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# Genetic diversity and iron metabolism of *Staphylococcus hominis* isolates originating from bovine quarter milk, rectal feces, and teat apices

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## ABSTRACT

*Staphylococcus hominis*, a member of the non-*aureus* staphylococci (NAS) group, is part of the human and animal microbiota. Although it has been isolated from multiple bovine-associated habitats, its relevance as a cause of bovine mastitis is currently not well described. To successfully colonize and proliferate in the bovine mammary gland, a bacterial species must be able to acquire iron from host iron-binding proteins. The aims of this study were (1) to assess the genetic diversity of *S. hominis* isolated from bovine quarter milk, rectal feces, and teat apices, and (2) to investigate the capacity of bovine *S. hominis* isolates belonging to these different habitats to utilize ferritin and lactoferrin as iron sources. To expand on an available collection of bovine *S. hominis* isolates (2 from quarter milk, 8 from rectal feces, and 19 from teat apices) from one commercial dairy herd, a subsequent single cross-sectional quarter milk sampling (n = 360) was performed on all lactating cows (n = 90) of the same herd. In total, 514 NAS isolates were recovered and identified by MALDI-TOF mass spectrometry; the 6 most prevalent NAS species were *S. cohnii* (33.9%), *S. sciuri* (16.7%), *S. haemolyticus* (16.3%), *S. xylosus* (9.6%), *S. equorum* (9.4%), and *S. hominis* (3.5%). A random amplified polymorphic DNA (RAPD) analysis was performed on 46 *S. hominis* isolates (19 from quarter milk, 8 from rectal feces, and 19 from teat apices). Eighteen distinct RAPD fingerprint groups were distinguished although we were unable to detect the presence of the same RAPD type in all 3 habitats. One *S. hominis* isolate of a distinct RAPD type unique to a specific habitat (8 from quarter milk, 3 from rectal feces, and 4 from teat apices) along with the quality control strain *Staphylococcus aureus* ATCC 25923 and 2 well-studied *Staphylococcus chromogenes* isolates ("IM" and "TA") were included in the phenotypical iron test. All isolates were grown in 4 types of media: iron-rich tryptic soy broth, iron-rich tryptic soy broth deferrated by 2,2'-bipyridyl, and deferrated tryptic soy broth supplemented with human recombinant lactoferrin or equine spleen–derived ferritin. The growth of the different strains was modified by the medium in which they were grown. *Staphylococcus chromogenes* TA showed significantly lower growth under iron-deprived conditions, and adding an iron supplement (lactoferrin or ferritin) resulted in no improvement in growth; in contrast, growth of *S. chromogenes* IM was significantly recovered with iron supplementation. *Staphylococcus hominis* strains from all 3 habitats were able to significantly utilize ferritin but not lactoferrin as an iron source to reverse the growth inhibition, in varying degrees, caused by the chelating agent 2,2'-bipyridyl.
**Key words:** dairy cow, mastitis, non-*aureus* staphylococci, iron deprivation, *Staphylococcus hominis*

## INTRODUCTION

Non-*aureus* staphylococci, generally considered minor mastitis pathogens, are a large and heterogeneous group with variable effects on bovine udder health (Piepers et al., 2009; Supre et al., 2011; Fry et al., 2014) and milk production (Pearson et al., 2013; Piepers et al., 2013; Valckenier et al., 2019). In many countries, they have become the most common etiological agents of bovine subclinical mastitis in well-managed dairy herds (Tenhagen et al., 2006; Piepers et al., 2007; Ruegg, 2009). *Staphylococcus chromogenes*, *Staphylococcus xylosus*, and *Staphylococcus simulans* are assumed to be the NAS species most relevant to udder health (Supre et al., 2011; Fry et al., 2014; De Visscher et al., 2017), and

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*S. chromogenes* is the most commonly identified species (Gillespie et al., 2009; Fry et al., 2014; Wuytack et al., 2020a).

*Staphylococcus hominis* is an important human pathogen generally associated with nosocomial (catheter-related) bloodstream infections (Kloos et al., 1998; Chaves et al., 2005; Zhang et al., 2013), meningitis (Azimi et al., 2020), and food poisoning (Khater et al., 2021). Its role in bovine udder health is currently not well described, although a study by Fry et al. (2014) on the influence of NAS species on mean SCC concluded that the impact of *S. hominis* appeared minimal. *Staphylococcus hominis* has been recovered in small proportions from teat apices (De Visscher et al., 2014) and in the cow's environment (Piessens et al., 2011). However, it is also one of the predominant species isolated from milkers' skin (elbow bends) and gloves (De Visscher et al., 2014). It is possible that *S. hominis* is being isolated somewhat more frequently from bovine mammary quarters with (sub)clinical signs of mastitis (Bochniarz et al., 2013; Jenkins et al., 2019; Wuytack et al., 2020a). Additionally, in a preliminary study (Wuytack et al., 2019), we revealed the presence of *S. hominis* in bovine rectal feces and discovered that, in a cross-sectional sampling of 8 dairy herds in Flanders, *S. hominis* was one of the overall predominant NAS species isolated from 3 bovine-associated habitats: quarter milk, rectal feces, and teat apices (Wuytack et al., 2020a). This might indicate that *S. hominis* causing IMI could originate from fecal shedding into the environment, presumably after teat apex colonization. A strain-typing approach should be applied (Wuytack et al., 2020b) to investigate this possible association and additional characteristics potentially clarifying the presence of *S. hominis* in different habitats.

