Non-*aureus* staphylococci and mammaliicocci (NASM): their role in bovine mastitis and One Health

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Abstract

Non-*aureus* staphylococci (NAS) are gaining importance in mastitis and public health, and some NAS have been reclassified as mammaliicocci (NASM). Bovine milk production has a major influence on the world economy, being an essential source of income for small, medium and large producers, and bovine mastitis caused by NASM can cause an economic impact. Mastitis generates financial losses due to reduced revenue, increased veterinary costs and expenses associated with animal slaughter. However, it is also a public health issue involving animal health and welfare, human health and the ecosystem. Furthermore, it is an increasingly common infection caused by NASM, including antimicrobial-resistant strains. Despite all these adverse effects that NASM can cause, some studies also point to its protective role against mastitis. Therefore, this review article addresses the negative and positive aspects that NASM can cause in bovine mastitis, the virulence of the disease and resistance factors that make it difficult to treat and, through the One Health approach, presents a holistic view of how mastitis caused by NASM can affect both animal and human health at one and the same time.

Introduction

Milk and milk products are important global dietary products consumed by more than 6 billion people worldwide. In 2021, recorded milk consumption was 928 million tons, making the dairy industry a very profitable market (Food and Agriculture Organization of the United Nations, 2021). However, an infection of the mammary gland caused mainly by bacteria, mastitis, is a major problem affecting animal welfare, productivity and the economy, especially in dairy production, which can lead to losses for the dairy industry (Abera *et al.*, 2013).

Among the main microorganisms involved in mastitis, we can mention *Staphylococcus* sp., of which *S. aureus* is the most significant mastitis pathogen, whilst the NAS (non-*aureus* staphylococci) are regarded as minor mastitis pathogens. Among all NAS found among cattle, *S. haemolyticus*, *S. chromogenes, S. epidermidis, S. warneri, S. cohnii, S. simulans, S. hominis, S. capitis, and S. xylosus* are the most prevalent species (Traversari *et al.*, 2019). Besides NAS, it is important to highlight the new genus reclassified from *Staphylococcus*, the *Mammaliicoccus*. A study published by Madhaiyan *et al.* (2020) suggests the reassignment of five species of *Staphylococc*us (*S. sciuri*, *S. fleurettii*, *S.lentus*, *S. stepanovicii* and *S. vitulinus*) to this novel genus, with *Mammaliicoccus sciuri* as the type species. By whole genome sequences, these mammaliicocci species are as distant from staphylococci (64.2–67.3% amino acid identity – AAI) as *Macroccus* is distant from *Staphylococcus* (61.1–64.3%). Non-*aureus* staphylococci and mammaliicocci (NASM) have become a concern among dairy producers, as they are the most common microorganisms isolated from aseptically collected quarter milk samples (Fergestad *et al.*, 2021).

According to the World Health Organization (WHO, 2024) 420 000 lives are lost due to food poisoning, and *Staphylococcus* spp. are characterized as important agents that can cause foodborne diseases. Poisoning occurs due to ingesting enterotoxins produced in food, including those produced by NASM, and symptoms include vomiting, diarrhea and cramps (da Silva Cândido *et al.*, 2020). But there are other problems as well. Political agendas, legislation, development of therapies and educational initiatives are essential to mitigate the increasing rate of antibiotic resistance amongst pathogenic organisms. In addition to bacteria having the ability to form biofilms, other genes are also involved in antibiotic resistance, and the process of becoming resistant can also occur through mutation at the genetic level (Zaman *et al.*, 2017). In addition, it has been suggested that NASM might act as a potential reservoir for resistance

genes, which can be transferred and integrated into the genome of *S. aureus* (Vitali *et al.*, 2014). Despite being under debate, it is essential to highlight that there are species of *Mammaliicoccus* that are suggested to be the evolutionary origin of the *mec*A gene in *S. aureus* (Lakhundi and Zhang, 2018).

For the most part, NASM can be regarded as minor pathogens that do have the ability to cause mastitis. However, some studies have demonstrated that NAS can reduce infection by *S. aureus* and other pathogens. Therefore, this review article aims to address the negative and positive aspects that NASM can have in bovine mastitis, in addition to addressing the importance of adopting the concept of One Health.

Non-*aureus* staphylococci

Differences among and within bovine NAS species have been reported regarding epidemiology and ecology (Souza *et al.*, 2016), antimicrobial resistance (Fergestad *et al.*, 2021), virulence (Wuytack *et al.*, 2020), potential protective traits (Toledo-Silva *et al.*, 2021a, 2021b), host interaction (Piccart *et al.*, 2016) and impact on udder health and milk yield (Valckenier *et al.*, 2019, 2020, 2021) as recently reviewed by De Buck *et al.* (2021). Non-*aureus* staphylococci can be present in a broad range of ecological habitats, which includes bovine teat canals (Traversari *et al.*, 2019) and teat apices (Adkins *et al.*, 2018) as well as the farm environment including bulk tank milk, bedding (Adkins *et al.*, 2022) and feces (Wuytack *et al.*, 2020). *S. chromogenes* and *S. epidermidis* are predominantly isolated from milk. Therefore, they are considered host-adapted species, whereas *S. equorum* and *S. devriesei* are mostly found in the environment and are classified as environmental NAS species (Adkins *et al.*, 2022). Other species, such as *S. haemolyticus,* originate from various (extra)mammary sites and so are labeled opportunistic (Adkins *et al.*, 2022). Unfortunately, multiple studies contradict this current species-stratification (Thorberg *et al.*, 2009; Supré *et al.*, 2011; De Visscher *et al.*, 2014; Vanderhaeghen *et al.*, 2015), and these confounding observations could be attributed to strain differences within NAS species. Wuytack *et al.* (2019), for example, suggested in a recent study on strain distribution of NAS in multiple bovine-associated habitats that different NAS strains within a species appear to be habitat-specific or site-specific.

