Interaction between bovine-associated coagulase-negative staphylococci species and strains and bovine mammary epithelial cells reflects differences in ecology and epidemiological behavior

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ABSTRACT

Bacteria adherence seems to be an essential first stage for the internalization of bacteria into the cytoplasm of the host cell, which is considered an important virulence strategy enabling bacteria to occupy a micro-environment separated from host defense mechanisms. Thus, this study aimed to explore the difference in the capacity of 4 bovine-associated staphylococci species or strains to adhere to and internalize into bovine mammary epithelial cells (MEC). Three different isolates of coagulase-negative staphylococci (CNS) were used: one strain of *Staphylococcus fleurettii* isolated from sawdust and considered an environmental opportunistic bacterium, and 2 dissimilar *Staphylococcus chromogenes* isolates, one cultured from a heifer's teat apex (*Staph. chromogenes* TA) and the other originating from a chronic intramammary infection (*Staph. chromogenes* IM). Also, one well-characterized strain of *Staphylococcus aureus* (Newbould 305) was used for comparison with a major mastitis pathogen. The CNS species and strains adhered to and internalized into MEC slower than did *Staph. aureus*. Still, we observed high variation in adhesion and internalization capacity among the different CNS, with *Staph. chromogenes* IM showing a greater ability to adhere to and internalize into MEC than the 2 CNS strains isolated from extramammary habitats. In conclusion, the 3 well-characterized bovine-associated CNS species and strains originating from distinct habitats showed clear differences in their

capacity to adhere to and internalize into MEC. The observed differences might be related to their diversity in ecology and epidemiological behavior.

Key words: coagulase-negative staphylococci, *Staphylococcus aureus*, mastitis, dairy cow

INTRODUCTION

Mastitis is one of the most common and detrimental diseases that dairy cows can experience. Moreover, mastitis threatens the income of farmers as well as the image of the entire dairy sector because of animal welfare issues and issues related to milk quality and public health due to an increased risk of antimicrobial residues and the emergence of resistant bacteria (Andrew et al., 2009; Huijps et al., 2009). Staphylococcal bacteria remain an important cause of bovine mastitis. The genus is divided into the coagulase-positive staphylococci, with *Staphylococcus aureus* remaining the most significant mastitis pathogen among the staphylococci, and the heterogeneous group of the CNS, which have become the most commonly isolated bacteria from milk samples of dairy cows and heifers (Fox, 2009; Piepers et al., 2009; De Vliegher et al., 2012), as well as small ruminants (Souza et al., 2012), in many regions and countries around the world. Despite their high prevalence as a cause of IMI, we have only started to learn about differences between species in epidemiological behavior, virulence, and interactions with the host.

Some authors have associated CNS with chronic IMI (Taponen et al., 2007; Thorberg et al., 2009; Piessens et al., 2011; Supré et al., 2011; Fry et al., 2014) and an increase in milk SCC (Supré et al., 2011; Fry et al., 2014; Tomazi et al., 2015), although their clinical relevance is still under debate (Schukken et al., 2009;

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Piepers et al., 2010, 2013). Nevertheless, conflicting results as to the importance of CNS as mastitis-causing agents is likely due to the failure to acknowledge variations within and between species (Fry et al., 2014). The development and validation of molecular identification techniques allow accurate speciation and strain-typing of bovine-associated CNS (Piessens et al., 2011, 2012a; Supré et al., 2011; De Visscher et al., 2014), identifying variations among species in effect on SCC, and different traits such as persistence of infection, antimicrobial resistance, virulence, and epidemiological behavior (Taponen et al., 2007; Piessens et al., 2011, 2012a,b; Supré et al., 2011; Avall-Jääskeläinen et al., 2013; De Visscher et al., 2014; Fry et al., 2014; Vanderhaeghen et al., 2014, 2015; Breyne et al., 2015; Tomazi et al., 2015). Bacterial adherence seems to be an essential first stage for the internalization of bacteria into the cytoplasm of the host cell, whereas internalization into host cells is considered an important virulence strategy. It allows bacteria to occupy a micro-environment protected from the host defense mechanisms operable at the mucosal surface (Almeida and Oliver, 2001; Peton et al., 2014). Apart from 3 studies (Burriel, 1999; Almeida and Oliver, 2001; Hyvönen et al., 2009), no information is available on the interaction between different staphylococci species and mammary epithelial cells (MEC). Also, none of the latter studies investigated the potential link between the adherence and internalization capacity, on the one hand, and the ecology and epidemiological behavior of CNS, on the other hand.