Iron is a strict nutritional requirement for most bacteria and, when restricted, it plays a key role in triggering the expression of bacterial virulence factors to facilitate iron acquisition (Diarra et al., 2002; Beasley and Heinrichs, 2010). The mammalian host is able to withhold free ionic iron by binding it in the form of iron–protein complexes such as transferrin in serum, lactoferrin in bodily secretions (e.g., milk and mucosal surfaces) and polymorphonuclear leucocytes, ferritin (intracellular iron storage protein) inside cells, and hemoglobin in erythrocytes (Martínez et al., 1990; Wooldridge and Williams, 1993; Hill et al., 1998). During an IMI, lactoferrin production from epithelial cells of the mammary gland and its release from leukocytes into the milk are intensified, albeit in varying concentrations dependent on the invading mastitis pathogen (Galfi et al., 2016). Furthermore, the increase in milk ferritin concentration during IMI can be attributed to mammary epithelial cell damage associated with the breakdown of the blood–milk barrier, the release of reactive oxygen intermediates and proteolytic enzymes from polymorphonuclear neutrophils, and the production of toxins or intracellular invasion by bacteria (Burriel and Heys, 1997; Orino et al., 2006; Zhao and Lacasse, 2008).

Staphylococci, among other bacteria, have developed iron transport systems such as siderophores—small, high-affinity iron-chelating compounds—to circumvent low iron availability (Martínez et al., 1990; Beasley and Heinrichs, 2010; Yilmaz and Li, 2018). Siderophores enable the development of different virulence factors such as toxins (e.g., hemolysin), enzymes, and adhesins by scavenging and acquiring residual free iron from the environment, as well as sequestering it from host glycoproteins, such as lactoferrin, transferrin, and ferritin; they are integral to microbial iron acquisition (Trivier and Courcol, 1996; Ratledge and Dover, 2000; Sheldon and Heinrichs, 2015). The competition for iron between a host and bacteria can determine the course and severity of the inflammatory reaction in response to infections (Wooldridge and Williams, 1993; Diarra et al., 2002).

Despite the high prevalence of NAS causing bovine IMI, to our knowledge only a few studies have observed and characterized the iron-uptake capabilities of individual NAS species in humans (Beasley and Heinrichs, 2010; Sheldon and Heinrichs, 2012) and not in cows. The objective of this study was to assess the genetic diversity within *S. hominis* isolates originating from one commercial dairy herd with the use of random amplified polymorphic DNA (**RAPD**)-PCR, to investigate the strain relatedness of the isolates between 3 bovine-associated habitats (quarter milk, rectal feces, and teat apices), and to make intra-species comparisons. Furthermore, we investigated the capacity of *S. hominis* isolates to obtain iron from host iron-binding proteins ferritin and lactoferrin as a potential explanation for the presence or absence of bacteria in these 3 habitats.

## MATERIALS AND METHODS

### Herd, Cows, and Isolates

Ethical approval was not required for this study because milk samples were collected as part of the routine milking procedure for the animal subjects.

First, *S. hominis* isolates (n = 29) from a commercial dairy herd collected in August 2017 (bulk milk SCC: 116,000 cells/mL of milk) as part of a previous observational study (Wuytack et al., 2020b) were available. These isolates were recovered from quarter milk (**QM**; n = 2), rectal feces (**RF**; n = 8), and teat apices (**TA**; n = 19).

Second, the same commercial dairy herd, enrolled in the local dairy herd improvement program (Flemish Region, Belgium), was visited again for this study in March 2018 (bulk milk SCC: 92,000 cells/mL of milk) because of the relatively extensive collection of *S. hominis* acquired during the previous collection, although not from milk (2 isolates only). The average herd size in 2017/2018 was 106 cows, with an average yearly production of 9,271 kg of milk/cow, and cows were milked twice a day in a herringbone parlor. The single cross-sectional quarter milk sampling was performed in March 2018. All lactating cows (n = 90) were sampled aseptically for quarter milk (n = 360) according to the guidelines of the National Mastitis Council (Hogan et al., 1999). The samples were refrigerated (4°C) and subsequently transported to the Mastitis and Milk Quality Research Laboratory (Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium) for further analysis.

### Laboratory Analysis of Milk Samples

A 0.01-mL loop of milk was spread on a quadrant of mannitol salt agar (Chapman medium, Thermo Fisher Scientific), a semi-selective medium for NAS recovery, as described by De Visscher et al. (2013). All plates were incubated aerobically at 37°C, and bacterial growth was examined at 24 and 48 h. Per plate, all phenotypically unique colony types were differentiated based on morphological characteristics (shape and color) and subcultured on blood agar (Colombia agar with 5% sheep blood, Thermo Fisher Scientific) to obtain pure cultures. Gram staining and catalase tests were performed on all purified isolates to presumptively identify staphylococci. All colonies that phenotypically appeared to be staphylococci were selected.

All phenotypically identified staphylococci isolates were subsequently speciated by MALDI-TOF mass spectrometry. The preparation for MS analysis followed a standard extraction protocol with the recommended Bruker *Escherichia coli* bacterial test standard (Bizzini et al., 2010; Cameron et al., 2017; Mahmmod et al., 2018). Protein fingerprints of the isolates were compared with a commercial databank of bovine reference spectra (Bruker Daltonics Inc.), in-house custom microbial spectra provided by Cameron et al. (2018), and additional microbial spectra of field isolates from our laboratory covering 4 species of NAS (*S. jettensis*, *S. lentus*, *S. rostri*, and *S. saprophyticus*) and additional bovine isolates (*S. chromogenes*, *S. epidermidis*, *S. equorum*, *S. fleurettii*, *S. haemolyticus*, *S. hyicus*, and *S. simulans*). For NAS species, a cut-off value of ≥1.7 was considered valid (Cameron et al., 2018). The *S. hominis* isolates (n = 18) were preserved in Microbank vials (Pro-Lab Diagnostics) at −80°C until further processing.

