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**Communication**

[*Comunicação*]

# Inhibition of the growth of major mastitis-causing pathogens by non-*aureus Staphylococcus* isolates using the cross-streaking method

[*Comunicação: Inibição do crescimento dos principais patógenos causadores de mastite por isolados de estafilococos não* aureus *pelo método* cross-streaking]

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Mastitis is an inflammation of the mammary gland. This disease is typically caused by bacteria and has a significant economic impact on the global dairy industry. *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* are considered to be the main etiological agents of bovine mastitis (Taponen *et al*., 2017; Rainard *et al*., 2018). The prevention and control of mastitis is vital to the entire milk production chain, since this disease requires the highest use of antimicrobials in dairy herds. Excessive antimicrobial use has been shown to favor the emergence and selection of antimicrobial resistant bacteria (Stevens *et al*., 2018), reinforcing the urgency for alternatives in the prevention, control and treatment of mastitis.

Previous studies have demonstrated that a *Staphylococcus chromogenes* isolate from a heifer's teat apex inhibits the growth of important mastitis agents *in vitro* including *S. aureus*, *S. dysgalactiae*, *S. uberis*, and *S. agalactiae* (Vliegher *et al*., 2004; Braem *et al*., 2014). Furthermore, the isolation of non-*aureus* staphylococci (NAS) from the teat apex of dairy heifers prior to parturition has been shown to be related to protection against intramammary infections (IMIs) by major pathogens (Piepers *et al*., 2011). Thus, it is suggested that NAS species may compete with other bacterial genera (Vliegher *et al*., 2004). In the last years, one of the most important bets is bacteriocins produced by NAS, which play an important role in the establishment and safeguarding of an ecological niche. With this in mind, a promising future option to treat mastitis is the use of bacteriocins, which present no risk of antibiotic residues in milk and would decrease the selection for antimicrobial resistance among mastitis-causing pathogens (Braem *et al*., 2014). In this study we used the cross-streaking method to evaluate the growth inhibition of major mastitis pathogens by bovine associated NAS isolates that were isolated from different ecological niches (milk, environment, and teat apex).

A total of 38 NAS isolates from different ecological niches were used to evaluate the potential growth inhibition of *S. aureus*, *S. agalactiae*, and *S. uberis* isolated from cows with subclinical mastitis and *E. coli* isolated from a cow with clinical mastitis. Among the NAS isolates, 15 were identified as *Staphylococcus chromogenes* (five of which were isolated from the teat apex, five from transient IMIs and five from persistent IMIs), 10 isolates were identified as *Staphylococcus haemolyticus* (five isolates from the teat apex and five from transient IMIs)

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and 13 isolates were identified as *Staphylococcus fleurettii* (five isolates from transient IMIs and eight from the dairy cows' environment). All NAS isolates were obtained from the Faculty of Veterinary Medicine, Ghent University, Belgium and were previously identified using biochemical and molecular tests. The study was approved by the Committee on Ethics of Animal Use (CEUA) at the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo, number of the process – 8609270815. *In vitro* growth inhibition by NAS isolates was determined using the cross-streaking method, as previously described by Vliegher *et al*. (2004). Briefly, suspensions of each NAS isolate (MacFarland 0.5 standard) were plated using sterile and disposable handles on a 5mm wide central strip on a petri dish containing defibrinated sheep blood agar (5%). The plates were incubated at 37°C for 24h under aerobic conditions. The agar was detached from the bottom of the petri dish with the aid of sterile disposable spatulas and placed on top of the lid, such that the NAS isolates were in direct contact with the lid of the petri dish, thus allowing the reverse side of the agar to be exposed. Next, suspensions of the mastitis pathogens (MacFarland 0.5 standard diluted to $10^{-3}$) were plated in the reverse side of the agar with the use of a sterile swab. After incubation for 24h at 37°C under aerobic conditions, petri dishes were examined and measured (mm) to determine whether there was partial inhibition (P), total inhibition (T) or no growth inhibition (N). If present, all growth inhibition zones were determined and averaged on the left and the right sides along the axis across the plate, perpendicularly on the center streak zone. The total growth inhibition zone was regarded when no colony was observed, the partial growth inhibition zone was considered when smaller and/or less colonies was observed, and the zone of no growth inhibition were determined by the same size and numbers of colonies as on positive control plate. The results of this study are summarized in Table 1.