Recent work suggests that some bovine-associated CNS species, such as *Staphylococcus fleurettii*, are typically present in dairy cows' environment and rarely cause IMI; in contrast, others, such as *Staphylococcus chromogenes*, colonize the teat apices, are commonly isolated from milk causing IMI, yet are seldom found in dairy cows' environment (Piessens et al., 2011; De Visscher et al., 2014; Vanderhaeghen et al., 2014, 2015). In that respect, we recently demonstrated that epidemiologically different CNS species and strains induce a differential host innate immune response in the murine mammary gland (Breyne et al., 2015).

We hypothesized that differences in epidemiological behavior and ecology of different CNS species and strains are reflected in their interaction (adherence and internalization) with MEC.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Three different isolates of CNS belonging to 2 species were used, the same isolates as used in a recently published study (Breyne et al., 2015): 1 strain of *Staph.*

fleurettii isolated from sawdust and considered to represent *Staph. fleurettii* as an environmental or opportunistic species, and 2 dissimilar *Staph. chromogenes* isolates. The first *Staph. chromogenes* isolate was cultured from a heifer's teat apex (TA) and has in vitro protective effects against major pathogens such as *Staph. aureus*, *Streptococcus uberis*, and *Streptococcus dysgalactiae* (De Vlieghe et al., 2004; Breyne et al., 2015). The other strain of *Staph. chromogenes* originated from a chronic IMI (IM; Supré et al., 2011) and is considered to behave as an udder-adapted bacterium (Piessens et al., 2011, 2012a; Breyne et al., 2015). Also, one well-characterized and host-adapted strain of *Staph. aureus* (Newbould 305) associated with mild and chronic bovine mastitis (Peton et al., 2014) was included as a positive control, being a major mastitis pathogen.

The isolates were stored at -80°C and thawed at 37°C . First, the strains were grown on Columbia sheep blood agar plates (Oxoid, Wesel, Germany). Then, fresh colonies of each bacteria were grown overnight in brain heart infusion (BHI) broth at 37°C . Subsequently, all strains were diluted at 1:1,000 in fresh BHI broth and incubated until they reached their respective late-exponential growth phase. After bacterial growth, the bacterial broth was centrifuged at $2,500 \times g$ for 15 min and washed twice with $1 \times$ Dulbecco's phosphate buffered saline (DPBS; cat. no. 14190185, Gibco, Paisley, UK). Then, the bacteria were resuspended in Dulbecco's modified Eagle's medium (DMEM, cat. no. 42430-025, Gibco) supplemented with 10% fetal bovine serum, 5 $\mu\text{g}/\text{mL}$ insulin (cat. no. I3536, Sigma Aldrich, St. Louis, MO), and 1 $\mu\text{g}/\text{mL}$ hydrocortisone (cat. no. H0888, Sigma Aldrich). The inoculum was adjusted to 3×10^5 cfu/mL (Bonnetfont et al., 2012) and stored at -80°C until further processing.

The bacterial count for the assay was determined using spectrophotometry at absorbance 600 nm. The inoculum suspension was also cultured on trypticase soy agar in dilution series, and colonies were counted to confirm the final inoculum dose.

Bovine Mammary Epithelial Cells

A clonal bovine mammary epithelial cell line originating from primary bovine alveolar cells (MAC-T; Huynh et al., 1991) were cultured using MAC-T medium containing DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 0.25 $\mu\text{g}/\text{mL}$ Fungizone (cat. no. 15240-096, Gibco), 5 $\mu\text{g}/\text{mL}$ insulin, and 1 $\mu\text{g}/\text{mL}$ hydrocortisone in 6-well plates, and incubated in a humidified incubator with 5% CO_2 at 37°C . To obtain a confluent monolayer, the cell line was treated with 0.25% trypsin (cat. no. 154000-054, Gibco), resuspended in fresh MAC-T

medium, seeded in 6-well plates (5×10^5 cells/well), and incubated overnight at 37°C in 5% CO₂.