### RAPD Analysis

The DNA of the *S. hominis* isolates was extracted with the use of the DNeasy Blood and Tissue Kit (Qiagen) in accordance with the instructions of the manufacturer. All samples were analyzed by RAPD-PCR. One microliter of extracted DNA was added to 24 μL of PCR mix, which included a random 23-nucleotide-long primer (D11344). The cycling program was as described by Fitzgerald et al. (1997). The generated amplified DNA fragments were separated on ethidium bromide–stained agarose gel (1.5% wt/vol; GC Biotech) at 150 V and 500 mA for 3 h. The images were imported and analyzed with the use of BioNumerics software version 7.6.3 (Applied Maths) as described by Wuytack et al. (2019). The analyzed quarter milk isolates were added to an existing RAPD dendrogram (Wuytack et al., 2020b). Each RAPD type was assigned an arbitrary lowercase letter based on both clustering and visual assessment of band differences, with the cut-point for grouping based on 100% similarity. We were unable to reculture one *S. hominis* isolate (Hominis 5) from the Microbank; consequently, it was excluded from the RAPD analysis.

### Phenotypical Iron Test

For the phenotypical iron test, a subset of the *S. hominis* isolates (all randomly selected isolates of a distinct RAPD type, n = 15) were grown in 4 types of media using a 96-well microplate (Novolab) at 37°C for 24 h without shaking, bordering on anaerobic conditions: an iron-rich medium, trypticase soy broth (**TSB**; Thermo Fisher Diagnostics), TSB deprived of iron by adding a final concentration of 0.5 m*M* of the iron-chelating agent 2,2'-bipyridyl (**dTSB**; Sigma Aldrich; Samaniego-Barrón et al., 2016), iron-deprived TSB supplemented with a final concentration of 50 μ*M* ferritin from equine spleen (**dTSBF**; Sigma Aldrich; Diarra et al., 2002; Vermassen et al., 2016), and 0.4 mg/mL iron-saturated recombinant human lactoferrin (**dTSBL**; Sigma Aldrich). Sterility was controlled for the resulting media. Pure colonies, cultured overnight on Colombia blood agar, were added to a separate sterile 0.85% NaCl solution (Biomerieux) until 0.5 McFarland density was reached, followed by a $10^{-3}$ dilution in Dulbecco's PBS (ThermoFisher). Twenty microliters of this suspension was then added to 180 μL of TSB, dTSB, dTSBF, or dTSBL in the wells of the microplate. Quality control reference strain *Staphylococcus aureus* ATCC 25923 (Diarra et al., 2002; Treangen et

**Table 1.** Species distribution of NAS isolates from quarter milk samples collected from all lactating dairy cows (n = 90) during the cross-sectional sampling performed in March 2018

| NAS species | No. (%) of isolates[1] | No. (%) of quarters | No. (%) of cows |
|---|---|---|---|
| *Staphylococcus cohnii* | 174 (33.9) | 142 (39.4) | 74 (82.2) |
| *Staphylococcus sciuri* | 86 (16.7) | 81 (22.5) | 53 (58.8) |
| *Staphylococcus haemolyticus* | 83 (16.2) | 68 (18.9) | 50 (55.6) |
| *Staphylococcus xylosus* | 49 (9.5) | 45 (12.5) | 36 (40.0) |
| *Staphylococcus equorum* | 48 (9.3) | 46 (12.8) | 27 (30.0) |
| *Staphylococcus hominis* | 18 (3.5) | 15 (4.2) | 16 (17.8) |
| *Staphylococcus auricularis* | 13 (2.5) | 13 (3.6) | 12 (13.3) |
| *Staphylococcus epidermidis* | 9 (1.8) | 8 (2.2) | 6 (6.7) |
| *Staphylococcus devriesei* | 9 (1.8) | 9 (2.5) | 9 (10.0) |
| *Staphylococcus saprophyticus* | 8 (1.6) | 8 (2.2) | 8 (8.9) |
| *Staphylococcus chromogenes* | 5 (1.0) | 5 (1.4) | 4 (4.4) |
| *Staphylococcus succinus* | 4 (0.8) | 4 (1.1) | 4 (4.4) |
| *Staphylococcus arlettae* | 3 (0.6) | 3 (0.8) | 3 (3.3) |
| *Staphylococcus lentus* | 2 (0.4) | 2 (0.6) | 2 (2.2) |
| *Staphylococcus agnetis* | 1 (0.2) | 1 (0.3) | 1 (1.1) |
| *Staphylococcus capitis* | 1 (0.2) | 1 (0.3) | 1 (1.1) |
| *Staphylococcus simulans* | 1 (0.2) | 1 (0.3) | 1 (1.1) |
| Total | 514 (100) | | |

[1]Total number of isolates per species originating from quarter milk samples and proportion (%) of all isolates (n = 514).

al., 2014) served as a positive control, and 2 well-studied NAS isolates used in a separate phenotypical iron test performed by Wuytack et al. (2019), *S. chromogenes* IM and TA, were included for comparison (De Vliegher et al., 2004; Piccart et al., 2016; Wuytack et al., 2019). The samples in the microplate wells were agitated by pipetting the medium before the microplate was sealed with a transparent seal (to minimize contamination of the samples) and incubated for 24 h at 37°C in a MultiScanGo apparatus (Thermo Fisher Scientific). The optical density (600 nm) was measured 25 times at 1-h intervals to quantify bacterial growth. SkanIt 4.1 for Microplate Readers software (Thermo Fisher Scientific) was used for protocol input and recording of the results. The phenotypical iron test was performed in duplicate over 2 independent days (replicates), and the incubation conditions were the same for all the tested isolates.

