica ou crônica). Sinais como febre aguda, perda de apetite, apatia, dispneia e dificuldades de locomoção podem indicar mastite aguda ou crônica [97, 85]. A forma clínica da mastite apresenta sintomas visíveis, como a presença de grumos, pus ou sangue no leite, além de inchaço e inflamação no úbere, acompanhados por sinais sistêmicos como febre, depressão, desidratação e diminuição da produção de leite. A identificação de alterações visíveis no leite pode ser realizada através do teste da caneca telada (fundo preto), que consiste em coletar os três primeiros jatos de leite e observar a presença de anormalidades [27].

Os testes mais utilizados para o diagnóstico da mastite em ruminantes são: cultura bacteriana, contagem de células somáticas (CCS) eletrônica e California Mastitis Test (CMT). O CMT, que se baseia na CCS, é um dos testes mais comuns para a detecção da mastite subclínica [70, 8]. No entanto, em pequenos ruminantes, resultados positivos no CMT nem sempre indicam infecção intramamária [23, 73, 85].

O desenvolvimento de ferramentas digitais, como a Internet das Coisas (IoT), tem revolucionado a produção animal. A IoT permite a disseminação de informações técnicas geradas por pesquisadores para o campo, atendendo `a demanda por conhecimento atualizado em todos os segmentos da pecuária. O uso dessas ferramentas tem se tornado cada vez mais comum em fazendas de todos os portes.

A IoT possibilita a geração de grandes volumes de dados (metadados), que podem ser analisados para identificar padrões e comportamentos, auxiliando na tomada de decisões e no desenvolvimento de sistemas de aprendizado para a produção animal e o controle de doenças. Combinados com técnicas microbiológicas, esses dados podem ser utilizados para alimentar bancos de dados e algoritmos de inteligência artificial. Um exemplo é o estudo de Bobbo et al. (2021) [15], que utilizou aprendizado de máquina para estimar o status de CCS de vacas leiteiras no mês seguinte, com base em dados do mês atual, visando prever a saúde do úbere. Os metadados coletados (identificação individual, raça, estágio de lactação, produção de leite, composição do leite e CCS) de 14 mil vacas em 791 fazendas na Itália foram utilizados para treinar e validar modelos de predição. Essa capacidade de prever o status de saúde do úbere pode auxiliar na tomada de decisões sobre o manejo de animais com mastite, na estimativa de perdas de produção e na implementação de medidas de prevenção e controle. A combinação de

tecnologias digitais com estudos genômicos e proteômicos pode contribuir para o desenvolvimento de ferramentas de diagnóstico mais precisas e eficientes para a mastite [50, 59].

1.2.2 Impacto econômico e dificuldades no controle

Durante a infecção em ruminantes, a persistência de *S. aureus* no interior das células epiteliais da glândula mamária pode ser um fator que dificulta a eliminação do patógeno. A disseminação de clones bacterianos resistentes a antibióticos agrava o problema, causando epidemias e surtos em animais e humanos [49, 94].

Segundo a EMBRAPA (2019) [4], na última década (2007-2016), houve aumento no efetivo de caprinos e ovinos mundialmente (15,51% e 5,77%, respectivamente), com destaque para o crescimento da ovinocultura leiteira (9,74%). No entanto, a caprinocultura leiteira apresentou leve queda (2,04%). No Brasil, entre 2006 e 2017, o Censo Agropecuário do IBGE registrou aumento de 16,11% no rebanho caprino e diminuição de 2,80% no rebanho ovino, além de uma queda de 30% na produção de leite de cabra. È importante ressaltar que a Produção Pecuária Municipal (PPM/IBGE) não divulga dados anuais sobre a produção de carne e leite de ovinos e caprinos, sendo estas informações coletadas apenas no Censo Agropecuário, realizado a cada 10 anos[5]. O Nordeste lidera o efetivo de ambos os rebanhos e a produção de leite de cabra no país.

Apesar da importância da ovinocultura, ainda não há legislação e medidas de controle específicas para este setor, sendo as metodologias de controle de *S. aureus* adaptadas do setor bovino. Essa adaptação pode ser inadequada, resultando em doses e duração de tratamento com antibióticos inadequadas, o que contribui para a seleção de bactérias resistentes e a presença de resíduos antimicrobianos no leite [93, 18].

O tratamento inadequado da mastite pode levar à resistência bacteriana e à contaminação de humanos por meio do consumo de alimentos de origem animal [17]. Mesmo a pasteurização do leite não elimina o risco de intoxicação alimentar, pois *S. aureus* produz toxinas termoresistentes, reforçando o papel zoonótico desse patógeno. Estudos têm demonstrado a ineficácia das medidas de diagnóstico e controle de *S. aureus* em caprinos e ovinos, em comparação com bovinos [109, 23, 4]. Enquanto o perfil populacional e as características genômicas de *S. aureus* isolados de leite bovino são bem conhecidos, os dados sobre isolados de pequenos ruminantes s˜ao escassos [73], dificultando o desenvolvimento de estratégias de controle eficazes.

[5]EMBRAPA CAPRINOS E OVINOS. Centro de Inteligência e Mercado de Caprinos e Ovinos. Produção Nacional, disponível em: https://www.embrapa.br/cim-inteligencia-e-mercado-de-caprinos-e-ovinos/producao-nacional

1.3. Genômica de *S. aureus*

O sequenciamento de genomas bacterianos aumentou significativamente o conhecimento sobre a estrutura, função, variação e relação do genoma com os mecanismos genéticos envolvidos na inteiração patógeno-hospedeiro [55, 62]. O primeiro genoma completo de uma bactéria patogênica sequenciado foi o de *Haemophilus influenzae* [36]. Em 2001, foram publicados os primeiros genomas completos de *S. aureus*, pertencentes `as linhagens Mu50 e N315, isoladas de humanos e resistentes `a meticilina (MRSA) [54].

O genoma bacteriano é um mosaico de regiões estáveis e instáveis. A diversidade na estrutura do genoma, também conhecida como plasticidade genômica, resulta de eventos de aquisição (inserção) e perda (deleção) de sequências, além de duplicações, inversões e translocações [84]. A introdução das tecnologias de sequenciamento de nova geração (NGS) reduziu drasticamente o custo e aumentou a eficiência e precisão do sequenciamento de DNA. Estudos de evolução e genômica comparativa em larga escala têm sido realizados, contribuindo para o conhecimento de novos determinantes de virulência bacteriana e resistência a drogas, além de incentivar pesquisas metagenômica e metataxonômicas em diversos ecossistemas [81, 56, 111, 58].

O genoma de *S. aureus* consiste em um cromossomo circular de aproximadamente 2,8 Mb, com uma média de 2.707 genes e conteúdo GC de cerca de 32,7% [63]. Atualmente, mais de doze mil genomas de *S. aureus* e suas linhagens estão depositados no banco de dados do NCBI (*National Center for Biotechnology Information*), dos quais cerca de 582 são genomas completos. No entanto, entre os genomas completos com informações sobre o hospedeiro de origem, apenas três (ED133[6] (CP0019966), NCTC1803[7] (LR1343057) e NCTC9555[8] (LR1340908) estão associados à mastite ovina. Dentre estes, apenas o genoma ED133 é utilizado como referência em estudos representativos [41]. Genomas completos são essenciais para a genômica comparativa, pois contêm a sequência completa do genoma e fornecem uma representação mais precisa do repertório gênico da bactéria [92].

O genoma de *Staphylococcus* é classificado em biotipos por tipagem molecular, que se baseia na variação alélica de genes de manutenção (*housekeeping genes*) para determinar o tipo de sequência (ST). Em *S. aureus*, a tipagem molecular utiliza a variação alélica de sete genes (*arc*C, *aro*E, *glp*F, *gmk*, *pta*, *tpi*, *yqi*L) [30]. Isolados que compartilham pelo menos cinco dos sete alelos formam um complexo clonal [30, 66, 105]. Estudos que buscam correlacionar a

[6] https://www.ncbi.nlm.nih.gov/nuccore/CP001996.1/
[7] https://www.ncbi.nlm.nih.gov/nuccore/LR134305.1/
[8] https://www.ncbi.nlm.nih.gov/nuccore/LR134090.1/

diversidade genômica com o tipo de hospedeiro utilizam métodos fenotípicos, como a tipagem de fagos, a presença de plasmídeos e o perfil proteico [35].

As análises de pan-genômica buscam identificar o repertório completo de genes de uma espécie. O genoma central (*core*) contém genes essenciais compartilhados por todos os genomas, que podem ser alvos de vacinas e drogas. O genoma acessório inclui genes presentes em mais de um genoma, mas não em todos, e pode conter genes relacionados à adaptação ao ambiente e ao diagnóstico de grupos de linhagens de interesse. Os genes exclusivos são encontrados em apenas um genoma específico [72, 107].

Uma característica importante de *S. aureus* ´e a sua capacidade de adquirir genes de resistência a antibióticos, dificultando o tratamento [43, 35]. Isolados de *S. aureus* de ovinos apresentam alta taxa de sensibilidade em comparação com isolados de bovinos, confirmada por análises *in silico* e testes de disco-difusão e E-test [86].

Análises de genômica comparativa são úteis para entender a evolução intraespecífica e a patogênese, pois as linhagens podem ter características genéticas específicas adquiridas por transferência horizontal de genes (ilhas de patogenicidade e sequências de bacteriófagos) [11, 28]. Apesar do aumento no número de genomas completos de *S. aureus* depositados no NCBI, a maioria é de isolados humanos, com apenas quinze linhagens isoladas de ovinos. Pouco se sabe sobre as particularidades do genoma de isolados de pequenos ruminantes e as características genômicas envolvidas na mastite ovina [105, 13, 11, 3, 80].

2. JUSTIFICATIVA

A melhor compreensão dos determinantes moleculares de virulência e patogenicidade de *S. aureus* poderá contribuir para o desenvolvimento de novas terapias não apenas contra a mastite, mas também contra outras doenças causadas por este agente em diferentes organismos hospedeiros. Genomas de diversos isolados de *S. aureus* têm sido sequenciados e depositados no banco de dados do NCBI, sendo a maioria de origem humana. De acordo os metadados disponibilizados no NCBI[1], dentre os isolados com genoma completo e depositado, tem-se poucos representantes de origem ovina e amostras isoladas do Brasil, sua maior representatividade são de genomas isolados de humanos e bovinos do continente Europeu e América do Norte.

Apesar dos esforços dedicados ao conhecimento do processo infeccioso da mastite e estratégias de prevenção e tratamento, pouco se sabe sobre as características exclusivas dos genomas de linhagens de *S. aureus* isoladas de pequenos ruminantes de diferentes áreas geográficas do Brasil [110, 88]. Estudos prévios sugerem a existência de genes, em *S. aureus*, que podem estar envolvidos na associação específica com o hospedeiro ovino [11, 3, 69]. Além disso, a nível de diagnóstico preciso, sabe-se que existe uma carência de técnicas eficientes para diagnostico de *S. aureus* em pequenos ruminantes. Mesmo havendo grandes estudos com bovinos, ainda existe o falso diagnostico, desencadeando grandes perdas econômicas para o setor produtivo [50, 98]. O aumento na produção de caprinos e ovinos na última década tem aumentado e o consumo dos derivados contaminados pode levar à intoxicação alimentar, um exemplo é a ingestão do leite cru, devido à presença de enterotoxinas estafilocócicas e enzimas termoestáveis [4, 73].

Diante desse cenário, o presente estudo se propõe avaliar as características genômicas das linhagens isoladas de ovinos e bovinos a fim de analisar a especificidade pelo hospedeiro, identificar a relação evolutiva entre os isolados e características que podem estar associadas à virulência, a fim de contribuir para novas estratégias de controle e tratamento dessa infecção. Outro viés desse trabalho são as análises genômicas voltadas para a prospecção de genes para diagnóstico de *S. aureus* coagulase negativa, já que estes têm se tornado emergentes. O alcance deste trabalho pode ir além da genômica, uma vez que os genomas das linhagens de ovinos e bovinos, aqui utilizadas, estão montadas, anotadas e disponíveis em banco de dados públicos contribuindo para novos estudos envolvendo outras abordagens ômicas.

[1] NCBI: https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=1280

3. OBJETIVO GERAL

Caracterizar amostras de *S. aureus* isoladas de mastite em ovinos e *S. aureus* coagulase negativa em bovinos, utilizando como ferramentas o sequenciamento e genômica comparativa a fim de entender sua epidemiologia genômica.

3.1 OBJETIVOS ESPECÍFICOS

Capítulo I:

1. Obter as sequências genômicas completas e anotadas das linhagens O55, O17, O82, O217, O267, O268, O322, O326, O331, O408, O11 e O46 de *S. aureus* isoladas de ovinos;
2. Proceder com a tipagem molecular *in silico* das linhagens de *S. aureus* utilizadas no estudo por meio da identificação de *Sequence Types* (STs) e complexos clonais (CCs);
3. Avaliar como as linhagens de *S. aureus* isoladas de ovinos se relacionam filogeneticamente entre si e com as demais linhagens selecionadas de outros hospedeiros;
4. Definir um padrão de segregação filogenômica capaz de distinguir grupos de linhagens oriundos de ovinos de grupos de linhagens encontradas em outros hospedeiros;
5. Avaliar os elementos genômicos, incluindo genes, ilhas genômicas, fatores de virulência, resistência e profagos, presentes apenas nas linhagens ovinas de *S. aureus* em comparação com as linhagens de outros hospedeiros;

Capítulo II:

1. Sequenciar, montar, anotar e depositar, no banco de dados do NCBI, os genomas das seis amostras 23S, 42S, 48S, 56S, 62S, 73S de *S. aureus* isoladas de bovinos;
2. Identificar a presença de plasmídeos;
3. Verificar a taxonomia por meio de Identidade Média de Nucleotídeos (ANI);
4. Avaliar como as linhagens de identificadas como *Staphylococcus* coagulase negativa se relacionam filogeneticamente com as *S. aureus* positivas e outras espécies;
5. Avaliar como os genes relacionados a coagulação, as estafilocoagulases, estão agrupados;
6. Avaliar o uso das técnicas de MALDI-TOF, teste de coagulase e sequenciamento na identificação de *S. aureus*.
7. Predizer fatores de virulência, patogenicidade e resistência nos genomas das linhagens de *S. aureus* isoladas de casos de mastite em ovinos e bovinos.
8. Observar a presença e ausência de fatores de virulência, patogenicidade e resistência entre os grupos de *S. aureus* coagulase positiva e negativa;

CAPÍTULO I

4. CAPÍTULO I

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Comparative genomic analysis of ovine and other host associated isolates of *Staphylococcus aureus* exhibit the important role of mobile genetic elements and virulence factors in host adaptation

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ABSTRACT

Staphylococcus aureus is the main etiological agent of mastitis in small ruminants worldwide. This disease has a difficult cure and possible relapse, leading to significant economic losses in production, milk quality and livestock. This study performed comparative genomic analyses between 73 *S. aureus* genomes from different hosts (human, bovine, pig and others). This work isolated and sequenced 12 of these genomes from ovine. This study contributes to the knowledge of genomic specialization and the role of specific genes in establishing infection in ovine mastitis-associated *S. aureus*. The genomes of *S. aureus* isolated from sheep maintained a higher representation when grouped with clonal complexes 130 and 133. The genomes showed high genetic similarity, the species pan-genome consisting of 4200 genes (central = 2008, accessory = 1559 and unique = 634). Among these, 277 unique genes were related to the genomes isolated from sheep, with 39.6 % as hypothetical proteins,

Abbreviations: list: AMR, Antimicrobial Resistance; ANI, Average Nucleotide Identity; ANIM, Average Nucleotide Identity Mummer; ARG-ANNOT, Antibiotic Resistance Gene-Annotation; BLASTN, Basic Local Alignment Search Tool Nucleotide; BLAZ, PC1 Beta-Lactamase; BPGA, Bacterial Pan Genome Analysis Tool; BRIG, Blast Ring Image Generator; BV-BRC, Bacterial and Viral Bioinformatics Resource Center; CAPES, Agencies Coordination for The Improvement Of Higher Education Personnel; CARD, The Comprehensive Antibiotic Resistance Database; CC, Clonal Complex; CCs, Clonal Complexes; CHIPS, Chemotaxis Inhibitory Protein of Staphylococcus; *cna*, Collagen-Binding Protein; *coa*, Coagulase; COG, Cluster of Orthologous Groups; *entb*, Staphylococcal Enterotoxin B; FAPEMIG, Minas Gerais Research Funding Foundation; GC, Genomic Content; GEIs, Genomic Islands; GIPSY, Genomic Island Prediction Software; HYSA, Hyaluronate Lyase Precursor; INRAE, Institut National de Recherche pour L'agriculture, L'alimentation et L'environnement; KEGG, Kyoto Encyclopedia Of Genes And Genomes; LB, Luria Bertani; LUKS-PV, Panton-Valentine Leukocidin; MATE, Multidrug And Toxic Compound Extrusion; MEGARES, An Antimicrobial Database for High-Throughput Sequencing; MFS, Major Facilitator Superfamily; MLSA, Multilocus Sequence Analysis; MLST, Multi-Locus Sequence Typing; MRSA, Methicillin-Resistant *Staphylococcus aureus*; MSSA, Methicillin-Susceptible *Staphylococcus Aureus*; NCBI, National Center for Biotechnology Information; PAIs, Pathogenicity Islands; RECOM, Omics Science Network; *S. aureus*, *Staphylococcus aureus*; *sak*, Staphylokinase; SCCmec, Chromosome Cassette *mec*; *scn*, Staphylococcal Complement Inhibitor; *selk*, Staphylococcal Enterotoxin K; SIs, Symbiosis Islands; SNP, Single Nucleotide Polymorphism; VFDB, Virulence Factors of Pathogenic Bacteria; vWpb, Secreted Von Willebrand Factor-Binding Protein Precursor.

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6.4 % as phages, 6.4 % as toxins, 2.9 % as transporters, and 44.7 % as related to other proteins. Furthermore, at the pathogen level, they showed 80 genes associated with virulence factors and 19 with antibiotic resistance shared in almost all isolates. Although *S. aureus* isolated from ovine showed susceptibility to antimicrobials *in vitro*, ten genes were predicted to be associated with antibiotic inactivation and efflux pump, suggesting resistance to gentamicin and penicillin. This work may contribute to identifying genes acquired by horizontal transfer and their role in host adaptation, virulence, bacterial resistance, and characterization of strains affecting ovine.

1. Introduction

Mastitis is an inflammation of the mammary gland, most often due to a bacterial infection, responsible for substantial economic losses in milk production worldwide (Halasa et al., 2009; Ruegg, 2017). That disease may vary from subclinical to clinical and can become chronic in some cases. Occasionally, mastitis can also become systemic. This infection can be caused by different bacterial species, among which *Staphylococcus aureus* is one of the most prevalent (Oviedo-Boyso et al., 2007; Le Maréchal, Hernandez, et al., 2011; Haag, Fitzgerald and Penadés, 2019a). However, antibiotics are often inefficient in curing the infection caused by *S. aureus;* in addition, their disease is prone to resurgence, as bacteria may remain viable inside the epithelial cells of the mammary gland (Haag, Fitzgerald and Penadés, 2019a; Oget, Tosser-Klopp and Rupp, 2019; Astrup, Pedersen and Farre, 2022). Additionally, some differences between the ovine and bovine epithelium lead to different interactions during treatment, such as the distribution of serotonin receptor subtypes in the mammary gland (Suárez-Trujillo et al., 2019; Hughes, 2020).

S. aureus strains are commonly categorized into clonal complexes (CCs) according to Multi-locus Sequence Typing (MLST). The MLST method has extensively been used to characterize *S. aureus* isolates and showed host-specificity within CCs, such as CC133 for ovine isolates (Smith et al., 2005; Aires-de-Sousa et al., 2007a; Zakour and Loir, 2007; Smyth et al., 2009; Merz, Stephan and Johler, 2016; Hoekstra et al., 2019). In addition, previous studies also showed host-specific variations in terms of genome content (mobile genetic elements) and differential expression of regulatory genes in the *S. aureus* species (Alves et al., 2009; Guinane, ben Zakour, et al., 2010; Richardson et al., 2018).

Comparative genomic analyses are conducted to understand better the basis of intraspecific evolution and pathogenesis of microorganisms since bacteria can have specific genetic characteristics of strains such as single nucleotide polymorphism (SNP) and mobile genetic elements (e.g., islands of pathogenicity, bacteriophages, plasmids, transposons, and integrative elements) (Dobrindt, 2001; ben Zakour et al., 2008). The mobile elements can carry copies of genes that confer additional characteristics in the recipient genome, such as resistance to antibiotics, heavy metals, or greater pathogenicity. The comparison allows the identification of specificities in hosts, as the *seh* gene was identified only in cow isolates. The *cap8* genotype is present in almost any type of host in a study involving isolates of mastitis *S. aureus* in cattle and goats (Acosta et al., 2018; Sheppard, Guttman and Fitzgerald, 2018; Oget, Tosser-Klopp and Rupp, 2019).

At the genomic sequence level, although the number of complete *S. aureus* genomes deposited in the NCBI genome database has increased considerably in recent years, most are related to human isolates. More recently, only three ovine strains (ED133, NCTC1803, and NCTC9555) have been isolated from mastitis (Guinane, ben Zakour, et al., 2010). The information about peculiarities of the genome of small ruminant isolates is rare, and there is no knowledge of the genomic characteristics that may be involved in the onset of mastitis in sheep (Aires-de-Sousa et al., 2007b; Peton et al., 2014; Bosi et al., 2016a; Oget, Tosser-Klopp and Rupp, 2019).

This study contributes to the knowledge of genomic specialization and the role of specific genes in establishing infection in ovine mastitis-associated *S. aureus.* Comparative genomic analyses were performed among 73 *S. aureus* genomes from different hosts (human, bovine, ovine, pig and others). In addition, this works isolated and sequenced 12 of these genomes collected in France in cases of ovine mastitis.

2. Material and methods

2.1. Ovine S. aureus strains and genomic DNA extraction

Twelve *S. aureus* strains were isolated on a Luria Bertani (LB) agar plate from different forms of mastitis in flocks of French sheep (Table 1). The genomic DNA was extracted after cultivating the strain on 30 mL of LB broth at 37 °C for 12 h. Every culture was centrifuged for bacterial pellet formation, and the supernatant was discarded. The pellet was suspended in 600 μL of solution (Tris-HCI pH 7.0, 0.5 M EDTA pH 8.0, NaCl 5 M, and distilled H2O sufficient to obtain 50 mL) and transferred to a tube of 2 mL containing glass beads (VK01) (Bertin Technologies). Next, 3 μL of RNase A solution (20 mg/mL) was added before subjecting the bacteria to mechanical lysis. Two homogenization cycles of 15 *sec* each, at 6,500 rpm, were performed using Precellys 24 (Bertin Technologies). Subsequently, 1 mL of phenol/chloroform/isoamyl alcohol (25:24:1) solution was added to the tube, and the mixture was homogenized and centrifuged at 13,000 rpm 7 min. Next, the upper aqueous phase of the mixture was transferred to a new tube, and the second round of phenol/chloroform/isoamyl alcohol purification was performed. Next, the upper aqueous phase was recovered and mixed with 1 mL of chloroform. Following centrifugation at 13,000 rpm for 7 min, the upper aqueous phase was transferred to a new tube. Next, 1 mL of ethyl alcohol, 40 μL of 3 M NaAc, and 4 μL of 20 mg/mL glycogen were added. The mixture was placed at −20 °C overnight following gentle inversion for DNA precipitation. Following centrifugation at 13,000 rpm, for 15 min, the supernatant was discarded. Next, 1 mL of 70 % ethyl alcohol was added to rehydrate the DNA pellet. The second round of 70 % ethyl alcohol wash was performed using centrifugation at 13,000 rpm for 15 min. Then, the DNA pellet was dried at 60 °C. Finally, the DNA precipitate was suspended in 50 μL of sterilized ultra-pure water. DNA quantity and quality assessments were conducted using NanoDropTM 2000 (Thermo ScientificTM), Qubit Fluorometer (Thermo ScientificTM), and 1 % agarose gel electrophoresis.

Table 1
S. aureus strains isolated from ovine mastitis sequenced for the present study.

Strain	Mastitis range	Isolation Year	Locality isolation (France)
O268	Clinical	1998	Pyrénées-Atlantiques
O17	Clinical	2003	Alpes Maritime
O322	Clinical	2008	Alpes de Haute-Provence
O326	Clinical	2008	Alpes de Haute-Provence
O217	Chronic	2002	Aveyron
O11*	Gangrenous	2002	Southeast of France
O408	Gangrenous	2010	Alpes de Haute-Provence
O46*	Subclinical	2002	Southeast of France
O82	Subclinical	2002	Unspecified
O267	Subclinical	1998	Pyrénées-Atlantiques
O331	Subclinical	2008	Alpes de Haute-Provence
O55	Subclinical	2002	Unspecified

* The O11 and O46 strains were not considered in the DNA extraction, since their genomes were previously sequenced.

2.2. Sequencing, assembly, and annotation of the ovine S. Aureus genomes

Sequencing of all total DNA the strains were performed using Hiseq 2500 platform, 150 bp, paired end (Illumina, San Diego, CA, USA). The two genomes previously sequenced (O11 and 046) was obtained with using an Illumina Genome Analyzer GAII (Le Maréchal, Hernandez, et al., 2011). The reads quality was checked with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

SPAdes, version 3.9.1 (Bankevich et al., 2012), was used for the *ab initio* assembly of all genomes, including O46 and O11. CONTIGuator version 2.7 (Galardini et al., 2011) generated scaffolding. The gaps resulting from assemblies were manually filled using the CLC Genomics Workbench software, version 7.0 (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench). Reads were mapped against the reference genome to generate the consensus sequences for gap filling. All genomes were submitted to an automatic annotation using the PROKKA version 3.0 (Seemann, 2014).

2.3. Genome average nucleotide identity analysis in the selected S. aureus strains

To determine the genome similarity among the ovine strains and the strains isolated from various hosts, we used the Average Nucleotide Identity (ANI) analysis, specifically ANIm, based on MUMmer calculations (Konstantinidis, Ramette and Tiedje, 2006; Kim et al., 2014a). JSpeciesWS Online Service Taxonomic Thresholds (available at jspecies.ribohost.com/jspeciesws/) (Richter et al., 2016) were used with BLASTn (Altschul et al., 1990). This analysis method performs pairwise comparisons through the identity and similarity values implemented (Goris et al., 2007). The similarity heatmap was screened using Morpheus software (available at https://software.broadinstitute.org/morpheus/).

3. Molecular typing and phylogenetic analysis of the selected *S. aureus* strains

To determine the Sequence Type (ST) of each selected *S. aureus* strain, the allelic prediction was performed using MLST (Multi-locus sequence typing) server version 1.8 (Larsen et al., 2012). The sequence typing of *S. aureus* is routinely performed with allelic variants of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi,* and *yqiL*) (Enright et al., 2000a), and the combination of these variants defines a specific sequence type. It compares allelic diversity based on approximately 500 bp internal gene fragments (available at cge.CBS.dtu.dk//services/MLST). PHYLOViZ, version 1.1 (Francisco et al., 2012), was used to form a minimum spanning tree (MST) through the goeBURST algorithm (Francisco et al., 2009) and clonal complex (CC) formation (available at https://online.phyloviz.net/index).

3.1. Phylogenomic analyses of the selected S. aureus strains

The phylogenomic tree was generated based on the nucleotide alignment of the core genome from seventy-three *Staphylococcus aureus* strains predicted by the Roary pangenome pipeline (Page et al., 2015). FastTree (Price, Dehal and Arkin, 2010) and iTOL version 3 (https://itol.embl.de/) were used to construct the maximum likelihood tree.

