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Review Article

Staphylococcus aureus Strains Isolated from Cattle Livestock and Possible Use of Anti-Virulence Strategies

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Abstract

Staphylococcus aureus is known for its ability to acquire resistance to commonly used antibiotics and causes mastitis in cows. Bacterial strains belonging to this species can spread in humans and animals, through diffusion in different contexts, including workplaces and the environment. The antibiotic-resistance in *S. aureus* strains evidenced the need for novel therapeutic approaches that do not exert selective pressure on the evolutionary adaptation of the bacteria. Alternative approaches can be represented by anti-virulence therapies that interfere with virulence factors, or relative pathways that regulate the production of toxins. Various *S. aureus* toxins and regulatory systems involved in secreting these toxins can be investigated. The potential of targeting *S. aureus* toxins and virulence-mediated pathways as anti-virulence strategies can be a substantial and important alternative, in contrast to traditional antibiotics directed at pathogen viability but triggering the mechanisms of antibiotic-resistance. Thus, the antivirulence approach must be aimed to reduce the production of virulence factors without affecting bacterial growth. Strategies to reduce bacterial virulence include compounds able to inhibit quorum sensing, disassemble bacterial membranes, disrupt biofilm formation, or neutralize the bacterial toxin, thus reducing the spread of the infection. Virulence factors eventually related to the infectiousness of *S. aureus* strains can offer new insights into vaccine development and possible identification of new vaccine targets. The benefits of the antivirulence-antibiotic combination during the treatment against *S. aureus* infections have been enhanced by virtue of the synergistic action between antibiotics and the antivirulence compounds. The characteristics of the spread of antibiotic resistant *S. aureus* and its virulence characteristics for anti-virulence strategies have been described.

Introduction

Staphylococcus aureus is an important opportunistic pathogen that can cause several diseases both in humans and animals, including mastitis in cows and food poisoning with the production of heat-stable enterotoxins [1]. *Staphylococcus aureus* is a Gram-positive bacterium present in multiple body sites, with nares as the most frequent colonized sites [2]. Considering healthy humans, about 20% are persistent carriers of *S. aureus* , about 30% are intermittent carriers, and about 50% are not colonized by *S. aureus* . In humans, *S. aureus* represents the most important cause of nosocomial infections, with clinical consequences comprehending skin infections to most important infections [3,4]. In animals, *S. aureus* is one of the three major pathogenic *Staphylococcus* species, together with *S. hyicus* and the *Staphylococcus intermedius* group – SIG [5]. The SIG group is subdivided into SIG members [6] comprising of four closely related but distinct coagulase-positive species: *S. intermedius* [7], *S. pseudintermedius* [8], *S. delphini* [9], and a human-originated *S. cornubiensis* [10].

Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Around 1945, penicillin was introduced into medicine and soon after most of the *Staphylococcus aureus* population became resistant to penicillin through the production of β-lactamase, an enzyme that hydrolyzes penicillin. In 1960, methicillin was introduced into clinical use for the treatment of infections caused by penicillin-resistant *Staphylococcus aureus* , as the first semi-synthetic penicillin derivative evidencing resistance to hydrolysis by staphylococcal β-lactamase [11]. However, the first isolate of methicillin-resistant *S. aureus* (MRSA) was reported within a year [12]. Methicillin is no longer in clinical use, however, the acronym MRSA has continued to be used. The MRSA strain was disseminated and subsequently declined during the late 1970s [13]. Throughout the 1990s, there was a marked resurgence in the prevalence of MRSA, often reflecting the emergence of epidemic strains (so-called EMRSA) that spread both within and between hospitals. Isolates of EMRSA-15 and -16 are commonly resistant to erythromycin and ciprofloxacin in addition to β-lactams, and a study at one affected hospital showed a temporal relationship between the rates of MRSA infection and the use of macrolides, third-generation cephalosporins and fluoroquinolones, suggesting that the use of antimicrobials to which an outbreak strain is resistant is an important contributory factor for the persistence of that strain [14,15]. Methicillin resistance is determined by the acquisition of the mecA gene, which encodes an alternative penicillin-binding protein, called PBP2A, which has a low affinity for β-lactam antibiotics. The mecA gene belongs to a large mobile genetic element, the staphylococcal cassette chromosome mec (SCCmec). The latter can be integrated at a specific site in the chromosome of methicillin-susceptible *S. aureus* (MSSA). SCCmec carries a set of cassette chromosome recombinase genes (ccrA, ccrB or ccrC) for excision and integration into the host chromosome. The various SCCmec elements differ from each other in the antibiotic resistance markers to antimicrobials other than β-lactams [5].