### Statistical Analysis

All data were entered in an electronic spreadsheet program (Excel 2016, Microsoft Corp.) and checked for unlikely values. The growth of the different bacterial strains was expressed as the area under the curve (**AUC**). The AUC was calculated based on the optical density measured per time interval over 24 h. To obtain a normal distribution, a $\log_{10}$-transformation of the AUC was performed (**$AUC_{log}$**).

A linear mixed regression model was used to determine the association between $AUC_{log}$ (outcome variable) and the growth medium (categorical predictor variable: TSB, dTSB, dTSBF, and dTSBL) and the bacterial strains (categorical predictor variable: *S. aureus* ATCC 25923, *S. chromogenes* IM, *S. chromogenes* TA, and *S. hominis* from QM, RF, and TA). The interaction between the growth medium and strains was tested as well (PROC MIXED, SAS version 9.4; SAS Institute Inc.). Isolate was incorporated as a random effect to account for the fact that experiments were repeated on 2 different days. The significance level was set at $P \leq 0.05$.

## RESULTS

### NAS Isolates from Milk

Five hundred fourteen NAS isolates were recovered and identified by MALDI-TOF MS in 77.5% (n = 279) of the 360 quarter milk samples from the 90 lactating cows collected during the cross-sectional herd visit. In 45.5% (n = 127) of the 279 NAS-positive quarters, more than 1 NAS species was recovered. *Staphylococcus cohnii*, *S. sciuri*, *S. haemolyticus*, *S. xylosus*, *S. equorum*, and *S. hominis* were the most prevalent of the 17 identified NAS species (see Table 1). It is important to note that in a recent phylogenomic analysis, it is suggested that *S. sciuri* and *S. lentus* should be reassigned to the novel genus *Mammaliicoccus* gen. nov. (Madhaiyan et al., 2020). In total, 18 *S. hominis* isolates were retrieved and added to the existing collection of 29 isolates.

### *Genetic Diversity of Staphylococcus hominis*

A total of 46 *S. hominis* isolates (19 from quarter milk, 8 from rectal feces, and 19 from teat apices) were included in the RAPD analysis. The RAPD-PCR subdivided the isolates into 18 distinct fingerprint groups at 36.3% similarity (RAPD types a–r, Figure 1). Six RAPD types (a, e, f, k, m, and o) were isolated more than once; RAPD type o was isolated most often (n = 15), predominantly from TA (n = 10) and occasionally from QM (n = 5). The RAPD types b, d, and e were found exclusively in RF; types c, g, h, i, m, and q exclusively in QM; and types j, l, n, p, and r exclusively from TA. The RAPD types a and o were the only fingerprint groups found both in QM and TA; RAPD type f was the only fingerprint group found in both RF and TA. Cows J, D, M, and P harbored one or more RAPD types in RF samples but no *S. hominis* isolates in their QM or TA. For each habitat, 1 *S. hominis* isolate per RAPD type (n = 15) was included in the phenotypical iron test.

### *Phenotypical Iron Test*

Average growth of *S. aureus* ATCC 25923, *S. chromogenes* IM, *S. chromogenes* TA, and the 15 *S. hominis* isolates (8 from QM, 3 from RF, and 4 from TA) on different media is depicted in Figure 2.

Across all media, there was a significant strain effect ($P = 0.0244$), with *S. aureus* ATCC 25923 growing the best across all media [least squares means (**LSM**) of $AUC_{log} = 0.85$] but not significantly different from *S. chromogenes* IM (LSM = 0.77; $P = 0.45$) and *S. hominis* from TA (LSM = 0.72; $P = 0.16$; Table 2).

Over all strains, there was a considerable effect of medium ($P < 0.0001$), with the best growth observed on TSB (LSM = 0.90; Table 2). Growth was significantly better in media with ferritin (dTSBF; LSM = 0.70) as the iron source compared with dTSB (LSM = 0.54; Bonferroni-corrected $P = 0.0001$) and dTSBL (LSM = 0.61; Bonferroni-corrected $P = 0.0002$). Additionally, lactoferrin as a supplement (dTSBL) made a significant difference in growth compared with dTSB (Bonferroni-corrected $P = 0.0157$).

Thus, growth of the different strains was modified by the different media used, as indicated by the highly significant interaction term in the statistical model between medium and strain ($P < 0.0001$; Figure 2 and Table 2). *Staphylococcus aureus* showed overall comparable growth on the different media (Figure 2A). In contrast, for *S. chromogenes* IM, a significant reduction in maximum growth was observed in dTSB (Bonferroni-corrected $P < 0.0001$); however, growth was significantly recovered when adding ferritin (dTSBF; Bonferroni-corrected $P = 0.0001$) or lactoferrin (dTSBL; Bonferroni-corrected $P = 0.0249$) to the deferrated medium (Figure 2B; Supplemental Table S1, https://doi.org/10.6084/m9.figshare.20455191.v4, Reydams et al., 2022).