3.2. Pangenome analysis of ovine mastitis-associated S. Aureus genes and proteins

Bacterial Pan Genome Analysis Tool (BPGA) version 1.3 (Chaudhari, Gupta and Dutta, 2016a) determines the ovine's core, accessory, and singleton genes. In addition, a group of complete genomes isolated from other hosts was added to the dataset for comparative purposes of the presence and absence of genes between hosts. This work considered two groups of genomes for pangenome analysis. The first group comprised twelve ovine strains (isolated from ovine, sequenced and assembled from herds in France), and three more ovine strains (ED133, NCTC1803, and NCTC9555) genomes sequences were downloaded from NCBI for the analysis. Moreover, genomes sequences of fifty-eight strains of *S. aureus* isolated from different hosts were retrieved from the NCBI database. In total this work, seventy-three genomes were considered for comparative genomic analyses. These strains were selected based on the diversity of hosts and the availability of complete and high-quality genomes, thus seeking their greatest population representability (**Table S1**).

3.3. Genome plasticity analyses in the selected S. aureus strains

The Genomic (GEIs), Pathogenicity (PAIs), and Symbiosis (SIs) Island predictions were performed using GIPSy (Genomic Island prediction Software), version 1.1.2 (Soares et al., 2016), with the genome of *Staphylococcus warneri* strain 16A (CP031269.1), retrieved from the NCBI genome database, used as the reference. BLAST Ring Image Generator (BRIG), version 0.95 (Alikhan et al., 2011), was used to generate a circular image of all ovine genomes with the predicted prophages, GEIs, and PAIs. Similar to the two clades (CC133 and CC130), strains 0268 and 082 were used (**Table S1**). The PHASTER (Arndt et al., 2016) was used to predict and annotate prophages in all strains' genomes. Considering the *S. aureus* methicillin resistance presence, we tried to identify the cassette chromosome *mec* (SCCmec) elements (Zafalon et al., 2018). A search for SCCmec IV, a cassette chromosome carrying the *mecA* or *mecC* gene, was performed by SCCmecfinder version 1.2 (Kaya et al., 2018). For this analysis, the thresholds of 90 % and 60 % for minimum identity and minimum length were used to compare the genome against the reference database (Kaya et al., 2018).

3.4. Resistance genes analysis in the ovine S. aureus strains

The Abricate (https://github.com/tseemann/abricate) tool was used to mass screen the genomes to find resistance: NCBI AMRFinderPlus (Feldgarden et al., 2019) CARD (Jia et al., 2017), Resfinder (Feldgarden et al., 2019), ARG-NNOT (Gupta et al., 2014) and MEGARES 2.00 (Doster et al., 2019) and for virulence genes, using the Virulence Factors of Pathogenic Bacteria (VFDB) (Chen et al., 2016).

4. Results

4.1. Genomic statistics from ovine S. aureus strains

The chromosomal genomes of the study's fifteen ovine *S. aureus* strains ranged from 2.7 to 2.8 Mb in size, with approximately 33 % of GC content. The PROKKA annotation predicted between 2,465 and 2,755 gene coding sequences. All genomes showed at least one region associated with prophages, with a maximum of seven regions on some chromosomes Table 2.

4.2. Phylogenomic analyses provide better clustering of ovine S. aureus strains

Phylogenomic analyses evaluated the clustering of *S. aureus* strains from different hosts on a genomic scale. The phylogenomic tree built showed a bootstrap between 0.9 and 1.0, as shown in Fig. 1, represented by the taxon's internal circles. It is possible to observe the grouping of populations by their type of clonal complex. The strains belonging to CC130 (O82, O46, O326, O11, and O408) formed a single clade (Fig. 1). The phylogenomic tree shows a common ancestor for all lineages proven to be ovine. The strains of CC130, CC133, CC425, CC59, and CC30 comprise five distinct clades. Two ovine clusters were formed from two major clonal complexes with similarities ranging from 97 to 100 % (CC130 and CC133). The phylogenomic analysis also revealed that these clusters did not group three strains from ovine (O217, O55, and O331).

Table 2
General genomic features of fifteen ovine *S. aureus* strains analyzed.

	Strain	Genomes length (bp)	GC content (%)	CDS Predicted	Number of Phages	NCBI Accession Number
1	B119	2,768,115	32.82	2597	3	CP038460
2	O11	2,770,352	32.72	2610	3	CP024649
3	O17	2,772,340	32.89	2598	4	CP032051
4	O217	2,764,228	32.86	2572	4	CP038461
5	O267	2,864,632	32.93	2755	6	CP034102
6	O268	2,841,948	32.91	2707	7	CP038612
7	O326	2,778,005	32.85	2616	3	CP032481
8	O331	2,695,389	32.91	2465	1	CP038269
9	O408	2,778,443	32.85	2618	3	CP038270
10	O46	279,141	32.80	2644	3	CP025395
11	O55	2,794,042	32.89	2650	4	CP038268
12	O82	2,761,328	32.89	2593	3	CP038819
13	NCTC1803	2,836,670	32.90	2692	5	LR134305
14	NCTC9555	2,843,795	32.94	2685	6	LR134090
15	ED133	2,832,478	32.92	2701	6	CP001996

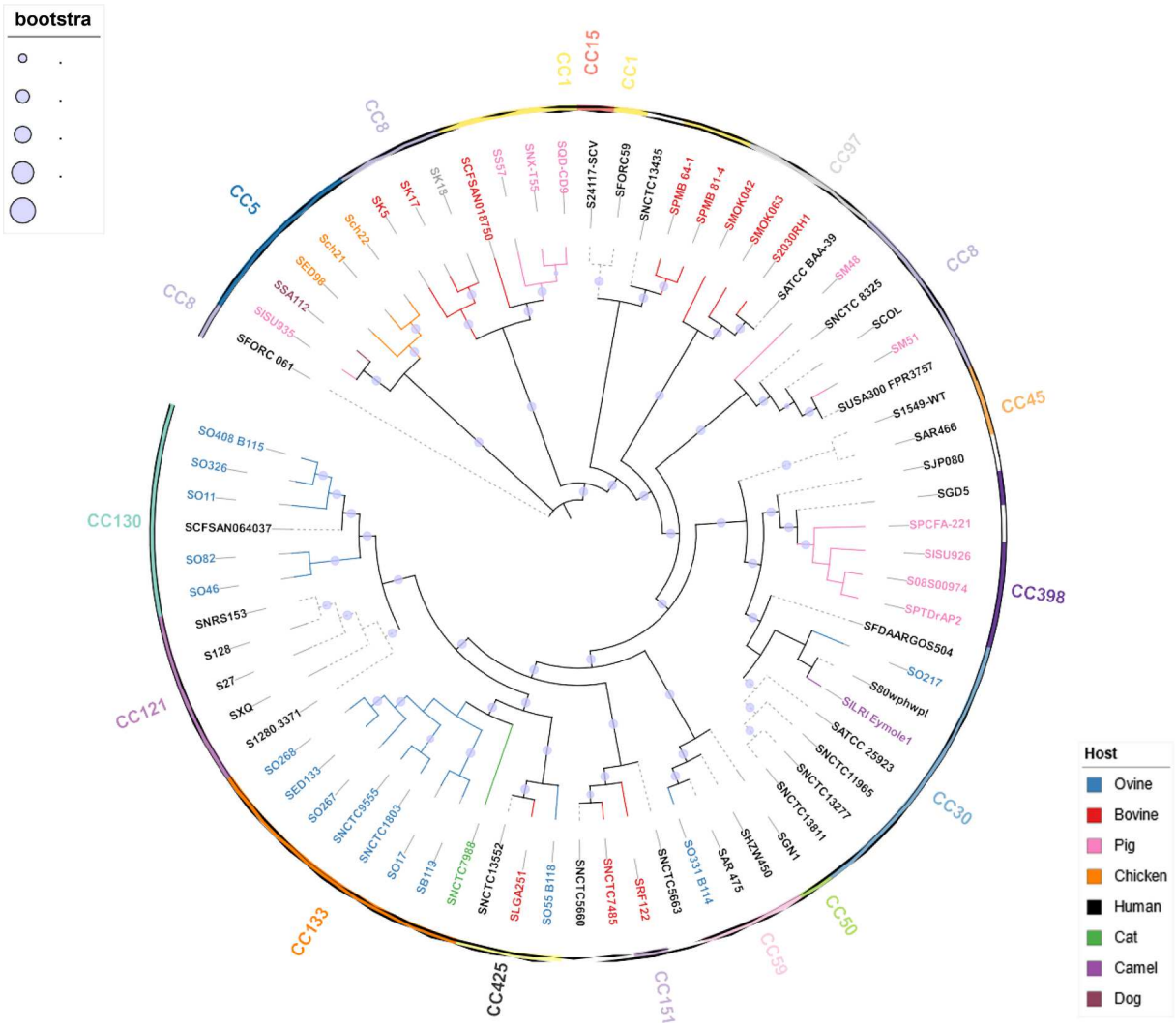

Fig. 1. Molecular phylogenomic analysis based on the nucleotide alignment of the core *Staphylococcus aureus* strain genomes. The hosts are in the following colours, and each complex clonal (CC) is highlighted with a coloured line.

However, strain O55 (CC425) was grouped with the strains isolated from cattle and humans. The strain O331 (CC59) is isolated from humans, and the strain O217 (CC30) forms a single clade with the strains from humans and camels.

4.3. ANI analysis evidence ovine S. aureus genomic relatedness

ANI predictions were conducted using all selected *S. aureus* strains to understand how the ovine strains genetically correlate with each other and with the strains from the other hosts. The genome of ovine *S. aureus*

strain ED133 was used as the reference for the ovine sequence. All genomes presented a high degree of similarity with the genome of the reference strain ED133 (ANI > 97 %). Most ovine *S. aureus* genomes were clustered in the highest similarity group (Fig. 2). The genomes NCTC9555, B119, O17, O267, O268, NCTC1803, and NCTC7988, presented the most significant similarities (nearly 100 %) with ED133. Interestingly, NCTC7988 was the non-ovine strain with the most considerable similarity with the ED133 reference, corroborating the previous phylogenomic clustering in this work (Fig. 1). In this analysis, all ovine genomes presented more than 98 % similarity regarding the ED133 genome, except for O217, which showed a minor similarity among sheep strains. In the same way, this strain was not grouped with any ovine genomes in MLST analysis. This study showed that it belongs to CC30, which is important in human and animal infections, and suggests no-host-specialization by this strain. On the other hand, genomes isolated from milk or mastitis in cows (LGA251 and RF122) present a similar level of 98 % regarding ED133 (isolated from ovine).

4.4. Molecular typing and phylogenetic analysis revealed large clonal complexes

The MLST analysis revealed that most of the sheep strains considered in this study had a cluster of two large clonal complexes (CC), CC130 and CC133. The exceptions are strain O55, which was grouped in CC425, together with the strains cattle (LGA251) and human (NCTC13552). The strain O217, which comprises CC30 together with the strains isolated from humans (ATCC25923, 80wphwpl, SFAARGOS_504, NCTC11965, NCTC13277, and NCTC13811), and camel (ILRI_Emoyle1/1). The strain O331 shares the clonal complex, only the human strains (SAR475 and SHZW450). All alleles predicted in these analyses are shown in **Table S1**.

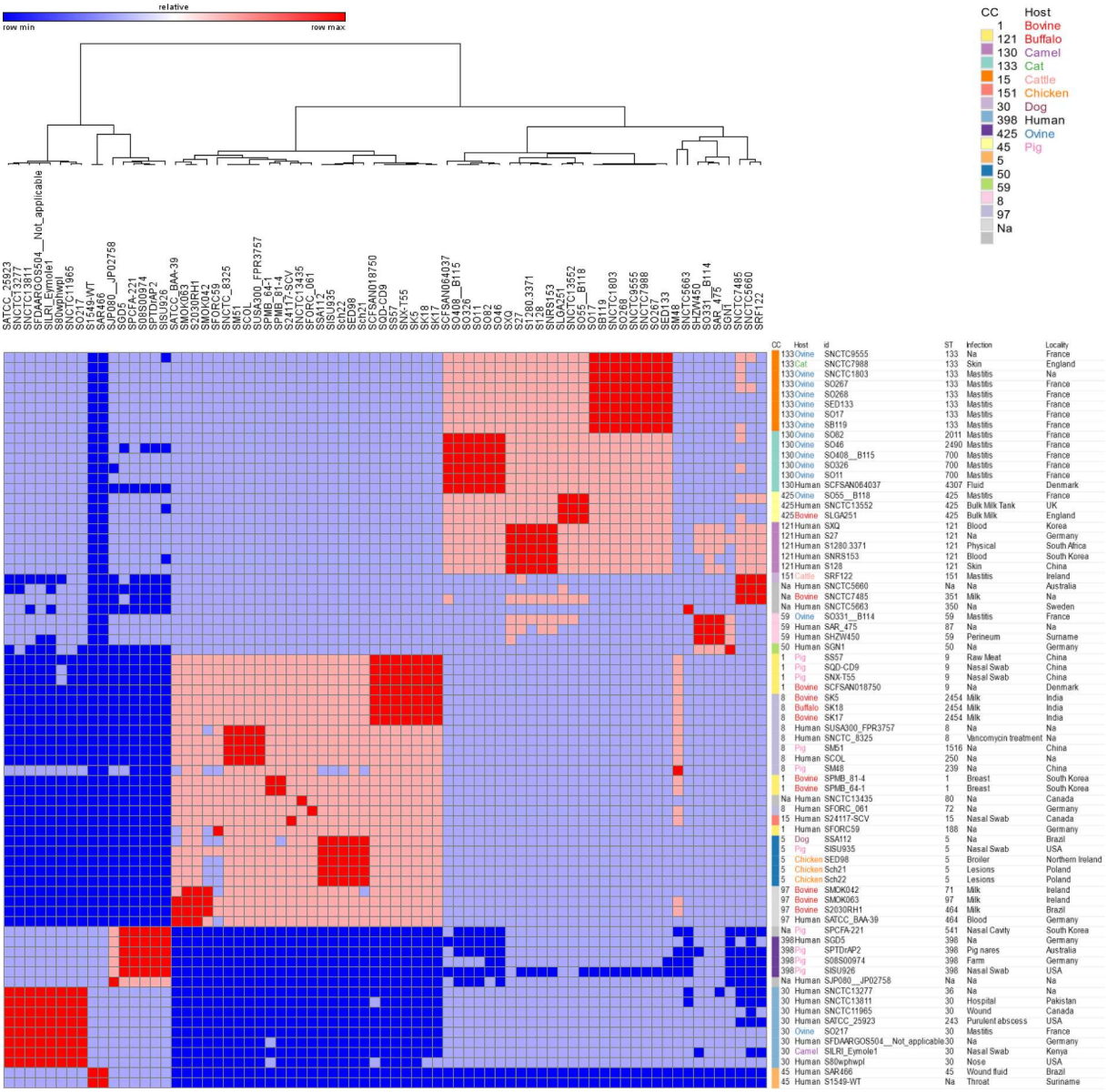

Fig. 2. The study investigates the heatmap of pairwise ANIb values of the 73 *Staphylococcus aureus* genomes. Red a high similarity; Blue a low similarity and light colors (blue and red) have an intermediate similarity. The value of similarity level intensity is based on the ANI analysis by JSpeciesWS. Metadata for cluster correlation: Sequence type (ST), Clonal complex (CC), Host, Sample, and Isolation locality. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.5. Identification of core, accessory, and exclusive genes in selected S. aureus strains

The 73 *S. aureus* genomes were separated into two groups for the comparative genomics analysis. The first group consisted of genomes isolated from the ovine host, and the second group was composed of genomes isolated from other hosts (Table 2). Using 50 % sequence identity as the cutoff value, the BPGA yielded 4200 distinct gene clusters. Out of which 2008 clusters respectively contain at least one gene from each genome and thus construct the core genome, 1559 of these genes are accessory genes (i.e., genes that are present in at least two genomes but not in all the genomes) and 634 unique genes (exclusive genes) of *S. aureus*. The genome statistics, such as the gene family distribution and new gene distribution of the *S. aureus* pangenome, are shown in Fig. 3. In addition, the number of genes unique or exclusively present and absent in each strain of the *S. aureus* dataset is shown in the last two columns of **Table S2**.

At the level of functional genome analysis, the genes belonging to each group's core, accessory, and exclusive genomes were categorized into cellular processes and functions by Cluster of Orthologous Groups (COG) distribution (Fig. 3**c**). Most of the significant spotted genes were associated with cellular metabolism, mainly the transport and metabolism of amino acids and unknown proteins. The accessory and unique genes were classified in greater representativeness with the following COG functional groups, replication (information storage) and recombination and repair (processing activities), general function, defense mechanisms, transcription, and unknown proteins. Out of 4200 gene clusters, BPGA could map 1866 (44.5 %) to KEGG pathways. From a perspective, KEGG assignments from BPGA showed an overall higher representation of metabolism-related pathways (Fig. 3**d**) and **Fig S4** (more detailed version).

The highest values of the number of exclusively absent genes in strains SMOK042 (bovine), SATCC_BAA-39 (human), and SM51(pig). The first two genomes belong to the CC97 and the third to CC8. When compared in this scenario against other hosts, the genomes of the ovine-associated strains did not show exclusively absent genes. Among the 634 unique genes seen, 28 genes were exclusive to ovine-associated strains; we highlight the species SO55, SNCTC9555 (18 and 6), and the SB119, SO46, SO82, and SO268 strains, with only one exclusive gene in each genome in TableS2. When these sequences are characterized *in silico* prediction, primarily hypothetical proteins, phage proteins, and only four proteins with related functions, such as DNA binding, cytosolic resistance, hyperosmolarity, and centrosome B protein, they are related to molecular function, cellular components, and biological processes.

Therefore, analyzing the inter-host (ovine) in subsets was necessary. In the subset analysis conducted, the pangenome profile analysis for fifteen ovine hosts and fifty-eight other hosts of the *S. aureus* dataset may help identify host-specific or virulence-specific genes in *S. aureus*. The results achieved for gene family distribution in these two subsets are shown in Table S3 and Table S4. We observed the presence of 277 unique genes in sheep strains. The genomes that presented the most exclusive genes were the genes corresponding to the complex clonal singletons, the SO55, SO217, and SO331 (72, 54, and 41) exclusive genes, respectively. The functional characterization resulted in products such as hypothetical proteins (39.6 %), phages (6.4 %), transporter (2.9 %), toxins (6.4 %), and others (44.7 %). A very high percentage of exclusive ovine *S. aureus* genes encode proteins with unknown functions, evidencing the need for further studies. Also, genes encode proteins involved in different cellular functions, such as iron acquisition proteins, transferases, DNA helicases, and coagulases. Interestingly the exclusive ovine genes are found in prophages and mobile genetic elements, suggesting that many of the genetic traits related to the ovine host specificity might be horizontally disseminated.

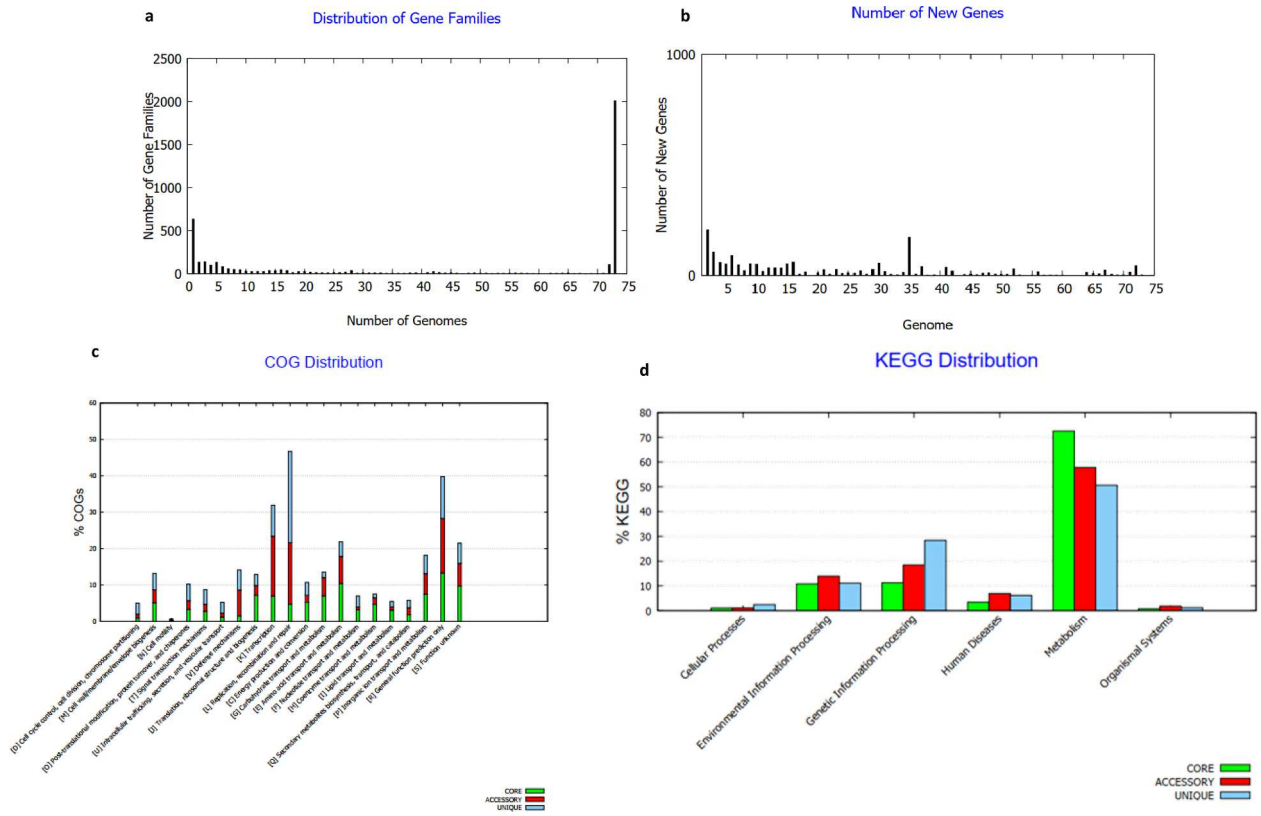

Fig. 3. Graphical representations of profiles of the genes assessed in the BPGA comparative analysis of all 73 selected *S. aureus* strains. (**a**) The gene family frequency spectrum. (**b**) New gene family distribution after sequential addition of each genome to the analysis. (**c**) COG distribution of core, accessory, and unique genes. (**d**) KEGG distribution of core, accessory, and unique genes.

4.6. Virulence genes in the S. aureus strains

Eighty genes were observed, considered encoders of the virulence factors, and 57 to 72 genes were present in all seventy-three genomes. All virulence proteins predicted using the VFDB database are listed in (Fig. 4**a** & **4b**).

The exciting thing is the sharing of genes related to the type of clonal complex, regardless of its type of host. However, there are some signatures regarding the suborder, as in the case of ruminants and small ruminants (Fig. 4**b**). When analyzed at the ovine subgroup level, we observed 59 to 70 virulence genes. CC130 and CC133 (large ovine clusters) have a repertoire of similar genes compared with singletons: CC30, CC59, and CC425. Some genes will be clustered according to the CC (twenty-nine genes), genes related to enzymes, *chp* (chemotaxis inhibitory protein of *Staphylococcus* - CHIPS) and *sak* (staphylokinase), and toxins such as the *lukS-PV* gene (Panton-Valentine leukocidin), at the level of clonal complexes shared with ovines, are seen only in strains isolated from humans. Some of these genes share only an ovine strain, such as the *cna* (collagen-binding protein) and the *scn* (staphylococcal complement inhibitor) genes, in the SO217 and SO331 strains, respectively. Three genes involved in the secretion system, Type VII, *esaC*, *essC,* and *esxB* relationship, were predicted only in CC130 and CC133 compared to other complexes shared with them. The overall comparison level (n = 73) had a significant presence and absence when comparing the genes associated with the secretion system (Fig. 4**b**).

Toxin.

Stress protein Secretion system Iron uptake Immune evasion.

Enzyme Capsular Adherence.

Pig.

4.7. Resistance genes in the ovine S. Aureus strains

The analysis of the presence and absence of resistance genes was performed via ABRicate with the support of the five databases and other relevant data available in PATRIC, such as AMR phenotypes for genomes, AMR genes, and AMR regions, aimed at predicting resistance to some antibiotic (Fig. 5 **and** Fig. 6). ABRicate predicted 37 genes related to some antimicrobial resistance for the 73 genomes. PATRIC is classified into eight classes of antibiotic drugs (ciprofloxacin, clindamycin, erythromycin, gentamicin, methicillin, penicillin, tetracycline, and trimethoprim-sulfamethoxazole) from a database containing 97 antibiotics. Analyzing the repertoire of resistance-related genes for ovines, each database yielded different predictions, with some of these genes varying in nomenclature. MEGARES 2.00 (18), CARD (10) ARG-NNOT (8), NCBI AMRFinderPlus (5) and Resfinder (2). All ovine strains did not show the presence of the chromosomal cassette SCCmec, referring to phenotypic resistance to methicillin; however, some genomes showed resistance to penicillin and gentamicin *in silico* analysis (Fig. 5). The *bla* operon genes (*blaI*, *blaR*, and *blaZ*) were predicted only in isolates SO217 (CC30) and SO55 (CC425). The *fosB* gene was present only in B119, ED133, NCTC1803, NCTC9555, O17, O267, O268, O217, and O55. Eight genomes showed the same nucleotide variations in the *GlpT* (A100V) and *murA* (E291D and T396N) genes, and both mutations confer resistance to fosfomycin. Only the SO217 genome showed specific variations in *GlpT* (A100V and V213I) and *murA* (D278E and E291D) (Table 3).

4.8. Prophages

In this study, in the 73 bacteria of *S. aureus* species, 299 phage-like elements were detected; 114 were classified as intact prophages, 133 as incomplete, and 52 were questionable using the PHASTER tool. The distribution of completeness among hosts can be visualized in Fig. 7 and Table S5. All genomes had at least one predicted prophage in the chromosomal region and were considered lysogenic bacteria (bacteria containing the integrated prophage) or poly-lysogenic bacteria (bacteria containing more than one integrated prophage). Table S5 can see more details about these regions, such as the number of predicted regions in each genome, totally predicted proteins, region position, most common phage, and GC% content. Of the prophages considered intact or complete (1 1 4), the smallest was found in *S. aureus* O408_B115 (ovine) with 17.8 kb, and the largest was in M48 (pig) with 134.2 kb. In the 52 considered questionable, the lowest was found in NCTC13811, 8 kb, and the highest in O46 (ovine), 61.4.5 kb. For the prophages considered

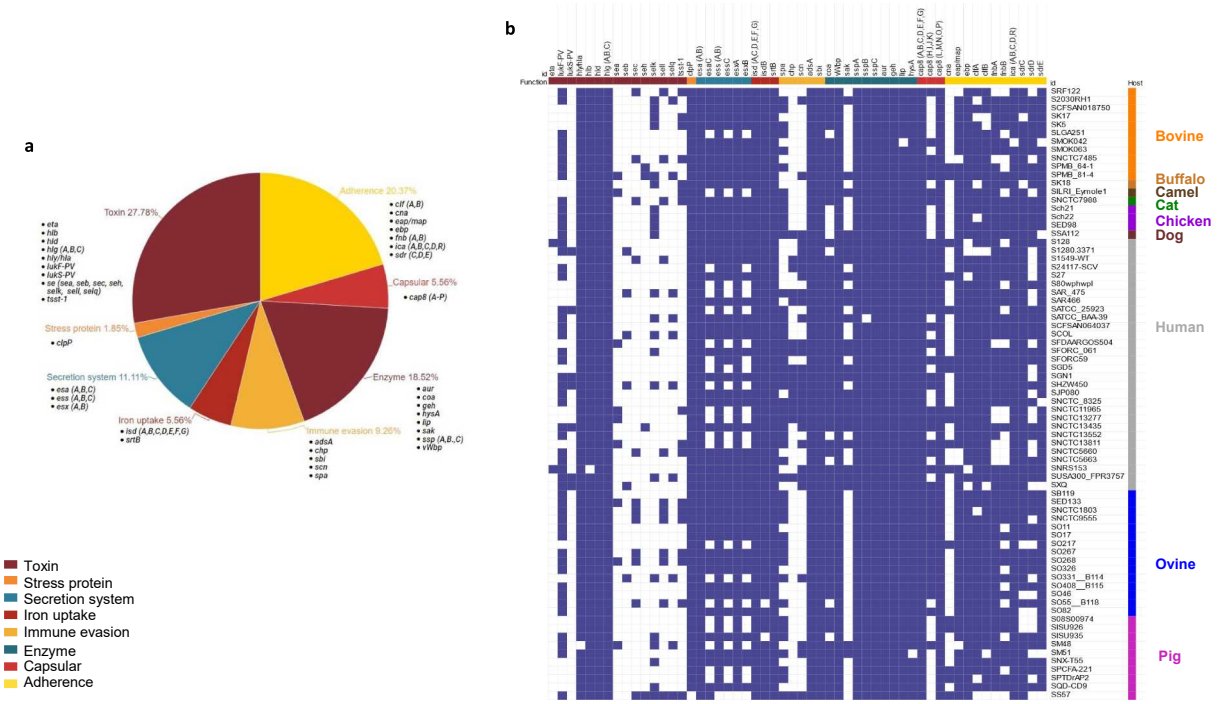

Fig. 4. Visualization of core virulome genes of the *S. aureus* strains according to the VFDB prediction (**a**) Graphical representations of virulence factor classes of these genes. (**b**) Heatmap with presence and absence associated with virulence factors that *S. aureus* correlates to strains and hosts.