Since 2004, MRSA has emerged in animals, and MRSA from this reservoir has been referred to as Livestock Associated-MRSA (LA-MRSA). LA-MRSA can be transmitted to humans in close contact with LA-MRSA colonized animals. LA-MRSA is mainly present in pigs and calves, however other animals can also be carriers. Moreover, workers in direct contact with LA-MRSA-positive animals, have an increased risk for LA-MRSA carriage (Figure 1). However, the positive association between the MRSA carrier status of family members and the MRSA carriage of the farmer indicates that human-to-human transmission cannot be excluded. Farm hygiene, including cleaning and disinfection of stables between production cycles, seems to be associated with a lower prevalence of MRSA. Antimicrobial use contributes to presence and diffusion of MRSA

in animals. Like any other microorganism, LA-MRSA is expected to be able to adapt to new hosts and may change over time in the potential to colonize and to produce toxins [5]. A novel pig-associated strain of MRSA was identified in the early part of the 21st century with sequence type 398 (ST398). The CC398 strain was first identified in pigs and pig farmers, but has since been found in other animals, including cattle, poultry and dogs, as well as humans, in several countries in Europe, Asia, North and South America and Australia. The discovery of this strain led to the addition of livestock-associated MRSA (LA-MRSA) to the lexicon to complement hospital-associated (HA) and community-associated (CA) strains. CC398 remains the most commonly identified type of LA-MRSA in most European countries. Studies conducted in Asia showed that a different strain of MRSA, ST9, appears to be the prominent type of LA-MRSA. CC398 seems to be frequently shared between animals and humans and is capable of causing active symptomatic infections in both species (Figure 1) [16]. Bidirectional transmission of strains of *S. aureus* between humans and livestock is not rare (Figure 1). Further information on the movement of CC398 between animals and humans, have suggested that a human pandemic clone, named CC97, originated from cattle. Moreover, it was suggested that antibiotic resistance genes, including *mecA* and *mecC* have an animal origin, thus reinforcing the animal role in development of these bacteria [16].

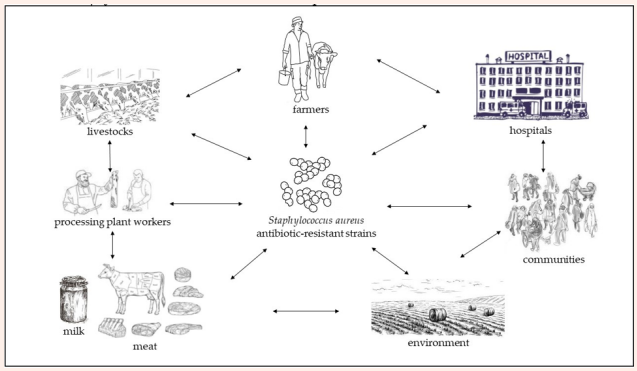

Figure 1: Antibiotic-resistant *Staphylococcus aureus* can spread in livestock operations and hospitals where antibiotics are regularly used. These antibiotic-resistant organisms can then spread into communities and the environment.

In Europe and Northern America, LA-MRSA belongs predominantly to clonal complex CC398 whereas in Asia ST9 seems to be dominant in pigs. In contrast with its success in animals, it seemed that MRSA CC398 is a poor persistent colonizer in humans. MRSA ST398 can, however, cause serious and invasive infections [5]. The epidemiology of CC398 and other livestock-associated *S. aureus* in the US appears to be notably different than in European countries. Studies on farms and of meat-identified CC398 strains in animals, farm workers, and meat products, contemporaneous studies also documented CC398 in populations with no obvious livestock contact. In one Texas study carried out in a jail setting rather than on a farm, CC398 isolates made up a significant portion of all methicillin-susceptible *S. aureus* (MSSA) identified within this population. While CC398 can have livestock as well as human versions, other human strains of *S. aureus* have also been found in US livestock. Studies carried out on swine farms in the US have identified human strains within the noses of live animals or as components of environmental samples of farm dust [16].

Distribution of *Staphylococcus aureus* and Infections

The distribution of *S. aureus* strains and infections can show seasonal variations [1]. *S. aureus* surveillance is most commonly carried out within a human clinical or hospital setting, likely, other spillover events of *S. aureus* from livestock to humans or vice versa are involved. The relationship between public health and the agricultural and food industry has importance [16]. It was reported that as much as 80% of total antibiotic production in the United States is used in agriculture, with a substantial portion of this used for the nontherapeutic purpose of growth promotion. Antibiotic-resistant bacteria have been found in farm animals where antibiotics are heavily used in associated food products, environments contaminated by animal waste, and farm workers (Figure 1). Furthermore, antibiotics used therapeutically in animals may also generate a reservoir of antibiotic-resistant bacteria. Antibiotic-resistant bacteria in food animals threaten the efficacy of human drugs if antibiotic-resistant bacteria or related genes become incorporated into bacteria populations colonizing humans [17]. Human-to-human transmission of zoonotic pathogens is rare, although it may occur in settings where humans are immuno-compromised or where the gut community has been disturbed by heavy medical antibiotic use. Therefore, the incidence of antibiotic-resistance in zoonotic infections of humans is directly related to the prevalence of antibiotic-resistance bacteria in food animals [17].