After several taxonomic reclassifications, introducing the coagulase test enabled differentiation between CoNS and CoPS (Fairbrother, 1940). Non-*aureus* staphylococci is a group of Gram-positive bacteria that were before known as coagulase-negative staphylococci (CoNS); they are usually found in the normal flora of humans and different animals, and some species are recognized as human and facultative animal pathogens (Belhout *et al.*, 2022). For a long time, only *S. aureus* was considered to be harmful to its host, however, as a consequence of patient- and procedure-related changes, NAS now includes a large number of nosocomial microorganisms, with *S. epidermidis* and *S. haemolyticus* being the most significant ones (Pereira-Ribeiro *et al.*, 2019).

Some of the pathogenicity of NAS strains is probably due to their ability to adhere to surfaces and form biofilm on polymer surfaces of medical devices used during the hospitalization process in hospitals (Le *et al.*, 2019). In addition, NAS were also recognized to be capable of 'hiding' from the immune system by being internalized by nonprofessional phagocytes, a mechanism that is mediated by the AtlE gene that helps the

microorganism not only bind but also be phagocyted by human endothelial cells (Christner *et al.*, 2010). Besides that, NAS showed antimicrobial resistance and carried multiple resistance genes, which can be shared with other species (Lee *et al.*, 2020).

NAS brings issues not only to human health but also to animals. They are often found as the majority in bovine intramammary infection, especially in dairy heifers, and several studies in different areas of the globe have found NAS in different proportions in bovine udder, mainly in non-bred or first lactation animals (De Buck *et al.*, 2021). This could have happened because the NAS group was considered to comprise minor pathogens and so were typically not treated. However, grouping these species contributed to a better understanding of their distribution and importance to the animal's health care (Condas *et al.*, 2017).

Despite NAS being a large group, we can highlight some individual species. *S. epidermidis* is recognized as a pathogen and is the most important cause of nosocomial infection (Hugh and Ellis, 1968). This species can also adapt quickly to extreme conditions. For example, to survive in a place with a high salt concentration, the species has a different number of ion/proton exchangers and systems for osmo protectants (Gill *et al.*, 2005), and carries many genes that allow it to survive in these severe situations (Otto, 2009). Also, it is known that *S. epidermidis* is capable of producing an extracellular polysaccharide adhesin, termed polysaccharide intercellular adhesin (PIA) or polymeric N-acetyl-glucosamine, by ica operon-encoded enzymes. This is a significant virulence factor of the specie, helping them to colonize medical devices and leading to device-related infections (Le *et al.*, 2019). Besides this, they can also produce different toxins (Namvar *et al.*, 2014). However, studies suggest that this bacterium has evolved to play an important role in maintaining the microbiomes of humans and other mammals (Otto, 2009).

Due to its potential to produce biofilm, *S. haemolyticus* is also a critical species for nosocomial infections in humans, especially from blood infections. However, there is not as much information for them as for *S. epidermidis* (Barros *et al.*, 2015). When the resistance to β-lactam antibiotics is considered, *S. aureus* and *S. haemolyticus* present enough similarity to say that they have the exact same resistance mechanism (Czekaj *et al.*, 2015).

In several countries, *S. chromogenes* has been identified as the most prevalent species in milk samples from healthy cows and those with subclinical mastitis and clinical mastitis (Valckenier *et al.*, 2020). *S. chromogenes* could overcome the physical barrier of the udder and form biofilms. In addition, this species is more adapted to the cow's mammary gland and may become a microbial reservoir and possible source of infection (Tomazi *et al.*, 2015; Isaac *et al.*, 2017). Molecular studies have also shown that *S. chromogenes* has several antibiotic resistance genes, in addition to having this resistance tested and confirmed in phenotypic tests (Qu *et al.*, 2019).

Mammaliicoccus

Mammaliicoccus are Gram-positive, non-motile, non-spore-forming, paired cocci cells with aerobic to facultative anaerobic growth. They are catalase-positive and possess variable oxidase activities. The G + C content varies from 31.6 to 35.7% in genomes that range from 2.44 to 2.81 Mbp. Based on the analyses of 16S rRNA gene sequences, five species (*M. sciuri*, *M. fleurettii*, *M. lentus*, *M. stepanovicii*, and *M. vitulinus*), are now in this novel genus (Madhaiyan *et al.*, 2020) and are as distant from *Staphyloccus* species as *Macrococcus* (another genus) is from

Staphylococcus. Recognized to colonize humans and animals, they are also considered potentially opportunist pathogens capable of affecting the immune system (Beuckelaere *et al.*, 2021). Like the NAS, this group may also cause mastitis in different species, and/or may serve to transmit resistance genes to other more pathogenic species, as suggested by the study of Lienen *et al.* (2022) where *M. sciuri, M. lentus, M. fleurettii* and *M. vitulinus* were isolated from milk and swab samples collected from German dairy cows.