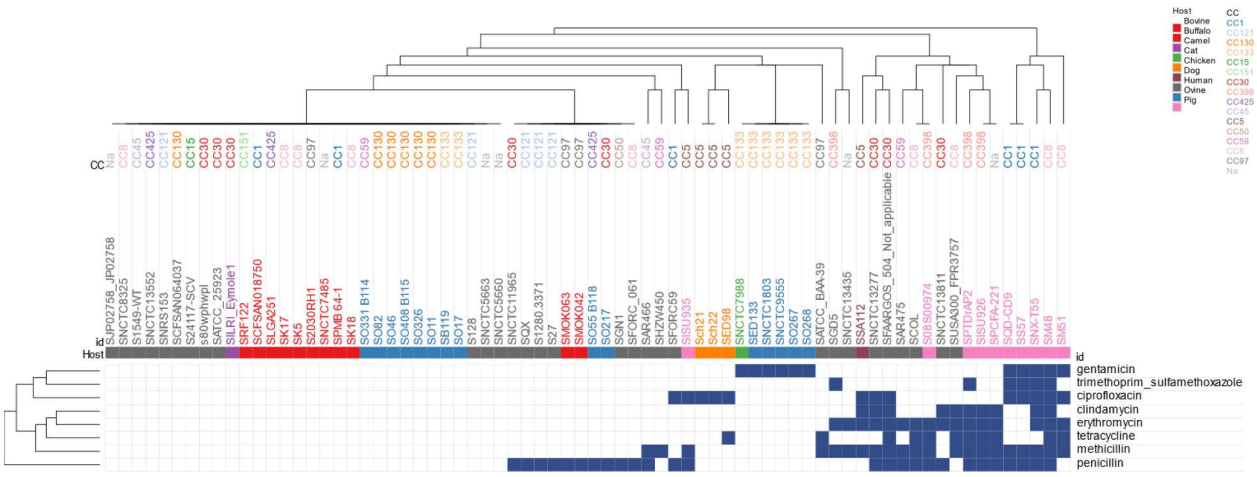

Fig. 5. Visualizing antimicrobial resistance (antibiotic) of the *S. aureus* strains according to the PATRIC prediction. Heatmap with presence (blue) and absence associated with resistance genes that *S. aureus* correlates to strains, hosts, and complex clones (CC). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

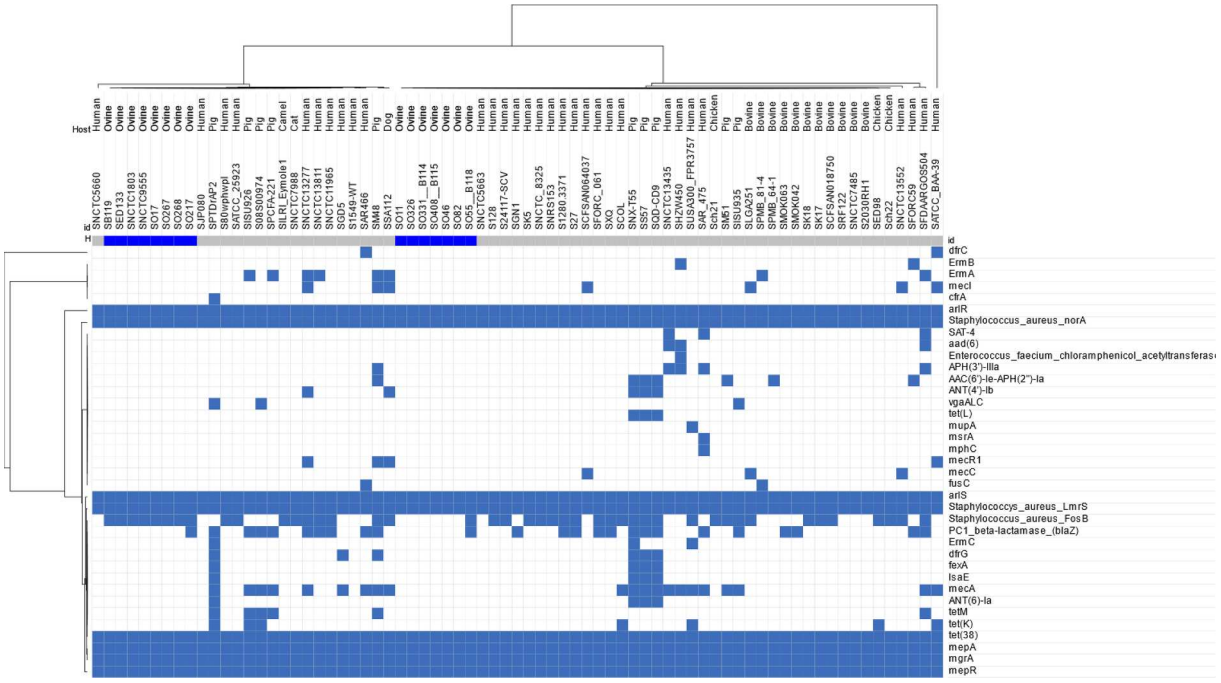

Fig. 6. Visualization of core resistome genes of the *S. aureus* strains according to the CARD prediction. Heatmap with presence (blue) and absence associated with resistance genes that *S. aureus* correlates to strains and hosts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

incomplete (degenerate prophages), the smallest size was M48 (pig) with 5.8 Kb and the largest MOK063 (bovine) 45.5 Kb. At the ruminant level, ovine showed approximately twice as many prophages classified as intact (33), while in bovine, only 12 can be observed as a percent in Fig. 7. All ovine genomes showed regions referring to prophages, and 68 regions were identified (intact = 33, incomplete = 28 and questionable = 7). Each isolate showed at least three to seven regions; only strain O331_B114 showed one region (PHAGE_Staphy_PT1028_NC_007045), and even then, with questionable completeness. The most common phages (20 types) were identified as bacteriophage sequences for *Staphylococcus phage* (16) and other types such as *Paenibacillus phage* (1) *Clostridium phage* (1), *Streptococcus prophage* (1) and *Thermus phage* (1). Only PHAGE_Staphy_PT1028_NC_007045 was predicted in all ovine isolates and PHAGE_Staphy_phiPV83_NC_002486 in almost all except O217, O331_B114 and O55_B118.

All prophages had their ORFs predicted for virulence factors (128 genes) using the VFDB; 349 genes were identified in 67 genomes, and only six strains (CFSAN018750, K17, LGA251, K18, 08S00974, ED98, NCTC13552) did not identify virulence factors in their prophage sequences Table S6. In ruminants (ovine and bovine), virulence genes associated with a toxin (32 and 30), immune evasion (13 and 3), and enzyme (12 and 24), respectively, were identified. It is noted that there was an absence of genes related to adherence and secretion system carried by prophages in these hosts in Fig. 8. The complete list of virulence genes predicted in prophage-related sequences can be seen in Table S6.

In the circular comparative genome mapping representation (Figs. 9 and 10), it is possible to visualize the regions related to prophages and

Table 3
Resistance genes identified by *in silico* prediction against ABRIcate of fifteen ovine *S. aureus* strains.

AMR Gene	SNP	Detection Criteria	AMR Gene Family	Drug Class	Resistance Mechanism
mepA			multidrug and toxic compound		
mepR			extrusion (MATE) transporter	glycylcycline, tetracycline antibiotic	
arlR					
arlS				fluoroquinolone antibiotic, acridine dye	
***Staphylococcus aureus* norA**				fluoroquinolone antibiotic	
***Staphylococcus aureus* LmrS**		protein homolog model	major facilitator superfamily (MFS) antibiotic efflux pump	macrolide antibiotic, aminoglycoside antibiotic, oxazolidinone antibiotic, diaminopyrimidine antibiotic, phenicol antibiotic	antibiotic efflux
tet (38)				tetracycline	
PC1 beta-lactamase (blaZ)			blaZ beta-lactamase	penam	
***Staphylococcus aureus* FosB**			fosfomycin thiol transferase		antibiotic inactivation
GlpT*	A100V		antibiotic-resistant GlpT	fosfomycin	
murA*	E291D, T396N	protein variant model	antibiotic-resistant murA transferase		antibiotic target alteration

* *Staphylococcus aureus* GlpT /murA with mutation conferring resistance to fosfomycin.

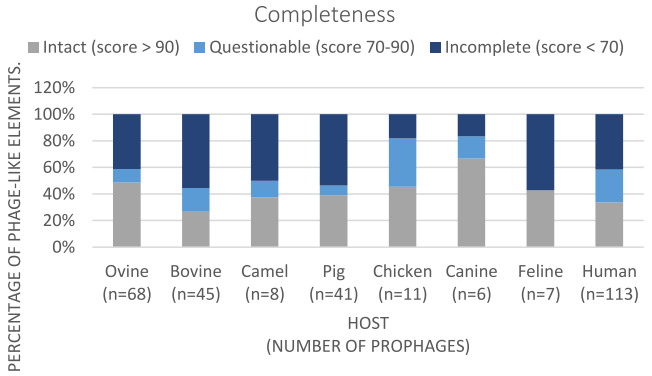

Fig. 7. . Distribution of the prophage-like elements within *S. aureus* in a different host. The values of the number of features found (n) are shown below each host's name. Completeness is divided into three stages represented by different colours (intact = grey, questionable = light blue and incomplete = dark blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

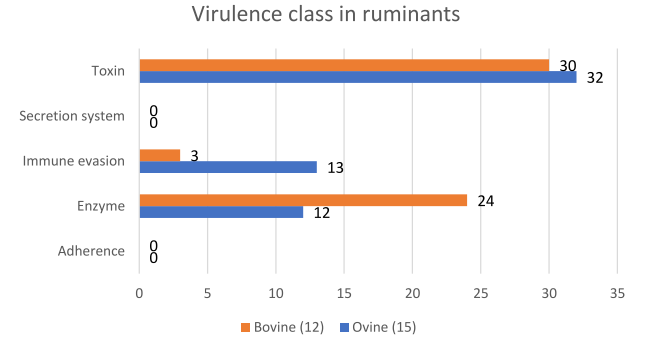

Fig. 8. Distribution of virulence class predicted with VFDB tool in prophage sequences in ruminants. Axis × is composed of the number of predicted genes. Axis y is categorized into all predicted virulence classes. The small ruminant (ovine) is the blue bar, while the orange bar represents the large ruminant (bovine). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

predicted virulence genes positioned along the genomes.

5. Discussion

S. aureus is an important pathogen of ovine mastitis (Le Maréchal et al., 2011; Smith et al., 2014). This infection is difficult to control since *S. aureus* can spread within the herd and become resistant to antibiotic treatment (Oviedo-Boyso et al., 2007). In addition, the number of ovine-associated *S. aureus* genomes available in the NCBI database and genomic studies involving these strains are scarce. Therefore, more information about *S. aureus* genomic characteristics is necessary to efficiently develop new strategies to control infection (Peton et al., 2014).

The isolates from France are of different herds, cities, and years, yet they share a high similarity. For example, the strains SAR475 and O331 are grouped in the MLST analysis. Considering that SAR475 was isolated from humans and O331 from ovine mastitis, this result shows that these two strains are closely related. Additionally, LGA251 and O55 are once more grouped, reinforcing the close phylogenetic relationship between these two strains isolated from different hosts and countries. These two strains present mammary gland tropism since LGA251 was isolated from a cow's bulk milk and O55 from ovine mastitis. In turn, the grouping of O217, IRLI_Emoyle1/1, and ATCC25923 is not supported in the phylogenomic approach, in contrast to the result provided by the MLST analysis. This result suggests that although these three strains belong to the same clonal complex (CC30), they do not share an extremely high level of similarities in their proteomes. Two ovine clusters were formed in the phylogenomic analysis with similarities ranging from 97 to 100 %. It is possible to observe clonal behavior among them compared with strains from the other hosts. However, these clusters did not group three strains (O217, O55, and O331) from ovine. O217 was grouped with genomes isolated from humans, agreeing with the CC formation. The same clustering agreement is observed for O55, which belongs to the same clonal complex of LGA251.

The ANI method is another way to measure evolutionary relatedness among closely related bacterial strains through the identity and similarity values of the total genome sequence (Konstantinidis, Ramette and Tiedje, 2006; Kim et al., 2014b). In this analysis, all ovine genomes presented more than 98 % similarity regarding the ED133 genome, except for O217, which showed a minor similarity among sheep strains. In the same way, this strain was not grouped with any ovine genomes in MLST analysis. This study showed that it belongs to CC30, which is important in human and animal infections, and suggests no-host-specialization by this strain. On the other hand, genomes isolated from

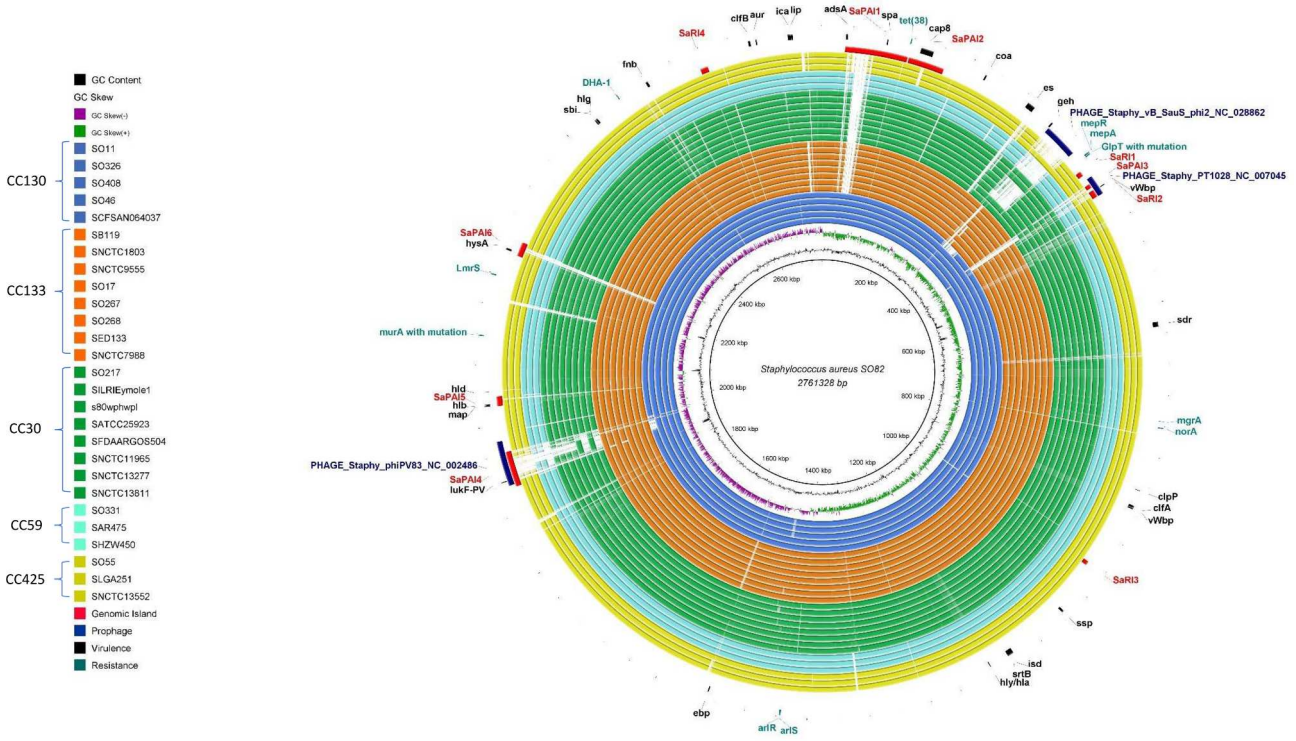

Fig. 9. Comparative circular genome maps the representation of the BRIG software with 28 genomes of *Staphylococcus aureus* isolates of ovines and other hosts. The O82 strain was the reference in the central position, with the first three inner rings showing its size, GC content, and GC skew, mapping all prophages, genes associated with resistance, virulence, and pathogenic islands found in the O82 strain.

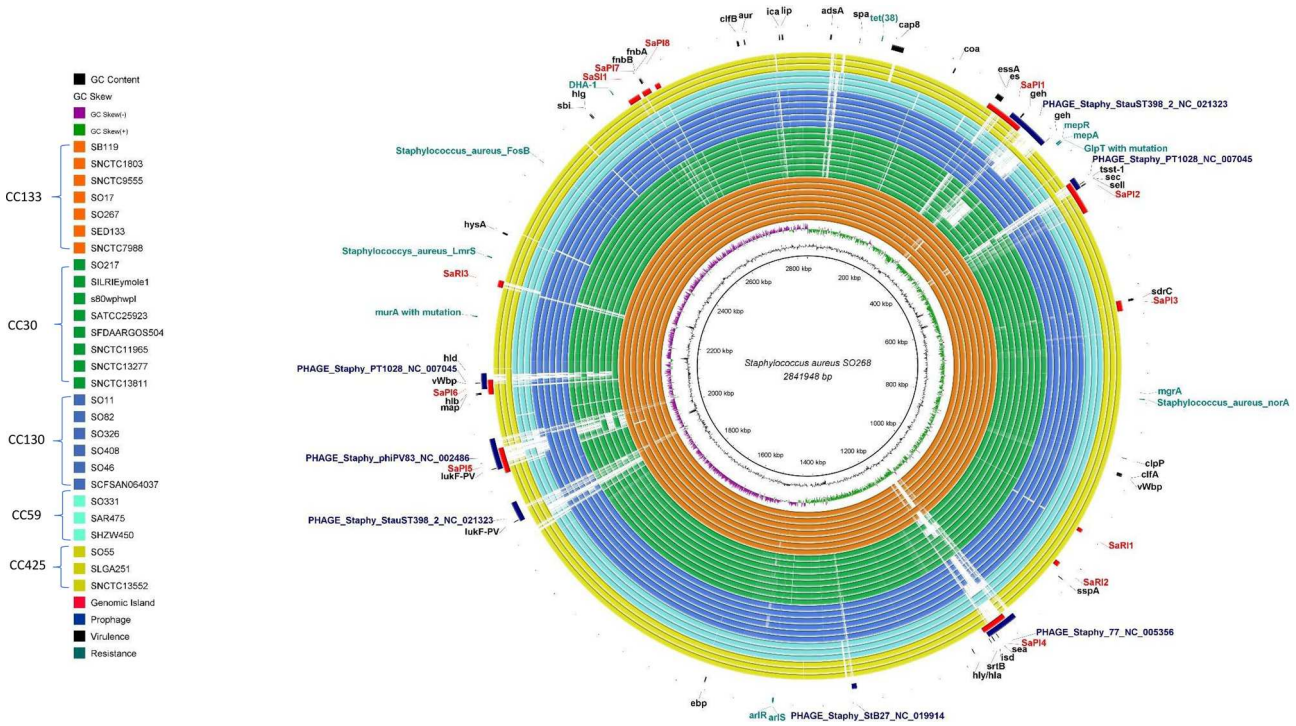

Fig. 10. Comparative circular genome maps the representation of the BRIG software with 28 genomes of *Staphylococcus aureus* isolates of ovines and other hosts. The O268 strain was the reference in the central position, with the first three inner rings showing its size, GC content, and GC skew, mapping all prophages, genes associated with resistance and virulence, and pathogenic islands found in the O268 strain.

milk or mastitis in cows (LGA251 and RF122) present a similar level of 98 % regarding ED133 (isolated from ovine). Previous studies reported that some CCs originated from humans and acquired genetic adaptation to infect ruminants (Guinane, Ben Zakour, et al., 2010; Sakwinska et al., 2011). Furthermore, the evolutionary separation of some ovine and bovine strains is unclear in the present study.

Additionally, three of the selected ovine strains belonged to the CC30 (STs 30 and 243). Studies have associated these STs with human, bovine, and ovine infections in Europe and Asia (Rabello et al., 2007; Monecke, Slickers and Ehricht, 2008; Smith et al., 2014; Haag, Fitzgerald and Penadés, 2019b). The strains ED133, O268, O17, O322, and O267 present the same ST and belong to the CC133, which is associated with intramammary infections in ovine (Smyth et al., 2009; Smith et al., 2014). Interestingly, only strain O331 presents the ST59. *S. aureus* ST59 has already been isolated from bovine milk (Hata et al., 2010; Richardson et al., 2018) and dairy goat mastitis (Chu et al., 2012). Therefore, the strain O331 is likely an atypical clone among the ovine strains considered in this study. Studies have shown that ST59 isolates originated in the United States and have spread to Asia. Furthermore, ST59 *S. aureus* has been isolated in Oceania and northern Europe (Tristan et al., 2007; Mediavilla et al., 2012; Smith et al., 2014). Further studies are necessary to track the origin of the ST59 isolates in France. A previous study suggested that the Multilocus Sequence Analysis (MLSA) can accurately determine the evolutionary relationships among the strains of *Flavobacterium columnare* (Kayansamruaj et al., 2017). However, a comparative genomics approach can provide a much higher strain typing resolution than the MLST analysis since more genes are considered (Hall, Ehrlich and Hu, 2010). In the MLST-based phylogenetic tree generated, the group of CC130 is divided into two different clades, one formed by the STs 2490 (O46) and 2011 (O82) and the other formed by ST700 (Fig. 1). In turn, the phylogenomic analysis based on the amino acid variation in the proteomes of the *S. aureus* strains resulted in the grouping of all strains belonging to the CC130, with a bootstrap value of 1. However, the phylogenomic analysis also reinforced that STs 2490 and 2011 are more closely related than ST700. This result indicates that the determination of phylogeny benefitted from a proteome-scale analysis compared to the limited number of gene *loci* considered in the MLST-based phylogenetic analysis.

A very conserved synteny level is observed on the ovine *S. aureus* strains. However, a few regions of DNA inversion were found. Differences caused by gene acquisition from phages and DNA recombination events likely explain the size variation among the ovine strains' genomes. One example involves PHAGE_Staphy_phiPV83_NC_002486, which was absent only in the O217, O331_B114 and O55_B118 genomes (Figs. 9 and 10). To evaluate the clonal relationship of the ovine *S. aureus* strains, MLST analysis was conducted. If at least 5 out of the seven alleles (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi,* and *yqiL*) in the different loci are shared between two *S. aureus* isolates, these isolates form a single clonal complex (Maiden et al., 1998; Enright et al., 2000b; Tettelin et al., 2005). The CC130, which had already been associated with ovine mastitis (Guinane, Ben Zakour, et al., 2010; Smith et al., 2014 Haag, Fitzgerald and Penadés, 2019b), encompassed 5 of the ovine strains considered in this study (STs 2411, 2490, and 700).

The genomic diversity of multiple strains can be uncovered by determining the core, accessory, and single genomes (Bosi et al., 2016b; Chaudhari, Gupta and Dutta, 2016b). The pangenome curve almost reached a plateau, indicating that it is open, and the *S. aureus* pangenome will probably close soon (Fig. S1). All strains used in this comparative analysis have an average of resulting genes predicted by the PATRIC server. The analysis of proteins exclusively shared among genomes of *S. aureus* involved in a single form of mastitis resulted in predicting hypothetical and phage-related proteins. However, exclusive genes are shared by at least two mastitis group strains and not by all strains (Table S4). Therefore, further studies are necessary to elucidate the molecular basis of *S. aureus* virulence that might lead to the development of different forms of mastitis. Additionally, specific traits such as susceptibility to ovine and interference with the environment and intestinal microbiota dysbiosis could contribute to developing a determined form of mastitis caused by *S. aureus* (Vonaesch, Anderson and Sansonetti, 2018; Oikonomou, Addis, Chassard, Nader-Macias, Grant, Delbès, Bogni, le Loir, et al., 2020).

The comparative genomics performed using BPGA software yielded 4200 genes. In turn, the core genome is formed by 2008 genes among all strains. It represents 48 % of the *S. aureus* genes average predicted in this study and a core genome 1,7-fold proportionally higher than a previous study of comparative genomic analysis in 64 *S. aureus* strains, which resulted in a core genome of 56 % on average of the predicted genes (Bosi et al., 2016b). The core genome analysis was also performed among ovine and other host strains, resulting in 2149 and 2119 genes, respectively. It represents 79 % (ovine strains) and 81 % (other hosts) of the average genes predicted by the PATRIC server. This study's number of core genes suggests low diversification in the core genome of *S. aureus* strains used for comparison. The functions of core genes were classified by COG categories, playing a role in amino acid transport and metabolism. These data are expected because the core genome belongs to the group of housekeeping functions (Tettelin et al., 2005 Medini et al., 2005).

The accessory genes predicted in this present study (n = 1559) are primarily involved in replication, recombination, and repair. These genes play a role in lateral gene transfer events from mobile genetic elements, such as transposons and bacteriophages (Tettelin et al., 2005Bosi et al., 2016b). Of the exclusive genes found in ovine genomes (n = 277), the central part consists of hypothetical proteins (39.6 %), which are proteins of unknown function, and can contribute to many activities in the genome. For instance, a functional assignment of hypothetical proteins in the *S. aureus* study predicted these as binding proteins, helicases, transporters, and virulence factors (Prava, G and Pan, 2018). Mobile genetic elements represent 25 % of the exclusive ovine genes. They are essential in bacterial diversity due to their capacity to transduce host genes and confer novel genetic information for bacteria, such as genes involved in virulence (Kwan et al., 2005). Some genes are predicted to play a role in virulence and are carried by phage, *entB* (Staphylococcal enterotoxin B), *selk* (Staphylococcal enterotoxin K), and *vWpb* (Secreted von Willebrand factor-binding protein precursor). Genes encoding cell wall proteins represent 3 % of the genes found in all ovine genomes. These proteins are essential for adherence (Silhavy, Kahne and Walker, 2010) and antibiotic resistance (Assis, Nedeljković and Dessen, 2017).