Studies of individuals close to concentrated animal feeding operations (Figure 1) support that non livestock strains may spread within areas proximal to farms. Two independent studies carried out in Iowa and Pennsylvania that examined the relationship between animal farms and MRSA found an increased risk of MRSA colonization or infection in those living close to farms or in areas where manure was spread on fields. Thus, either strain other than livestock isolates are evolving on farms, or it may be the presence of antibiotic resistance genes and antibiotic residues on farms causing a shift toward antibiotic-resistant strains in these populations, or perhaps a combination of both mechanisms [16]. Figure 1 reports possible different contexts involved in *S. aureus* antibiotic-resistant spread.

Virulence Factors in *Staphylococcus aureus*

Bacterial strains of the species *S. aureus* include commensal pathogens with a wide variety of infections that can be caused, from superficial skin and soft tissue infections to life-threatening septicemia. *S. aureus* infections are serious public health problems in hospital and community settings, as well as animal welfare and economic challenges. In dairy cows, *S. aureus* causes mastitis, a major problem in the dairy industry, affecting animal health and causing economic losses. Antibiotic treatment represents a solution, albeit an unfavorable one due to high costs and the risk of development of antibiotic resistance and is not suitable for addressing the problem of long-term persistence of pathogenic *S. aureus* in breast tissue [18]. Strains of the species *S. aureus* can invade host cells and persist intracellularly for various periods. Intracellular persistence would provide *S. aureus* with an ideal strategy to escape from professional phagocytes and extracellular antibiotics and promote recrudescent infection [19]. *Staphylococcus aureus* is recognized worldwide as one of the major agents of contagious bovine mastitis and is a frequent reason for the therapeutic and prophylactic use of antibiotics on dairy farms. It causes subclinical infections resulting in increased somatic cell count and reduced milk production but can also cause clinical mastitis. Subclinical mastitis caused by *S. aureus* is a major concern for dairy producers, affecting animal health and causing economic losses due to its negative impact on milk yield and quality. Subclinical mastitis caused by *S. aureus* tends to become chronic and can be difficult to cure by conventional antimicrobial therapies [20], due to the establishment of deep-seated pockets of infection in the milk-secreting cells (alveoli) followed by abscess formation, intracellular survival within neutrophils and biofilm formation [21].

Successful establishment of infection depends in part on virulence factors produced by *S. aureus* [22]. Depending on the stimuli from the infection site, *S. aureus* may activate or suppress the expression of its multiple virulence factors, and this produces different phenotypes from the same bacterial strain. Many of the virulence genes encode toxins that are harmful to humans and can cause severe gastrointestinal illness. *Staphylococcus aureus* is considered the third most important cause of disease in the world among the reported foodborne illnesses [23]. Growth of *S. aureus* in foods leads to the production of staphylococcal enterotoxins (SEs) and results in food poisoning when these foods are consumed. Contaminated milk and milk products have frequently been implicated in staphylococcal food poisonings (Table 1) [21].

Strains of *S. aureus* survive host immune responses and are etiologic agents of several diseases, due to their high versatility and ability to activate their virulence determinants. The virulence characteristics of *S. aureus* infections depend on several factors: the production of surface proteins that mediate bacterial adhesion to host tissues; the secretion of a variety of extracellular toxins and enzymes that destroy host cells and tissues; the ability to nullify the host's immune defense response; and the ability of these bacteria to grow and spread in host cells [24]. These strains produce toxins, virulence factors consisting of proteins secreted into their extracellular matrix during the post-exponential and initial stationary phases. These proteins play a role in tissue penetration and enable bacterial cells to invade hosts. Furthermore, these proteins are also cytolytic and promote bacterial growth, for example by complexing some essential nutrients, such as iron, starting from lysed cells. Toxins secreted by *S. aureus* strains include hemolysin, leukotoxin, exfoliative toxin, enterotoxin, and toxic shock syndrome toxin-1 (TSST-1). Virulence factors of bacterial strains of *S. aureus* also include surface proteins and enzymes. The secretion of enzymes, such as coagulase, protease, and staphylokinase, aids bacterial evasion of host defenses, as well as invasion and penetration of host tissues. Most of these enzymes function

by degrading host molecules or by interfering with signaling cascades and metabolic pathways in the host [25,26]. Surface proteins of *S. aureus* , including aggregation factors, fibronectin proteins, protein A, and collagen adhesion, may also play a role in bacterial adhesion, tissue invasion, and cell evasion host defense [27].