Adherence and Internalization Assay

The adherence and internalization assay was performed as described elsewhere (Almeida and Oliver, 2001) with slight modifications. After removing the culture medium, the confluent monolayers of MAC-T cells were washed 3 times with 1× DPBS, and 4 mL of the staphylococcal suspension (3×10^5 cfu/mL; 1:1 ratio of staphylococci to cells) was added per well. First, the number of bacteria in the MAC-T medium (supernatant) was assessed at each sampling time (0 h, before the staphylococcal suspension was added, and 1, 3, 6, and 12 h thereafter) by the drop-plate method (Herigstad et al., 2001) wherein 10 µL of each diluted sample is dropped onto trypticase soy agar until the bacteria are dilute enough to count viable bacteria accurately (between 3 and 30 cfu). Counting the number of bacteria in the MAC-T medium allowed for estimating the bacterial growth in the MAC-T medium at each incubation time. The obtained number of bacteria at each incubation time was used to correct for the number of bacteria in the cell-culture environment in the calculation of the percentage of adherent and internalized bacteria.

After 0, 1, 3, 6, and 12 h of incubation at 37°C in 5% CO₂, the supernatants were removed and the monolayers were washed 3 times with 1× DPBS. Subsequently, the cells were treated with a mixture of 0.25% trypsin and 0.025% Triton X-100 (cat. no. 93443, Sigma Aldrich) in sterile distilled water to lyse the cells. Then, the lysates and supernatants were mixed, serially diluted 10-fold with sterile saline, plated onto square plates with trypticase soy agar, and incubated for 16 to 18 h at 37°C. The number of cell-associated bacteria; that is, adherent and internalized bacteria (cfu/mL), was determined by the drop-plate method.

In another well, a parallel assay was performed to determine the number of internalized bacteria. After removing the supernatants and washing the monolayers 3 times with 1 × DPBS, a similar assay including an additional step to kill the noninternalized bacteria (i.e., adherent bacteria) was performed to determine the internalization capacity of the different staphylococcal bacteria. To kill the noninternalized bacteria, a cell culture medium with lysostaphin (20 µg/mL; cat. no. L7386, Sigma Aldrich) was added to the monolayer. After an incubation of 20 min at 37°C, the cell culture medium containing lysostaphin was removed, and the monolayers were washed 3 times with 1× DPBS. Subsequently, the cells were treated with a mixture of 0.25% trypsin and 0.025% Triton X-100 suspended in sterile

distilled water to lyse the cells. Then, the lysates and supernatants were mixed and serially diluted 10-fold with sterile saline to determine the number of internalized bacteria (cfu/mL), as described above. To confirm the killing of noninternalized bacteria by lysostaphin, 100 µL of the MAC-T medium was dropped onto trypticase soy agar as well.

Outcome Variables

The number of adherent bacteria was determined by subtracting the number of internalized bacteria from the corresponding number of cell-associated bacteria. Nonspecific binding of bacteria to plastic was estimated in parallel by placing the suspension of each bacteria inoculum into wells without MAC-T cell monolayers, and then used to corrected values of adherent and internalized of bacteria to MAC-T cells (Almeida and Oliver, 2001). The percentage of adherent bacteria was calculated by dividing the count (cfu/mL) of adherent bacteria by the count (cfu/mL) of total bacteria (i.e., sum of the number of cell-associated bacteria and the number of bacteria in the MAC-T medium) × 100. The number of internalized bacteria equals the number of intracellular bacteria. Similar to the percentage of adherent bacteria, the percentage of internalized bacteria was calculated by dividing the count (cfu/mL) of intracellular bacteria by the count (cfu/mL) of total bacteria × 100. Each strain was tested in triplicate and the assays were repeated 5 times.

Statistical Analyses

To determine the differences between the different isolates in adherence and internalization capacity, respectively, linear mixed regression models were fit with the number of adherent bacteria, the percentage of adherent bacteria, the number of internalized bacteria, and the percentage of internalized bacteria as continuous outcome variables, and isolate (*Staph. aureus*, *Staph. chromogenes* TA, *Staph. chromogenes* IM, and *Staph. fleuretti*), sampling time, and the interaction between isolate and sampling time as categorical independent variables (PROC MIXED, SAS 9.4; SAS Institute Inc., Cary, NC). A first-order autoregressive correlation structure was used to adjust for clustering of repeated measurements within assays. The numbers of adherent (cfu/mL) and internalized (cfu/µL) bacteria were log₁₀-transformed to normalize the data. The percentages of adherent and internalized bacteria were arcsine-transformed to normalize the data. The results are reported as the mean ± standard error. Significance was set at $P \leq 0.05$. A Bonferroni-correction was applied to adjust for multiple comparisons.