Staphylococci are significant causes of drug-resistant infections, and phages that infect and destroy these organisms have enormous therapeutic potential (Nayeemul Bari and Hatoum-Aslan, 2019). The five prophages (intact = 1 and incomplete = 4) were found in only ovine strains, not in strains from other host groups. The PHAGE_Staphy_42E_NC_007052, present only in NCTC9555, was predicted to be an intact phage with a PHASTER score of 150 and produces 69 proteins. The phage gene set is functionally classified into integrase, tail, portal, head and capsule. Of note, (Kwan et al., 2005) showed that gene transfer between *S. aureus* phages is more prevalent than between *S. aureus* and other species. However, four incomplete regions were found only in ovine, more commonly in other species different from *S. aureus*. The PHAGE_Strept_EJ_1_NC_005294 (O217) in the *Myoviridae* morphology family and found an atypical *Streptococcus pneumoniae* (Romero, López and García, 2004), the PHAGE_Thermu_OH2_NC_021784 (O268), in *Geobacillus kaustophilus* (Doi et al., 2013) and the PHAGE_Clostr_phiMMP03_NC_028959 (NCTC9555) in *Clostridioides difficile* (Rashid et al., 2016). The protocols described in Nayeemul Bari and Hatoum-Aslan, 2019, hoped to advance the basic understanding of *Staphylococcus* phages while allowing the development of more powerful phage-based antimicrobials. Likewise, 32 genes were predicted to be toxins precursors in phage regions. Toxins have an essential role in pathogenicity; in this context, *S. aureus* exotoxins are a leading cause of gastroenteritis in humans from the consumption of contaminated food, principally raw milk and raw milk cheese, which can infect animals(Balaban and Rasooly, 2000; Le Loir, Baron and Gautier, 2003; Oikonomou, Addis, Chassard, Nader-Macias, Grant, Delbès, Bogni, le Loir, et al., 2020; van den Brom et al., 2020). Additionally, exfoliative toxins were found in *S. aureus* isolated from cows, and these proteins are an agent of a scalded-skin syndrome in humans (Vautor

et al., 2009; Que and Moreillon, 2014). In addition, the exclusive ovine genes were compared to the VFDB database through BLASTp. They resulted from 14 predicted proteins, such as the coagulase (*coa*), secreted by *S. aureus* and causes clotting in the host's plasma. As a result of mechanisms of escape from the immune system (Salaberry et al., 2015; Javid et al., 2018), hyaluronate lyase precursor (*hysA*) plays a role in subcutaneous infection (Makris et al., 2004; Ibberson et al., 2014) and toxins (Le Maréchal, Seyffert, et al., 2011; Monistero et al., 2018). It was previously reported that different levels of iron metabolism, transcriptional regulators, and exoprotein production could contribute to ovine mastitis severity, and different levels of toxin expression were related to the pathogenic potential of genomes isolated from bovine mastitis (ben Zakour et al., 2008; Le Maréchal et al., 2011).

These strains were predicted to be sensitive to methicillin. However, the horizontal acquisition of resistance genes could increase the relevance of ovine mastitis in human infection with methicillin-resistant *S. aureus*. Although the SCCmec chromosome cassette was not found in the genomes of the ovine strains considered in this study, these genomes present multiple chromosomal factors that can contribute to the methicillin resistance phenotype, such as the *fmtB* gene (Komatsuzawa et al., 1997). It has already been shown that the *fmtB* gene indirectly affects methicillin resistance, but further biochemical studies are necessary to elucidate the role of *fmtB* in this resistance mechanism (Komatsuzawa et al., 2000).

6. Conclusion

The comparative genomics among the ovine and other host-associated isolates of *S. aureus* showed genetic differences. Our results suggest that the accessory genes encoding virulence factors and unknown proteins that might be essential in establishing infection are exclusively found in the ovine genomes. Although it is possible to observe that the *S. aureus* genomes are very similar at the genomic level, independent of the host, clonal complexes CC130 and CC133, CC425, CC59, and CC30 are associated with small ruminants, where CC130 and CC133 are considered representative of the host, ovine. As for the invasion and permanence of the bacteria in the cell, it showed a vast repertoire of virulence-associated genes. All strains showed genes related to antimicrobial resistance. However, at the level of antibiotic resistance, some highly resistant representatives were found predicted *in silico*, as observed in the genomes isolated from swine. The cassette chromosome *mec* was not detected in strains isolated from the ovine. However, it showed genes associated with resistance in specific genomes, which may be associated with the reservoir of resistance genes for other hosts. This work shows new evidence of genomic specialization in *S. aureus* associated with ovine mastitis.

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Data availability statement

Genome sequences are available in the NCBI database.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2022.147131.

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5.1.2 *Supplementary material*

Supplementary material

Figure S1: Pan-genome profile curves for 73 strains of *Staphylococcus aureus*.

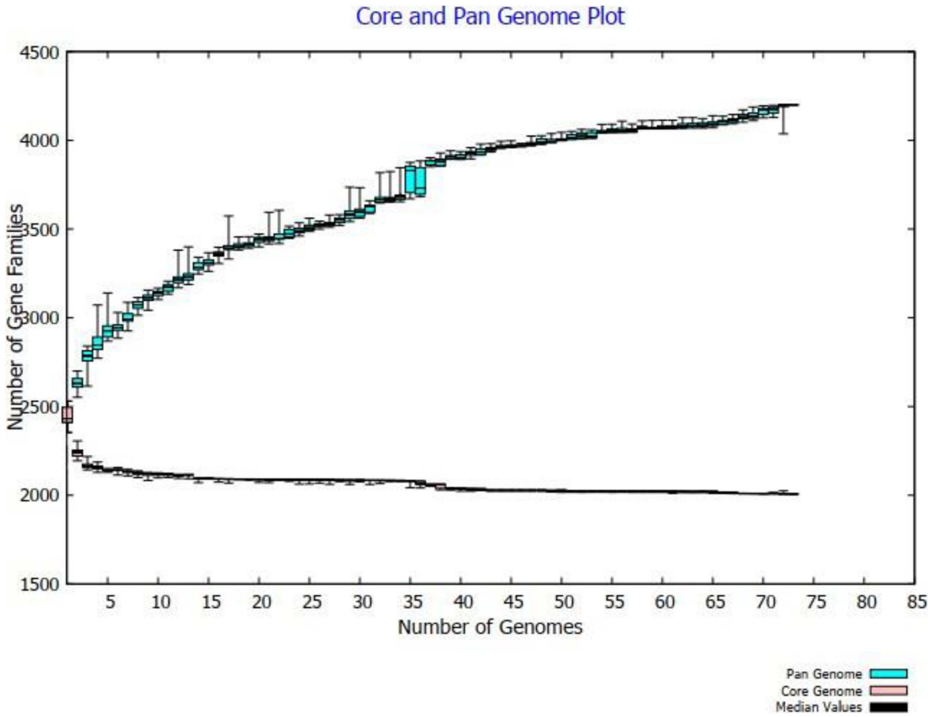

Figure S2: Matrix representation of the Whole Genome Sequence-Based Phylogenetic Tree and heatmap of the presence-absence of genes acquired through ROARY for 73 strains of *Staphylococcus aureus*.

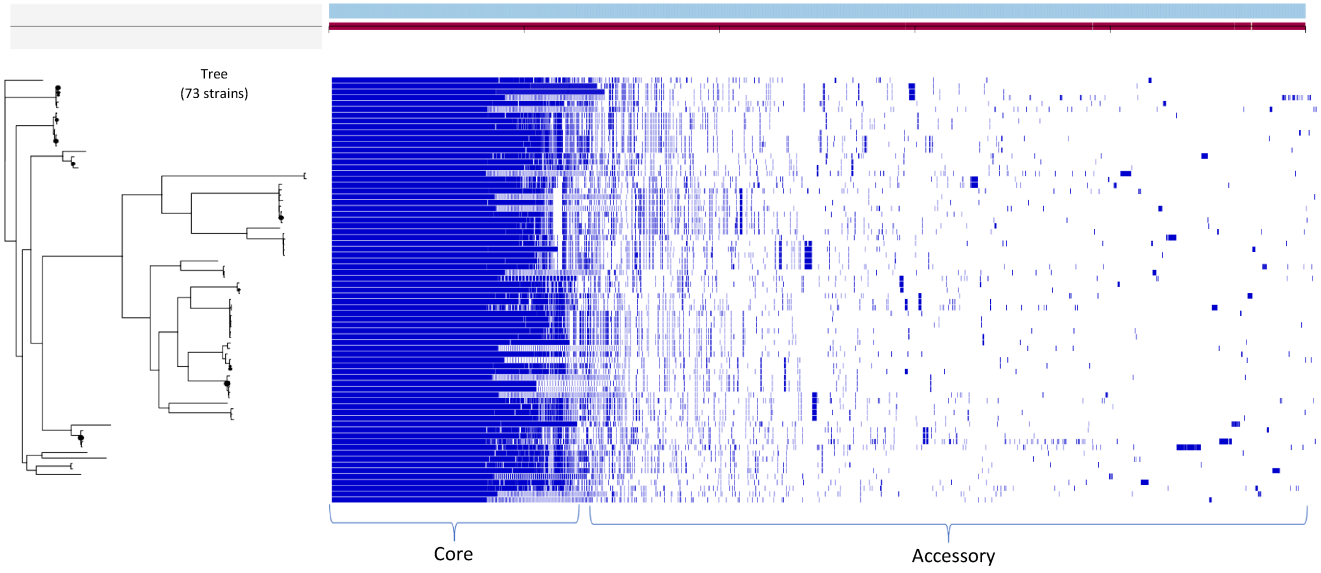

Figure S3: KEGG details pathway distribution for 73 strains of *Staphylococcus aureus*.

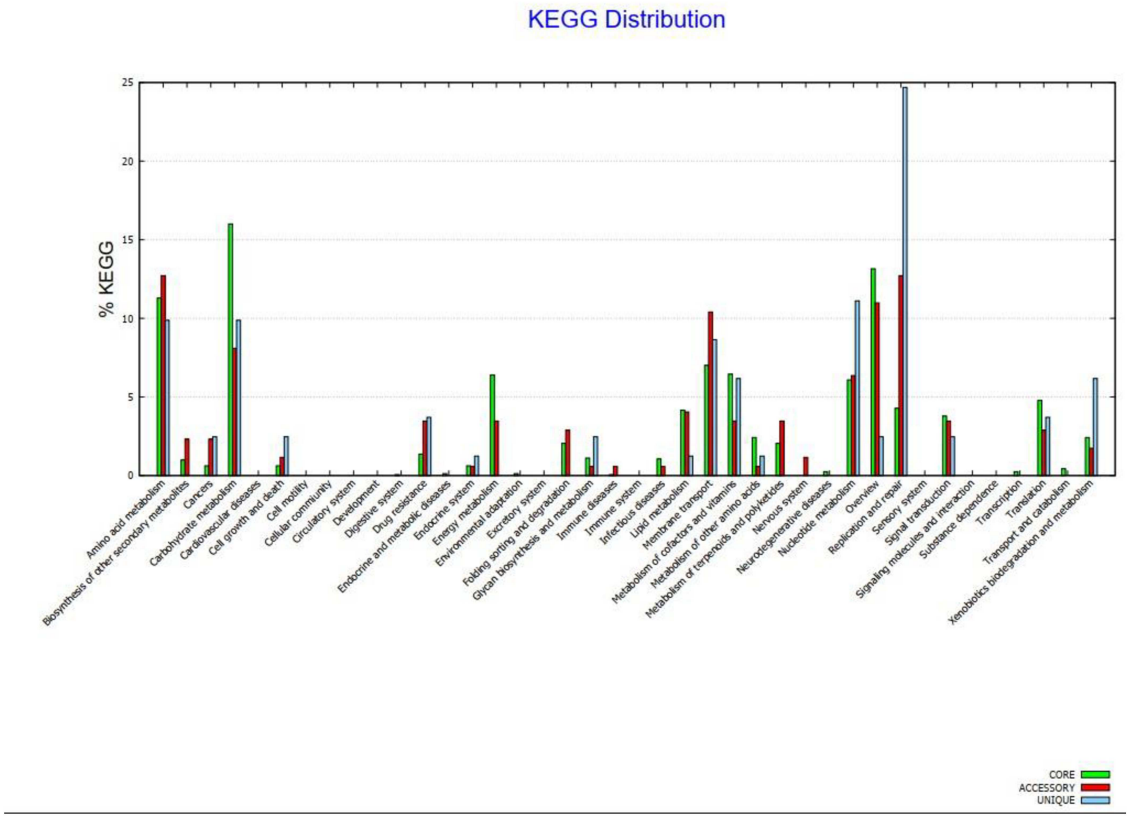

Figure S4: COG distribution of *Staphylococcus aureus* pan-genome

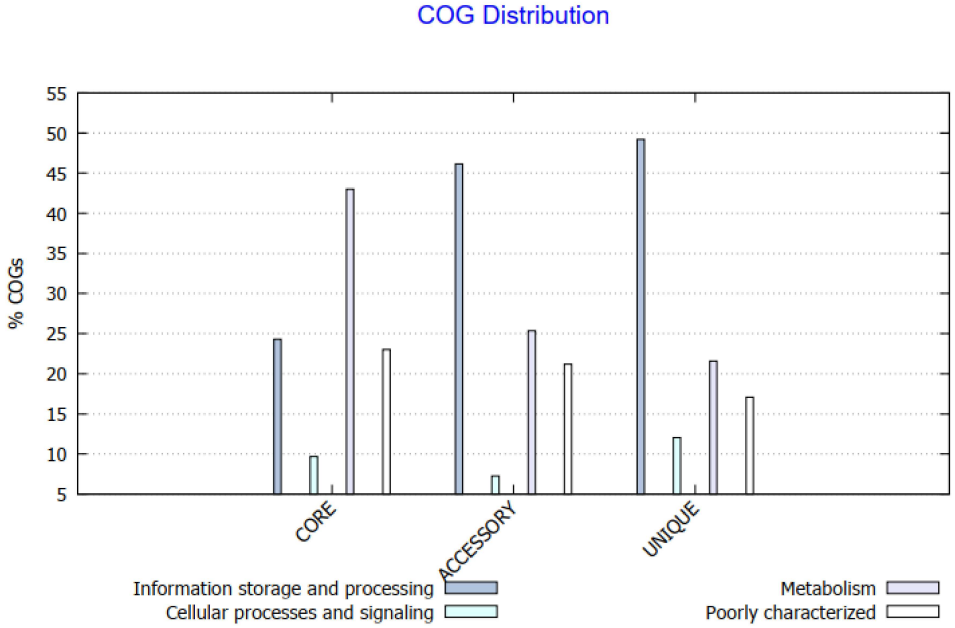

Table S1: *Staphylococcus aureus* strains isolated from different hosts used in this study (dataset).

nº	Genome name	Strain	Host
1	*Staphylococcus aureus* strain O217	O217*	Ovine
2	*Staphylococcus aureus* strain O331 strain B114	O331 B114*	Ovine
3	*Staphylococcus aureus* O11	O11*	Ovine
4	*Staphylococcus aureus* strain O326	O326*	Ovine
5	*Staphylococcus aureus* strain O408 strain B115	O408 B115*	Ovine
6	*Staphylococcus aureus* O46	O46*	Ovine
7	*Staphylococcus aureus* strain O82	O82*	Ovine
8	*Staphylococcus aureus* strain B119	B119*	Ovine
9	*Staphylococcus aureus* strain NCTC1803	NCTC1803	Ovine
10	*Staphylococcus aureus* strain NCTC9555	NCTC9555	Ovine
11	*Staphylococcus aureus* strain O17	O17*	Ovine
12	*Staphylococcus aureus* strain O267	O267*	Ovine
13	*Staphylococcus aureus* strain O268	O268*	Ovine
14	*Staphylococcus aureus* subsp. aureus ED133	ED133	Ovine
15	*Staphylococcus aureus* strain O55 strain B118	O55 B118*	Ovine
16	*Staphylococcus aureus* strain CFSAN018750	CFSAN018750	Bovine
17	*Staphylococcus aureus* strain PMB 64-1	PMB 64-1	Bovine
18	*Staphylococcus aureus* strain PMB 81-4	PMB 81-4	Bovine
19	*Staphylococcus aureus* strain K5	K5	Bovine
20	*Staphylococcus aureus* strain K17	K17	Bovine
21	*Staphylococcus aureus* strain 2030RH1	2030RH1	Bovine
22	*Staphylococcus aureus* strain MOK042	MOK042	Bovine
23	*Staphylococcus aureus* strain MOK063	MOK063	Bovine
24	*Staphylococcus aureus* subsp. aureus LGA251	LGA251	Bovine
25	*Staphylococcus aureus* strain NCTC7485	NCTC7485	Bovine
26	*Staphylococcus aureus* RF122	RF122	Cattle
27	*Staphylococcus aureus* strain K18	K18	Buffalo
28	*Staphylococcus aureus* strain ILRI_Eymole1/1	ILRI_Eymole1	Camel
29	*Staphylococcus aureus* strain NX-T55	NX-T55	Pig
30	*Staphylococcus aureus* strain QD-CD9	QD-CD9	Pig
31	*Staphylococcus aureus* strain S57	S57	Pig
32	*Staphylococcus aureus* strain ISU935	ISU935	Pig
33	*Staphylococcus aureus* strain M48	M48	Pig
34	*Staphylococcus aureus* strain M51	M51	Pig
35	*Staphylococcus aureus* strain 08S00974	08S00974	Pig
36	*Staphylococcus aureus* subsp. *aureus* strain ISU926	ISU926	Pig
37	*Staphylococcus aureus* strain PTDrAP2	PTDrAP2	Pig
38	*Staphylococcus aureus* strain PCFA-221	PCFA-221	Pig
39	*Staphylococcus aureus* strain ch21	ch21	Chicken
40	*Staphylococcus aureus* strain ch22	ch22	Chicken
41	*Staphylococcus aureus* subsp. aureus ED98	ED98	Chicken
42	*Staphylococcus aureus* strain SA112	SA112	Dog

43	*Staphylococcus aureus* strain NCTC7988	NCTC7988	Cat
44	*Staphylococcus aureus* strain 27	27	Human
45	*Staphylococcus aureus* strain 128	128	Human
46	*Staphylococcus aureus* strain 24117-SCV	24117-SCV	Human
47	*Staphylococcus aureus* strain ST20130938	12803371	Human
48	*Staphylococcus aureus* strain 1549-WT	1549-WT	Human
49	*Staphylococcus aureus* subsp. aureus strain 80wphwpl	80wphwpl	Human
50	*Staphylococcus aureus* strain AR_475	AR475	Human
51	*Staphylococcus aureus* subsp. aureus strain ATCC 25923	ATCC_25923	Human
52	*Staphylococcus aureus* strain ATCC BAA-39	ATCC_BAA-39	Human
53	*Staphylococcus aureus* strain CFSAN064037	CFSAN064037	Human
54	*Staphylococcus aureus* subsp. aureus COL	COL	Human
55	*Staphylococcus aureus* strain FDAARGOS_504	FAARGOS_504	Human
56	*Staphylococcus aureus* strain FORC_061	FORC_061	Human
57	*Staphylococcus aureus* strain FORC59	FORC59	Human
58	*Staphylococcus aureus* strain GD5	GD5	Human
59	*Staphylococcus aureus* strain GN1	GN1	Human
60	*Staphylococcus aureus* strain HZW450	HZW450	Human
61	*Staphylococcus aureus* strain 466	466	Human
62	*Staphylococcus aureus* strain NCTC11965	NCTC11965	Human
63	*Staphylococcus aureus* strain JP080 strain *JP02758*	JP02758_JP02758	Human
64	*Staphylococcus aureus* strain NCTC13277	NCTC13277	Human
65	*Staphylococcus aureus* strain NCTC13435	NCTC13435	Human
66	*Staphylococcus aureus* strain NCTC13552	NCTC13552	Human
67	*Staphylococcus aureus* strain NCTC13811	NCTC13811	Human
68	*Staphylococcus aureus* strain NCTC5660	NCTC5660	Human
69	*Staphylococcus aureus* strain NCTC5663	NCTC5663	Human
70	*Staphylococcus aureus* subsp. aureus NCTC8325 strain NCTC8325	NCTC8325	Human
71	*Staphylococcus aureus* strain NRS153	NRS153	Human
72	*Staphylococcus aureus* subsp. aureus USA300_FPR3757	USA300_FPR3757	Human
73	*Staphylococcus aureus* strain XQ	XQ	Human
	***contribution of this study**		

ST	CC	Sample	Isolation locality
30	30	mastitis	France
59	59	mastitis	France
700	130	mastitis	France
700	130	mastitis	France
700	130	mastitis	France
2490	130	mastitis	France
2011	130	mastitis	France
133	133	mastitis	France
133	133	mastitis	NA
133	133	NA	NA
133	133	mastitis	France
133	133	mastitis	France
133	133	mastitis	France
133	133	mastitis	France
425	425	mastitis	France
9	1	NA	Denmark
1	1	breast	South Korea
1	1	breast	South Korea
2454	8	milk	India
2454	8	milk	India
464	97	milk	Brazil
71	97	milk	Ireland
97	97	milk	Ireland
425	425	bulk milk	England
351	NA	milk	NA
151	151	mastitis	Ireland
2454	8	milk	India
30	30	nasal swab	Kenya
9	1	nasal swab	China
9	1	nasal swab	China
9	1	raw meat (slaughter)	China
5	5	nasal swab	United States
239	8	NA	China
1516	8	NA	China
398	398	fatting pig at farm	Germany
398	398	nasal swab	United States
398	398	pig nares	Australia
541	NA	nasal cavity	South Korea
5	5	deep wounds and lesions	Poland
5	5	deep wounds and lesions	Poland
5	5	broiler chicken	Northern Ireland
5	5	NA	Brazil

133	133	skin	England
121	121	NA	Germany
121	121	skin	China
15	15	Nasal swab	Canada
121	121	Human physical	South Africa
	45	Throat	Suriname
30	30	Nose from military trainee	United States
87	59	NA	NA
243	30	Purulent abscess from military	United States
464	97	blood	Germany
4307	130	biological fluid-human	Denmark
250	8	NA	NA
30	30	NA	Germany
72	8	NA	Germany
188	1	NA	Germany
398	398	NA	Germany
50	50	NA	Germany
59	59	Perineum	Suriname
45	45	wound fluid	Brazil
30	30	wound	Canada
	NA	NA	NA
36	30	NA	NA
80	NA	NA	Canada: Calgary
425	425	Bulk Milk Tank	United Kingdom
30	30	hospitals	Pakistan
	NA	NA	Australia:
350	NA	NA	Sweden
121	121	vancomycin treatment	NA
8	8	blood	South Korea
8	8	NA	NA
121	121	blood	Korea

Genbank Accession
CP038461
CP038269
CP0246491
CP032481
CP038270
CP0253951
CP038819
CP038460
LR134305
LR134090
CP032051
CP034102
CP038612
CP001996
CP038268
CP028189
CP034486
CP034441
CP020656
CP0207131
CP039848
CP029627, CP029628
CP029629
FR821779, FR821780
LS483300
AJ938182
CP0207141
LN626917
CP031839, CP031840
CP031838
CP030136
CP017090
CP030138
CP030137
CP020019
CP017091
CP029172, CP029168, CP029169, CP029170,CP029171
CP035003, CP035004
CP017804, CP017805, CP017806
CP017807, CP017808, CP017809
CP001781, CP001783, CP001784, CP001782
CP020553

LR134271
CP021907, CP021908
CP019563, CP019564, CP019565, CP019566, CP019567
CP015447
CP010952
CP014423, CP014424, CP014425
CP016855, CP016853, CP016854
CP030323, CP030324
CP016858, CP016857, CP016859
CP022903
CP028165
CP000046, CP000045
LT992464
LT992463
LT992465
LT992469
LT992472
CP014362, CP014363, CP014364
CP027486 ... CP027494
CP020956
CP033865
LS483484
CP020960
LR134084
CP039160, CP039161
LR134088
LS483317
CP000736, CP000737
CP029198, CP029199
CP000255, CP000256, CP000257, CP000258
CP013957, CP013958

Table S2: Distribution of core, accessory, unique, and exclusively absent genes among all 73 *S. aureus* strains

Organism name	No. of core genes	No. of accessory genes	No. of unique genes
S08S00974	2008	424	0
S27	2008	444	0
S80wphwpl	2008	331	0
S128	2008	455	0
S1280.3371	2008	472	13
S1549-WT	2008	390	3
S2030RH1	2008	403	0
S24117-SCV	2008	340	6
SAR_475	2008	408	10
SAR466	2008	455	8
SATCC_25923	2008	433	0
SATCC_BAA-39	2008	403	27
SB119	2008	418	1
SCFSAN018750	2008	329	0
SCFSAN064037	2008	395	1
Sch21	2008	467	1
Sch22	2008	470	6
SCOL	2008	428	7
SED98	2008	473	0
SED133	2008	465	0
SFDAARGOS504	2008	513	10
SFORC_061	2008	359	7
SFORC59	2008	345	14
SGD5	2008	363	1
SGN1	2008	391	8
SHZW450	2008	425	6
SILRI_Eymole1	2008	491	9
SISU926	2008	466	1
SISU935	2008	453	13
SJP080	2008	366	49
SK5	2008	402	0
SK17	2008	413	0
SK18	2008	415	2
SLGA251	2008	387	0
SM48	2008	519	164
SM51	2008	421	4
SMOK042	2008	503	35
SMOK063	2008	438	0
SNCTC_8325	2008	467	2
SNCTC1803	2008	427	0
SNCTC5660	2008	353	13
SNCTC5663	2008	427	17

SNCTC7485	2008	343	0
SNCTC7988	2008	484	3
SNCTC9555	2008	469	6
SNCTC11965	2008	433	2
SNCTC13277	2008	520	2
SNCTC13435	2008	427	11
SNCTC13552	2008	383	7
SNCTC13811	2008	534	6
SNRS153	2008	511	6
SNX-T55	2008	472	2
SO11	2008	421	0
SO17	2008	418	0
SO46	2008	410	1
SO55_B118	2008	428	18
SO82	2008	408	1
SO217	2008	387	0
SO267	2008	496	0
SO268	2008	475	1
SO326	2008	417	0
SO331_B114	2008	307	0
SO408_B115	2008	420	0
SPCFA-221	2008	352	15
SPMB_64-1	2008	316	10
SPMB_81-4	2008	468	8
SPTDrAP2	2008	435	26
SQD-CD9	2008	482	2
SRF122	2008	401	2
SS57	2008	480	3
SSA112	2008	479	16
SUSA300_FPR3757	2008	483	46
SXQ	2008	458	2

using USEARCH clustering tool.