Hemolysins are toxins that lyse red blood cells and include many classes such as α, β and γ-hemolysins. δ-Hemolysin has been classified as a phenol-soluble form that does not require a receptor for its hemolytic activity. α-Hemolysin is the most studied member of the hemolysins produced by *S. aureus* . This toxin lyses red blood cells and leukocytes, not neutrophils, and their action is usually receptor-mediated. The toxin activity disrupts cellular homeostasis by inducing the formation of pores on cell membranes and causing Ca^{2+} influx and K^{+} efflux, thus leading to cell death [28]. β-hemolysin are non-pore-forming toxins that have been characterized as sphingomyelinases. The toxin β-hemolysin hydrolyses sphingomyelin and lyses monocytes. It is capable to only lyse erythrocytes at low temperatures and is not cytolytic to lymphocytes and granulocytes [29]. γ-hemolysin is hemolytic to rabbit erythrocytes, and its membrane-damaging activity is also evident in leukocytes, such as neutrophils, monocytes, granulocytes, and macrophages [30], and this group of hemolysins are bi-component and made up of polypeptides designated as S and F [31]. The toxin δ-hemolysin is a phenol-soluble modulin that are hemolytic to erythrocytes, various organelles, bacterial protoplasts, and spheroplasts [32]. The toxins are small and amphipathic with a high affinity to lipids [30]. Leukotoxins represent a group of toxins able to lyse white blood cells and most likely require receptors for their mode of action, although the receptors have remained mostly uncharacterized until recently. They come from the two-component Luk toxin family and consist of Panton-Valentine leukocidin (PVL) (LukS and LukF proteins), LukDE, and LukAB (also known as LukGH), with PVL reported to be 100 times more potent than the others [33]. Staphylococcal exfoliative toxins (ETs) are serine proteases and the causative agent of Staphylococcal Scalded Skin Syndrome (SSSS), a disease predominantly affecting infants and children. Adults with renal impairment and immunodeficiency are also at risk [34]. In patients infected with staphylococcal exfoliative toxins, blistering of the skin is evident along with shedding of the upper layers of the skin, with dehydration and secondary infections. Exfoliative toxins target the protein desmoglein 1 and cleave it to disrupt desmosomal cell connections, resulting in detachment of the epidermal layer of the skin [35]. The rupture of the epidermal layer of the skin triggers the progression of the infection. Exfoliative toxins are also considered superantigens but with a milder effect than other superantigens [36].

Staphylococcal enterotoxins (SE) cause vomiting and diarrhea and often represent foodborne illness. Enterotoxigenic foodborne strains of *S. aureus* secrete these toxins which are heat stable and resist degradation by cooking processes. These toxins are differentiated according to antigenic heterogeneity (SEA-SElV) [37]. Staphylococcal enterotoxins are superantigens that trigger T-cell activation and proliferation. Their mode of action may include cytokine release and cell death via apoptosis, resulting in toxic shock syndrome which is life-threatening [38]. Staphylococcal enterotoxins function has been renamed as TSST-1 (toxic-shock syndrome toxin-1), with secretion that lead to severe morbidity and mortality. The gene encoding this toxin is carried by only a limited number of strains. The lethality of TSST-1 does not depend on T-cell proliferation but instead involves other types of host cell receptors. TSST-1 stimulates the release of chemokines, such as IL-8 and MIP-3α, IL-2, and TNFα [39]. The activation of immune cells is able to enhance inflammation and cause mucosal cell barrier disruption, allowing further interaction of the toxin with T-cells and macrophages, leading towards toxic shock syndrome [40,41]. The effectiveness of infection in both humans and animals is determined by the virulence factors produced by *S. aureus* . A broad spectrum of secreted and cell surface-associated virulence factors can be expressed to promote adhesion to host extracellular matrix components, damage host cells, and combat the immune system [22]. Several toxins, microbial surface-recognizing adhesive matrix molecules important for tissue adhesion, immune evasion molecules, and many other virulence factors are known, and some new virulence factors have been recently identified in mastitis and *S aureus* [18]. Data on the presence of virulence genes in *S. aureus* strains isolated from cattle are reported in Table 1. The presence of the superantigen genes *sae–see*, *seg–seo*, and *seq*, as well as the toxic shock syndrome toxin 1 gene, was investigated in isolates from a number of animals, including cow isolates [42] (Table 1). A larger collection of virulence genes in *S. aureus* strains isolates from clinical cases of bovine mastitis from all over The Netherlands was studied [43] (Table 1). Variations in the presence of the genes encoding the different superantigens were moreover evidenced. The presence of the genes for several additional virulence factors, including adhesins, proteases, and capsule type, was investigated. Regarding adhesins, the genes for fibronectin-binding protein A, elastin-binding protein and extracellular fibrinogen-binding protein were almost always present, as was the gene for capsule type. Only one isolate encoded staphylococcal complement inhibitor, chemotaxis inhibitory protein of *S. aureus* , and staphylokinase. This suggests that the isolate may have a human origin because these virulence factors show activity only against the human innate immune system [44]. The *sec3/sel/tst* signature of the bovine staphylococcal pathogenicity island (SaPIbov) was present in few isolates, whereas a little portion of the isolates lacked one of the genes, evidencing to contain an incomplete or variant SaPIbov [18].