Here, it was observed that a *S. chromogenes* isolate with persistent IMI origin showed total inhibition against *S. aureus*, being the only isolate that showed total inhibition in our study, together with an extended partial inhibition zone. Although both partial and total inhibition of major mastitis pathogens by NAS were regarded here, it was hypothesized that the width of the inhibition zone and the type of inhibition (partial or total) are closely related to the concentrations of inhibitory substances produced by each NAS isolate that diffuse through the agar, such as bacteriocins. Altogether, it suggests that the *S. chromogens* isolate from a persistent IMI described above produced higher amounts of inhibitory substances than other studied NAS isolated here. Furthermore, it was observed that another isolate of *S. chromogenes* with the same origin and a *S. chromogenes* isolate from the teat apex presented partial inhibition against *S. aureus*.

Lastly, four isolates of *S. fleurettii* of environmental origin partially inhibited *S. aureus*. When analyzing *E. coli*, we could see that it was partially inhibited by an *S. chromogenes* isolate originating from transient IMI and also from an environmental isolate of *S. fleurettii*. *Streptococcus uberis* was partially inhibited by a *S. chromogenes* isolate originating from a persistent IMI, as well as a *S. haemolyticus* isolate from the teat apex. In addition, *S. uberis* was partially inhibited by isolates of *S. fleurettii*, one from an environmental origin and one from a transient IMI. Finally, *S. agalactiae* was partially inhibited by three isolates of *S. chromogenes* from the teat apex, one isolate of *S. haemolyticus* from the teat apex, and seven isolates of *S. fleurettii* (one from a transient IMI and six of environmental origin). In all, we had nineteen NAS isolates (50 %) from different origins among the 38 tested that demonstrated at least partial inhibition against at least one major mastitis-causing pathogen. The NAS isolates tested here that did not show any zone of growth inhibition of major mastitis-causing pathogens are not listed in Table 1. We emphasize that the cross-streaking method is easy to perform and interpret, and allows for the selection of isolates capable of inhibiting the growth of mastitis-causing pathogens. This method should be followed up with an evaluation of the mechanism of inhibition *in vitro*, such as the possible production of bacteriocins.

Table 1. *In vitro* inhibition of growth of major mastitis-causing pathogens by staphylococci non-*aureus* of different origins (apex teat, persistent and transient intramammary infections and environmental isolates) with a total of 19 isolates that demonstrated an inhibitory effect as determined by the *cross-streaking* method