No. of exclusively absent genes
1
0
0
1
8
0
0
0
0
0
0
20
0
0
0
0
0
0
0
0
0
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0
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2
17
22
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9
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0
0
0
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0
0
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0
0
6
5
0
1
0
2
0
0
0
0

Table S3: Distribution of core, accessory, unique, and exclusively absent genes among the su using the USEARCH clustering tool

Organism strain	No. of core genes	No. of accessory genes	No. of unique genes
SB119	2146	280	1
SED133	2146	325	2
SNCTC1803	2146	289	0
SNCTC9555	2146	320	17
SO11	2146	278	5
SO17	2146	280	0
SO46	2146	271	2
SO55_B118	2146	236	72
SO82	2146	269	2
SO217	2146	195	54
SO267	2146	339	19
SO268	2146	337	1
SO326	2146	279	0
SO331_B114	2146	128	41
SO408_B115	2146	281	1

bset 1 (15 *S. aureus* ovines strains)

No. of exclusively absent genes
0
0
0
2
0
0
1
15
0
15
1
0
0
48
0

Table S4: Distribution of core, accessory, unique, and exclusively absent genes among subset 2 (5

Organism strain	No. of core genes	No. of accessory genes	No. of unique genes
S08S00974	2008	424	0
S27	2008	444	0
S80wphwpl	2008	331	0
S128	2008	455	0
S1280.3371	2008	472	13
S1549-WT	2008	390	3
S2030RH1	2008	403	0
S24117-SCV	2008	339	7
SAR_475	2008	408	10
SAR466	2008	455	8
SATCC_25923	2008	433	0
SATCC_BAA-39	2008	403	27
SCFSAN018750	2008	329	0
SCFSAN064037	2008	388	8
Sch21	2008	467	1
Sch22	2008	470	6
SCOL	2008	428	7
SED98	2008	473	0
SFDAARGOS504	2008	513	10
SFORC_061	2008	359	7
SFORC59	2008	345	14
SGD5	2008	363	1
SGN1	2008	391	8
SHZW450	2008	425	6
SILRI_Eymole1	2008	488	12
SISU926	2008	466	1
SISU935	2008	453	13
SJP080	2008	366	49
SK5	2008	402	0
SK17	2008	413	0
SK18	2008	415	2
SLGA251	2008	385	2
SM48	2008	519	164
SM51	2008	421	4
SMOK042	2008	503	35
SMOK063	2008	438	0
SNCTC_8325	2008	467	2
SNCTC5660	2008	351	15
SNCTC5663	2008	427	17
SNCTC7485	2008	343	0
SNCTC7988	2008	476	11
SNCTC11965	2008	433	2

SNCTC13277	2008	520	2
SNCTC13435	2008	427	11
SNCTC13552	2008	383	7
SNCTC13811	2008	534	6
SNRS153	2008	511	6
SNX-T55	2008	472	2
SPCFA-221	2008	352	15
SPMB_64-1	2008	316	10
SPMB_81-4	2008	467	9
SPTDrAP2	2008	435	26
SQD-CD9	2008	482	2
SRF122	2008	401	2
SS57	2008	480	3
SSA112	2008	479	16
SUSA300_FPR3757	2008	483	46
SXQ	2008	458	2

58 *S. aureus* different hosts) using

No. of exclusively absent genes
1
0
0
1
8
0
0
1
0
0
0
20
0
0
0
0
0
0
0
0
0
2
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0
0
1
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1
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0
0
2
17
22
1
1
9
0
0
0
0

0
0
6
0
0
0
6
5
0
1
0
2
0
0
0
0

Host	Strain	Region	Region Length	Completeness	Score
Pig	QD-CD9	1	10.4Kb	incomplete	20
Pig	M48	9	10.5Kb	incomplete	50
Bovine	NCTC7485	1	10.8Kb	incomplete	40
Bovine	RF122	1	10.8Kb	incomplete	20
Human	FAARGOS_504	3	10.8Kb	incomplete	60
Human	NCTC5660	1	10.8Kb	incomplete	50
Human	FORC_061	1	11.3Kb	incomplete	10
Ovine	O46	3	11.4Kb	incomplete	10
Human	12803371	3	11.4Kb	incomplete	50
Dog	SA112	1	13.1Kb	incomplete	40
Human	HZW450	6	13.3Kb	incomplete	40
Human	NCTC11965	3	13.3Kb	incomplete	60
Pig	QD-CD9	5	14.1Kb	incomplete	60
Ovine	O11	1	14.3Kb	incomplete	20
Human	466	4	14.3Kb	incomplete	20
Human	NCTC13811	7	14.3Kb	incomplete	30
Human	NRS153	5	14.3Kb	incomplete	40
Camel	ILRI_Eymole1	3	14.9Kb	incomplete	30
Human	ATCC_BAA-39	2	15.5Kb	incomplete	40
Human	1549-WT	1	15.8Kb	incomplete	20
Ovine	NCTC1803	4	15.9Kb	incomplete	40
Ovine	NCTC9555	5	15.9Kb	incomplete	40
Cat	NCTC7988	5	15.9Kb	incomplete	40
Ovine	O217	4	16.2Kb	incomplete	30
Bovine	MOK042	3	16.2Kb	incomplete	40
Human	24117-SCV	1	16.2Kb	incomplete	40
Human	12803371	2	16.2Kb	incomplete	40
Pig	PCFA-221	1	16.6Kb	incomplete	40
Ovine	O217	3	16.7Kb	incomplete	50
Ovine	O267	2	16.7Kb	incomplete	30
Pig	NX-T55	4	16.8Kb	incomplete	40
Pig	QD-CD9	4	16.8Kb	incomplete	40
Pig	S57	5	16.8Kb	incomplete	40
Ovine	O326	3	16Kb	incomplete	10
Bovine	MOK042	7	16Kb	incomplete	20
Human	FORC59	3	16Kb	incomplete	30
Human	NCTC11965	2	17.1Kb	incomplete	30
Pig	08S00974	1	17.5Kb	incomplete	30
Camel	ILRI_Eymole1	4	17.6Kb	incomplete	30
Human	ATCC_25923	5	17.6Kb	incomplete	20
Human	CFSAN064037	1	17.8Kb	incomplete	40
Ovine	NCTC1803	2	17Kb	incomplete	30
Ovine	NCTC9555	2	17Kb	incomplete	30
Ovine	O268	2	17Kb	incomplete	30
Ovine	ED133	2	17Kb	incomplete	30
Cat	NCTC7988	2	17Kb	incomplete	30
Bovine	MOK063	3	18.3Kb	incomplete	20
Chicken	ch21	3	18.8Kb	incomplete	20
Chicken	ch22	3	18.8Kb	incomplete	20
Human	ATCC_BAA-39	1	19.1Kb	incomplete	30

Human	1549-WT	2	19.2Kb	incomplete	50
Human	80wphwpl	3	19.5Kb	incomplete	50
Bovine	2030RH1	5	19.6Kb	incomplete	20
Human	NCTC13811	8	19.7Kb	incomplete	50
Human	USA300_FPR3757	1	20.6Kb	incomplete	20
Pig	NX-T55	3	21.2Kb	incomplete	20
Pig	QD-CD9	2	21.2Kb	incomplete	20
Pig	S57	4	21.2Kb	incomplete	20
Bovine	MOK042	2	21.3Kb	incomplete	20
Bovine	MOK063	2	21.3Kb	incomplete	20
Bovine	MOK042	6	21.6Kb	incomplete	50
Ovine	O267	5	21.9Kb	incomplete	40
Ovine	O55 B118	5	21.9Kb	incomplete	30
Ovine	O267	7	22.2Kb	incomplete	30
Ovine	O268	7	22.2Kb	incomplete	30
Ovine	B119	3	22.3Kb	incomplete	30
Ovine	O17	4	22.3Kb	incomplete	30
Ovine	ED133	6	22.3Kb	incomplete	30
Ovine	NCTC1803	6	22.4Kb	incomplete	30
Ovine	NCTC9555	7	22.4Kb	incomplete	30
Cat	NCTC7988	7	22.4Kb	incomplete	30
Human	AR475	4	22Kb	incomplete	50
Bovine	2030RH1	3	23.2Kb	incomplete	60
Camel	ILRI_Eymole1	6	23.4Kb	incomplete	50
Human	HZW450	2	23.5Kb	incomplete	50
Human	AR475	2	23.7Kb	incomplete	20
Human	1549-WT	4	24.2Kb	incomplete	50
Human	NCTC11965	5	26.4Kb	incomplete	30
Pig	M48	5	28.6Kb	incomplete	50
Ovine	O55 B118	2	28.9Kb	incomplete	20
Bovine	2030RH1	2	29.1Kb	incomplete	50
Human	AR475	1	29.6Kb	incomplete	30
Bovine	MOK063	4	29.8Kb	incomplete	50
Human	GN1	3	29Kb	incomplete	40
Bovine	K5	2	30.5Kb	incomplete	40
Pig	S57	1	32.2Kb	incomplete	60
Bovine	CFSAN018750	1	35Kb	incomplete	30
Bovine	K17	1	36.5Kb	incomplete	40
Bovine	K5	1	36Kb	incomplete	50
Bovine	K18	1	36Kb	incomplete	40
Bovine	MOK042	5	40.3Kb	incomplete	60
Bovine	MOK063	5	45.5Kb	incomplete	60
Pig	M48	4	5.8Kb	incomplete	50
Pig	M51	3	5.8Kb	incomplete	50
Human	FORC59	2	5.8Kb	incomplete	60
Human	80wphwpl	2	5.9Kb	incomplete	20
Human	ATCC_25923	2	5.9Kb	incomplete	20
Human	FAARGOS_504	2	5.9Kb	incomplete	20
Human	HZW450	7	5.9Kb	incomplete	40
Pig	ISU926	1	6.1Kb	incomplete	30
Human	GD5	1	6.7Kb	incomplete	20
Pig	PTDrAP2	2	6.8Kb	incomplete	30

Ovine	NCTC1803	3	6.9Kb	incomplete	40
Ovine	NCTC9555	3	6.9Kb	incomplete	40
Ovine	O268	4	6.9Kb	incomplete	20
Cat	NCTC7988	4	6.9Kb	incomplete	40
Human	FORC59	5	7.1Kb	incomplete	40
Human	80wphwpl	4	7.3Kb	incomplete	40
Bovine	PMB 64-1	1	7.4Kb	incomplete	40
Human	GN1	4	7.5Kb	incomplete	50
Human	FORC59	1	7.7Kb	incomplete	40
Ovine	O17	2	7.8Kb	incomplete	40
Ovine	O267	4	7.8Kb	incomplete	20
Ovine	ED133	4	7.8Kb	incomplete	40
Pig	M51	4	7Kb	incomplete	50
Human	COL	3	7Kb	incomplete	30
Human	HZW450	4	8.1Kb	incomplete	50
Human	GN1	1	8.2Kb	incomplete	40
Bovine	2030RH1	6	8.3Kb	incomplete	50
Bovine	MOK063	6	8.3Kb	incomplete	50
Pig	M48	3	8.3Kb	incomplete	30
Pig	M51	1	8.3Kb	incomplete	50
Human	HZW450	1	8.4Kb	incomplete	50
Human	ATCC_25923	3	8.6Kb	incomplete	50
Human	GN1	2	8.7Kb	incomplete	50
Bovine	NCTC7485	3	9.2Kb	incomplete	30
Bovine	RF122	3	9.2Kb	incomplete	30
Human	NCTC5660	3	9.2Kb	incomplete	30
Bovine	MOK042	8	9.6Kb	incomplete	40
Camel	ILRI_Eymole1	5	9.6Kb	incomplete	30
Human	FAARGOS_504	6	9.9Kb	incomplete	40
Pig	NX-T55	1	9Kb	incomplete	10
Pig	S57	3	9Kb	incomplete	10

Total Proteins	Region Position
13	916311-926728
13	3033854-3044446
14	311405-322272
13	311933-322800
20	776444-787287
15	309795-320658
12	1820862-1832244
10	1876007-1887453
12	2709786-2721193
19	372560-385713
16	1924393-1937758
22	1333963-1347328
15	2763261-2777369
16	27239-41569
22	2078078-2092411
22	1890968-1905311
19	2640243-2654598
20	423494-438486
26	1315445-1330962
25	295336-311145
10	1555584-1571514
11	1907077-1922989
10	1911608-1927538
15	2027447-2043649
16	421565-437812
16	2259703-2275955
18	1856123-1872374
19	2007478-2024145
20	1868824-1885591
20	447603-464365
19	2352244-2369043
18	2413513-2430312
18	2363601-2380400
18	1862736-1878826
20	2113131-2129215
18	1828764-1844782
16	836932-854067
14	421296-438862
19	754992-772644
24	2038708-2056385
20	585555-603452
18	124158-141195
18	425302-442339
18	431681-448718
20	431632-448668
18	432710-449747
20	717828-736156
22	1883833-1902662
22	2117797-2136611
19	870203-889361

16	846112-865396
26	1822895-1842474
16	2316737-2336422
37	2934948-2954740
20	879301-899940
27	1455673-1476971
27	1476123-1497421
27	1475597-1496895
16	389151-410529
16	402543-423922
25	1945908-1967529
20	1930239-1952229
16	2057565-2079465
14	2118256-2140549
15	2099035-2121329
15	2024522-2046871
15	2028025-2050374
15	2089669-2112018
15	1746903-1769310
15	2098440-2120903
15	2115227-2137633
16	1962839-1984927
32	1475026-1498272
18	1088627-1112097
18	399009-422598
11	1363855-1387592
29	2554481-2578707
22	2084339-2110797
28	1919066-1947722
18	410771-439725
25	838418-867560
13	102704-132335
25	878185-907990
29	744140-773186
16	513979-544508
23	708481-740768
26	284313-319343
50	798885-835418
49	364139-400170
50	163340-199370
65	1362348-1402680
66	1438071-1483636
14	1912256-1918140
14	922343-928227
7	1687958-1693801
9	1098431-1104417
8	1119373-1125359
9	603950-609936
10	2822674-2828626
11	1324108-1330282
11	1306575-1313334
8	1455607-1462442

12	1004099-1011092
12	1613070-1620063
12	1351996-1358989
12	1363454-1370447
11	2694955-2702079
11	2701338-2708638
7	1267691-1275150
17	1385980-1393535
17	1282371-1290165
17	1289287-1297178
12	1368695-1376585
15	1351863-1359754
18	1461187-1468208
17	1348415-1355435
20	1340155-1348313
7	244468-252763
13	2700297-2708619
13	2794912-2803233
19	1363103-1371455
14	5566-13889
15	2279-10747
14	1291825-1300486
7	568381-577175
9	849736-859024
10	846053-855330
10	842427-851698
12	2829261-2838944
9	882421-892114
12	2334424-2344334
12	851306-860391
12	916048-925133

Most Common Phage	GC %
PHAGE_Staphy_SPbeta_like_NC_029119(3)	27.50%
PHAGE_Bacill_IEBH_NC_011167(2)	32.20%
PHAGE_Staphy_Ipla35_NC_011612(6)	31.87%
PHAGE_Staphy_Ipla35_NC_011612(5)	31.90%
PHAGE_Staphy_SA97_NC_029010(2)	27.03%
PHAGE_Staphy_Ipla35_NC_011612(6)	31.90%
PHAGE_Strept_315.5_NC_004588(1)	26.74%
PHAGE_Staphy_phiPV83_NC_002486(1)	31.04%
PHAGE_Bacill_Waukesha92_NC_025424(1)	32.26%
PHAGE_Staphy_AJ_2017_NC_048644(5)	30.60%
PHAGE_Staphy_StauST398_4_NC_023499(3)	29.50%
PHAGE_Staphy_StB27_NC_019914(2)	27.79%
PHAGE_Staphy_SPbeta_like_NC_029119(7)	32.01%
PHAGE_Staphy_phi7247PVL_NC_048624(2)	29.57%
PHAGE_Staphy_PT1028_NC_007045(10)	32.34%
PHAGE_Staphy_PT1028_NC_007045(10)	32.32%
PHAGE_Staphy_PT1028_NC_007045(4)	30.86%
PHAGE_Staphy_PT1028_NC_007045(10)	33.18%
PHAGE_Staphy_187_NC_007047(4)	30.42%
PHAGE_Staphy_187_NC_007047(4)	29.94%
PHAGE_Staphy_phi2958PVL_NC_011344(2)	29.03%
PHAGE_Bacter_Diva_NC_028788(2)	29.01%
PHAGE_Bacter_Diva_NC_028788(2)	29.04%
PHAGE_Staphy_PT1028_NC_007045(3)	31.25%
PHAGE_Staphy_PT1028_NC_007045(4)	32.02%
PHAGE_Staphy_phi2958PVL_NC_011344(2)	30.14%
PHAGE_Lactob_Lj965_NC_005355(2)	30.13%
PHAGE_Staphy_PT1028_NC_007045(5)	31.88%
PHAGE_Strept_EJ_1_NC_005294(1)	28.97%
PHAGE_Staphy_PT1028_NC_007045(6)	31.49%
PHAGE_Staphy_PT1028_NC_007045(4)	31.71%
PHAGE_Staphy_PT1028_NC_007045(4)	31.71%
PHAGE_Staphy_PT1028_NC_007045(4)	31.70%
PHAGE_Staphy_phi7247PVL_NC_048624(2)	30.31%
PHAGE_Staphy_PT1028_NC_007045(10)	31.87%
PHAGE_Staphy_37_NC_007055(2)	30.48%
PHAGE_Staphy_PT1028_NC_007045(3)	31.16%
PHAGE_Staphy_PT1028_NC_007045(3)	32.71%
PHAGE_Staphy_PT1028_NC_007045(9)	32.36%
PHAGE_Staphy_PT1028_NC_007045(10)	32.03%
PHAGE_Lactob_Lj965_NC_005355(2)	29.81%
PHAGE_Staphy_PT1028_NC_007045(5)	31.50%
PHAGE_Staphy_PT1028_NC_007045(5)	31.52%
PHAGE_Staphy_PT1028_NC_007045(5)	31.51%
PHAGE_Staphy_PT1028_NC_007045(6)	31.52%
PHAGE_Staphy_PT1028_NC_007045(5)	31.51%
PHAGE_Staphy_PT1028_NC_007045(10)	31.33%
PHAGE_Staphy_phi2958PVL_NC_011344(2)	27.65%
PHAGE_Staphy_phiPV83_NC_002486(2)	27.66%
PHAGE_Staphy_PT1028_NC_007045(9)	30.99%

PHAGE_Paenib_Shelly_NC_041909(2)	29.58%
PHAGE_Lactob_BH1_NC_048737(1)	29.24%
PHAGE_Staphy_StB20_like_NC_028821(2)	33.36%
PHAGE_Staphy_phi2958PVL_NC_011344(16)	33.21%
PHAGE_Staphy_PT1028_NC_007045(5)	30.80%
PHAGE_Staphy_187_NC_007047(5)	30.04%
PHAGE_Staphy_187_NC_007047(5)	30.03%
PHAGE_Staphy_187_NC_007047(5)	30.03%
PHAGE_Staphy_StB20_like_NC_028821(2)	32.73%
PHAGE_Staphy_StB20_like_NC_028821(2)	32.72%
PHAGE_Staphy_StauST398_5_NC_023500(2)	29.54%
PHAGE_Staphy_X2_NC_007065(6)	32.53%
PHAGE_Staphy_PT1028_NC_007045(3)	30.44%
PHAGE_Staphy_PT1028_NC_007045(3)	30.37%
PHAGE_Staphy_PT1028_NC_007045(3)	30.34%
PHAGE_Staphy_PT1028_NC_007045(3)	30.43%
PHAGE_Staphy_PT1028_NC_007045(3)	30.43%
PHAGE_Staphy_PT1028_NC_007045(3)	30.40%
PHAGE_Staphy_PT1028_NC_007045(3)	30.47%
PHAGE_Staphy_PT1028_NC_007045(3)	30.51%
PHAGE_Staphy_PT1028_NC_007045(3)	30.45%
PHAGE_Escher_503458_NC_049341(2)	30.77%
PHAGE_Staphy_187_NC_007047(5)	29.92%
PHAGE_Staphy_StB27_NC_019914(2)	28.02%
PHAGE_Staphy_StB20_like_NC_028821(2)	32.54%
PHAGE_Staphy_AJ_2017_NC_048644(4)	30.75%
PHAGE_Staphy_PT1028_NC_007045(10)	30.27%
PHAGE_Staphy_PT1028_NC_007045(10)	31.31%
PHAGE_Lactob_Lj965_NC_005355(2)	28.95%
PHAGE_Staphy_PT1028_NC_007045(5)	32.89%
PHAGE_Staphy_phi2958PVL_NC_011344(2)	29.31%
PHAGE_Entero_fiAA91_ss_NC_022750(2)	32.93%
PHAGE_Staphy_phi2958PVL_NC_011344(2)	29.34%
PHAGE_Staphy_P282_NC_048634(14)	32.91%
PHAGE_Staphy_SPbeta_like_NC_029119(3)	34.73%
PHAGE_Staphy_PT1028_NC_007045(5)	30.45%
PHAGE_Staphy_187_NC_007047(5)	31.17%
PHAGE_Staphy_187_NC_007047(19)	30.40%
PHAGE_Staphy_187_NC_007047(19)	30.46%
PHAGE_Staphy_187_NC_007047(19)	30.46%
PHAGE_Staphy_187_NC_007047(19)	30.07%
PHAGE_Staphy_187_NC_007047(19)	29.96%
PHAGE_Paenib_PBL1c_NC_048689(2)	31.11%
PHAGE_Bacter_Sitara_NC_028854(2)	31.11%
PHAGE_Bacill_SP_10_NC_019487(1)	31.62%
PHAGE_Staphy_phiPV83_NC_002486(2)	28.58%
PHAGE_Staphy_JS01_NC_021773(2)	28.55%
PHAGE_Staphy_JS01_NC_021773(2)	28.65%
PHAGE_Bacill_IEBH_NC_011167(2)	31.72%
PHAGE_Staphy_StauST398_3_NC_021332(2)	25.36%
PHAGE_Staphy_vB_SauS_phi2_NC_028862(1)	25.44%
PHAGE_Staphy_SPbeta_like_NC_029119(3)	27.50%

PHAGE_Staphy_vB_SpsS_QT1_NC_048192(1)	26.34%
PHAGE_Clostr_phiMMP03_NC_028959(1)	26.35%
PHAGE_Thermu_OH2_NC_021784(1)	26.32%
PHAGE_Escher_520873_NC_049344(1)	26.32%
PHAGE_Bacill_IEBH_NC_011167(2)	31.54%
PHAGE_Bacill_IEBH_NC_011167(2)	31.76%
PHAGE_Staphy_SPbeta_like_NC_029119(3)	31.21%
PHAGE_Staphy_StB27_NC_019914(2)	26.60%
PHAGE_Staphy_StB27_NC_019914(2)	26.49%
PHAGE_Staphy_StB27_NC_019914(2)	26.20%
PHAGE_Staphy_StauST398_5_NC_023500(1)	26.18%
PHAGE_Staphy_StB27_NC_019914(2)	26.19%
PHAGE_Aeriba_AP45_NC_048651(1)	26.08%
PHAGE_Staphy_55_NC_007060(1)	26.06%
PHAGE_Staphy_StB27_NC_019914(2)	26.71%
PHAGE_Staphy_Pvl108_NC_008689(1)	32.73%
PHAGE_Plankt_PaV_LD_NC_016564(1)	32.73%
PHAGE_Bacill_SPbeta_NC_001884(1)	32.72%
PHAGE_Staphy_SPbeta_like_NC_029119(3)	27.69%
PHAGE_Vibrio_VBP47_NC_020848(1)	32.71%
PHAGE_Bacill_IEBH_NC_011167(2)	32.87%
PHAGE_Staphy_StB27_NC_019914(2)	26.55%
PHAGE_Staphy_StB27_NC_019914(1)	33.69%
PHAGE_Staphy_phiPV83_NC_002486(4)	31.77%
PHAGE_Staphy_phiPV83_NC_002486(4)	31.80%
PHAGE_Staphy_phiPV83_NC_002486(4)	31.84%
PHAGE_Pseudo_pYD6_A_NC_020849(1)	31.89%
PHAGE_Staphy_SA780ruMSSAST101_NC_048711(3)	32.59%
PHAGE_Bacill_IEBH_NC_011167(2)	32.14%
PHAGE_Staphy_phiSa119_NC_025460(1)	26.23%
PHAGE_Staphy_phiSa119_NC_025460(1)	26.22%

Vfclass	Virulence Factors	Related genes	O217*	O331 B114*
		349	11	3
Adherence	Autolysin	atl	-	-
	Cell wall associated fibronectin binding protein	ebh	-	-
	Clumping factor A	clfA	-	-
	Clumping factor B	clfB	-	-
	Collagen adhesion	cna	-	-
	Elastin binding protein	ebp	-	-
	Extracellular adherence protein/MHC analogous protein	eap/map	-	-
	Fibrinogen binding protein	efb	-	-
	Fibronectin binding proteins	fnbA	-	-
		fnbB	-	-
	Intercellular adhesin	icaA	-	-
		icaB	-	-
		icaC	-	-
		icaD	-	-
		icaR	-	-
	Ser-Asp rich fibrinogen-binding proteins	sdrC	-	-
		sdrD	-	-
		sdrE	-	-
		sdrF	-	-
		sdrG	-	-
		sdrH	-	-
	Staphylococcal protein A	spa	-	-
Enzyme	Cysteine protease	sspB	-	-
		sspC	-	-
	Hyaluronate lyase	hysA	-	-
	Lipase	geh	-	-
		lip	-	-
	Serine V8 protease	sspA	-	-
	Serine protease	splA	-	-
		splB	-	-
		splC	orf0093	-
		splD	orf0092	-
		splE	orf0091	-
		splF	orf0088	-
	Staphylocoagulase	coa	-	-
	Staphylokinase	sak	-	-
	Thermonuclease	nuc	-	-
Immune evasion	AdsA	adsA	-	-
	CHIPS	chp	-	-
	Capsule	Undetermined	-	-
	SCIN	scn	orf0111	-

	Sbi	sbi	-	-
Secretion system	Type VII secretion system	esaA	-	-
		esaB	-	-
		esaD	-	-
		esaE	-	-
		esaG	-	-
		essA	-	-
		essB	-	-
		essC	-	-
		esxA	-	-
		esxB	-	-
		esxC	-	-
		esxD	-	-
	Alpha hemolysin	hly/hla	-	-
	Beta hemolysin	hlb	-	-
	Delta hemolysin	hld	-	-
	Enterotoxin A	sea	-	-
	Enterotoxin B	seb	-	orf026
	Enterotoxin C	sec	-	-
	Enterotoxin D	sed	-	-
	Enterotoxin E	see	-	-
	Enterotoxin G	seg	orf0102	-
	Enterotoxin H	seh	-	-
	Enterotoxin I	sei	-	-
	Enterotoxin J	sej	-	-
	Enterotoxin Yent1	yent1	-	-
	Enterotoxin Yent2	yent2	-	-
	Enterotoxin-like K	selk	orf0105	orf005
	Enterotoxin-like L	sell	-	-
	Enterotoxin-like M	selm	orf0106	-
	Enterotoxin-like N	seln	orf0103	-
	Enterotoxin-like O	selo	orf0107	-
	Enterotoxin-like P	selp	-	-
	Enterotoxin-like Q	selq	-	orf006
	Enterotoxin-like R	selr	-	-
	Enterotoxin-like U	selu	orf0104	-
	Exfoliative toxin type A	eta	-	-
	Exfoliative toxin type B	etb	-	-
	Exfoliative toxin type C	etc	-	-
	Exfoliative toxin type D	etd	-	-
		set10	-	-
		set11	-	-
		set12	-	-
		set13	-	-
		set14	-	-
		set15	-	-
		set16	-	-

Toxin	Exotoxin	set17	-	-
		set18	-	-
		set19	-	-
		set1	-	-
		set20	-	-
		set21	-	-
		set22	-	-
		set23	-	-
		set24	-	-
		set25	-	-
		set26	-	-
		set2	-	-
		set30	-	-
		set31	-	-
		set32	-	-
		set33	-	-
		set34	-	-
		set35	-	-
		set36	-	-
		set37	-	-
		set38	-	-
		set39	-	-
		set3	-	-
		set40	-	-
		set4	-	-
		set5	-	-
		set6	-	-
		set7	-	-
		set8	-	-
		set9	-	-
	Gamma hemolysin	hlgA	-	-
		hlgB	-	-
		hlgC	-	-
	Leukocidin M	lukF-like	-	-
		lukM	-	-
	Leukotoxin D	lukD	-	-
	Leukotoxin E	lukE	-	-
	Panton-Valentine leukocidin	lukF-PV	-	-
		lukS-PV	-	-
	Toxic shock syndrome toxin	tsst	-	-

Ovine									
O11*	**O326***	**O408 B115***	**O46***	**O82***	**B119***	**NCTC1803**	**NCTC9555**	**O17***	**O267***
10	**10**	**3**	**8**	**3**	**3**	**8**	**7**	**3**	**6**
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orf0010	orf0102	-	orf0106	-	-	-	-	-	-
orf0011; orf0012	orf0100; orf0101	-	orf0105	-	-	-	-	-	-
orf0013	orf0099	-	orf0104	-	-	-	-	-	-
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orf0014	orf0098	-	-	-	-	-	-	-	-
orf0015	-	-	-	-	-	-	-	-	-
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-	orf0082	orf0079	orf0086	orf0086	orf0134	orf0203	orf0269	orf0152	orf0318

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orf0004	orf0108	-	-	-	-	-	-	-	-
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orf0093	orf0113	orf0092	orf0116	orf0107	orf0060	orf0131	orf0196	orf0080	orf0245
orf0005; orf0092	orf0107; orf0112	orf0091	orf0109; orf0115	orf0106	orf0059	orf0122; orf0130	orf0187; orf0195	orf0079	orf0244
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			Bovine							
O268*	**ED133**	**O55 B118***	**CFSAN018750**	**PMB 64-1**	**PMB 81-4**	**K5**	**K17**	**2030RH1**	**MOK042**	**MOK063**
8	**8**	**4**	**0**	**2**	**5**	**1**	**0**	**13**	**10**	**10**
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-	orf0011	-	-	-	-	-	-	-	orf0003	orf0003
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-	-	-	-	-	-	-	-	orf0098	orf0244	orf0130
-	-	-	-	-	-	-	-	orf0099	orf0243	orf0131
-	-	-	-	-	-	-	-	orf0100	orf0242	orf0132
-	-	-	-	-	-	-	-	orf0101	orf0241	orf0133
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orf0300	orf0271	orf0196	-	-	orf0117	-	-	orf0063	orf0103	-

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-	-	orf047	-	-	-	-	-
-	-	orf048	orf0069	-	-	-	orf0163
-	-	-	orf0068	-	-	-	-
-	-	-	-	-	-	orf0029	-
-	-	-	-	-	-	orf0030	-
-	orf0292	orf036	-	-	-	-	-

CAPÍTULO II

5. CAPÍTULO II

Artigo não submetido em processo de escrita:

Título: "*Genome-Wide analysis of Coagulase-negative Staphylococcus aureus"*

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Genome-Wide analysis of Coagulase-negative *Staphylococcus aureus*

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Abstract:

Staphylococcus aureus is an important pathogen of ruminant's mastitis. The identification of Coagulase-negative *S. aureus* is considered an atypical event. Coagulation is an innate defense mechanism against pathogens, immobilizing invading bacteria in a clot. It happens through the cascade of serine protease-driven reactions. In addition, *S. aureus* can produce the extracellular enzyme coagulase, classified as staphylocoagulases, namely coagulase (coa) and von Willebrand factor binding protein (vWbp). In this study, comparative genomic analyzes were performed with 34 genomes of *Staphylococcus* spp. the six strains of CoNS isolated in this work, identified as *S. aureus*, isolated from mastitis in bovine from different farms and regions of southeastern Brazil, and another 28 genomes, with diversity in the host, species, and presence/absence of coagulase, retrieved from the public genomic database. As a result, of the six samples identified by the MALDI-TOF MS as CoNS *S. aureus*, four strains were genomically confirmed to belong to *S. aureus,* and two were characterized as *S. capitis*. Phylogenetically, the staphylocoagulases present three important branching points. The vWbp gene presents two clades differing between strains of *S. aureus* and other *non-aureus Staphylococcus* and a clade referring to the coa gene. In the vWbp alignment (499 aa), it is possible to observe the proteins related to the CoNS *S. aureus* fragmentation genomes, and the two vWbp proteins have a size of 304 aa or 188 aa. Coagulase-negative *S. aureus* can occur in the context of mastitis in bovines, which can result in the misdiagnosis of CoNS in the routine diagnosis of mastitis, at least in rare cases. To ensure the correct diagnosis of the species, genome sequencing and amplification of specific genes such as coa and vWbp are necessary in these cases.