The presence of genes of virulence factors associated to adhesion to host cells (*fnbA, fnbB, clfA* and *clfB*), toxins production (*sea, seb, sec, sed, seg, seh, sei, tsst, hla* and *hlb*), and capsular polysaccharide (*cap5* and *cap8*) was evaluated in 123 *S. aureus* strains isolated from cows in Brazil. The *seh* gene was identified in isolated strains. The *cap5* genotype predominated (Table 1), thus suggesting that *S. aureus* may pose a potential threat to human health [45]. *Staphylococcus aureus* can express a wide spectrum of pathogenic factors used to attach, colonize, invade and infect the host. New genotypes were observed for South African strains while for all the other countries new variants of existing genotypes were detected. For each country, a specific genotypic pattern was found. Among the virulence factors, *fmtB, cna, clfA* and leucocidins genes were the most frequent. The *sea* and *sei* genes were present in seven out of eight countries; *seh* showed high frequency in South American countries (Brazil, Colombia, Argentina), while *sel* was harboured especially in one Mediterranean country (Tunisia). The *etb, seb* and *see* genes were not detected in any of the isolates, while only two isolates were MRSA (Germany and Italy) confirming the low diffusion of methicillin resistance microorganisms among bovine mastitis isolates (Table 1) [46].

Table 1: Virulence genes in *Staphylococcus aureus* isolated from cow livestocks.

Genes	Products	Origins	References
hlgA, hlgB, hlgC, hlb, lukD, lukE, aur, splA, splB, splE, seb, sec, sed, seg, seh, sei, sek, sel, sem, sen, seo, seq, seu, tst, sak, scn	hemolysins and leukocidins, proteases, superantigenic toxins, staphylokinase, staphylococcal complement inhibitor	bulk tank milk of dairy farms	[21]
tst, sec, sed, seg, sei, sej, sel, sem, sen, seo	superantigenic toxins	cows	[42]
tst, sea, seb, sec, sed, seg, seh, sei, sej, sel, sem, sen, seo, hla, lucF, lucS, lucM, hlgA	superantigenic toxins, α-haemolysin, leukocidins, γ-haemolysin component A	cattle	[47]
tst, sec, seg, seh, sei, sel, sem, sen, lukE, chp, scn, sak, fnbA, fnbB, clfA, sdrE, cna, ebps, efb, cap5A, cap8, icaB, icaC, icaD, sspA, sspB, slpA, slpB, map	superantigenic toxins, leukocidins, staphylokinase, staphylococcal complement inhibitor, binding proteins, adhesins, protease-like proteins, MHC class 2 analogue proteins	bovine mastitis	[43]
hla, lukF, lukS, hlgA	α-haemolysin, leukocidins, γ-haemolysin component A	calves, mastitis milk	[48]
se, sel, set, hly, luk, adhesins, eta, spa	superantigenic toxins, hemolysin, leukocidin, adherence genes, exfoliants gene, immune evasion gene	raw milk samples collected from cow with subclinical mastitis, western regions of Russia	[49]