RESULTS

Adhesion

The dynamics of adherence to bovine MEC of the different staphylococci strains are summarized in Figure 1. No adherent bacteria to MEC were found at time zero. The overall logarithm number (cfu/mL) of bacteria adhering to MEC increased from 3.43 ± 0.16 after 1 h of incubation over 3.85 ± 0.15 and 4.71 ± 0.15 after 3 and 6 h of incubation, respectively, up to 6.00 ± 0.16 after 12 h of incubation ($P \leq 0.001$). Except for *Staph. chromogenes* TA (3.02 ± 0.20 , $P = 0.04$), no substantial differences were observed in the number of adherent CNS (3.48 ± 0.20 for *Staph. fleurettii*, and 3.22 ± 0.20 for *Staph. chromogenes* IM) and the number of adherent *Staph. aureus* (3.48 ± 0.20) at 1 h (Figure 1A). The strength of the increase depended on the isolate and was most pronounced for *Staph. chromogenes* IM (Figure 1A). *Staphylococcus chromogenes* IM (5.34 ± 0.20 at 6 h; 6.72 ± 0.20 at 12 h) showed a higher number of adherent bacteria to MEC than did *Staph. fleurettii* (4.52 ± 0.20 at 6 h, $P = 0.003$; 5.84 ± 0.20 at 12 h, $P = 0.001$) or *Staph. chromogenes* TA (4.08 ± 0.20 at 6 h, $P \leq 0.001$; 5.16 ± 0.20 at 12 h, $P \leq 0.001$) after 6 and 12 h of incubation (Figure 1A). At 6 and 12 h after incubation, the number of *Staph. chromogenes* IM adhering to MEC was even higher than the number of adherent *Staph. aureus* (4.90 ± 0.20 at 6 h; 6.29 ± 0.20 at 12 h), although those differences were not statistically significant (Figure 1A). Still, all 3 CNS isolates (*Staph. chromogenes* IM: 3.74 ± 0.20 , $P = 0.05$; *Staph. chromogenes* TA: 3.23 ± 0.20 , $P \leq 0.001$; and *Staph. fleurettii*: 3.85 ± 0.20 , $P = 0.02$) adhered more slowly to MEC, particularly evidenced after 3 h of incubation, than did *Staph. aureus* (4.56 ± 0.20 ; Figure 1A).

The percentage of adherent bacteria to MEC only slowly increased from 0.63 ± 0.09 after 1 h of incubation over 1.24 ± 0.08 and 1.93 ± 0.08 after 3 and 6 h of incubation, respectively, up to 8.56 ± 0.17 after 12 h of incubation ($P \leq 0.001$; Figure 1B). Agreeing well with the findings obtained for the number of adherent bacteria to MEC, the percentage of adherent *Staph. aureus* to MEC (6.73 ± 0.16) increased slightly faster than the percentage of adherent CNS species (*Staph. chromogenes* IM: 0.42 ± 0.16 , $P = 0.02$; *Staph. chromogenes* TA: 0.30 ± 0.16 , $P = 0.02$; and *Staph. fleurettii*: 0.45 ± 0.16 , $P = 0.001$), as evidenced by the substantial differences after 3 h of incubation (Figure 1B). No significant differences were observed between any of the CNS species and *Staph. aureus* at any other time point (Figure 1B). Still, after 12 h of incubation, the percentages of adherent *Staph. chromogenes* IM (14.92 ± 0.17 , $P \leq 0.001$) and *Staph. fleurettii* (11.18 ± 0.17 , $P =$

0.05) bacteria were substantially higher than for *Staph. chromogenes* TA (2.35 ± 0.18 ; Figure 1B).