Keywords: Coagulase-negative *Staphylococcus aureus*; vWbp; bovine mastitis

1. Introduction

Staphylococcus aureus is an important pathogen of ruminant's mastitis, a Gram-positive bacterium that is 0.5-1.5µm in diameter, usually fast-growing, with 1.0 to 2.0 mm in diameter after 24h of incubation. The cells have the shape of cocci, resemble a bunch of grapes, can be found singly or in pairs, and are immobile, non-spore-forming, and facultative anaerobes, able to perform both aerobic respiration and anaerobic fermentation. They are usually hemolytic, can produce a yellow or yellow-orange pigmentation, and are opaque and convex (Murray, 1995; Harris *et al.,* 2002). Currently, the genus *Staphylococcus* corresponds to 81 species and subspecies (https://lpsn.dsmz.de/genus/staphylococcus), among which most have clinical importance and are considered commensal bacteria or opportunistic pathogens colonizing in places such as the skin, nasopharynx and several mucous membranes of humans and other animals (Haag, Fitzgerald and Penadés, 2019). *S. aureus*, in comparison with other staphylococcal species, behaves related to the specific host. For example, *S. aureus* isolated from a bovine host will have more similarities among bovines than with *S. aureus* isolated from humans and other hosts such as ovine, swine, and poultry (Sheppard, Guttman and Fitzgerald, 2018; Haag, Fitzgerald and Penadés, 2019). The coagulase test gives the main classification within the *Staphylococcus* genus, subdividing into two significant groups, coagulase-positive (CoPS) and coagulase-negative (CoNS). They are associated with the ability of bacteria to produce coagulase (Coa) and von Willebrand factor binding protein (vWbp), two proteins that help coagulation. Both promote similar changes in the coagulation cascade (McAdow, Missiakas and Schneewind, 2012). Coagulase production is considered synonymous with invasive pathogenic potential. The CoPS is almost exclusively represented by *S. aureus*; it is considered a differentiating factor among other staphylococcus species. The identification of coagulase-negative *S. aureus* is considered an atypical event (Becker, Heilmann and Peters, 2014; Bonar, Międzobrodzki and Wladyka, 2018). A few studies are found in the literature that seek to characterize the staphylocoagulase genes (Coa and vWbp) molecularly and their functions (Javid et al. 2018; Chmagh and Abd Al-Abbas 2019; Maddur et al. 2022).

Genomics investigations available on coagulase-negative *S. aureus* are still rare. However, recently Custodio, 2019 part of the group, unexpectedly identified, six samples initially identified as coagulase-negative *S. aureus*. The strategy of large-scale DNA sequencing and the use of genetic tools has demonstrated a great potential to elucidate the correct identification of these samples in species and are essential for the possible understanding of the genetic mechanism associated with this phenomenon. This study contributes to the knowledge of

genomic specialization and the role of coagulase-negative strains in establishing infection in *S. aureus* associated with bovine mastitis, presenting comparative genomic analyses of four coagulase-negative *S. aureus*. In addition, four samples characterized as coagulase-negative *S. aureus* were isolated from bovine mastitis from Brazilian strains to infer genomic characteristics and phylogenetic relationships.

2. Materials and Methods

2.1. Bacterial strains

The strains used in this study were isolated and identified by Custodio, 2019. Six strains isolated from the milk of bovines with subclinical mastitis, collected in 2004 in Campo das Vertentes (mesoregions of the Brazilian state of Minas Gerais) TableS4, were initially identified as Coagulase-negative *S. aureus*, based on the methodology described by Quinn et al., 1994. These strains were grown on 5% sheep blood agar for 24h at 37°C; after the growth period, presumed functional tests of primary identification were performed (Gram, morphology, catalase, and mannitol test). After this, they were cultivated in BHI broth (Brain Heart Infusion) (Difco, USA) at 37°C for 24h and subjected to a coagulase test (mac Faddin, 1980). The coagulase test was performed in a tube containing 250 µL of rabbit plasma, and the same amount of culture was added, followed by incubation for 24h, 48h and 72 h at 37°C. *Staphylococcus aureus* ATCC 25923 and *Staphylococcus chromogenes*, isolated from bovine mastitis belonging to the Collection of Microorganisms from Laboratório de Bacteriologia, Departamento de Medicina Veterinária, Universidade Federal de Lavras, were used as positive and negative controls in all assays, respectively. The presence of a clot was considered coagulase positive.

All six Coagulase-negative *S. aureus* isolates were performed by protein profile, using the MALDI TOF technique, according to Sauer et al., 2008. The BioTyper TM software (Bruker Daltonics, USA) was used in an Autoflex III mass spectrometer. The characterization of microorganisms was performed by reading the fingerprints using an Autoflex III mass spectrometer (Bruker Daltonics, USA).

The prospection of virulence genes encoding biofilm expression (*icaAD*) was performed according to Fegjun et al.,2003 . The identification of the enterotoxin genes *sea, seb, sec, sed,* and *see* was performed by multiplex PCR, according to Mehrotra et al., 2000, with minor modifications described by Pérez et al., 2020. The presence of the hemolysin *hla* and *hlb* genes was investigated by multiplex PCR, according to Jarraud et al., 2002 TableS5.

All the PCR products were separated by 1.0% agarose gel electrophoresis stained with ethidium bromide (0.5 mg / mL), using the Tris-borate- EDTA (TBE) (89 mM Tris Base, 89 mM boric acid and 2 mM EDTA pH 8.0). The 100 bp DNA Ladder (KASVI, Brazil) was used in all assays. The images were developed and recorded in a transilluminating device (L-Pix Chemi Photo Digitizer - Loccus Biotecnologia, Brazil) for further analysis.

The antimicrobial susceptibility test was performed using the broth microdilution technique to assess the minimal inhibition concentration (MIC), according to the Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2018). Seven antimicrobials were tested in different concentration ranges. All tests were duplicated; *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality controls in all assays. The results were interpreted according to CLSI [18]. The expression phenotypic resistance profile aminoglycosides (GEN), tetracycline (TET), cephems (CEF), macrolides (ERY), folate pathway antagonist (SXT), quinolones (CIP), aminocoumarin, (NOV) as described in work by Custodio, 2019 TableS6.

2.2. Sequencing, assembly, and annotation of the genomes

Chromosomal sequencing of each strain performed in Hiseq Illumina platforms using 2 x 150bp paired-end technology (Illumina, San Diego, CA, USA). The read quality was checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Isolate reads were used for the ab initio assembly, Unicycler, version 0.4.5:37(Wick *et al.,* 2017), Edena v3.131028:56 (Hernandez *et al.,* 2014), and SPAdes v3.15.0:46 (Bankevich *et al.,* 2012) of all genomes. The QUAST (Gurevich *et al.,* 2013) tool was used to identify the best assembly. CONTIGuator version 2.7 (Galardini *et al.,* 2011) generated scaffolding. After sorting, the six sequences were submitted to the automatic gap-closing step, using other assemblies as a fill guide, thus obtaining the scaffolds. All sequences underwent coverage validation (chromosome versus reads) and no low coverage regions (≤ 30). The gaps resulting from assemblies were automatically closed in the G-Finisher v1.4 (Guizelini *et al.,* 2016), and the Benchmarking Universal Single-CFopy Orthologs (BUSCO) v5.3.0 (Manni *et al.,* 2021) tool was used to identify the integrity of the genome against Bacteria and *Bacillales* database (odb10). The mapping generated the read's CLC Genomics Workbench software, version 7.0 (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench), with a low coverage cut-off lower than ≥ 30. The reference and lineage confirmation of the genomes selected to the resulting contigs for each strain were submitted to the Basic Local Alignment Search Tool for nucleotides (BLASTn) (Alikhan *et al.,* 2011) and JSpeciesWS Online Service

Taxonomic Thresholds (available at https://jspecies.ribohost.com/jspeciesws/) (Richter *et al.,* 2016). MOB-suite version 3.0.3 (available at https://github.com/phac-nml/mob-suite) (Robertson *et al.,* 2020) reconstructs individual plasmid sequences from draft genome assemblies and separates them into different files.

2.3. Comparative genomic analysis of classical CoNS strains and coagulase-negative *S. aureus*

Twenty-eight sequence data from different *Staphylococcus* species used for comparison are available from NCBI Genbank, selected based on complete, representative genomes and phenotypic coagulase tests confirmed in the literature. All information about these sequences, such as strain, host, origin, isolation locality and Genbank accession, is present in Table S1. All genomes (n=34) were submitted to an automatic annotation using the PROKKA server, version 3.0 (Seemann, 2014) (available at https://github.com/tseemann/prokka).

2.3.1. Prediction of orthologous proteins

The prediction of orthologous groups and the species trees from 34 *Staphylococcus* spp. strains predicted by OrthoFinder, version 2.5.4 (Emms and Kelly, 2017, 2019) (available at https://github.com/davidemms/OrthoFinder).

2.3.2. Phylogenomic analysis

The phylogenomic tree was generated based on Roary's nucleotide alignment of the core genome(Page *et al.,* 2015). FastTree (Price, Dehal and Arkin, 2010) was used to construct the maximum likelihood tree. The iTOL version 3 was used to customize the tree (Letunic and Bork, 2016) (available at https://itol.embl.de/).

2.3.3. Virulence factors and antimicrobial resistance genes identification

The ABRicate, with a cut-off of ≥75 coverage and ≥80 identify (Seemann, *Abricate*) (available at https://github.com/tseemann/abricate) tool, was used to mass screen the genomes to find resistance genes with the CARD tool (Jia *et al.,* 2017). Moreover, for virulence genes, using the VFDB (Virulence Factors of Pathogenic Bacteria) (Chen *et al.,* 2016). All databases used were updated (2022-Mar). In addition, the similarity heatmap was screened using Morpheus online software (available at https://software.broadinstitute.org/morpheus/) with metric (Euclidean distance), linkage method (complete), and cluster (columns and rows).

2.3.4. Sequence alignment and phylogenetic analysis of Staphylocoagulases enzymes

The orthologous genes' results made it possible to identify the orthogroups referring to the staphylocoagulases genes. The phylogenetic relationship, distribution, and identical coagulase genes (*coa* and *vwb*) were accomplished in the rooted Neighbour Joining

phylogenetic tree using the JTT model and bootstrap method (resamples = 1000), performed with MEGA-X [24]. The tree was edited in iTOL version 3 (Letunic and Bork, 2016) (available at https://itol.embl.de/). It is constructed from MAFFT-aligned (Rozewicki *et al.,* 2019) amino acid sequences with default parameters and visualized using Jalview version 2.11.2 (Troshin *et al.,* 2018).

3. Results

3.1. Genomic analysis

3.1.1. Genomic characterization (Sequencing, assembly, and submission of the genomes)

The six strains showed excellent sequencing quality without the sequence duplication level of overrepresentation. Unicycler was the assembler for all assemblies that best-returned results regarding contigs values, N50 and L50. The BLAST determined the references for sorting, and the best similarity values of ANI, *Staphylococcus aureus* RF122 (AJ938182.1), were used for the four strains (23S, 42S, 48S, and 56S) of *S. aureus*. Strains 62S and 73S showed an ANI ≥ 95 for *Staphylococcus capitis* species; the most representative genome was *S. capitis* BN2 (CP042341.1). After sequencing, they did not (62S and 73S) belong to the same species. The MALDI-TOF technique identified all isolates as *S. aureus*; however, the 62S and 73S identified other species. Table S2 describes the genomes used to JSPECIES and BLASTn tools to confirm the species for each strain belonging. The total BLASTn of the contigs generated by the assembly made it possible to identify the most similar species for each sample. Complete genomes and representatives for the species of *S. aureus* and *S. capitis* are from the NCBI. The strains 23S, 42S, 48S, and 56S showed identity and coverage greater than ≥ 95% for *S. aureus*. While strains 62S and 73S showed more significant similarity with *S. capitis*, the related organisms do not belong to the same species indicated by MALDI-TOF.

The chromosomal genomes of the six coagulase-negative strains sequenced in this study ranged from 2.5 to 2.6 Mb in size, with approximately 33% of GC content and 2,603 and 2,620 gene coding sequences (Table 1). All genomes used in this study were re-annotated using the Prokka tool. In addition, two replicon types predict the plasmid sequences for the *S. aureus* samples and six for the *S. capitis* samples (Table S3).

Table 1. General genomic features of strains analyzed.

Strain	Genomes length (bp)	GC content (%)	Gene predicted	Contigs	Plasmid*	NCBI Accession Number
Staphylococcus aureus 23S	2731001	33.6	2607	32	2	
Staphylococcus aureus 42S	2726742	33.6	2603	32	2	
Staphylococcus aureus 48S	2727207	33.6	2604	29	2	

Staphylococcus aureus 56S	2727149	33.6	2604	29	2
Staphylococcus capitis 62S	2668873	33.5	2612	42	6
Staphylococcus capitis 73S	2691222	33.5	2620	40	6

*** Identification MOB-suit**

3.2. Comparative genomics of coagulase-negative *Staphylococcus* spp

3.2.1. Prediction of pan-genome and identification of orthologous proteins

The pan-genome represents the entire set of genes that characterizes a given species, consisting of the core genome (sequences shared among all individuals of the species) and the accessory genome (shared among two or more species). This analysis made it possible to identify genomic clusters based on gene sharing. The pan-genome analysis was performed with the Roary tool, identified in the group of 21 isolates of *S. aureus*., seeking to identify the presence and absence between the CoPS and CoNS. The comparison resulted in 4,305 genes, with 1,967 related to the core genome and 2,383 accessory genomes. The accessory genes represent less than 95% of the isolates. In addition, Orthofinder identified 3,844 orthogroups, 85,481 genes in orthogroups, and 1014 unassigned genes for the 34 genomes (*S. aureus* and others *Staphylococcus* spp.).

3.2.2. Phylogenomic analysis

The Orthofinder-generated phylogram of the 34 genomes shows two nodes separating *S. aureus* (blue branch) from the other species of *Staphylococcus* spp. (red dotted branch) with bootstrap support of 1000 resamples and showing branches with distant lengths. Strains 23S, 42S, 48S, and 56S clustered firmly in the *S. aureus* branch, while the Sta_62S and Sta_73S share a branch with *S. capitis* BN2 (Figure 1. a), and the cladogram of *S. aureus* (Figure 1. b) can identify the grouping between *S. aureus* CoNS (bold) and CoPS since they are similar.

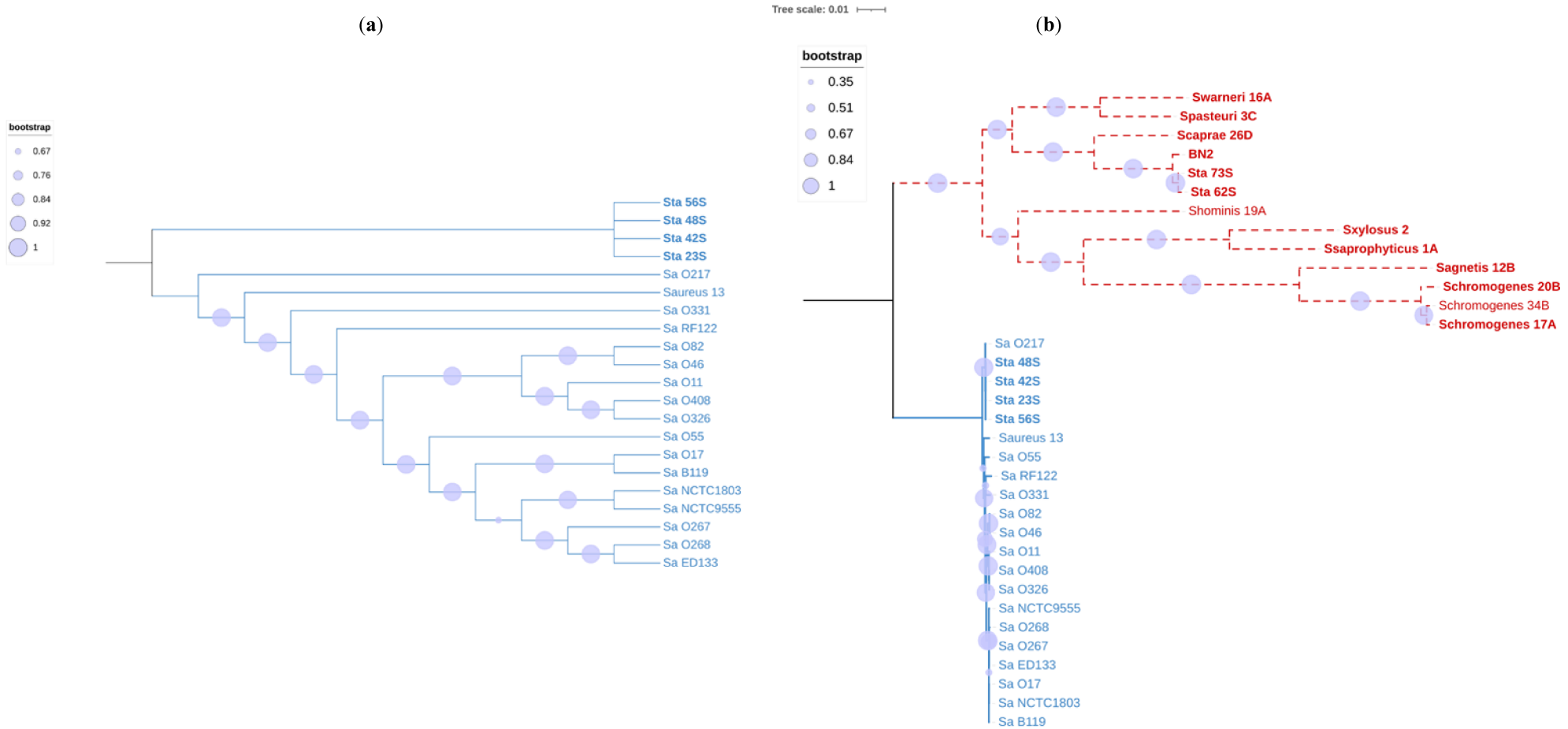

Figure 1. Phylogenomic analysis, molecular phylogenomic analysis based on the nucleotide alignment of the core: (**a**) 21 *Staphylococcus aureus* strains genomes; (**b**) 34 *Staphylococcus* spp. strains genomes. The coagulase-negative strains are in the bold, purple balls representing the bootstrap (1000 replicons), the blue is *S. aureus*, and the red others *Staphylococcus* spp.

3.2.3. Virulence factors and antimicrobial resistance genes identification

The VFDB tool identified in the 34 isolates of *Staphylococcus* spp., seventy-six genes associated with virulence factors and functionally characterized as toxin, enterotoxins *se*, adherence, enzyme, secretion system, immune evasion, iron uptake, stress protein, and capsular genes. Through this analysis, it is possible to identify the particularities of the groupings of each species based on virulence factors. In addition, it is also possible to identify the presence or absence of genes confirmed in molecular tests (Table S5). All predicted genes can be followed in Figure S1, which shows the dendrogram clusters in two large groups of genes' presence and absence; each colour in the figure represents a profile. One group of genomes found 59 to 70 genes, generally related to *S. aureus* (n=21), and another identified at least 1 ≤ 4 genes per genome, represented by another *Staphylococcus* spp. (n=13). The *S aureus* CoNS in Figure S1 (id 28,29,30 and 31) strains were predicted by 60 virulence factors, except for the 42S isolate's absence of the *cna* gene, which was found only in two other isolates (*S. aureus* 13 and *S. aureus* O217). Compared with other *S. aureus*, the CoNS have fewer genes, mainly in the absence of genes associated with toxins (*luk*F-PV), adherence (*fnb*AB, *sdr*D), and secretion system (*esa*C, *ess*C, and *esx*B).

Different antibiotic resistance profiles were identified through antimicrobial susceptibility testing of the strains isolated from milk of cattle with subclinical mastitis used in the present study (Table S6). All strains (23S, 42S, 48S, 56S, 62S and 73S) are susceptible to sulfa-trimethoprim and novobiocin, and only strain 23S showed resistance to ciprofloxacin. Utilizing the CARD tool found 19 genes related to antimicrobials for resistance gene prediction. Each genome presented between 1 to 10 genes. Figure S2 shows genes and isolates in rows and columns. Each profile is a colour identified in eight clusters of presence and absence profiles between the columns (strains). For example, the *S. aureus* isolates showed the same profile, differing only with two genes (PC1_beta-lactamase_(blaZ) and *Staphylococcus_aureus*_FosB) identified in some species. No profile was unique to CoNS.

3.2.4. Sequence alignment and phylogenetic analysis of Staphylocoagulases

The alignment and phylogram of the orthologous referring to the staphylocoagulases genes (*coa* and *vwb*) identified points of origin (nodes), similarities, and inter and intra-species modifications. Phylogenetically, in Figure 2.a, it is possible to identify 3 points considered as points of origin and their bifurcations, with bootstrap greater than or equal to 78% (lilac circles). In bold are the sequences extracted from the *S. aureus* coagulase negative genomes. The *coa* gene was predicted only in strains of *S. aureus*, identified in 21 sequences (strains = 21) with only one gene per isolate, belonging to the group OG0002195 (identified by OrthoFinder).

Figure S3 shows the multiple alignments of the *coa* gene and the conservation degree graph. It demonstrates the high similarity of sequences in the *coa* gene family. The gene alignment varies from 550 aa (minimum sequence length) to 714 aa (maximum sequence length), with an average of 672 aa (average length). The initial positions and highly conserved regions are observed throughout the sequence, represented by "*" in Figure S3 and along the sequence, identifying 46 variants in the *coa* gene. The sequences referring to the coagulase-negative *S. aureus* isolated in this study present a smaller size (663 aa), but this characteristic is shared with all other *S. aureus* isolates from bovine, differing missing regions only from the strains isolated from ovine.

Using *S. aureus* 13 (CoPS) with a reference, 19 variants are characterized as synonymous (SYN) and 27 as not synonymous. At the relative level between *S. aureus* CoNS (4 strains) and CoPS (17 strains), only variation at site 471 (AAR=K; AAA=N). The phylogram in Figure 2.b shows the diversity between the *coa* gene sequences (green branch) of the negative-coagulase strains (bold) and the others, only the isolate SaO217 shares the same grouping.

The representatives of the *vWb* gene from other species (red dotted branches) are clustered externally of the genes (coagulase and *vWb*) predicted in *S. aureus*.

The *vWb* gene belongs to strains of *S. aureus* and other *Staphylococcus* spp. (*S. cromogenes* and *S. agnetis*), and 44 sequences were identified (strains = 25). Each isolate presents one or more coding sequences of the gene belonging to the OG0000069 group (identified by OrthoFinder), as seen in Figure 2.c, the *vWb* genes predicted in *S.aureus* (blue branch) are primarily present in pairs and groups in different branches. However, four strains (SaO11, SaO331, SaRF122, and Saureus13) showed only one gene copy. The outer group (red dotted branches) contains three representative genes in each species (*S. cromogenes* and *S. agnetis*). The size of this enzyme is approximately greater than 450 amino acid residues. The gene alignment varies from 188 aa (minimum sequence length) to 508 aa (maximum sequence length); the alignment presented an average of 448 aa (average length). All *S. aureus* CoPS isolates have two copies of *vWb* with a size of ≥ 499 aa. In contrast, the *S. aureus* CoNS isolates have a protein with a size of 304 aa and another 188aa, presenting the fragmented form of the gene, as seen in Figure 3, in the alignment, this fragment is in both copies in the region between site 185 and 202, in individuals (18,19,20 and 21).