coa, hla, hlb, sea, seb, sec, sed	coagulase, haemolysins, enterotoxins	milk samples from the clinical mastitis cases	[50]
sea, sed, sej, ser, lukD, lukE, hlb probe *3, sak, scn, splA, splB, splE, aur, fib, ebpS* probe 612, *clfB, fnbA, fnbB, sasG, sdrC, sdrD, vwb*	superantigenic toxins, leukocidins, haemolysin beta, staphylokinase, staphylococcal complement inhibitor, serin-proteases, aureolysin, fibrinogen-binding protein, cell surface elastin-binding protein, clumpimg factor B, fibronectin-binding proteins, *S. aureus* surface protein G, serine-aspartate repeat protein D, van Willebrand factor-binding protein	milk samples from dairy cow mastitis	[51]
hla, hlb, hlc, hld, clfA, clfB, fnbA, fnbB, icaA, icaD, tsst, sea, seb, sec, see, seg, sei	haemolysins, leukocidins, adhesins, superantigenic toxins,	bovine mastitis in Chinese dairy herds	[52]
fmtB, cna, clfA and leucocidins genes the most frequent	adhesins	bovine mastitis, the *sea* and *sei* genes were present in seven out of eight countries; *seh* showed high frequency in South American countries (Brazil, Colombia, Argentina), while *sel* was harboured especially in one Mediterranean country (Tunisia). The *etb, seb* and *see* genes were not detected in any of the isolates, while only two isolates were MRSA (Germany and Italy) confirming the low diffusion of methicillin resistance microorganism among bovine mastitis isolates. A wide variety of *S. aureus* genotypes was found in dairy cattle worldwide	[46]
lukM, lukD, clfA, icaD, clfB, sea, sdrC, eno	leukocidins, adhesins, biofilm-related genes,	milk samples were collected from dairy cattle with subclinical mastitis from various farms located in northeast Poland	[53]
fnbA, fnbB, clfA, clfB, sea, seb, sec, sed, seg, seh, sei, hla, hlb, cap5, cap8	adhesins, toxins, innate immune defence	milk of cows with mastitis	[45]
coa, spA, tst, clfA	coagulase, *S. aureus* protein A, toxic shock syndrome gene, haemolysins, clumbing factor	milk samples collected from clinically and sub clinically infected cases from Egyptian farms	[54]
icaA, icaD, fnbpA, clfB, spa	biofilm, internalization	milk samples were collected from udder quarters affected by mastitis	[55]
lukM-lukF′	leukocidins	isolates from clinical mastitis	[56]
eno, fib, fnbA, fnbB, ebps, splA, sspA, sei, sem, sen, seg, seo	encoding adhesins, proteases and superantigenic toxins	isolates from subclinical mastitis in cows in eastern Poland	[57]

Forms of Anti-Virulence Theraphy

Staphylococcus aureus is known for its ability to acquire resistance to the commonly used antimicrobial agents as typified by MRSA, Vancomycin-Intermediate *S. aureus* (VISA), and Vancomycin-Resistant *S. aureus* (VRSA) [41]. Antibiotic-resistance development poses an urgent need for the discovery of novel prospective approaches in the fight against multidrug-resistant bacteria. Along with the search for new antibiotics, there is a growing interest in novel non-traditional approaches. Such non-traditional approaches are the attempts to suppress bacterial virulence and the development of virulence-related phenotypes instead of killing the bacteria [58]. *Staphylococcus aureus* is an important etiological agent of ruminant intramammary infections and its eradication from dairy cattle and dairy small ruminants has proven to be difficult. This bacterium can cause a broad range of diseases due to an abundance of virulence factors that facilitate attachment, colonization, tissue invasion, toxinosis, and immune evasion, including adhesion proteins, enterotoxins and capsular polysaccharides [59]. Adhesion is hypothesized to be a prerequisite and crucial early step for Intramammary infections development. Two fibronectin-binding proteins (FnBPs), FnBPA and FnBPB, are involved in not only adhesion to cells but also internalization by cells [19]. Other two important adhesion factors involved in the pathogenesis of *S. aureus* are the clumping factors, ClfA and ClfB [19]. Staphylococcal enterotoxins (SEs) are an important group of virulence factors. They play a significant role in modulating the host immune response and may contribute to maintaining a suitable environment for colonization. In addition, SEs and toxic shock syndrome toxin 1 (TSST1) are superantigens, which have the ability to stimulate large populations of T cells that have a particular Vβ element of the T-cell receptor [60]. Other important toxins are hemolysins, which can negatively affect a wide range of host cells including erythrocytes, epithelial cells, endothelial cells, T cells, monocytes and macrophages [61]. Capsular polysaccharide (cap) is a cell wall bacterial component that protects bacterium from phagocytic uptake and enhances microbial virulence [62]. Cap5 and cap8 were the predominant capsular types in *S. aureus* isolated from clinical bovine mastitis in different countries [63]. The strong pathogenicity of *S. aureus* strains is driven by multifactorial and complex virulence factors. Appropriate molecular typing methods and information about the genetic diversity of *S. aureus* strains in a particular region may contribute to the development of effective strategies for epidemiological control [45].