M. sciuri (previously *Staphylococcus sciuri*) is usually found on the skin of wild and domestic animals. Based on their ribotype pattern, the species was divided into three different subspecies called *S. sciuri* subsp. *sciuri, S. sciuri* subsp. *carnaticus* and *S. sciuri* subsp. *rodentium* (Nemeghaire *et al.*, 2014). In addition, some studies suggest this species as the origin of the *mecA* gene, which is located on a mobile genetic element called staphylococcal cassette chromosome mec (SCCmec), and is the gene responsible for methicillin-resistance in *S. aureus* (MRSA) or more generally in *Staphylococcus* spp (MRSS: Couto *et al.*, 1996; Fuda *et al.*, 2007). Whilst being characterized as a typical colonizer of a wide range of animals, this species has also been associated with various health problems including skin disease in goats and pigs (Nemeghaire *et al.*, 2014). Despite displaying various virulence factors (lipolytic, proteolytic and hemolytic), these bacteria are not related to infection in humans, except after previous infection due to a pathogen (Nemeghaire *et al.*, 2014).

M. lentus (previously *S. lentus*) was first characterized as a subspecies of *S. sciuri*. However, after analysis of DNA-DNA hybridization, it was placed into a new species (Schleifer *et al.*, 1983). It was originally isolated from poultry and other animals and can produce enterotoxins, but only to a minor extent (less than 100 ng/ml supernatant: Irlinger, 2008). Guerre *et al.* (1999) showed that the enterotoxigen strain of *M. lentus* inoculated in milk for the production of Camembert-type cheeses does not produce enterotoxins despite high levels of the microorganisms.

For *M. fleurettii* (previously *S. fleurettii*), it was also possible to identify enterotoxin production, and some studies suggest that this species, rather than *S. sciuri,* is the origin of the mecA gene in MRSA. However, the exact origin is still debated (Lakhundi and Zhang, 2018). In this context, *M. stepanovicii* was also reported with the presence of the gene (Loncaric *et al.*, 2013). Table 1 provides a complete list of NASM and the different samples from which they were isolated.

NASM as a cause of mastitis

NAS forms a heterogeneous group of more than 50 species, of which approximately ten are associated with intramammary infections in dairy cattle and are abundantly present in bulk milk (Vanderhaeghen *et al.*, 2015). As other species of Staphylococci become more commonly associated with the disease, it is of interest to learn more about the distribution of NAS on dairy farms. They have many virulence factors, and mastitis control is complicated because this group contains many species (Thorberg *et al.*, 2009).

The main ways to diagnose subclinical mastitis from NAS are from the results of somatic cell count (SCC) and culture. Tomazi *et al.* (2015) observed that mammary quarters infected by NAS had elevated SCC (> 306 000 *vs.* < 65 000 in contralateral uninfected quarters), without any affect on milk yield or contents of fat, crude protein, casein, lactose, total solids, and solids-not-fat.

Although considered as pathogens with a lower clinical incidence, NAS infections have been identified as relatively common (Persson Waller *et al.*, 2011; Gao *et al.*, 2017; Heikkilä *et al.*, 2018), clinical cases being due mainly to *S. chromogenes, S. simulans* and *S. haemolyticus* (Persson Waller *et al.*, 2011). In subclinical mastitis cases, NAS are the second most commonly identified pathogen after *S. aureus* in Swedish dairy cows, *S. epidermidis* followed by *S. chromogenes, S. simulans,* and *S. haemolyticus* being the most common (Persson Waller *et al.*, 2011). Another study isolated *S. chromogenes, S. fleurettii, S. haemolyticus, S. sciuri, S. simulans, S. succinus* and *S. xylosus* from mastitic bovine milk (Khazandi *et al.*, 2018).

The main pathogenic factors of NASM

According to Vanderhaeghen *et al.* (2014), there are three virulence factor groups involved in the pathogenesis of staphylococcal infections: secreted proteins (e.g., superantigens, cytotoxins and tissue-degrading enzymes), cell surface-bound proteins (e.g. microbial surface components recognizing adhesive matrix molecules: MSCRAMM) and cell wall components, with the polysaccharide capsule and lipoteichoic being examples of those. Naushad *et al.* (2019), using whole genome sequencing analyzed the distribution of 191 virulence factors in 441 genomes of 25 NAS species; the authors reported virulence factors in functional categories such as toxins ($n = 93$), iron metabolism ($n = 29$), adherence ($n = 28$), exoenzymes ($n = 21$) and immune evasion ($n = 20$). Another study by Wuytack *et al.* (2020) compared the presence of virulence genes (*agr*A, *bap,* and cap5H and the *mec*A methicillin resistance gene) of NAS isolated from the milk samples of healthy quarters and quarters with clinical mastitis. Based on this comparison, they suggested that NAS has the potential to cause mild clinical mastitis.