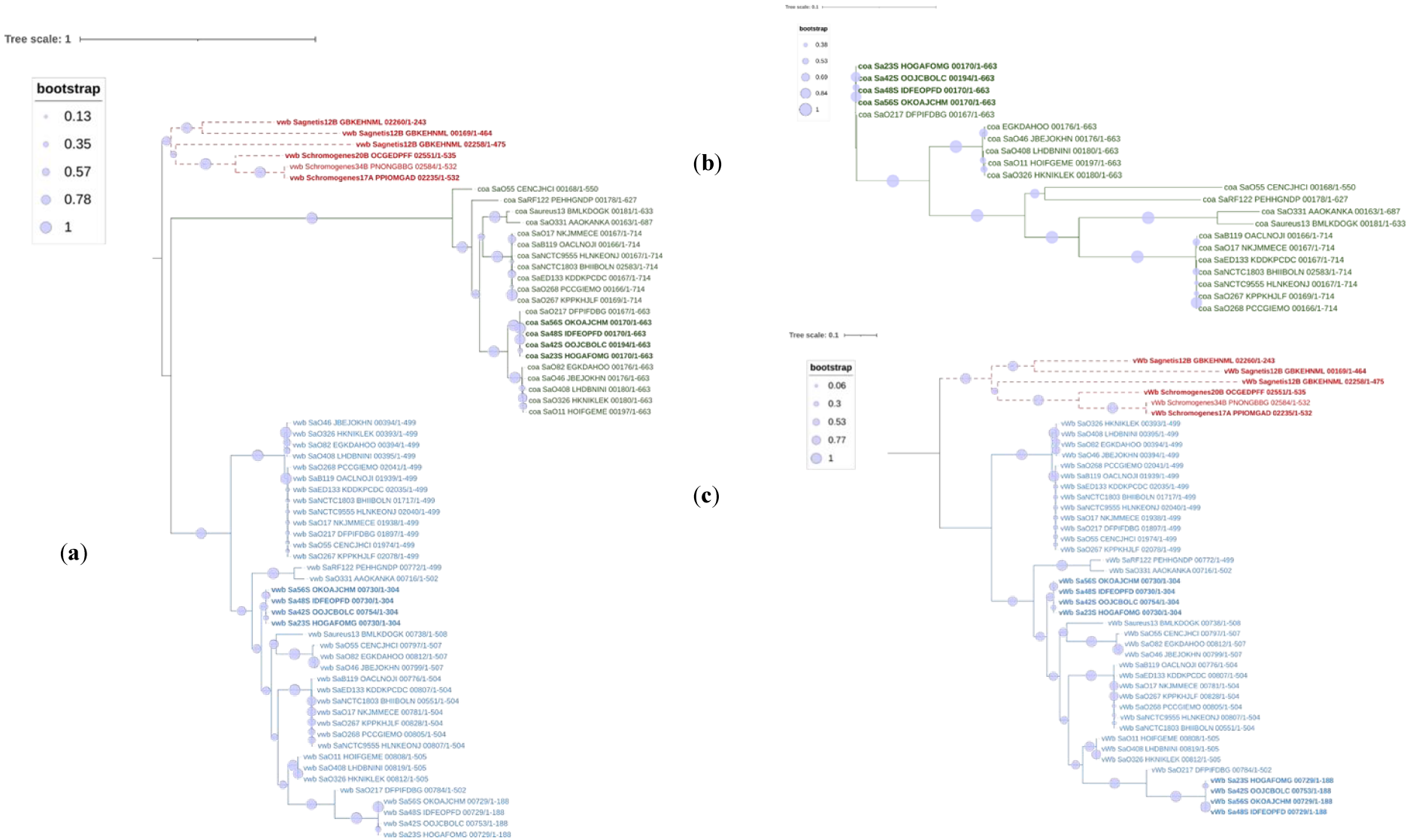

Figure 2. Phylogenetic tree of staphylocoagulases amino acid sequences. (**a**) 65 sequences of *coa* gene (OG0002195, identified by OrthoFinder) and 44 sequences of *vWb* gene (OG0000069, identified by OrthoFinder). Label name: gene/strain/locus-tag/aligned sequence length; Colors: dotted line red: *vWb* gene of others *Staphylococcus* ssp., blue: *vWb* gene of *Staphylococcus aureus*, and green: *coa* gene; Bold: *Staphylococcus* coagulase-negative.

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b

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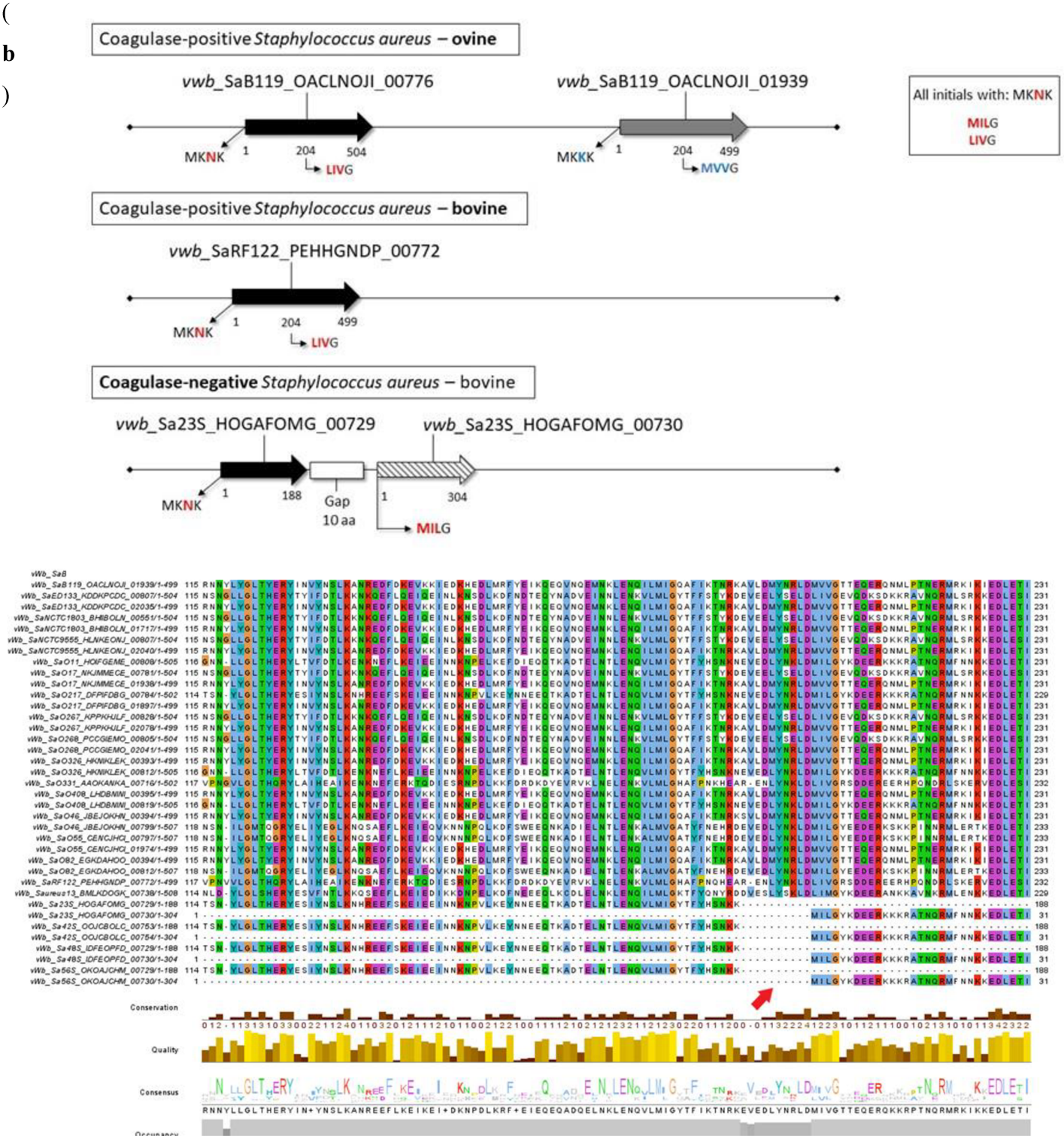

Figure 3. Representation of the *vWbp* gene: (**a**) Gene forms found in *S. aureus* coagulase-negative, coagulase-positive in different host; (**b**) Multiple alignment of the vwbp protein highlighting with the red arrow the region considered fragmented in coagulase-negative *S. aureus*.

4. Discussion

Staphylococcus bacteria group is the most common gram-positive bacteria isolated from clinical specimens. This bacterial genus comprises gram-positive cocci, i.e., spherical-shaped bacteria-stained violet by gram staining and may be present as single cells or in arrangements, in pairs or clusters. One main characteristic that differentiates them from other gram-positive

cocci is the enzyme catalase, evidenced by the formation of gas bubbles when colonies are in contact with hydrogen peroxide. Metabolically, they are facultative anaerobes, do not possess flagella, are immobile, and produce many exotoxins. The natural habitat of this bacterial group is the skin and mucosa of animals, which survive on environmental surfaces, able to grow at temperature extremes and in high salt concentrations. It is related to several animal infectious processes, such as skin infections in the community that may evolve with complications and dissemination (folliculitis, boils, and carbuncles, impetigo, mastitis). They can also be related to hospital infectious processes, such as wound infections (post-operative), bacteremia and endocarditis (dissemination of infectious focus), meningitis (a complication of bacteremia or trauma), pneumonia (community-acquired or hospital-acquired), osteomyelitis or septic arthritis (dissemination of infectious focus), pyomyositis (secondary to carbuncles). Detecting and identifying these zoonotic bacteria of human and animal medical importance is an essential and indispensable service. Improving control protocols over the years is necessary, contributing to a correct diagnosis and monitoring, leading to the ideal treatment. Colonies on blood agar medium are significant, opaque, with zones of hemolysis evident and whitish; some iso-lates have a slightly yellowish pigmentation, which gives the species its name "aureus." The laboratory identification of this staphylococcus species mainly involves determining the coagulase enzyme (positive), which induces clot formation on contact with the plasma. Other tests used for identification are observing the production of endonucleases on DNAse agar medium (positive) and yellow fermenting colonies on mannitol salt agar medium (Mannitol Fermenter). The use of routinely applied microbiological methods such as colony growth in the culture medium, morphological and biochemical characterization, the biofilm and coagulase tests, and the MALDI-TOF MS technique led to the identification of *S. aureus*. However, the MALDI-TOF MS technique needs a reference database to identify the micro-organism spectrum and its association. Even though it is a very effective technique in identifying pathogens that cause mastitis compared to conventional biochemical methods, presenting high sensitivity and specificity, the MALDI-TOF MS technique can generate false positives depending on the database. In addition, spectrum reference data presents difficulty in identifying species with very similar protein profiles, as with the strains identified as *S. aureus* but genomically characterized as *S. capitis*.

3.1. Coagulase-negative *Staphylococcus aureus* diagnosis and genomic

The genus *Staphylococcus* has 56015 genomes deposited at the NCBI (June 2022), with 75% (42080) of *S. aureus* year released between 1980 and 2022; in the last year alone (2022), 2367 genomes were submitted. Regardless of the isolation host, the *S. aureus* genome usually

has only one chromosome, a size of ≅ 2.8 megabases (Mb), low GC content (≅ 32.8%), and ≅ 2,743 genes (https://www.ncbi.nlm.nih.gov/datasets/genomes/?taxon=1280). A genomic reference of bovine *S. aureus* is RF122, also known as ET3-1, the first animal lineage of *S. aureus* with a fully sequenced genome, providing insights into studies in population genetic analysis among isolates of humans and ruminants (Fitzgerald and Holden, 2016). In addition, with the sequencing and deposit in a public database, our study contributed to four genomes of Coagulase-negative *S. aureus* isolated in bovine from the mesoregions of the Brazilian state of Minas Gerais, confirmed by the coagulase test. Routinely, classification of *Staphylococcus* spp. into *S. aureus* and *S. non-aureus* is based on the coagulase test. The coagulase test is a gold method for routine identifying *S. aureus* positive. The coagulase-negative diagnoses are considered minority pathogens, especially in the case of *S. aureus*. Even atypical, it is possible to observe Coagulase-negative *S. aureus* and too associated with bovine mastitis (Młynarczyk *et al.*, 1998; HELTBERG and BRUUN, 2009; Akineden *et al.*, 2011). The interpretation of the coagulation test results in this work was compared with reference intervals between 24h, 48h, and 72h. Since in culture versus literature, between clotting time versus literature, do the host's blood or plasma, a visualization of clotting can be seen immediately or later (Siller-Matula *et al.*, 2008; HELTBERG and BRUUN, 2009; Bonar, Międzobrodzki and Wladyka, 2018). The lack of correct diagnoses and diagnostic quality control can lead to the wrong treatment choice and thus hamper the prudent use of antibiotics. Therefore, there is a need for guidelines that guarantee quality in the diagnosis of mastitis (Astrup, Pedersen and Farre, 2022). In the public genome databases, no *S. aureus* genome, characterized as coagulase-negative, has been identified. Since 1986, only the literature has reported the diagnosis in production animals and clinical cases in humans, some of these presenting misdiagnoses, not considering *S. aureus* as coagulase-negative (Ruane *et al.*, 1986; Neville *et al.*, 1991; Wanger *et al.*, 1992; Vandenesch *et al.*, 1993; HELTBERG and BRUUN, 2009; Akineden *et al.*, 2011).

3.2. Phylogenomics analysis

A phylogenetic analysis of the sequenced strains grouped isolates of different manifestations into separate branches, suggesting that some particularities among isolates may be related to their coagulase-negative profile. In interspecies of *S. aureus,* it is possible to identify the coagulase-negative grouping. Note that *S. aureus* genomes are very close and similar and can be grouped by isolation host. Compared to other *Staphylococcus,* there is great diversity.

The phylogenetic analysis clearly showed the separation of genomes related to *S. aureus* and other staphylococci, presenting a high similarity among species with reliable bootstraps.

When analyzing the clusters between Coagulase-positive and negative *S. aureus*, we can observe the segregation between them. The Coagulase-negative genome group separated from the other *S. aureus*, grouping only with the isolate SaO217 (*S. aureus*), as seen in Figure 1.b. Regardless of the clustering methodology, Coagulase-negative *S. aureus* isolates clustered separately at the genomic level and the level of staphylocoagulase genes. In order to identify the shared points of origin between the staphylocoagulases genes (coa and vWbp), it is noted that even though the genes present similar regions at the coagulase function level, the gene encoding coagulase (coa) was observed only in *S. aureus* isolates and did not share a point of origin close to the vWbp gene. The von Willebrand factor-binding protein, on the other hand, was predicted in non - *S. aureus*. However, these genes have different points of origin when related to *S. aureus* and non-*S. aureus*. Thus, both genes (coa and vwbp) can be treated independently, although in the literature, the functional annotation in public sequence databases, these are named as coagulase, with no distinction. They are making necessary a curatorship in the nomenclature and a greater sensitivity in annotating these genes.

3.3. Virulence factors and antimicrobial resistance genes identification

S. aureus may present a great diversity of virulence factors, contributing to its capacity to cause complicated infectious processes. Among them is the ability to form biofilms, the external capsule of polysaccharides that hinders phagocytosis by leukocytes. Furthermore, the teichoic acids of the bacterial network facilitate adherence to tissues and may induce septic shock in the host. In addition, the presence of surface protein A, encoded by the spa gene, hinders the recognition of the bacterial cell by the immune system, the production of various enzymes contributes to the dissemination of bacteria by the tissues, and the secretion of exotoxins induces cell destruction and intense immune response. They can produce toxins in food (ham, poultry, egg dishes, pastries), causing food poisoning (ingestion of enterotoxins) or the toxic shock syndrome resulting from infection processes producing the toxic shock syndrome toxin, also known as TSST-1 (presence of superantigens encoding for tsst-1 gene). All these factors contribute to the pathogenicity of this micro-organism.

All coagulase-negative *S. aureus* isolates were identified to produce biofilm, also known as water-soluble film consisting of monosaccharides, proteins, and small peptides. This extracellular substance binds the bacteria to tissues and foreign bodies such as catheters, grafts, prosthetic valves and joints, and shunts. It is significant for the survival of relatively avirulent coagulase-negative staphylococci. CasuL-tetracycline and CasuL-ampicillin combinations inhibited biofilm formation. In Figure S1 it is possible to identify the predicted presence and absence of genes related to *Staphylococcus aureus* fibronectin-binding proteins (FnBPs) play a

critical role in *S. aureus* pathogenesis. FnBPs mediate adhesion to fibronectin and invasion of mammalian cells, including epithelial, endothelial, and fibroblastic cells, by fibronectin bridging to the host cell (fibronectin receptor integrin). A stop codon was observed in both FnBPs (fnbA and fnbB genes), making them truncated and not expressed. Furthermore, they were secre-ted into the culture medium and not anchored to the cell wall since they lacked the sorting motif [42]. Consequently, this led to a loss of FnBPs dependent functions such as solid adhesion to immobilized fibronectin and fibrinogen binding. In our work, the genes related to FnBps were predicted only in *S. aureus*. All coagulase-positive *S. aureus* had the presence of fnbA or both, but in isolates referring to coagulase-negative *S. aureus*, neither of the two genes were predicted (Figure S1).

Bacterial resistance is the ability of bacterial strains to resist the action of antimicrobials and continue to reproduce. Today it is a large-scale worldwide problem, causing several inconveniences, such as increased hospitalization time, treatment costs, and morbidity and mortality rates of patients. Furthermore, it can affect the health of animals, humans, and even the environment (soil and water). The microbial resistance arises with the contribution of several facts; however, the main one is the indiscriminate use of antimicrobials, which exerts enormous selective pressure on the maintenance and expansion of this resistance. In animal husbandry, it is mainly used for therapeutic purposes and in administering antimicrobials as a performance enhancer. For the treatment of bovine mastitis, antimicrobial agents were first used starting in the 1940s. Antimicrobials are mainly used to help the host's defense, eliminate the pathogens, and reduce the negative consequences of the infection. Besides being a fundamental measure for curing cases of clinical mastitis, antimicrobials are used in dairy cows to control, prevent, and reduce the spread of contagious mastitis among cows in the herd. To acquire resistance needs to modify its DNA. This process can occur in two ways: inducing mutation in the native DNA and introducing external DNA containing genes of resistance, which can be acquired between genera or different species of bacteria. In most cases, the acquisition of resistance genes occurs by the incorporation of DNA present in extrachromosomal plasmids, which can be transfer-red between micro-organisms in a process denominated horizontal transfer horizontal transference. The incorporation of foreign DNA can still be realized in a process called transformation, a type of horizontal transfer that results in exchanges of chromosomal DNA between species, consequently leading to interspecies recombination.

Besides virulence factors, *S. aureus* is also known for its potential resistance to antimicrobials, either by the presence of mutated proteins, such as the PBP2a protein (MRSA) or acquisition of a resistance gene such as the VanA-Type Vancomycin-Resistant

Staphylococcus aureus the vanA gene (VRSA), which fortunately is still very rare, among other diver-se mechanisms that this bacterium can present. The emergence of multi-resistant pathogens, the failure of antibiotic therapy in mastitis treatment in cattle, and the presence of antibiotic residues in milk and milk products have led to the emergence of alternative treatments, such as the use of Calliandra surinamensis pinnulae lectin (CasuL). This work de-monstrates the efficiency of CasuL-tetracycline and CasuL-ampicillin combinations in inhibited biofilm formation. Suggesting, CasuL is a bacteriostatic and antibiofilm agent against some mastitis isolates and displayed a synergistic potential when combined with either ampicillin or tetracycline [43]. Although the use of antibiotics cannot be abolished, the rational administration of these compounds requires a careful selection of the drug and duration of use, and its correct indication.

The CoNS can produce enterotoxins that are resistant to enzymatic proteolysis, resistant to heat treatment, and do not lose biological activity, becoming a consumer health risk and a public health problem. In addition, they tend to be more resistant to antimicrobials than *S. aureus* and quickly develop multidrug resistance (el Haddad *et al.,* 2019). All strains of this work did not show the identification of enterotoxins in vitro or predictions in the functional annotation. That is, neither the presence of genes was observed throughout the genome.

3.4. Staphylocoagulases

S. aureus has a significant proclivity to activate coagulation and fibrinolytic procedures. For coagulation to occur in *S. aureus*, the presence of two factors is observed in the literature: coa and vWbp. vWbp is more unknown than Coa. The von Willebrand factor binding protein expressed by *S. aureus* facilitates the onset of septic arthritis (Na *et al.,* 2020). Perhaps this justifies being treated as a hypothetical protein during predictions. In both PFAM, Interpro-scan and Prokka are identified as "coagulase family" or "Fragmented Staphylocoagulase." In BLAST, we identify von Willebrand factor binding protein Vwbp, and in Interproscan, the entire prediction of the domains for a protein is even considered fragmented or gene truncated and functional.

Coagulation is promoted by two secreted enzymes (coagulases), coagulase (Coa) and von Willebrand factor-binding protein (vWbp), that activate host prothrombin and consequently cleave fibrinogen to fibrin. Coa and vWbp promote clotting of soluble fibrinogen, plasma, or blood by forming a stable complex with prothrombin. Apart from its coagulation promoting property, von Willebrand-binding protein also has a high affinity to von Willebrand factor that can form ultra-large multimers retained on the endothelial cell sur-face upon activation of endothelial cells. Furthermore, it has been shown that interaction between the von Willebrand

factor and vWbp contributes to vascular adhesion of *S. aureus*. Show the contribution of Coa and vWbp for joint-specific invasiveness by *S. aureus* in septic arthritis (Na *et al.,* 2020).

A prospective study was conducted evaluating the utility of polymyxin susceptibility testing in discovering coagulase-negative strains of *S. aureus*. During a six-month study period, 14 staphylococcal isolates from four patients were initially found to have no coagulase activity and to be resistant to polymyxin; in comparison, approximately 1,500 common coagulase-positive, polymyxin-resistant *S. aureus* isolates were found during the same period. One isolate from each of the four patients and a coagulase-negative *S. aureus* strain previously isolated from our own collection was further characterized. Two strains showed delayed coagulase activity in the retest, while the other three failed to produce coagulation in any coagulase assay. Judged by thermostable nuclease activity, phage typing, and biochemical profiles, these three coagulase-negative strains are genuine *S. aureus* strains. The polymyxin test, therefore, appears to be helpful in revealing *S. aureus* variants not identified as *S. aureus* by routinely performed coagulase assays.

5. Conclusions

The MALDI-TOF MS technique can be a handy tool for rapidly and accurately diagnosing CoNS species. However, the method should be used cautiously when evaluating veterinary strains, as the strain profile may not be available in databases. In addition, the classification of *Staphylococcus* should not be based solely on the coagulase test due to false identifications. Therefore, the *coa* and *vwbp* genes appear to help reveal *S. aureus* variants not identified as *S. aureus* by routinely performed coagulase assays. In conclusion, these genes can be used for primer design and conventional or real-time PCR to complement (or, if not available, replace) other techniques for CoNS *S. aureus* identification.

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Supplementary material

Table S1. *Staphylococcus* spp. strains used for genomic comparison, obtained from the NCBI (dataset).

	Strain	Host	Origin	Coagulase positive or negative [1]	Isolation locality[2]	GenBank Accession
1	*Staphylococcus aureus* strain O217	ovine	abscess/clinical mastitis	+	Aveyron/FR	CP038461
2	*Staphylococcus aureus* strain O331	ovine	abscess/subclinical mastitis	+	Alpes de Haute-Provence/FR	CP038269
3	*Staphylococcus aureus* O11	ovine	abscess/gangrenous mastitis	+	Southeast of France/FR	CP0246491
4	*Staphylococcus aureus* strain O326	ovine	abscess/clinical mastitis	+	Alpes de Haute-Provence/FR	CP032481
5	*Staphylococcus aureus* strain O408	ovine	abscess/gangrenous mastitis	+	Alpes de Haute-Provence/FR	CP038270
6	*Staphylococcus aureus* O46	ovine	abscess/subclinical mastitis	+	Southeast of France/FR	CP0253951
7	*Staphylococcus aureus* strain O82	ovine	abscess/subclinical mastitis	+	Unspecified	CP038819
8	*Staphylococcus aureus* strain B119	ovine	abscess/clinical mastitis	+	Alpes de Haute-Provence/FR	CP038460
9	*Staphylococcus aureus* strain O17	ovine	abscess/clinical mastitis	+	Alpes Maritime/FR	CP032051
10	*Staphylococcus aureus* strain O267	ovine	abscess/subclinical mastitis	+	Pyrénées-Atlantiques/FR	CP034102
11	*Staphylococcus aureus* strain O268	ovine	abscess/clinical mastitis	+	Pyrénées-Atlantiques/FR	CP038612
12	*Staphylococcus aureus* subsp. aureus ED133	ovine	clinical mastitis	+	France	CP001996
13	*Staphylococcus aureus* strain O55	ovine	abscess/subclinical mastitis	+	Unspecified	CP038268
14	*Staphylococcus aureus* strain NCTC1803	ovine	mastitis	+	Unspecified	LR134305
15	*Staphylococcus aureus* strain NCTC9555	ovine	Unspecified	+	Unspecified	LR134090

16	*Staphylococcus aureus* RF122	bovine	Bulk milk/clinical mastitis	+	Ireland	AJ938182
17	*Staphylococcus aureus* 13	buffalo	Milkers' hands	+	São Paulo/BR	CP031265.1
18	*Staphylococcus caprae* 26D	buffalo	Milk/ healthy buffalo	-	São Paulo/BR	CP031271.1
19	*Staphylococcus chromogenes* 34B	buffalo	Milk/subclinical mastitis	+	São Paulo/BR	CP031470.1
20	*Staphylococcus chromogenes* 17A	buffalo	Milk/ healthy buffalo	-	São Paulo/BR	CP031274.1
21	*Staphylococcus chromogenes* 20B	buffalo	Milk/subclinical mastitis	-	São Paulo/BR	CP031471.1
	Strain	**Host**	**Origin**	**Coagulase positive or negative [1]**	**Isolation locality[2]**	**GenBank Accession**
22	*Staphylococcus hominis* 19A	buffalo	Milk/subclinical mastitis	+	São Paulo/BR	CP031277.1
23	*Staphylococcus pasteuri* 3C	buffalo	Milkers' hands	-	São Paulo/BR	CP031280.1
24	*Staphylococcus saprophyticus* 1A	buffalo	Milkers' hands	-	São Paulo/BR	CP031196.1
25	*Staphylococcus agnetis* 12B	buffalo	Milk/subclinical mastitis	-	São Paulo/BR	CP031266.1
26	*Staphylococcus warneri* 16A	buffalo	Milk/ healthy buffalo	-	São Paulo/BR	CP031269.1
27	*Staphylococcus xylosus* 2	buffalo	Milkers' hands	-	São Paulo/BR	CP031275.1
28	*Staphylococcus capitis* BN2	cat	Unspecified	-	Wachtberg/DE	CP042341.1

[1] + positive; -, negative. / [2] BR – Brazil; FR – France; DE – Germany

Table S2. *Staphylococcus* spp. strains used for species identification from the NCBI (dataset).

	Strain	Level	Assembly	Size(Mb)
1	*Staphylococcus capitis* XZ03	Complete	GCA_020883475.1	2.5
2	*Staphylococcus capitis* TW2795	Complete	GCA_002356175.1	2.4
3	*Staphylococcus capitis* FDAARGOS_753	Complete	GCA_013267675.1	2.4
4	*Staphylococcus capitis* BN2	Complete	GCA_015243435.1	2.5
5	*Staphylococcus capitis* FDAARGOS_378	Complete	GCA_002591175.1	2.4
6	*Staphylococcus capitis* AYP1020	Complete	GCA_001028645.1	2.5
7	*Staphylococcus aureus* RF122	Complete	AJ938182	
8	*Staphylococcus aureus* subsp. aureus ED133	Complete	CP001996	
9	VSF435 – 23S[1]	Draft		2.6
10	VSF436 – 42S[1]	Draft		2.6
11	VSF437 – 48S[1]	Draft		2.6
12	VSF438 – 56S[1]	Draft		2.6
13	VSF439 – 62S[1]	Draft		2.5
14	VSF440 – 73S[1]	Draft		2.6

Table S3. General plasmid features of strains analyzed.

Strain	Plasmid*	Size	Predicted mobility	Mash neighbor identification	Primary cluster_id	NCBI Accession Number
S. aureus 23S	1	3794	mobilizable	*S. cohnii*	AB633	
	2	3909	non-mobilizable	*S. aureus*	AE018	
S. aureus 42S	1	3096	non-mobilizable	*S. aureus*	AE018	
	2	1668	non-mobilizable	*S. aureus*	AE387	
S. aureus 48S	1	3096	non-mobilizable	*S. aureus*	AE018	
	2	1668	non-mobilizable	*S. aureus*	AE387	
S. aureus 56S	1	3096	non-mobilizable	*S. aureus*	AE018	
	2	1668	non-mobilizable	*S. aureus*	AE387	
S. capitis 62S	1	3794	mobilizable	*S. cohnii*	AB633	
	2	6342	mobilizable	*S. lugdunensis*	AB557	

	3	3658	mobilizable	*S. aureus*	novel_0ae65f4dd2816eaaee30cfc758487ae4
	4	6238	non-mobilizable	*S. epidermidis*	AB724
	5	2508	non-mobilizable	*S. capitis*	novel_abfcf46887c23682311779a6121532c8
	6	2844	non-mobilizable	*S. aureus*	AE018
S. capitis 73S	1	3794	mobilizable	*S. cohnii*	AB633
	2	6342	mobilizable	*S. lugdunensis*	AB557
	3	3658	mobilizable	*S. aureus*	novel_1d2f25a192a62f84de5959166700ae0c
	4	6238	non-mobilizable	*S. epidermidis*	AB724
	5	2508	non-mobilizable	*S. capitis*	novel_672ead7c1d615b08e7b8e0570da08b31
	6	2844	non-mobilizable	*S. aureus*	AE018

* Identification MOB-suit

Table S4. *Staphylococcus aureus* strains isolated from the milk of bovines with subclinical mastitis were used in the present study.