Internalization

The dynamics of the internalization into bovine MEC of the different staphylococci strains are summarized in Figure 1. No internalized bacteria into MEC were found at time zero. Furthermore, at no time were any bacteria detected in the MAC-T medium after lysostaphin treatment, which confirmed the killing of noninternalized bacteria. The overall logarithm number (cfu/ μ L) of internalized bacteria into MEC increased from 0.18 ± 0.14 after 1 h of incubation over 0.54 ± 0.13 and 1.19 ± 0.13 after 3 and 6 h of incubation, respectively, up to 2.10 ± 0.14 after 12 h of incubation ($P \leq 0.001$). All CNS species (*Staph. chromogenes* IM: 0.50 ± 0.16 , $P \leq 0.001$; *Staph. chromogenes* TA: 0.14 ± 0.16 , $P \leq 0.001$; and *Staph. fleurettii*: 0.28 ± 0.16 , $P \leq 0.001$) internalized more slowly into MEC, particularly after 3 h of incubation, than did *Staph. aureus* (1.25 ± 0.16 ; Figure 1C). After 6 h of incubation, significantly more *Staph. chromogenes* IM bacteria (1.30 ± 0.16) were internalized into MEC than *Staph. fleurettii* (0.79 ± 0.16 , $P \leq 0.001$) and *Staph. chromogenes* TA (0.81 ± 0.16 , $P = 0.002$) bacteria. At this time point, *Staph. aureus* (1.86 ± 0.16 , $P \leq 0.02$) still had the highest number of internalized bacteria into MEC (Figure 1C). After 12 h of incubation, all staphylococci strains differed among each other, with *Staph. chromogenes* IM (3.10 ± 0.16 , $P \leq 0.0002$) exhibiting the highest number of internalized bacteria into MEC, followed by *Staph. aureus* (2.38 ± 0.16 , $P \leq 0.001$), *Staph. fleurettii* (1.87 ± 0.16 , $P \leq 0.001$), and *Staph. chromogenes* TA (1.05 ± 0.17 , $P \leq 0.001$; Figure 1C).

The percentage of bacteria internalized into MEC ranged from 0.08 ± 0.03 after 1 h of incubation to over 0.40 ± 0.03 and 0.51 ± 0.03 after 3 and 6 h of incubation, respectively, and up to 1.27 ± 0.03 after 12 h of incubation ($P = 0.006$). Changes in the percentage of internalized bacteria into MEC over time for each of the staphylococcal species were not as straightforward as for the other variables (Figure 1D).

DISCUSSION

This is the first study that describes differences in adherence to and internalization capacity into MEC among bovine-associated CNS from distinct habitats and with different epidemiological behaviors. Overall, the CNS isolates adhered to and internalized more slowly into MEC than did *Staph. aureus*. Still, these traits varied strongly among the different CNS species and strains, suggesting that the adherence and inter-

Figure 1. Adherence and internalization of different species and strains of staphylococci to and into mammary epithelial cells. Depicted are the logarithmic number (cfu/mL) of adherent (A) and internalized (cfu/μL) (C) *Staphylococcus aureus* Newbould 305, *Staphylococcus fleurettii* isolated from sawdust, *Staphylococcus chromogenes* isolated from a chronic IMI (*Staph. chromogenes* IM) and *Staph. chromogenes* isolated from a heifer's teat apex (*Staph. chromogenes* TA); the percentage of adherent (B) and internalized (D) bacteria are also shown. Different lowercase letters designate means with statistically significant differences ($P \leq 0.05$) among isolates across time; uppercase letters indicate statistically significant differences ($P \leq 0.05$) at a specific time point within an isolate.

nalization capacities of CNS depend on their habitat and epidemiological behavior. In contrast to previously published studies that focused on the interaction of staphylococci species and bovine MEC (Almeida and Oliver, 2001; Hyvönen et al., 2009), we decided to account for the growth of bacteria in the MAC-T medium when calculating the percentage of adherent and inter-

nalized bacteria into MAC-T. This approach allowed us to precisely picture the kinetics of adherent and internalized bacteria over a period of 12 h. In addition, the concentration of inoculum used in the present study was equivalent to an initial multiplicity of infection of 1 cfu per cell (Bonnefont et al., 2012), better resembling the pathogenesis of a natural infection during the early

stages of inflammation than in other studies (Almeida and Oliver, 2001; Hyvönen et al., 2009) that used a high inoculum dose.

The kinetics of adherence to and internalization into MEC apparently did not follow the growth of the staphylococcal isolates in the cell culture environment. The latter was derived from the fact that the percentage of adherent to and notably of internalized bacteria into MEC did not always increase over time. This phenomenon might, at least in part, be explained by a shift in the expression of surface proteins toward the secretion of proteins. During the early stages of *Staph. aureus* infections, surface proteins involved in the attachment of bacteria (i.e., collagen- and fibronectin-binding protein) and defense (i.e., protein A) predominate. However, once a high cell density is achieved at the infection site, the expression of *Staph. aureus* surface proteins is decreased and the secretion of proteins is preferred instead. Thus, bacteria can regulate their behavior according to their population density via cell-to-cell communication or quorum sensing systems (de Kievit and Iglewski, 2000).