Enterotoxins

Staphylococcal poisoning is widespread and mainly transmitted by food products, as it can affect healthy people. NAS is also able to produce enterotoxins, which can be one of the causes of many food diseases (da Silva Cândido *et al.*, 2020). Enterotoxins belong to a group of extracellular single-chain proteins that are water-soluble and resistant to proteolytic enzyme action in the digestive system. This means that they remain active after ingestion and can compromise human health and, consequently, the public health system. These proteins are also thermostable and resist thermal treatments such as pasteurization (Vanderhaeghen *et al.*, 2014). There are more than 25 types of staphylococcal enterotoxins (SE) and enterotoxin-like (SEL) toxins; the former have an emetic effect in primates, whilst the latter do not. The most known of the enterotoxins are the classical serotypes (SEA through SEE: Bergdoll *et al.,* 1971), followed by the non-classical SEG through SET as well as SEY and more recently SE02 (Su and Lee Wong, 1995; Munson *et al.*, 1998; Ono *et al.*, 2008, 2015, 2019; Omoe *et al.*, 2013; Suzuki *et al.*, 2020). The SELs identified until today are SELJ and then SELU through SELX, SELZ and SEL 01 (Zhang *et al.*, 1998; Letertre *et al.*, 2003; Thomas *et al.*, 2006; Wilson *et al.*, 2011; Spoor *et al.*, 2015; Hisatsune *et al.*, 2017). NASM are capable of producing the majority of these (Rodrigues *et al.*, 2017; da Silva Cândido *et al.*, 2020). However, this has largely been ignored by authorities as NAS can establish a commensal relationship with humans and

Table 1. NASM isolated from different samples (human, animal, environment, and food) and different countries

Specie	Prevalence (%)	Sample collection	Number of samples	Presence of enterotoxin	Place	Reference
S. arlettae	12	Subclinical mastitis milk samples	209	*seb* (16.6%)	China	Xu *et al.* (2015)
S. xylosus	12			No presence		
M. sciuri	12			*sei* (8.3%)		
S. chromogenes	49	Intramammary infection	5,434	Not analyzed	Canada	Condas *et al.* (2017)
S. simulans	17					
S. xylosus	12					
S. equorum	7	Rectal feces	80	Not analyzed	Belgium	Wuytack *et al.* (2020)
S. chromogenes	29	Quarters from 82 heifers	324	Not analyzed		Valckenier *et al.* (2020)
S. xylosus	9					
S. vitulinus	9					
S. chromogenes	49	82 dairy cows	328	Not analyzed	Finland	Taponen *et al.* (2008)
S. simulans	23					
S. warneri	5					
S. chromogenes	47	Composite milk samples	441	Not analyzed	Argentina	Raspanti *et al.* (2016)
S. haemolyticus	32					
S. warner	7					
S. chromogenes	30	Quarter milk samples	4220	Not analyzed	Netherlands	Sampimon *et al.* (2009)
S. epidermidis	13					
S. capitis	11					
S. warneri	37	Udder quarters	46	Not analyzed	Poland	Malinowski *et al.* (2006)
S. chromogenes	33					
S. xylosus	23					
S. chromogenes	48	Milk samples	604	Not analyzed	USA	Jenkins *et al.* (2019)
S. haemolyticus	18					
S. simulans	7					
S. epidermidis	75	Human Nasal swab	176	Not analyzed	Germany	Marincola *et al.* (2021)
S. hominis	15					
S. warneri/ S. pasteuri	7.1					
S. epidermidis	57.9	Prosthetic joint infection	113	Not analyzed	USA	Tande *et al.* (2014)
S. capitis/caprae	5.3					
S. lugdunensis	7.9					

Species	%	Source	n	Toxin	Country	Reference
S. epidermidis	83.3	Prosthetic joint infection	6	Not analyzed	Poland	Bogut et al. (2014)
S. warneri	16.7					
S. epidermidis	56.7	Blood, pus, wound swab, drain fluid, tracheal aspirate, peritoneal fluid, and pleural fluid	120	Not analyzed	India	Bora et al. (2018)
S. haemolyticus	21.7					
S. lugdunensis	11.7					
S. epidermidis,	80.7	Human nasal swab among patients undergoing haemodialysis	71	Not analyzed	Poland	Kozioł-Montewka et al. (2006)
S. lugdunensis	7.7					
S. haemolyticus	3.8					
S. warneri	3.8					
S. capitis	3.8					
M. sciuri	58.8	Environment	17	Not analyzed	German	Schnitt et al. (2021)
S. lentus	17.6					
S. fleurettii	5.8					
S. epidermidis	5.8					
S. haemolyticu s	5.8					
S. cohnii	5.8					
S. haemolyticus	1	Ready-to-eat raw fish	200	Not analyzed	Japan	Hammad et al. (2012)
S. haemolyticus	1					
S. pasteuri	1					
S. intermedius	25	Meat	286	sec (12.5%)	Jordan	Al-Tarazi et al. (2009)
S. warneri	12.5			No presence		
S. lugdenesis	12.5			No presence		
S. epidermidis	6.3%			sec (66.6%)		
M. sciuri	3.1			sec (100%)		
S. intermedius	3.1			No presence		
S. haemolyticus	29.0	Bicycles/ Environment	79	Not analyzed	China	Xu et al. (2019)
S. hominis	29.0					
S. epidermidis	14.0					
S. saprophyticus	33.3	Cheese/ Raw milk/ Pasteurized milk/Food handlers /Environment/ Brine process, and Clot	229	seb (2.1%), see (2.1%) seg (3.2%) seh (1.1%), and sei (1.1%)	Brazil	da Silva Cândido et al. (2020)
S. warneri	15.8			sea (1.1%), see (1.1%), seg (1.1%), and seh (3.2%)		
S. xylosus,	12.3			sed (1.1%), see (1.1%), seg (2.1%), seh (1.1%), and sei (1.1%)		

(Continued)

Table 1. (Continued.)