| NAS species (identification number) | Ecological niche | Inhibition zone | | | |
|---|---|---|---|---|---|
| | | C | P (mm) | T (mm) | N (mm) |
| Escherichia coli | | | | | |
| S. chromogenes (6) | Transient IMIs | P | 3.5 | 0 | 39 |
| S. fleurettii (34) | Environment | P | 3.5 | 0 | 39 |
| Streptococcus uberis | | | | | |
| S. chromogenes (14) | Persistent IMI | P | 3.5 | 0 | 39 |
| S. haemolyticus (19) | Teat apex | P | 3.0 | 0 | 39.5 |
| S. fleurettii (27) | Transient IMI | P | 3.0 | 0 | 39.5 |
| S. fleurettii (38) | Environment | P | 2.0 | 0 | 40.5 |
| Streptococcus agalactiae | | | | | |
| S. chromogenes (2) | Teat apex | P | 2.0 | 0 | 40.5 |
| S. chromogenes (3) | Teat apex | P | 3.5 | 0 | 39 |
| S. chromogenes (5) | Teat apex | P | 3.0 | 0 | 39.5 |
| S. haemolyticus (19) | Teat apex | P | 3.0 | 0 | 39.5 |
| S. fleurettii (30) | Transient IMI | P | 3.0 | 0 | 39.5 |
| S. fleurettii (31) | Environment | P | 4.0 | 0 | 38.5 |
| S. fleurettii (32) | Environment | P | 3.0 | 0 | 39.5 |
| S. fleurettii (33) | Environment | P | 3.5 | 0 | 39 |
| S. fleurettii (35) | Environment | P | 2.5 | 0 | 40 |
| S. fleurettii (37) | Environment | P | 3.0 | 0 | 39.5 |
| S. fleurettii (38) | Environment | P | 3.0 | 0 | 39.5 |
| Staphylococcus aureus | | | | | |
| S. chromogenes (1) | Teat apex | P | 2.5 | 0 | 40 |
| S. chromogenes (11) | Persistent IMI | P | 4.0 | 0 | 38.5 |
| S. chromogenes (12) | Persistent IMI | T | 38.0 | 4.5 | 0 |
| S. fleurettii (34) | Environment | P | 2.5 | 0 | 40 |
| S. fleurettii (35) | Environment | P | 3.0 | 0 | 39.5 |
| S. fleurettii (36) | Environment | P | 3.0 | 0 | 39.5 |
| S. fleurettii (37) | Environment | P | 3.0 | 0 | 39.5 |

*C: central strip of NAS isolate, measuring (5mm), t: total inhibition and p: partial inhibition; P: zone of partial growth inhibition of major pathogens causing mastitis; T: zone of total growth inhibition of major pathogens causing mastitis; N: zone with no growth inhibition of the major pathogens causing mastitis. NAS: non-*aureus* staphylococci; *S. chromogenes: Staphylococcus chromogenes*; *S. fleurettii*: *Staphylococcus fleurettii*; *S. haemolyticus*: *Staphylococcus haemolyticus*; IMI: intramammary infection.

Our results are in agreement with current literature (Woodward *et al*., 1987; Vliegher *et al*., 2004; Braem *et al*., 2014) that suggest that some NAS isolates, particularly *S. chromogenes*, could confer protection against major mastitis-causing pathogens. Indeed, Vliegher *et al*. (2004) showed that *S. chromogenes* isolates from teat apices inhibited the *in vitro* growth of Gram-positive bacteria, but had no effect on Gram-negative bacteria (e.g. *E. coli*).

A previous study has suggested that the growth inhibition observed within and adjacent to the cross-streak zone could be explained by the production of antagonistic substances (e.g. bacteriocins) that diffuse through the agar and inhibit bacterial growth at the backside of the agar (Vliegher *et al*., 2004). The authors hypothesized that the degree of inhibition of major mastitis-causing pathogens by *S. chromogenes* was not identical against all species because the inhibitory effect of bacteriocins is usually more intense against phylogenetically related bacterial species. These facts may explain why we observed some inhibitory effect against Gram-positive bacteria, although, only a modest effect was observed against *E. coli*.

Similarly, Woodward *et al*. (1987) conducted an *in vitro* study showing that 25% of NAS that were isolated from the teat apex of heifers were able to inhibit the growth of *E. coli* and *S. aureus*. However, the degree of inhibition differed between Gram-positive and Gram-negative bacteria, with greater inhibition observed in Gram-positive bacteria, as found here.