The fact that all 3 CNS isolates adhered to and internalized slower into MEC than *Staph. aureus* corresponds well with the findings of Hyvönen et al. (2009), who reported that the intensity of internalization into MEC of CNS strains isolated from clinical and subclinical mastitis was generally weaker than that of *Staph. aureus* strains. The production of the extracellular adherence protein (Eap) by all isolates of *Staph. aureus* (but not by other staphylococcal species) significantly increases the adhesion capacity of *Staph. aureus*, followed by an even more pronounced potential to internalize into eukaryotic cells (Bur et al., 2013).

Both adherence and internalization traits strongly varied among the 3 CNS isolates originating from different habitats, representing different CNS species and strains. Almeida and Oliver (2001) also found significant differences in adherence to and internalization into MEC among *Staph. xylosus*, *Staph. epidermidis*, and *Staph. hyicus* isolated from mastitic milk. In the latter study, *Staph. xylosus* showed the highest potential to adhere to and internalize into MEC. Although *Staph. xylosus* is a species most likely environmental in nature (Piessens et al., 2012b), we hypothesize that some strains are host-adapted, explaining these findings. The fact that both *Staph. chromogenes* isolates in our study interacted somewhat differently with MEC suggests this is likely the case. Remarkably, Hyvönen et al. (2009) did not find any difference in adherence and internalization at 90 min among CNS strains isolated from transient and persistent IMI. Differences were either not present or not detected. The latter might be explained, in part, by the shorter time of incubation

and by the fact that, besides bacterial virulence factors, many other more cow-related factors determine persistence of IMI (Burvenich et al., 2003; Petzl et al., 2008).

One of the key findings of our study is that the *Staph. chromogenes* IM strain isolated from a persistently infected quarter adhered to and internalized better into MEC than did the CNS isolates isolated from the teat apex (*Staph. chromogenes* TA) and environment (*Staph. fleurettii*); that is, 2 extramammary habitats. The latter finding suggests that the epidemiology and ecology of CNS might partly affect the persistence in or elimination of invading bacteria from the udder. In fact, it has been reported that among the CNS species, *Staph. chromogenes* caused more persistent than transient IMI (Thorberg et al., 2009; Piessens et al., 2011; Supré et al., 2011), whereas *Staph. fleurettii* only caused transient IMI (Supré et al., 2011). Internalization of bacteria by host cells has already been proposed as an important virulence strategy because it allows bacteria to occupy a micro-environment separated from the host defense mechanisms (Almeida and Oliver, 2001; Peton et al., 2014). In this respect, Peton et al. (2014) demonstrated that the host-adapted *Staph. aureus* Newbould 305 strain had a higher capacity to adhere to and internalize into MAC-T cells than a *Staph. aureus* strain (RF122) isolated from a clinical case of mastitis. Furthermore, in-depth investigation revealed that the *Staph. aureus* Newbould 305 strain carried specific surface proteins that recognize adhesive matrix molecules, enabling the interaction with host proteins such as fibronectin, collagen, and elastin, and then triggering the invasion. With this in mind, further studies into fine-tuned characterization of the CNS isolates used in our study could be helpful.

Another intriguing finding of our study was the equally high percentage of adherent *Staph. fleurettii* and *Staph. chromogenes* IM to MEC after 12 h of incubation. Both CNS strains exhibited a higher percentage of adherent bacteria to MEC than *Staph. chromogenes* TA. Nevertheless, the percentage of internalized *Staph. fleurettii* and *Staph. chromogenes* TA into MEC at the same time was not different, and both strains displayed a lower percentage of internalized bacteria into MEC than *Staph. chromogenes* IM. Altogether, the results demonstrate the potential of *Staph. fleurettii* to cause IMI, particularly transient IMI, reinforcing the hypothesis that *Staph. fleurettii* isolated from sawdust should be regarded as an environmental or opportunistic species (Piessens et al., 2011). Based on these findings, we can speculate that, in case of either a high infection pressure or an impaired immunity of the cows or quarters, this CNS species may act as a mastitis pathogen (Piessens et al., 2011). In contrast, *Staph. chromogenes* TA showed the lowest percentage of adherent and

internalized bacteria into MEC over time, suggesting that this CNS strain is less likely to cause chronic IMI because it will most likely be cleared from the milk compartment rather than hiding from immune defenses in mammary epithelial cells.

In conclusion, our findings provide new insights into the capacity to adhere to and internalize into MEC by 3 CNS isolates with different epidemiological behaviors and originating from distinct habitats. First, all isolates adhered to and internalized into MEC more slowly than *Staph. aureus* did. Furthermore, the capacity to adhere to and internalize into MEC strongly differed among the different CNS isolates and potentially reflects intra-species diversity in ecology and epidemiological behavior.

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