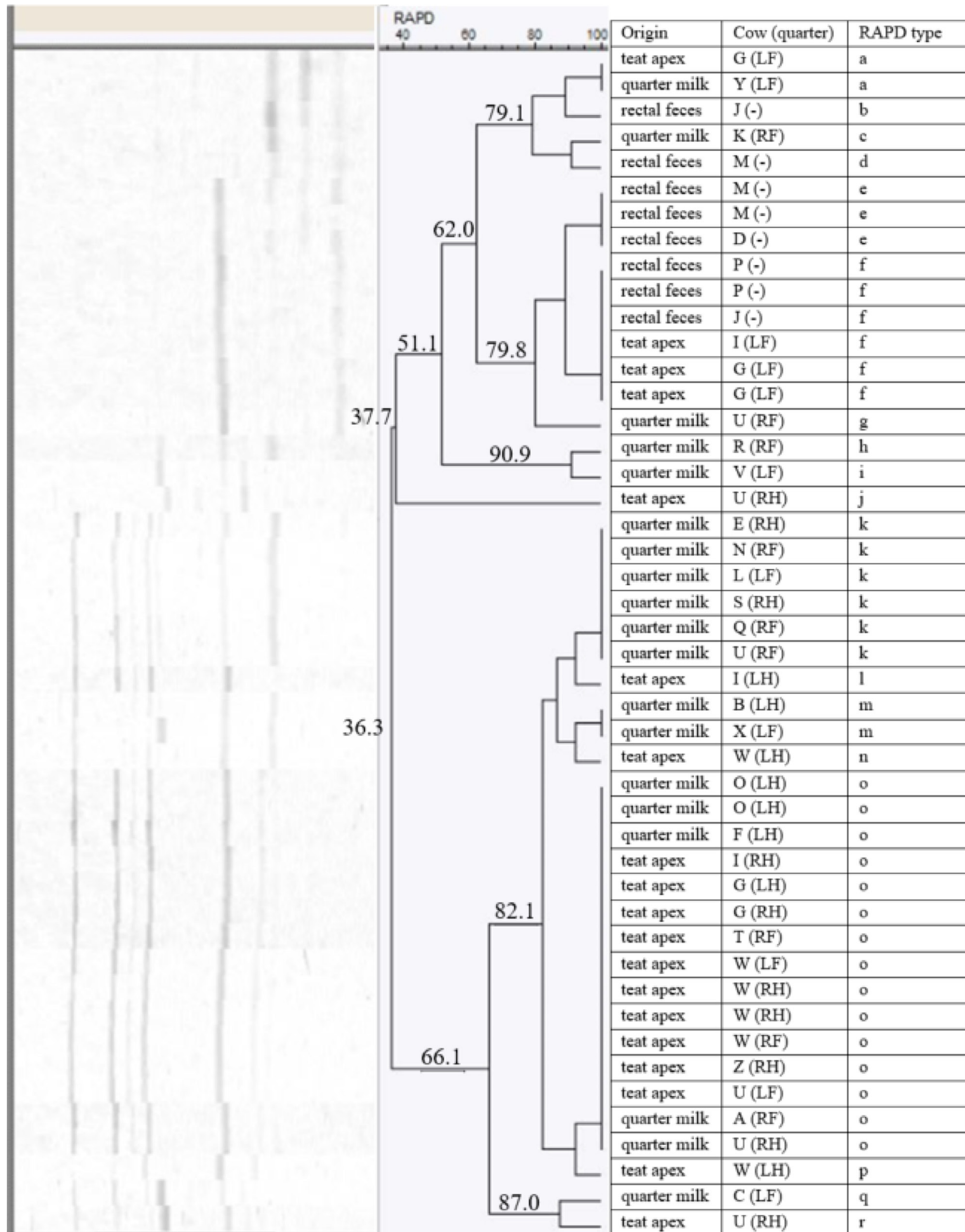
*Staphylococcus chromogenes* TA showed a significantly lower growth under iron-deprived conditions (Bonferroni-corrected $P = 0.0001$); adding ferritin or lactoferrin to deferrated TSB made no difference (Figure 2C; Bonferroni-corrected $P = 1$ for both dTSBF and dTSBL, Supplemental Table S1).

Overall, all *S. hominis* strains grew significantly less in deferrated media (Figure 2D–F; for all 3, Bonferroni-corrected $P < 0.0001$, Supplemental Table S1); however, a significant improvement in growth was observed when adding ferritin to dTSB for isolates from QM (Figure 2D; Bonferroni-corrected $P < 0.0001$, Supplemental Table S1), RF (Figure 2E; Bonferroni-corrected $P = 0.0007$, Supplemental Table S1), and TA (Figure 2F; Bonferroni-corrected $P = 0.0398$, Supplemental Table S1) but not for lactoferrin (Figure 2D–F; Supplemental Table S1).

## DISCUSSION

This study aimed to extend our knowledge on the genetic diversity and iron uptake capacity of bovine-associated *S. hominis* originating from different habitats (QM, RF, and TA). We did so because *S. hominis* was one of the overall predominant NAS species isolated from 3 bovine-associated habitats in a cross-sectional sampling of 8 dairy herds performed by Wuytack et al. (2020a) and has been identified as the cause of subclinical mastitis cases (Bochniarz et al., 2013; Wuytack et al., 2020a). We decided to further investigate this species and its potential importance for udder health, including making comparisons with a more relevant NAS species (*S. chromogenes*) and a major mastitis pathogen (*S. aureus*). In this herd, *S. hominis* isolates were relatively highly genetically diverse, an overlapping RAPD type for *S. hominis* from RF and QM isolates was not found, and *S. hominis* from all 3 habitats were significantly better suited to utilize equine ferritin as an iron source than recombinant human lactoferrin. In contrast to the *S. chromogenes* strains, where origin affected growth in different media, all *S. hominis* strains, despite variations in RAPD types, appeared to grow similarly in the different media regardless of origin.