As described above, NAS are capable of inhibiting the growth of primary mastitis-causing pathogens. Nascimento *et al*. (2005) have shown in an *in vitro* study that NAS isolated from bovine mastitis cases produce bacteriocins capable of inhibiting the growth of *S. agalactiae*, similar to our work using the *cross-streaking* method, suggesting that NAS can be used to inhibit the major pathogens causing mastitis. Braem *et al*. (2014) observed that some NAS isolated from bovine teat apex skin, when tested against major mastitis pathogens such as *S. aureus* and *S. uberis*, demonstrated considerable growth inhibition in an *in vitro* study. The same study was the first to identify a bacteriocin of *S. chromogenes* L217, referred to as nukacin L217. Bacteriocins are antimicrobial peptides synthesized by the ribosomes of Gram-positive bacteria and act primarily to inhibit bacterial growth of similar species (Nascimento *et al*., 2005; Carson *et al*., 2017). In another study, Carson *et al*. (2017) concluded that 21% of NAS isolated from bovine milk are capable of inhibiting *S. aureus*. Although bacteriocins produced by staphylococci show promise for antibacterial therapies, including reducing of antibiotic resistant strains of *Staphylococcus*, their use for treatment of mastitis is still limited (Braem *et al*., 2014).

In *in vivo* study by Vliegher *et al*. (2003), it was observed that prepartum teat apex colonization with *S. chromogenes* appeared to protect mammary quarters from having high somatic cell counts in the first few days of lactation. In previous *in vivo* studies, Matthews *et al*. (1990) and Poutrel and Lerondelle (1980) found that mammary quarters that had previous contact with *S. chromogenes* were less prone to become infected when subjected to *S. aureus* inoculation compared to mammary quarters that had no previous contact. Similarly, Piepers *et al*. (2011) conducted an epidemiological study showing that colonization of the heifer teat apex by NAS in the prepartum period conferred a protective effect against IMI by major causative agents of mastitis. Taken together, these results suggest that NAS may have a potential protective effect against other pathogens, such as *S. aureus.*

To the best of our knowledge, this study is the first that evaluates the potential inhibition of major mastitis pathogens by NAS isolated from different ecological niches (milk, environment and teat apex). Initially, we postulated that NAS strains isolated from the teat apex are more likely to produce antagonist substances against other bacteria due to evolutionary pressure and bacterial competition to colonize the teat apex. Conversely, we hypothesized that NAS isolated from the mammary gland environment (e.g. NAS isolated from persistent IMIs) are less likely to produce antagonist substances, as the pressure and selection may be mainly related to evading host immune defense mechanisms. Thus, we propose that the ecological niche influences the ability of the NAS to inhibit the growth of the major pathogen of mastitis. For example, we expected that NAS isolated from the teat apex is related to a greater chance of inhibiting mastitis-causing pathogens. However, this phenomenon was not observed in our research, therefore, more studies using larger numbers of isolates are needed.

Thus, here it was shown that some NAS isolates can inhibit the growth of major mastitis pathogens, especially against Gram-positive bacteria, however, the ecological niche did not determine this beneficial effect.

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*Keywords: dairy cows, coagulase-negative staphylococci, intramammary infection*

**RESUMO**

*O objetivo do presente estudo foi avaliar a capacidade de estafilococos não* aureus *(NAS) isolados de diferentes nichos ecológicos (leite, ambiente e ápice do teto), associados a vacas leiteiras, de inibir os principais agentes etiológicos da mastite bovina* (Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis e Escherichia coli). *Neste estudo, 38 isolados NAS de diferentes nichos ecológicos foram avaliados quanto à capacidade de inibir o crescimento* in vitro *de importantes patógenos causadores de mastite pelo método* cross-streaking. *No total, 19 (50%) isolados de NAS (oito isolados de* S. chromogenes, *10 de* S. fleurettii *e um de* S. haemolyticus) *apresentaram inibição contra os principais patógenos causadores de mastite. No entanto, a inibição dos patógenos causadores da mastite bovina por isolados de NAS foi maior contra bactérias Gram-positivas. Além disso, o presente estudo não sugeriu que os nichos ecológicos influenciam a capacidade do NAS de inibir os principais patógenos causadores da mastite bovina. Com base nesses resultados, concluiu-se que certos isolados de NAS apresentam potencial efeito protetor contra os principais patógenos da mastite, pelo menos* in vitro.

*Palavras-chave: bovinos de leite, estafilococos coagulase-negativos, infecção intramamária*

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