Specie	Prevalence (%)	Sample collection	Number of samples	Presence of enterotoxin	Place	Reference
S. epidermidis	10.5			see (1.1%) and seg (1.1%)		
S. chromogenes	8.8			see (2.1%) and seg (1.1%)		
S. hyicus	5.3			No presence		
S. haemolyticus	2			see (2.1%) and seh (1.1%)		
S. agnetis, S. capitis, S. kloosii, S. pasteuri, M. sciuri and S. simulans	1.8			seg (1.1%), seb (1.1%), and seh (1.1%)		

animals since they are also part of their normal microbiota (Podkowik et al., 2013).

When de Freitas Guimarães et al. (2013) studied 263 isolates of Staphylococcus spp. from mastitic bovine milk samples they were able to show that 49% were NAS, and of these 66% contained the gene responsible for enterotoxin production, with sea, seb and sec (producing the equivalent classical SEs) being the ones that appeared most. In addition, Ünal and Çinar (2012) found that 46% of isolated NAS tested positive for having one or more SE genes, with 63% out of the total presenting the seh-sej gene, which belongs to the non-classical SEs. Corroborating these results, Rodrigues et al. (2017) characterized NAS isolated from raw milk, Minas cheese, and Minas cheese production lines. They detected the genes seb, seg, seh, sek and seu in the NAS species identified.

Biofilm formation

Different species of bacteria can live in communities, adhere to an inert or living surface, and be protected by a polymeric matrix called a biofilm. This matrix is a self-product of its metabolism, consisting mainly of sugar and proteins (Shemesh and Ostrov, 2020). Biofilm formation in staphylococcal species is associated with a number of factors, and several studies in the literature show NAS as carriers of biofilm genes. Production of PIA is mediated by the genes icaA, icaB, icaC and icaD, representing an important contribution to cell–cell adhesion (Srednik et al., 2017). One study observed biofilm formation in 50% of the NAS strains studied (Rumi et al. (2013). Naushad et al. (2019), in a study based on whole-genome sequencing data for 441 isolates from 25 species, identified that the icaC gene of the ica operon, believed to be involved in biofilm formation, was the second most frequent gene, and it was present in 17 isolates out of all species studied. Other genes of the ica operon, namely icaA, were found in eight species, icaB in seven species and icaD in eight.

Bap is another protein involved in biofilm formation, which is responsible for adhesion. In their research, Piessens et al. (2012) showed that 11.2% (41/366) of NAS were bap gene positive. Zuniga et al. (2015) isolated 67 strains of NAS from subclinical bovine mastitis and tested for the presence of the bap gene; they reported that 45 (67.2%) isolates tested positive and could thus produce biofilms. Rohde et al. (2007) found that nearly 90% of S. epidermidis strains produced a biofilm, and up to 27% of biofilm-positive S. epidermidis isolates produced PIA-independent biofilms, which involves the expression and accumulation of the associated protein. This indicates that, besides Bap, other proteins are involved in ica-idependent biofilm formation. Also, NAS produces MSCRAMM proteins, which mediate staphylococcal adherence to the host's extracellular matrix components. In addition to the operon ica that produces PIA adhesion, these components also form a biofilm (Christner et al., 2010).

Microbial surface components recognizing adhesive matrix molecules (MSCRAMM)

Adhesion to host proteins is usually mediated by bacterial cell-wall-associated proteins called MSCRAMMs (Walsh et al., 2008). A gene related to biofilm formation is embp, which is a MSCRAMM that plays a role during primary attachment to conditioned surfaces (Christner et al., 2010). According to Turchi

et al. (2020), this was their study's most frequently detected gene, followed by *eno* and *fbe*, then *altE*. Srednik *et al.* (2017) showed in their research that NAS isolated from cows with mastitis can produce biofilm in vitro, and they also showed that 90% of the isolates tested positive for one of the above genes.

Zuniga *et al.* (2015) also studied the components on microbial surfaces that recognize the adhesive matrix and found the presence of the *can* gene in 17% of isolates, and the *eno* gene at 82.1% was the most prevalent gene among all. The *ebpS* gene was positive in 17.9%, and there were 72.6% strains positive for the *fnbA* gene and 5.7% for the *fnaB* gene. The *fib* gene was among the most prevalent, present in 76/106 (71.6%), and the *bap* gene was in 60/106 (56%). Darwish and Asfour (2013) identified the presence of the *eno* gene in 92.6% of all 68 NAS isolates. Simojoki *et al.* (2012) also showed a prevalence of the *eno* gene, which was positive in 75% of the 84 studied strains, and the genes *bbp* (1.2%), *cflA* (4.8%), *cflB* (3.6%), *ebpS* (4.8%), *fib* (4.8%), *fnbA* (3.6%), and *fnbB* (2.4%) were also detected. The *cflA* gene is involved with adhesion and immune evasion whilst the *ebpS* gene is responsible for encoding elastin-balding proteins present in the extracellular matrix. Fibrinogen-binding proteins are encoded by the *fib* gene, and fibronectin-binding protein A and fibronectin-binding protein B are encoded by the *fnbA* and *fnbB* genes respectively (Zuniga *et al.*, 2015).