A total of 18 different NAS species were isolated and identified from the extensive quarter milk sampling on the herd performed specifically to find as many *S. hominis* isolates as possible from milk. *Staphylococcus epidermidis*, *S. chromogenes*, and *S. simulans* accounted for only 1.8, 0.8, and 0.2% of the isolates, respectively,




| Origin | Cow (quarter) | RAPD type |
|---|---|---|
| teat apex | G (LF) | a |
| quarter milk | Y (LF) | a |
| rectal feces | J (-) | b |
| quarter milk | K (RF) | c |
| rectal feces | M (-) | d |
| rectal feces | M (-) | e |
| rectal feces | M (-) | e |
| rectal feces | D (-) | e |
| rectal feces | P (-) | f |
| rectal feces | P (-) | f |
| rectal feces | J (-) | f |
| teat apex | I (LF) | f |
| teat apex | G (LF) | f |
| teat apex | G (LF) | f |
| quarter milk | U (RF) | g |
| quarter milk | R (RF) | h |
| quarter milk | V (LF) | i |
| teat apex | U (RH) | j |
| quarter milk | E (RH) | k |
| quarter milk | N (RF) | k |
| quarter milk | L (LF) | k |
| quarter milk | S (RH) | k |
| quarter milk | Q (RF) | k |
| quarter milk | U (RF) | k |
| teat apex | I (LH) | l |
| quarter milk | B (LH) | m |
| quarter milk | X (LF) | m |
| teat apex | W (LH) | n |
| quarter milk | O (LH) | o |
| quarter milk | O (LH) | o |
| quarter milk | F (LH) | o |
| teat apex | I (RH) | o |
| teat apex | G (LH) | o |
| teat apex | G (RH) | o |
| teat apex | T (RF) | o |
| teat apex | W (LF) | o |
| teat apex | W (RH) | o |
| teat apex | W (RH) | o |
| teat apex | W (RF) | o |
| teat apex | Z (RH) | o |
| teat apex | U (LF) | o |
| quarter milk | A (RF) | o |
| quarter milk | U (RH) | o |
| teat apex | W (LH) | p |
| quarter milk | C (LF) | q |
| teat apex | U (RH) | r |

**Figure 1.** Dendrogram of the random amplified polymorphic DNA PCR fingerprints of 46 *Staphylococcus hominis* isolates. Cows were assigned an arbitrary uppercase letter; LF, RF, LH, RH = left front, right front, left hind, and right hind quarter, respectively. Random amplified polymorphic DNA (RAPD) types were assigned an arbitrary lowercase letter (a–r) based on clustering.