Virulence is the ability of an organism to infect the host and cause disease. Bacterial characteristics that contribute to disease are called 'virulence factors'. These can be secretory, membrane-bound or cytosolic. Cytosolic factors facilitate the bacterium to start adaptive structural and physiological shifts. Membrane-associated ones, like flagella and pili, are responsible for motility and promote adhesion and host colonization. Secretory products like toxins and enzymes cause harm to host cells and tissues and are important components of the armamentarium used by bacteria to evade the host's innate and adaptive immune response. Together with these, an important multifactorial virulence factor is the ability of pathogenic bacteria to form biofilms [64]. Virulence factors are specific for each bacterial species, with a typical evolution in relation to the characteristics of the occupied host niches and the relationship with the host [58]. An emerging but rapidly expanding area of research is the search for ways and compounds capable of suppressing bacterial virulence [65]. This strategy known as 'anti-virulence therapy' is a novel approach to fight against bacterial pathogens [65,66]. It aims at suppressing the expression of bacterial virulence-related phenotypes rather than killing the bacteria. Virulence factors are microbial components (biomolecules and structures) or more complicated behavioural phenotypes (like biofilm formation) used by pathogens to colonized, invade and persist in a susceptible host [67]. It is known that by applying a substance with strong antibacterial action to a genetically variable bacterial population, selective pressure is triggered by killing the sensitive bacteria leaving more sources for the reproduction and spread of the resistant bacteria. This can be achieved by both occupancies of the liberated living niches and the transfer of resistance genes from the resistant bacteria to some of the drug-sensitive ones. As an important alternative, by suppressing the bacterial virulence mechanisms without killing the microorganisms, the host could be protected against the harmful actions of the pathogens without promoting the emergence of resistant bacteria [58]. Innovative therapeutic strategies must be employed to restrict resistance. Among the innovative proposed strategies, anti-virulence therapy has been envisioned as a promising alternative for effectively controlling the emergence and spread of resistant pathogens. Alternative strategies focused on quench pathogen quorum sensing (QS) systems, disassemble of bacterial Functional Membrane Microdomains (FMMs), disruption of biofilm formation and bacterial toxin neutralization [68].

Quorum sensing can represent a target for interference with bacterial virulence, as this process is a cell-to-cell communication that allows bacteria to obtain information about microbial cell density. Bacterial cells release various signal molecules through their lifespan and once the quantity of these molecules in the environment reaches a threshold, this is a signal to bacterial cells that the living resources have changed, so it is necessary to respond and adapt to the changes by adjusting gene expression. Among the processes controlled by quorum sensing, which are of special importance for the interaction between the host and the pathogenic bacteria, is the expression of virulence factors [69]. For this reason, the quorum sensing system is considered as one of the important targets for antivirulence therapeutics. The approaches to quorum sensing inhibition are various and can reduce the selective pressure exerted by killing bacteria with antibiotics which leads to resistance [58]. Biofilms can be considered as a drug-tolerant virulence-related phenotype related to quorum sensing. This structure confers drug resistance and/or tolerance due to forming an impermeable biofilm composed of sessile bacterial communities. They are attached to surfaces, and the bacterial contamination of some characters may be associated with severe health hazards. Biofilm formation is a multifactorial process with several common steps in the biofilm-formation, starting from free-floating (planktonic) bacteria that may adhere to a surface and attach to it. Single bacterial cells then start reproducing and forming microcolonies. Together with increasing in number, the bacteria lose their motility and release extracellular polymeric substances (EPS) like polysaccharides, proteins, extracellular DNA and a variety of low-molecular-weight substances. Once the number of biofilm cells in the microcolonies increases to a threshold, QS regulation mechanisms start to operate in the control of the biofilm-formation cycle: increase in the size of the microbial colonies, formation of biofilm architecture, which is species- and even strain-specific, and, finally, the stage when some of the sessile bacteria regain their motility and detach from the biofilm to disseminate to new niches. Together with the high antibiotic tolerance, the biofilm structure creates a barrier that prevents the access of the host antibodies and immune cells, determining tolerance to the immune system. The biofilm phenotype of pathogenic bacteria is recently gaining more concern [58].

One promising approach, defined as 'antivirulence therapeutics', is based on the possibility for suppressing the virulence of bacteria rather than killing them. While the expression of virulence phenotypes like toxins, adhesins, biofilms, etc., may vary between bacterial species, they share principally similar regulatory pathways. Therefore, an important focus in antivirulence therapeutics is to interfere with the regulatory mechanisms of virulence expression [58]. A combination of anti-virulence compounds targeting various virulence factors would be a more effective solution than conventional treatments as proposed [70]. Therefore, the characterization of virulence gene profiles and clonal diversity among *S. aureus* populations are very important in the development of anti-virulence therapies. Alpha-hemolysin (Hla) toxin is the most emphasized and characterized virulence factor in *S. aureus* [71]. Changes in key amino acid residues may affect Hla activity. For example, a H35L substitution had no hemolytic or lethal activity, whereas a C259T substitution resulted in a premature stop codon and a significant reduction in Hla production. Promising results have been obtained using Hla as a candidate for developing a vaccine to prevent *S. aureus* infections [52,72]. Non-traditional approaches intent on circumventing bacterial drug resistance, targeting virulence via toxin production and virulence factor secretion, impeding bacterial adhesion to host cells and biofilm formation, interrupting or inhibiting bacterial communication, and downregulating virulence. Other strategies include immune evasion, microbiome-modifying therapies, and the employment of phages as treatments or carriers [67].