Resistance to antibiotics

Biofilm formation is also connected to antibiotic resistance. According to Srednik *et al.* (2017), NAS isolates growing within biofilms are less susceptible to antimicrobials commonly used on farms. Antibiotics are manufactured at an estimated scale of about 100 000 tons annually worldwide, and their use profoundly impacts bacterial life on Earth. More pathogenic strains have become antibiotic-resistant, and some have become resistant to multiple antibiotics, known as multidrug resistance (Nikaido, 2009). El-Seedy *et al.* (2017) tested how biofilm formation could affect antibiotic resistance, and a total of 95 NAS were identified and tested for biofilm formation, of which 95.8% were resistant to ampicillin, 77.9% were resistant to cefoxitin, 35.8% were resistant to cefuroxime and 31.6% were resistant to amoxycillin.

Besides biofilm formation, there are also genes involved in antibiotic resistance. Xu *et al.* (2015) studied the presence of some of these genes in 76 NAS isolates. They reported the following results: the most prevalent gene was *linA*, which gives resistance to lincosamides, followed by *tetK*, which is responsible for resistance to tetracycline. Conferring resistance to penicillin, the gene *blaZ* was found in 30.3% Also present were the genes *aacA* – *aphD* which provide resistance to aminoglycoside. Another gene studied was *msrA/B*, which was positive in 17.1 and 19.7% of isolates, respectively, conferring resistance to macrolide and erythromycin. The gene *mecA* is one of the most studied genes, and it is responsible for methicillin resistance and was positive in 17.1% of isolates; the genes *ermB/C* that confer resistance to erythromycin and clindamycin were present in 9.2 and 13.2% respectively whilst the genes *aac (6′)/aph (2′)*, responsible for resistance to streptomycin, were present in 10.5% of the strains (Kumar *et al.*, 2010).

Another study identified 109 NAS, all of which were positive for the *blaZ* gene, and 73% of isolates had the *mecA* gene, both of which resist β-lactam groups. The genes that grant resistance to tetracycline *tetK*, *tetL* and *tetM* all showed a high presence, and other genes identified in a significant amount of isolates

were the MLSb resistance genes, *ermB*, *inuA*, *msrA*, and *mphC*. In addition, *dfrG, aacA-aphD* and *aphA3* were present in 25, 24, and 43 NAS isolates (Qu *et al.*, 2019). Ahmed *et al.* (2020) found NASM isolates exhibited a high prevalence of the resistance genes *mecA, blaZ* and *tetK*. Approximately half of the isolates harbored the *aac-aphD*, *erm*(C), and *erm*(B) genes.

Klibi *et al.* (2018) studied 83 isolates; sixty-eight of these strains were identified as NAS. In the study, 29.41% showed oxacillin and cefoxitin resistance whilst some strains were *mec*A positive. Eleven of those twenty methicillin resistant non-*aureus* staphylococci (MRNAS) showed erythromycin-resistance and were positive for *ermB, ermT, ermC, mphC* or *msrA*. Four strains were *tetK* positive, which were resistant to tetracycline. Silva *et al.* (2014) also studied MRNAS, and they reported 26 strains with oxacillin and cefoxitin resistance that were positive for the *mecA* gene. In the same study, the SCC*mec* gene, a mobile genetic element that carries the central determinant for broad-spectrum beta-lactam resistance encoded by the *mecA* gene, was analyzed; it was present in 9 out of 26 strains. MRNAS isolates were also found to display additional resistance to tetracycline, streptomycin, tobramycin and gentamicin, and harbored the genes: *tet* (K), *str*, *ant*(4′)-I, and *aac*(6′)*aph*(2″).

Multidrug resistance

Studies suggest that the frequent use of drugs in veterinary practices, such as penicillin and tetracycline, especially among cattle, has increased the resistance of microorganisms over the years. The Food and Drug Administration (FDA) and the European Medicine Agency (EMA) show that tetracycline, penicillin, and sulfonamides are the classes of antibiotics most sold in the USA and Europe (Dorneles *et al.*, 2019). Several studies in the literature point to multidrug-resistant NAS and these are enumerated in Table 2. In addition, NAS are known to be more resistant to antibiotics when compared to *S. aureus*, and this resistance can be to different drugs and contribute to a low cure rate. The resistance can be due to NAS, typically on the cow's body, being more exposed to antimicrobials (Khazandi *et al.*, 2018).

NASM involvement in mastitis prevention

While studies show NASM as the cause of mastitis, some studies have demonstrated that NAS can reduce infection by *S. aureus* and other pathogens (Brouillette *et al.*, 2022). In another study by Toledo-Silva *et al.*, (2021a, 2021b), it was observed that NASM suppressed the biofilm dispersion of a strain of *S. aureus*. Some studies also point to a possible protective role of NASM, as described by Piepers *et al.* (2013), who in comparing infected heifers with uninfected heifers, observed that heifers infected with NASM had fewer cases of clinical mastitis throughout lactation.