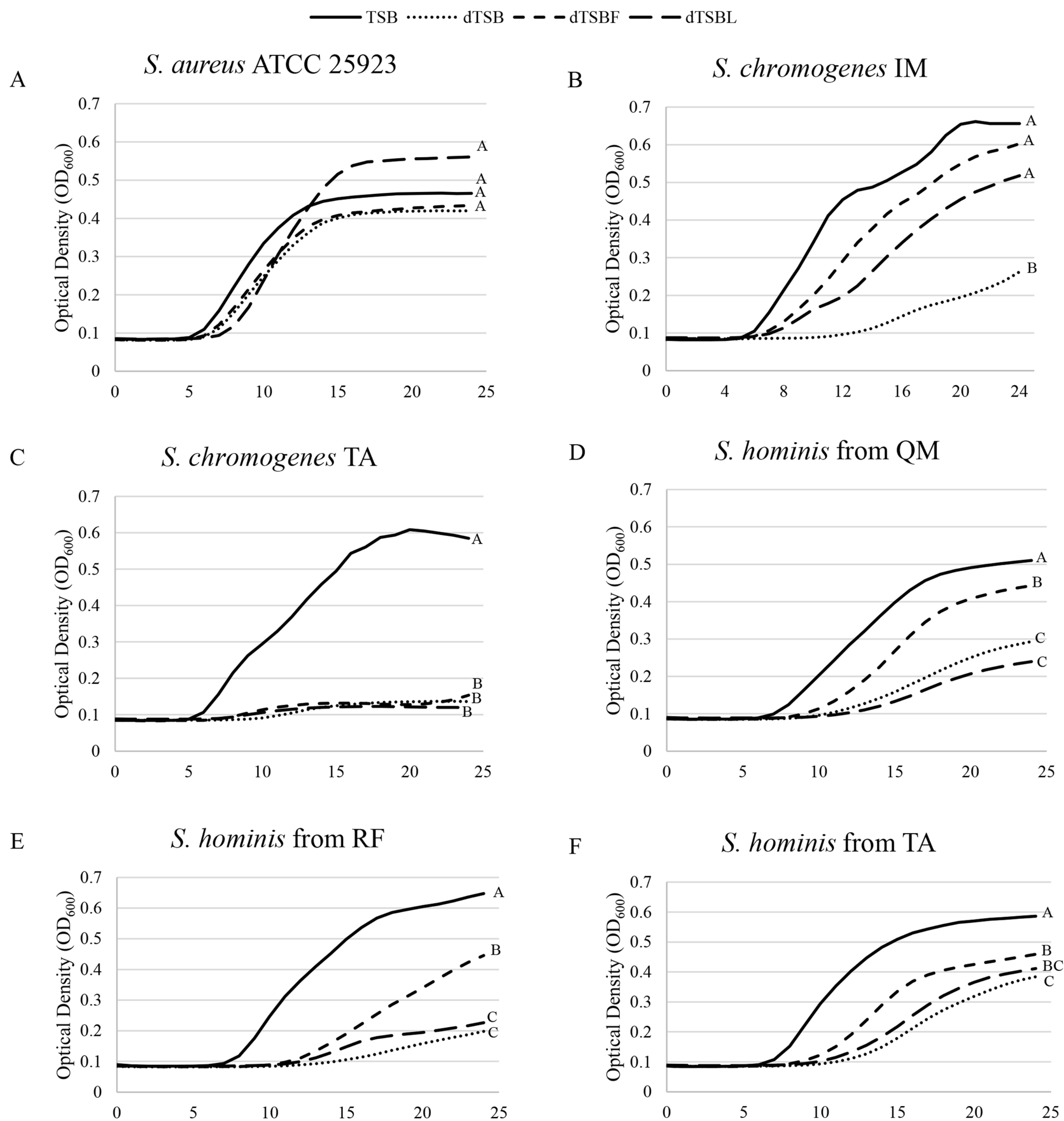



**Figure 2.** Overview of growth (optical density at 600 nm; $OD_{600}$) over 24 h in different media: tryptic soy broth (TSB), deferrated tryptic soy broth (dTSB), dTSB with ferritin from equine spleen (dTSBF), and dTSB with human recombinant lactoferrin (dTSBL) of (A) *Staphylococcus aureus* ATCC 25923, (B) *Staphylococcus chromogenes* "IM" (causing chronic IMI), (C) *S. chromogenes* "TA" (from teat apex of a heifer), (D) *Staphylococcus hominis* from quarter milk (QM), (E) *S. hominis* from rectal feces (RF), and (F) *S. hominis* from teat apices (TA). All experiments were performed in duplicate. Different letters within each figure (A–C) indicate significant differences when applying the Bonferroni correction between growth media within strains ($P \leq 0.05$).

**Table 2.** Linear mixed regression model for the log-transformed area under the curve

| Variable | β[1] | SE | LSM | *P*-value |
|---|---|---|---|---|
| Intercept | 0.88 | 0.10 | | <0.0001[2] |
| Medium[3] | | | | <0.0001[2] |
| TSB | Referent | | 0.90 | |
| dTSB | −0.06 | 0.08 | 0.54 | <0.0001[4] |
| dTSBF | −0.05 | 0.07 | 0.70 | <0.0001[4] |
| dTSBL | 0.02 | 0.07 | 0.61 | <0.0001[4] |
| Strain | | | | 0.0244[2] |
| *Staphylococcus aureus* ATCC 25923 | Referent | | 0.85 | |
| *Staphylococcus chromogenes* IM[5] | 0.08 | 0.13 | 0.77 | 0.45[4] |
| *S. chromogenes* TA[6] | 0.04 | 0.13 | 0.54 | 0.01[4] |
| *Staphylococcus hominis* from QM[7] | −0.07 | 0.10 | 0.63 | 0.01[4] |
| *S. hominis* from RF[8] | 0.02 | 0.11 | 0.60 | 0.01[4] |
| *S. hominis* from TA[9] | 0.05 | 0.11 | 0.72 | 0.16[4] |
| Medium × strain[10] | | | | <0.0001[2] |

[1]Regression coefficient.

[2]Overall *P*-value for fixed effect.

[3]TSB = tryptic soy broth; dTSB = deferrated tryptic soy broth; dTSBF = dTSB with ferritin from equine spleen; dTSBL = dTSB with human recombinant lactoferrin.

[4]*P*-value for differences of LSM.

[5]*S. chromogenes* isolate causing chronic IMI (Supré et al., 2011).

[6]*S. chromogenes* isolate from a teat apex of a heifer (De Vliegher et al., 2004).

[7]*S. hominis* isolates representing different strains from quarter milk only.

[8]*S. hominis* isolates representing different strains from rectal feces only.

[9]*S. hominis* isolates representing different strains from teat apices only.

[10]See Figure 2.

which was highly unexpected as these species are typically the predominant NAS species isolated from bovine mammary quarter milk samples (Gillespie et al., 2009; Pyörälä and Taponen, 2009; Nyman et al., 2018). The overall prevalence of NAS at the quarter level in the herd was 77.5%, which is 4 to 7 times higher than the prevalence of NAS (as a group) reported in other studies (Tenhagen et al., 2006; Taponen et al., 2008; Gillespie et al., 2009; Sampimon et al., 2009; Supre et al., 2011; Condas et al., 2017; Wuytack et al., 2020a). Differences in prevalence of NAS have to be attributed to herd- and region-specific distribution of NAS (Supre et al., 2011; De Visscher et al., 2017), the method used for NAS identification (phenotypic versus genotypic methods; Sampimon et al., 2009; Condas et al., 2017; Wuytack et al., 2019), the criteria for diagnosing a sample as NAS (Sampimon et al., 2009), herd management (Fox et al., 1995), and the study design (Condas et al., 2017). Although mannitol salt agar improves the differentiation of phenotypically different colonies from NAS (De Visscher et al., 2013) and the colonies selected from the same sample for further processing and identification were phenotypically unique, we recognize the small possibility that some isolates from the same sample belonging to the same NAS species were multiple colonies from the same clone.