Anti-virulence compounds offer an attractive option to conventional antibiotics and hold great promise as a new therapeutic paradigm. One of the pivotal characteristics of an anti-toxin or anti-virulence compound is that the compound does not affect bacterial viability or growth. Anti-virulence therapies that do not target bacterial viability are likely to target nonessential genes and impose reduced selective pressure minimising the probability of resistance development [41]. Several *S. aureus* -specific anti-toxins have shown promise in animal models of infection. Hemolysins are major *S. aureus* toxins expressed by most *S. aureus* strains. Hemolysin, particularly α-hemolysin (Hla), has received substantial attention as a target for anti-toxin neutralizing antibodies. Anti-α-hemolysin antibodies confer a high degree of protection against lethal staphylococcal pneumonia caused by diverse *S. aureus* clinical isolates in experimental animals and significantly reduced abscess formation in a *S. aureus* dermonecrosis model [73]. A β-hemolysin neutralizing single-domain

antibody that inhibits Hlb hemolytic activity *in vitro* has been isolated by antibody phage display. A single antibody with high affinity and cross-reactivity towards α-hemolysin and four bi-component leukocidins was shown to prevent the destruction of multiple human cells by both these toxins [74]. Several molecules or compounds that block the hemolytic activity of *S. aureus* α-hemolysin have been discovered. One example is β-cyclodextrin derivatives, which have been reported to inhibit α-hemolysis in vitro and protect the host during an *S. aureus* infection. Using in silico tools and simulation programs has facilitated the discovery of other α-hemolysin inhibitors [41]. Moreover, targeting *S. aureus* leukotoxins, staphylococcal enterotoxins, and pathways that govern toxin production are other aspects underlining the importance of targeting these vital regulons to render *S. aureus* avirulent or less virulent [41].

Conclusion

Staphylococcus aureus , one of the most prevalent etiologic agents, has an important role in clinical and subclinical mastitis, characterized by persistent and recurrent infections with low cure rates in response to antimicrobial therapy. *Staphylococcus aureus* is known for the ability to develop resistance to antimicrobial agents (*e.g.* methicillin-resistant *S. aureus* , vancomycin-intermediate *S. aureus* , and vancomycin-resistant *S. aureus*) and to secrete numerous virulence factors to exacerbate inflammation. As an alternative to antimicrobials, anti-virulence therapies interfere with bacterial toxins, virulence factors, and/or pathways that regulate their production. As an example, alpha-hemolysin (Hla) toxin is the most emphasized and characterized virulence factor in *S. aureus* . Changes in key amino acid residues may affect Hla activity. C259T substitution resulted in a premature stop codon and a significant reduction in Hla production. Additionally, promising results have been obtained using Hla as a candidate for developing a vaccine to prevent *S. aureus* infections. Important innovative aspects emerged from this investigation, including the need to focus on the virulence mechanisms of *S. aureus* , thus contributing to the development of new effective therapies and prevention programs for the future. Virulence factors could be related to the infectiousness of the *S. aureus* strain, offering new insights into vaccine development and the possible identification of new vaccine targets. Strategies to reduce bacterial virulence include looking for compounds that can inhibit quorum sensing, disassemble bacterial membranes, disrupt biofilm formation, or neutralize the bacterial toxin. Therefore, such strategies can be used to reduce the spread of the infection. The antivirulence approach must be aimed to reduce the production of virulence factors without affecting bacterial growth, as in the case of control or inhibition of staphyloxanthin, the golden carotenoid pigment of *S. aureus* strains, acting as an important virulence factor and whom biosynthesis pathway could represent a potential target to treat *S. aureus* infections. Benefits from antivirulence–antibiotic combinatorial treatment against *S. aureus* infections should be developed, thus providing new perspectives to improve antibiotic adjuvants. Several natural compounds have been found to target virulence gene expression in *S. aureus* , as in the case of solonamide isolated from the marine bacterium *Photobacterium halotolerance*. Further investigations and future research aimed at highlighting optimal virulence factors to be used in anti-virulence strategies against pathogens, may offer important insights into the possibility of counteracting *S. aureus* MRSA, without triggering the phenomenon of antibiotic resistance.

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