Some gram-positive bacteria can produce bacteriocins, inhibiting the growth of other bacterial strains. Bacteriocins produced by NASM include epidermin, hyicin 3682, nukacin ISK-1, nukacin 3299 and nukacin KQU-131. *Staphylococcus epidermidis* produces Tu 3298, *Staphylococcus hyicus* 3682, *Staphylococcus warneri* ISK-1, *Staphylococcus simulans* 3299, and *Staphylococcus hominis* KQU-131 (Yong *et al.*, 2019), so it can be seen that there is potential for NASM to negatively affect the growth of pathogenic bacteria. In this context, Ferronatto *et al.* (2019) used the cross-streaking method to test growth inhibition of mastitis-causing pathogens. Using this method, it is possible to determine if there was partial inhibition, total inhibition (no colony was

Table 2. Number of multidrug resistant non-*aureus* Staphylococci; antibiotic groups they have resistance to; percentage of resistant strains for each drug or group.

Number of MDR-NAS	Antibiotic resistance	% of MDR	Reference
73/152	Penicillin + ampicillin + erythromycin	51%	Phophi et al. (2019)
60/107	Enrofloxacin + oxacillin + penicillin + tetracycline	56%	Cheng et al. (2019)
14/37	Oxacillin + chloramphenicol + novobiocin + tetracycline	38%	Khazandi et al. (2018)
3/8	Ampicillin + clindamycin + oxacillin erythromycin + gentamycin + penicillin + sulfonamide + trimethoprim/sulfamethoxazole + tetracycline	37.5%	Dorneles et al. (2019)
2/8	Ampicillin + penicillin + trimethoprim/sulfamethoxazole	25%	
1/8	Ampicillin + gentamycin + oxacillin + penicillin + sulfonamide + trimethoprim/sulfamethoxazole + tetracycline	12.5%	
2/8	Ampicillin + gentamycin + oxacillin + penicillin + trimethoprim/sulfamethoxazole + tetracycline	25%	
18/170	Same group of antibiotics (beta-lactam or MLS compounds)	10.6%	Sampimon et al. (2011)
18/170	Different groups	10.6%	

observed), or no growth inhibition (same size and number of colonies as on the positive control plate). It was reported that all of the 19 NAS studied could inhibit growth partially, and in the case of *S. chromogenes* there was total inhibition. Another study showed the capacity of 38 strains of NAS to inhibit *Listeria innocua* growth, with the strain that most displayed this ability being *S. quorum* (Braem et al., 2014).

De Vliegher et al. (2004) also studied how *S. chromogenes* could inhibit the growth of some bacteria, such as *S. aureus, S. dysgalactiae* and *S. uberis*, and the result was positive. The NAS studied inhibited the growth of these strains but not that of *E. coli*, which is typically expected, as the inhibitory effect of bacteriocins is usually more intense against phylogenetically related bacterial species (Ferronatto et al., 2019). Furthermore, the same authors in a previous study suggested that colonization of the teat apex by *S. chromogenes* in pre-calving heifers protected the udder against increased SCC in the early postpartum period (De Vliegher et al., 2003).

According to Isaac et al. (2017), NASM is a group of bacteria classified as either minor mastitis pathogens or commensal microbiota. A study showed that *S. epidermidis, S. simulans, S. hominis, S. saprophyticus*, and *S. arlettae* isolated from milk were able to produce antimicrobial compounds to inhibit the growth of some mastitis pathogens, including *S. aureus* (Nascimento et al., 2005). Beuckelaere et al. (2021) observed a probiotic effect of *S. chromogenes* when colonizing dry quarters with this microorganism. As a result, the authors observed a shift to the Th1 response in late pregnancy and early lactation, increasing IgG2 concentration. In a study with a murine mastitis model, the authors observed that *S. aureus* intramammary colonization is reduced by exoproducts from *S. chromogenes* and *S. simulans* (Brouillette et al., 2022). Some NAS species (*S. chromogenes, S. simulans* and *S. epidermidis*) inhibited the growth of *S. aureus* and downregulated the expression of rnaIII, important molecule of the QS system (Toledo-Silva et al., 2021a, 2021b). In addition, other studies point out that NASM protects the mammary quarters against IMI caused by primary pathogens (De Buck et al., 2021). oth characteristics of NASM, to cause or prevent mastitis, could be explained by differences among the species and intraspecies once the host determines the infection, the virulence of microorganisms and biofilm formation capacity (Brouillette et al., 2022). However, much still needs to be understood about these ratehr complex interactions within the microbiota.

One health and public health

The One Health approach conceptualizes that we cannot truly understand human, animal and environmental health by only addressing each in isolation. Because of this, we must understand the connections of these areas using holistic interpretations to face public health challenges. In other words, One Health focuses equally or more on the relationships between the factors in the system rather than on the individual-level factors themselves (Dalton et al., 2020).

We can observe in Tables 1 and 2 NASM being isolated from food, clinical infections and environment, as well as their resistance to antibiotics. Several NASM strains from these studies (Kozioł-Montewka et al., 2006; Hammad et al., 2012; Xu et al., 2019; Schnitt et al., 2021) hve been confirmed as MRNAS. Also, the topics about virulence and part of Table 1 showed the capacity of these species to produce toxins and form biofilm. All these components place NASM as a concern for public health.