Strain*	Species*	Isolation Year	Locality isolation (Minas Gerais, Brazil)
23S	*S. aureus*	2004	Lavras
42S	*S. aureus*	2004	Itutinga
48S	*S. aureus*	2004	Ribeirão Vermelho
56S	*S. aureus*	2004	Ingaí
62S	*S. aureus*	2004	Ingaí
73S	*S. aureus*	2004	Bom sucesso

* Coagulase-negative *Staphylococcus aureus* isolates were identified using the Mass Spectrometry technique (MALDI-TOF).

Table S5. Virulence profile of *Staphylococcus* spp. strains isolated from the milk of bovines with subclinical mastitis were used in the present study.

Strain*	Virulence profile	
	Gene expression[1]	Phenotype
23S	*ica*AD (-), *hla (-)* and *hlb (-)*	biofilm
42S	*ica*AD (+), *hla (+)* and *hlb (-)*	biofilm and hemolysins
48S	*ica*AD (+), *hla (+)* and *hlb (+)*	biofilm and hemolysins
56S	*ica*AD (+), *hla (-)* and *hlb (-)*	biofilm
62S	*ica*AD (-), *hla (+)* and *hlb (+)*	biofilm and hemolysins
73S	*ica*AD (-), *hla (+)* and *hlb (+)*	biofilm and hemolysins

* Coagulase-negative *Staphylococcus aureus* isolates were identified using the Mass Spectrometry technique (MALDI-TOF).

[1] All absent expression in vitro for enterotoxins *sea,seb, sec, sed* and *see*; Genes: biofilm (*ica*AD), alpha (*hla*) and beta (*hlb*) hemolysins;

Table S6. Antimicrobial susceptibility of *Staphylococcus* spp. strains isolated from the milk of bovines with subclinical mastitis were used in the present study.

	Resistance profile[12]						
Strain*	**Antibiotics**	**Gentamicin**	**Tetracycline**	**Cetfiofur**	**Erythromycin**	**Ciprofloxacin**	**Trimethoprim-sulfamethoxazole**	**Novobiocin**
	Class	Aminoglycosides	Tetracycline	Cephems	Macrolides	Quinolones	FPA - Folate pathway antagonist	Others
	MIC range	0.25 to 64 µg/mL	0.5 to 128 µg/mL	0.025 to 32µg/mL	0.025 to 32 µg/mL	0.25 to 64 µg/mL	0.0625/1 to 16/30 µg/mL	1 to 256 µg/mL
23S		R	R	S	S	R	S	S
42S		S	S	R	S	S	S	S
48S		S	S	S	S	S	S	S
56S		R	S	S	R	S	S	S
62S		R	R	S	R	S	S	S
73S		R	S	R	S	S	S	S

***** Coagulase-negative *Staphylococcus aureus* isolates were identified using the Mass Spectrometry technique (MALDI-TOF).

[1] All are susceptible to Sulfa-Trimethoprim and novobiocin;

[2] Resistance (R) Susceptible (S).

Figure S1: Visualization of core virulome genes the 34 strains of *S. aureus* according to the VFDB prediction. Heatmap with presence (blue box) and absence associated with virulence factors that *S. aureus* correlates to strains. Different colors next to each strain name refer to the cut-off of the dendogram being 21 groups.

Figure S2: Visualization of core resistoma genes the 34 strains of *S. aureus* according to the CARD prediction. Heatmap with presence (blue box) and absence associated with resistance gene that *S. aureus* correlates to strains. Different colors next to each strain name refer to the cut-off of the dendogram being 8 groups.

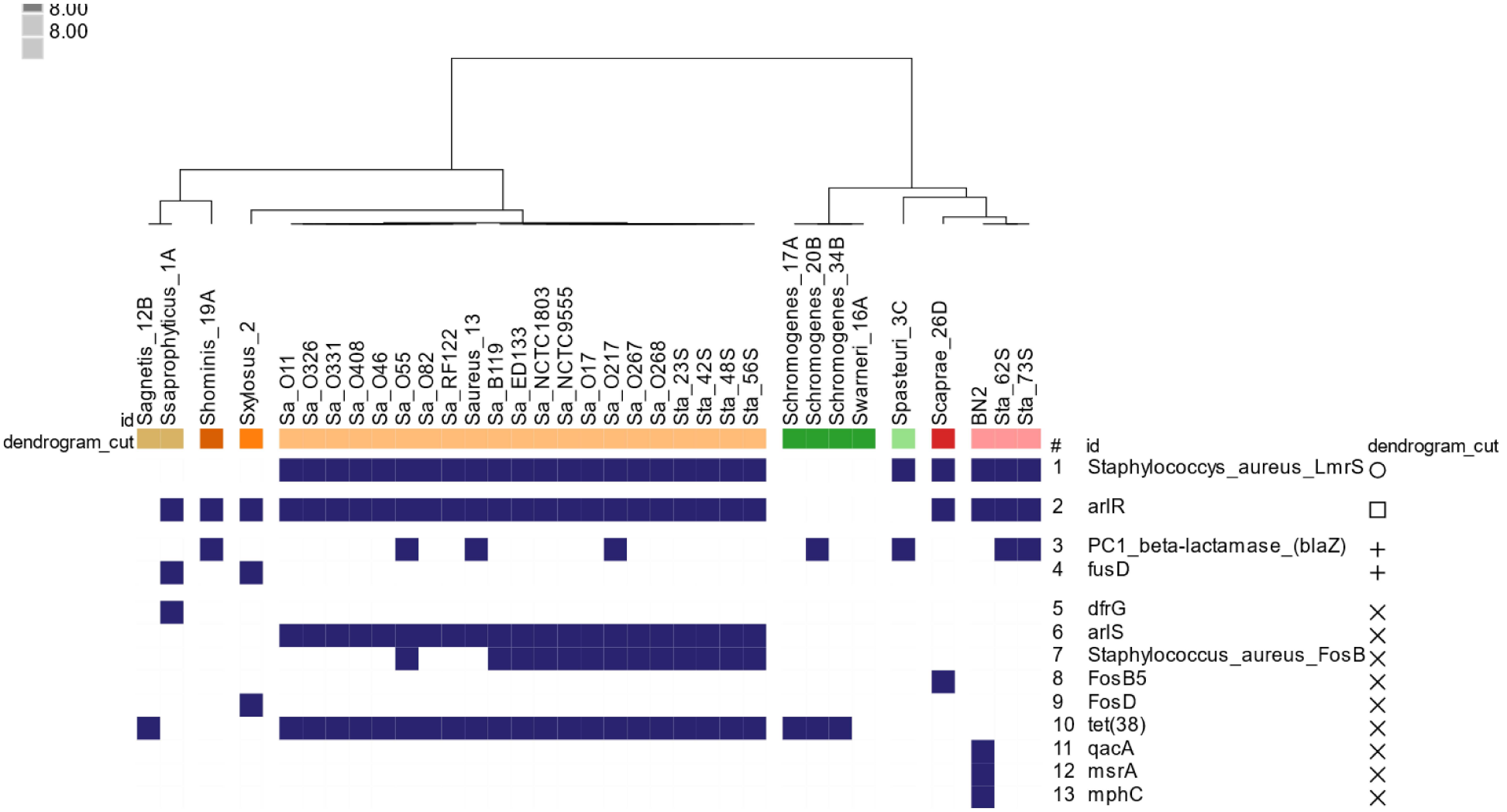

Figure S3: Multiple sequence alignment of 21 amino acid coa sequences of *Staphylococcus aureus* using Jalview software.

Figure S4: Multiple sequence alignment of 21 amino acid vWbp sequences of *Staphylococcus aureus* using Jalview software.

6. Conclusões

O presente trabalho buscou explorar a nível genômico a caracterização de genes e particularidades do comportamento entre nichos de hospedeiro e infecção. Utilizando como modelo de organismo *S. aureus* isolado de ruminantes diagnosticado com mastite. A primeira parte analisou o repertório e comportamento gênico de genomas isolados de mastites em ovinos oriundos de diferentes fazendas da França. Foram preditos dois grandes agrupamentos baseados em tipagem molecular (CC130 e CC133), identificados especificamente em ovinos, além disso, observou-se relação entre proximidade filogenômica e complexo clonal. Profagos, ilhas de patogenicidade e ilhas genômicas foram preditas e carregam importantes fatores de virulência bacteriana. No geral, o comportamento gênico a nível de virulência para *S. aureus* foram bem similares independente do hospedeiro. Todas as linhagens apresentaram genes relacionados a resistência a antimicrobianos, no entanto, a nível de resistência a antibióticos foram encontrados alguns representantes altamente resistentes preditos *in silico*, como observado nos genomas isolados de suínos. Quanto a invasão e permanência da bactéria na célula, apresentou um vasto repertório de genes associados a virulência. O cromossomo cassete mec não foi detectado em nenhuma das linhagens isoladas de ovinos. Porém mostrou genes associados a resistência em genomas específicos, podendo estar associado ao reservatório de genes de resistência para outros hospedeiros, o que demonstra novas evidências de especialização genômica em *S. aureus* associado à mastite ovina. A segunda parte do trabalho envolveu isolados de mastite em bovinos oriundos de diferentes fazendas e regiões do sudeste do Brasil, classificados como coagulase-negativa e identificados pelo espectro de massa MALDI-TOF MS como *S. aureus*. Por meio do sequenciamento genômico e análises comparativas, apenas 4 isolados foram confirmadas genomicamente pertencerem a *S. aureus* e 2 sendo caracterizados como *S. capitis*. Através da genômica comparativa foi possível correlacionar o comportamento atípico e a epidemiologia genômica. Para os isolados referentes a *S. aureus*, todas possuem as proteínas staphylocoagulases, a coagulase (Coa) foi predita apenas em *S. aureus* não apresentando diferenças significativas que distinguisse as *S. aureus* positiva e negativa. Já a proteína *von Willebrand factor-binding protein* (vWbp) foi predita em isolados de outros *Staphylococcus* (*S. aureus*, *S. agnetis* e *S. chromogenes*), independentemente de serem coagulase positiva ou negativa. Relacionando filogeneticamente essas proteínas apresentam *in silico* 3 pontos de ramificações importantes, a *vWbp* apresenta dois clados divergindo entre amostras de *S. aureus* e outros estafilococos (*S. agnetis* e *S. chromogenes*) e um clado referente ao gene *coa*. No

alinhamento de *vWbp* é possível observar nas proteínas relacionadas aos genomas de *S. aureus* coagulase-negativa uma fragmentação. Ambos os trabalhos buscaram explorar a nível genômico a caracterização de genes e particularidades do comportamento entre nichos de hospedeiro e infecção. Comparando o conteúdo gênico com a epidemiologia, contribuindo com possíveis novas estratégias de identificação de espécie e caracterização de *S. aureus* que acometem ovinos e se comportam como coagulase-negativa. Mais estudos são necessários para elucidar esse comportamento, pouco se sabe sobre características genômicas de linhagens isoladas de mastite em animais de pequeno porte como os ovinos.

7. Perspectivas

Com os genomas sequenciados e montado e depositados, descritos no Quadro 1, que não entraram no atual trabalho (nº 13 ao 111), pretendemos:´

Com os genomas isolados de bovinos no Brasil (Quadro 1):

- Realizar uma análise comparativa entre todos os genomas;
- Analisar o perfil de resistência e virulência nas amostras brasileiras, utilizando ferramentas de bioinformática;
- Gerar um panorama exploratório sobre os profagos preditos nas sequencias estudas.
- Fazer a predição de regiões relacionadas aos profagos nas amostras brasileiras, a fim de identificar assinaturas entre eles;
- Gerar uma anotação estrutural nas regiões referentes aos profagos, a fim de identificar genes associados a virulência e resistência.
- Amplificar as regiões relacionadas aos genes coagulase e a proteína vwbp (*von Willebrand factor-binding protein*) a fim de confirmar possíveis duplicações e especificidades encontradas *in silico*.

Com os genomas de ovinos (nº1 ao 12):

- Analisar perfil de resistência;
- Realizar a anotação funcional das proteínas hipotéticas encontradas exclusivamente em genomas isolados de ovinos, a fim de elucidar os possíveis papeis dessas proteínas na infecção e associação ao hospedeiro;
- Avaliar a presença de genes que podem contribuir para o tropismo em *S. aureus* isolados em ovinos; compreender se hospedeiros como os ovinos, estão agindo como um reservatório para o desenvolvimento e transferência de determinantes de resistência antimicrobiana. Buscando identificar a utilização de antibióticos na agropecuária e sua correlação com a saúde humana.

QUADRO 01: Linhagens que foram sequenciadas pela plataforma de sequenciamento Illumina Hiseqe, montadas, anotadas e depositadas até o momento pelo grupo LGCM em parceira com o INRAE e UFLA.

Nº	Espécie	Amostra	Hospedeiro	Local	Origem	Montador	Identificador depósito
1	*S. aureus*	O268	Ovino	Mastite	Pyrénées-Atlantiques – França	SPAdes v. 3.9.1	CP038612
2	*S. aureus*	O17	Ovino	Mastite	Alpes Maritime – França	SPAdes v. 3.9.1	CP032051

continua

Nº	Espécie	Amostra	Hospedeiro	Local	Origem	Montador	Identificador depósito
3	*S. aureus*	O322	Ovino	Mastite	Alpes de Haute-Provence – França	SPAdes v. 3.9.1	CP038460
4	*S. aureus*	O326	Ovino	Mastite	Alpes de Haute-Provence – França	SPAdes v. 3.9.1	CP032481
5	*S. aureus*	O217	Ovino	Mastite	Aveyron – França	SPAdes v. 3.9.1	CP038461
6	*S. aureus*	O11	Ovino	Mastite	Unespecified – França	SPAdes v. 3.9.1	CP0246491
7	*S. aureus*	O408	Ovino	Mastite	Alpes de Haute-Provence – França	SPAdes v. 3.9.1	CP038270
8	*S. aureus*	O46	Ovino	Mastite	Desconhecido – França	SPAdes v. 3.9.1	CP0253951
9	*S. aureus*	O82	Ovino	Mastite	Unespecified – França	SPAdes v. 3.9.1	CP038819
10	*S. aureus*	O267	Ovino	Mastite	Pyrénées-Atlantiques – França	SPAdes v. 3.9.1	CP034102
11	*S. aureus*	O331	Ovino	Mastite	Alpes de Haute-Provence – França	SPAdes v. 3.9.1	CP038269
12	*S. aureus*	O55	Ovino	Mastite	Unespecified – França	SPAdes v. 3.9.1	CP038268
13	*S. aureus*	Sta_1	Bovino	Mastite	Coronel Pacheco – MG / Brasil	Unicycler v.0.4.5	
14	*S. aureus*	Sta_2	Bovino	Mastite	Juiz de Fora – MG / Brasil	Unicycler v.0.4.5	
15	*S. aureus*	Sta_14	Bovino	Mastite	Além Paraíba – MG / Brasil	Unicycler v.0.4.5	
16	*S. aureus*	Sta_20	Bovino	Mastite	Santos Dumont – MG / Brasil	Unicycler v.0.4.5	
17	*S. aureus*	Sta_28	Bovino	Mastite	Mar de Espanha – MG / Brasil	Unicycler v.0.4.5	
18	*S. aureus*	Sta_29	Bovino	Mastite	Mar de Espanha – MG / Brasil	Unicycler v.0.4.5	

continua

Nº	Espécie	Amostra	Hospedeiro	Local	Origem	Montador	Identificador depósito
9	*S. aureus*	Sta_31	Bovino	Mastite	Paty do Alferes – RJ / Brasil	Unicycler v.0.4.5	
20	*S. aureus*	Sta_48	Bovino	Mastite	Coronel Pacheco – MG / Brasil	Unicycler v.0.4.5	
21	*S. aureus*	Sta_60	Bovino	Mastite	Coronel Pacheco – MG / Brasil	Unicycler v.0.4.5	
22	*S. aureus*	Sta_65	Bovino	Mastite	Barra Mansa – RJ / Brasil	Unicycler v.0.4.5	
23	*S. aureus*	Sta_68	Bovino	Mastite	Matozinhos – MG / Brasil	Unicycler v.0.4.5	
24	*S. aureus*	Sta_70	Bovino	Mastite	Juiz de Fora – MG / Brasil	Unicycler v.0.4.5	
25	*S. aureus*	Sta_75	Bovino	Mastite	Rio Novo – MG / Brasil	Unicycler v.0.4.5	
26	*S. aureus*	Sta_78	Bovino	Mastite	Matozinhos – MG / Brasil	Unicycler v.0.4.5	
27	*S. aureus*	Sta_85	Bovino	Mastite	Barra Mansa – RJ / Brasil	Unicycler v.0.4.5	
28	*S. aureus*	Sta_88	Bovino	Mastite	Coronel Pacheco – MG / Brasil	Unicycler v.0.4.5	
29	*S. aureus*	Sta_94	Bovino	Mastite	Três Rios – RJ / Brasil	Unicycler v.0.4.5	
30	*S. aureus*	Sta_95	Bovino	Mastite	Três Rios – RJ / Brasil	Unicycler v.0.4.5	
31	*S. aureus*	Sta_106	Bovino	Mastite	Vassouras – RJ / Brasil	Unicycler v.0.4.5	
32	*S. aureus*	Sta_114	Bovino	Mastite	Bem Posta – RJ / Brasil	Unicycler v.0.4.5	
33	*S. aureus*	Sta_137	Bovino	Mastite	Juiz de Fora – MG / Brasil	Unicycler v.0.4.5	
34	*S. aureus*	Sta_138	Bovino	Mastite	Matias Barbosa – MG / Brasil	Unicycler v.0.4.5	
35	*S. aureus*	Sta_139	Bovino	Mastite	Matias Barbosa – MG / Brasil	Unicycler v.0.4.5	

continua

Nº	Espécie	Amostra	Hospedeiro	Local	Origem	Montador	Identificador depósito
36	*S. aureus*	Sta_140	Bovino	Mastite	Matias Barbosa – MG / Brasil	Unicycler v.0.4.5	
37	*S. aureus*	Sta_23S	Bovino	Mastite	Lavras – MG / Brasil	Unicycler v.0.4.5	
38	*S. aureus*	Sta_42S	Bovino	Mastite	Itutinga – MG / Brasil	Unicycler v.0.4.5	
39	*S. aureus*	Sta_48S	Bovino	Mastite	Ribeirão Vermelho – MG / Brasil	Unicycler v.0.4.5	
40	*S. aureus*	Sta_56S	Bovino	Mastite	Ingaí – MG / Brasil	Unicycler v.0.4.5	
41	*S. capitis*	Sta_62S	Bovino	Mastite	Ingaí – MG / Brasil	Unicycler v.0.4.5	
42	*S. capitis*	Sta_73S	Bovino	Mastite	Bom sucesso – MG / Brasil	Unicycler v.0.4.5	
43	*Staphylococcus* spp.	Sta_153	Bovino	Mastite	Matozinhos – MG / Brasil		
44	*Staphylococcus* spp	Sta_154	Bovino	Mastite	Matozinhos – MG / Brasil		
45	*Staphylococcus* spp.	Sta_157	Bovino	Mastite	Belmiro Braga – MG / Brasil		
46	*Staphylococcus* spp.	Sta_159	Bovino	Mastite	Belmiro Braga – MG / Brasil		
47	*Staphylococcus* spp.	Sta_162	Bovino	Mastite	Rio das Flores – RJ / Brasil		
48	*Staphylococcus* spp.	Sta_163	Bovino	Mastite	Rio das Flores – RJ / Brasil		
49	*Staphylococcus* spp.	Sta_167	Bovino	Mastite	Soledade de Minas – MG / Brasil		
50	*Staphylococcus* spp.	Sta_170	Bovino	Mastite	Matozinhos – MG / Brasil		
51	*Staphylococcus* spp.	Sta_184	Bovino	Mastite	Pirapetinga – MG / Brasil		
52	*Staphylococcus* spp.	Sta_185	Bovino	Mastite	Pirapetinga – MG / Brasil		

continua

Nº	Espécie	Amostra	Hospedeiro	Local	Origem	Montador	Identificador depósito
53	*Staphylococcus* spp.	Sta_194	Bovino	Mastite	Coronel Pacheco – MG / Brasil		
54	*Staphylococcus* spp.	Sta_198	Bovino	Mastite	Campos dos Goitacazes – RJ / Brasil		
55	*Staphylococcus* spp.	Sta_202	Bovino	Mastite	Pedregulho – SP / Brasil		
56	*Staphylococcus* spp.	Sta_204	Bovino	Mastite	Pedregulho – SP / Brasil		
57	*Staphylococcus* spp.	Sta_213	Bovino	Mastite	Volta Grande – MG / Brasil		
58	*Staphylococcus* spp.	Sta_226	Bovino	Mastite	Itanhandu – MG / Brasil		
59	*Staphylococcus* spp.	Sta_228	Bovino	Mastite	Tebas – MG / Brasil		
60	*Staphylococcus* spp.	Sta_240	Bovino	Mastite	Vassouras – RJ / Brasil		
61	*Staphylococcus* spp.	Sta_241	Bovino	Mastite	Vassouras – RJ / Brasil		
62	*Staphylococcus* spp.	Sta_243	Bovino	Mastite	Eng Paulo de Frontin		
63	*Staphylococcus* spp.	Sta_257	Bovino	Mastite	Funilândia – MG / Brasil		
64	*Staphylococcus* spp.	Sta_256	Bovino	Mastite	Funilândia – MG / Brasil		
65	*Staphylococcus* spp.	Sta_274	Bovino	Mastite	Funilândia – MG / Brasil		
66	*Staphylococcus* spp.	Sta_275	Bovino	Mastite	Inhaúma – MG / Brasil		
67	*Staphylococcus* spp.	Sta_276	Bovino	Mastite	Inhaúma – MG / Brasil		
68	*Staphylococcus* spp.	Sta_280	Bovino	Mastite	Vassoiras – MG / Brasil		
69	*Staphylococcus* spp.	Sta_283	Bovino	Mastite	Itaperuna– RJ / Brasil		
70	*Staphylococcus* spp.	Sta_289	Bovino	Mastite	Matozinhos – MG / Brasil		

continua

Nº	Espécie	Amostra	Hospedeiro	Local	Origem	Montador	Identificador depósito
71	*Staphylococcus* spp.	Sta_290	Bovino	Mastite	Conservatória– RJ / Brasil		
73	*Staphylococcus* spp.	Sta_300	Bovino	Mastite	Rio das Flores– RJ / Brasil		
74	*Staphylococcus* spp.	Sta_301	Bovino	Mastite	Santos Dumont – MG / Brasil		
75	*Staphylococcus* spp.	Sta_315	Bovino	Mastite	Ouro Branco – MG / Brasil		
76	*Staphylococcus* spp.	Sta_317	Bovino	Mastite	Rio Casca – MG / Brasil		
77	*Staphylococcus* spp.	Sta_320	Bovino	Mastite	Alvinópolis – MG / Brasil		
78	*Staphylococcus* spp.	Sta_323	Bovino	Mastite	Rio Piracicaba – MG / Brasil		
79	*Staphylococcus* spp.	Sta_334	Bovino	Mastite	Juiz de Fora – MG / Brasil		
80	*Staphylococcus* spp.	Sta_328	Bovino	Mastite	Juiz de Fora – MG / Brasil		
81	*Staphylococcus* spp.	Sta_330	Bovino	Mastite	Uberlândia – MG / Brasil		
82	*Staphylococcus* spp.	Sta_340	Bovino	Mastite	Pirassununga – SP / Brasil		
83	*Staphylococcus* spp.	Sta_346	Bovino	Mastite	Pirassununga – SP / Brasil		
84	*Staphylococcus* spp.	Sta_347	Bovino	Mastite	Pirassununga – SP / Brasil		
85	*Staphylococcus* spp.	Sta_352	Bovino	Mastite	Cruzília – SP / Brasil		
86	*Staphylococcus* spp.	Sta_355	Bovino	Mastite	Pará de Minas– MG / Brasil		
87	*Staphylococcus* spp.	Sta_359	Bovino	Mastite	Bom Despacho– MG / Brasil		
88	*Staphylococcus* spp.	Sta_360	Bovino	Mastite	Bom Despacho– MG / Brasil		
89	*Staphylococcus* spp.	Sta_365	Bovino	Mastite	Santa Cruz da Conceição – SP / Brasil		

continua

Nº	Espécie	Amostra	Hospedeiro	Local	Origem	Montador	Identificador depósito
90	*Staphylococcus* spp.	Sta_372	Bovino	Mastite	Santa Cruz da Conceição – SP / Brasil		
91	*Staphylococcus* spp.	Sta_381	Bovino	Mastite	São Carlos – SP / Brasil		
92	*Staphylococcus* spp.	Sta_386	Bovino	Mastite	Lima Duarte– MG / Brasil		
93	*Staphylococcus* spp.	Sta_405	Bovino	Mastite	Inhaumas– MG / Brasil		
94	*Staphylococcus* spp.	Sta_411	Bovino	Mastite	Bom Jesus – GO / Brasil		
95	*Staphylococcus* spp.	Sta_412	Bovino	Mastite	Bom Jesus – GO / Brasil		
96	*Staphylococcus* spp.	Sta_414	Bovino	Mastite	Inhaumas– MG / Brasil		
97	*Staphylococcus* spp.	Sta_421	Bovino	Mastite	Inhaumas– MG / Brasil		
98	*Staphylococcus* spp.	Sta_423	Bovino	Mastite	Morrinhos – GO / Brasil		
99	*Staphylococcus* spp.	Sta_431	Bovino	Mastite	Inhaumas– MG / Brasil		
100	*Staphylococcus* spp.	Sta_1B	Bovino	Amostras Clínicas	Desconhecido		
101	*Staphylococcus* spp.	Sta_2B	Bovino	Amostras Clínicas	Desconhecido		
102	*Staphylococcus* spp.	Sta_9B	Bovino	Amostras Clínicas	Desconhecido		
103	*Staphylococcus* spp.	Sta_42GO	Bovino	Amostras Clínicas	Desconhecido		
104	*Staphylococcus* spp.	Sta_1MS	Bovino	Amostras Clínicas	Desconhecido		
105	*Staphylococcus* spp.	Sta_3MS	Bovino	Amostras Clínicas	Desconhecido		
106	*Staphylococcus* spp.	Sta_4MS	Bovino	Amostras Clínicas	Desconhecido		
107	*Staphylococcus* spp.	Sta_5MS	Bovino	Amostras Clínicas	Desconhecido		

continua

Nº	Espécie	Amostra	Hospedeiro	Local	Origem	Montador	Identificador depósito
108	*Staphylococcus* spp.	Sta_6MS	Bovino	Amostras Clínicas	Desconhecido		
109	*Staphylococcus* spp.	Sta_8MS	Bovino	Amostras Clínicas	Desconhecido		
110	*Staphylococcus* spp.	Sta_9MS	Bovino	Amostras Clínicas	Desconhecido		
111	*Staphylococcus* spp.	Sta_10MS	Bovino	Amostras Clínicas	Desconhecido		

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