Advances in molecular techniques allow us to relate strains from community and clinical infections, which helps us understand transmission among humans, animals and environments. The species most studied in *Staphylococcus* spp. is *S. aureus* and many studies have observed clones common in clinical infections, in animals or in the environment (Silva et al., 2013, 2014; Aires-de-Sousa, 2016; Zhou et al., 2018). But, we also observe the same route for NAS in studies that demonstrate the presence of identical clones in hospitals and communities (Martínez-Meléndez et al., 2016; Xu et al., 2019; Pinheiro-Hubinger et al., 2021). So, the prevalence of NASM in the dairy chain and the increasing prevalence of resistance genes raises further concern. The prevalence of antimicrobial-resistant pathogens has increased while the approval of new drugs has decreased, which is a public health challenge. However, the number of antimicrobials used in veterinary medicine is immense and should be discussed since they select resistant microorganisms. Animals are the reservoirs of these organisms and can transmit them through food (Gohl et al., 2016; FDA, 2018).

According to the FDA, each year in the United States at least 2.8 million antibiotic-resistant infections occur, and more than 35 000 people die as a result. Therefore, agencies around the world are trying to minimize the harm caused by the incorrect use of antibiotics, for example, using them for purposes for which they are not intended (to treat diseases caused by viruses, fungi or yeasts), in addition to the frequent use of broad-spectrum

antibiotics (Hildreth *et al.*, 2009). The FDA and the European Food Safety Authority (EFSA) are two organizations that have been playing a crucial role in the dissemination of vital information to preserve the effectiveness of currently available antimicrobial drugs and promote the development of new medical products that can help reduce the emergence and spread of antimicrobial resistant bacteria (EFSA, nd; FDA, 2018).

Stevens *et al.* (2018) analyzed the consumption of antimicrobials in dairy herds and its association with the diameters of the antimicrobial inhibition zone of NAS isolated from subclinical mastitis. They reported a high incidence of antimicrobial treatment for fourth generation cephalosporins, a class of antibiotics important for human health. Strains of MRNAS were identified in milk samples with mastitis in a study in Korea, and one of the strains persisted on one of the analyzed farms for more than two years (Kim *et al.*, 2019). The role of NAS as a cause of disease in humans has been described. Diseases such as endocarditis, tract infections, catheter-related sepsis, endophthalmitis, surgical site infections, peritonitis in patients with continuous ambulatory peritoneal dialysis and foreign body infections were due to infections caused by NAS (Piette and Verschraegen, 2009). Approximately 55–75% of nosocomial isolates are resistant to methicillin, and NASM is responsible for about 30% of all nosocomial bloodstream infections (Piette and Verschraegen, 2009).

In the context of One Health and mastitis, there is a solid recommendation for reducing antimicrobial use. Taking into acount that milk with antibiotic residues does not enter the supply chain, as it is withdrawn at source, this recommendation emphasizes the need to periodically search for antimicrobial residues in raw milk on farm. Combined with microbiome analysis and research on antimicrobial residues, we have the somatic cell count, which acts as an indicator of the health of the mammary gland, helping to reveal the situation of the dairy herd within the context of health (Gohl *et al.*, 2016). When milk contains residues, this indicates that it comes from unhealthy animals, so it can cause foodborne pathogens and be a potential allergen for consumers. This concern is even more significant in developing countries, where there is no supervision on using antimicrobials in animals or the opportunity to test milk and meat for drug residues. The presence of adulterating antimicrobials in milk can have a profound impact on the health of babies and children in regions that already face high rates of diarrheal diseases and malnutrition. Several studies have shown a relationship between the intestinal microbiome, health status and disease. Therefore, ensuring that milk does not contain antimicrobial residues significantly impacts public health and producers' economic livelihood (Mohan *et al.*, 2008; Gordon *et al.*, 2012; Garcia *et al.*, 2019). Since NASM is a large group that contains species with different levels of pathogenicity, virulence, resistance profile and host adaptation, analyses of the genetic profile of these species are necessary to understand the role of each NAS species in bovine mastitis (De Buck *et al.*, 2021). Thus, the One Health approach is also required when we are specifically talking about NAS.

This article gathers evidence that NASM has the potential to cause mastitis, and that all of this can cause public health problems since there is a lack of specific studies and strategies to combat these pathogens. Even with this evidence, NASM is still overlooked as causing disease in both humans and animals. With all these points raised, we must apply a One Health approach to address this critical public health problem, including implementing good antimicrobial administration practices in human and veterinary health settings to delay the development of resistance and prolong the life of useful antimicrobials (FDA, 2018).

In conclusion, since it is an important food at all stages of life, milk consumption increases each year along with population growth. We know that NASM mastitis can harm the health and welfare of animals and, consequently, human health, either by food poisoning or by the recurrent use of antimicrobials to treat the disease, which can leave residues in the milk that will be consumed in different ways. Studies related to NASM mastitis are extremely important to learn more about the role of these microorganisms in the disease and how their virulence factors can make treatment difficult. Questions that need more concrete answers are why some NASM strains that cause mastitis appear to have a protective role against other microorganisms and why some strains can harm milk production and animals.

In addition, the increased resistance of these microorganisms to antimicrobials is worrying, requiring the implementation of residue monitoring in milk and studies of NASM antimicrobial resistance used to combat mastitis. Thus, a holistic vision that encompasses safer food and solutions to improve animal, human and ecosystem health seems to be the best alternative.

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