*Staphylococcus hominis* was one of the most prevalent NAS species isolated from milk samples, with a prevalence of 4.2%, comparable to our previous study (Wuytack et al. (2020a). However, Condas et al. (2017) and Nyman et al. (2018) reported a prevalence of <1% and Gillespie et al. (2009) a prevalence of 2%. The quarter milk samples for this study were directly plated on mannitol salt agar to find *S. hominis* when present, and a sample was considered positive for NAS when at least 1 colony was found, meaning that samples were included even if >2 species were identified. This would explain, at least partially, the higher prevalence of NAS overall and specifically of *S. hominis* in this herd.

When comparing the RAPD types from QM, RF, and TA, a relatively high degree of polymorphism (genetic diversity) was found among the isolates, substantiating earlier studies reporting on *S. hominis* as a genetically highly diverse species (Jenkins et al., 2019; Wuytack et al., 2020b). Despite reports of *S. hominis* as being highly adaptable to various conditions (Jenkins et al., 2019; Wuytack et al., 2020b), the strains appear to be habitat- or site-specific, considering that only 1 of the 18 RAPD fingerprint types was retrieved from both RF and TA and 2 from both TA and QM. None of the RF RAPD types were recovered from QM, suggesting that these isolates are probably not adapted to the mammary gland, at least not in this herd, corroborating results of an earlier study by Wuytack et al. (2020b) that used a smaller sample size of *S. hominis* from QM. The latter finding could be attributed to the limited number of isolates originating from one herd only or

could confirm earlier studies reporting that strains are host- and niche-specific.

We initially applied human recombinant lactoferrin on the bovine NAS isolates for the phenotypical iron test in our study because of the degree of iron saturation and associated cost; however, we decided to also perform the test with bovine lactoferrin to ensure the suitability of the previous preparation (data not shown). Comparable growth curves were found compared with human recombinant lactoferrin. Although species differences exist in receptor-mediated iron acquisition, siderophore-mediated processes are known to acquire iron from iron-bound protein regardless of species (Modun et al., 1998), and bovine and human lactoferrin forms have high sequence homology with similar antibacterial effects (Teraguchi et al., 2004; Rosa et al., 2017; Kell et al., 2020). Additionally, the incubation conditions of the phenotypical iron test bordered on anaerobic because the microplates plates were sealed throughout the 24-h incubation period. Decreased oxygen availability can suppress the transcription of iron-regulated genes (Ledala et al., 2014) and influence the overall growth of some NAS species, as seen in a study on the growth of different NAS species in iron-poor or iron-rich medium in (an)aerobic conditions by Wuytack et al. (2019). Still, because all isolates were tested under the same conditions, we believe the comparisons between isolates remain valid. During an IMI, the oxygen concentration in the mammary gland decreases (Wittek et al.,2019), making the results of our study even more relevant.

*Staphylococcus aureus* is able to grow in the presence of extremely low (0.04 $\mu M$) iron concentrations (Trivier and Courcol, 1996), and its success as a pathogen in causing persistent IMI is partly attributable to its ability to exploit multiple sources of iron (Diarra et al., 2002; Sheldon and Heinrichs, 2015). Accordingly, we showed the ability of this bacterium, strain ATCC 25923, to grow similarly regardless of the medium. Later, we tested an additional laboratory *S. aureus* strain 8325-4 (Toledo-Silva et al., 2021) under the same conditions (using human recombinant lactoferrin and ferritin from equine spleen) and achieved similar results (data not shown). In contrast, in a study by Diarra et al. (2002), lactoferrin inhibited the growth of all tested *S. aureus* strains. However, staphylococci have shown variable and strain-dependent susceptibility to both lactoferrin and ferritin. In accordance with a phenotypical iron test performed by Wuytack et al. (2019), *S. chromogenes* IM showed small differences in growth on different growth media, in contrast to *S. chromogenes* TA. This suggests that this specific strain of *S. chromogenes* (IM) competes better with ferritin and lactoferrin for iron and can, to some extent, utilize these iron proteins as sources of iron. Beuckelaere et al. (2021) was able to successfully colonize dry udder quarters with *S. chromogenes* IM, an involution period of the mammary gland when lactoferrin is present in high concentrations (Welty et al., 1976; Galfi et al., 2016). This finding substantiates our in vitro work presented here and the fact that this particular strain was isolated from a multiparous cow with a persistent IMI lasting over 300 d (Piessens et al., 2011) and is considered to be a true udder-adapted strain (Wuytack et al., 2019). On the other hand, *S. chromogenes* TA was unable to compete with ferritin and lactoferrin for iron. This strain was isolated from the TA of a heifer with no signs of mastitis (De Vliegher et al., 2004), and behaved very differently compared with *S. chromogenes* IM in several in vitro and in vivo experiments conducted over the years (Breyne et al., 2015; Piccart et al., 2016; Souza et al., 2016). The results of the phenotypical iron test combined with the aforementioned studies on the in vitro and in vivo traits of both strains indicate, to a certain degree, that the improved ability to withstand and thrive in the mammary gland is strain-dependent and reflects their habitat and epidemiological behavior; studying NAS at the species level without considering strain differences is no longer sufficient.

The ability of *S. hominis* from QM, RF, and TA to grow to some degree in the presence of ferritin could elucidate the opportunistic nature of *S. hominis* (Soroush et al., 2017); however, none of the *S. hominis* isolates were able to significantly utilize lactoferrin as an iron source, suggesting that this species likely does not favor the mammary gland. This finding needs to be substantiated in further research by performing repeated monthly samplings in order to define a stringent IMI definition, and by measuring the SCC (Supre et al., 2011) to help determine the effects of *S. hominis* on udder health. Although *S. hominis* is usually considered part of the normal human skin microbiota, cows also harbor *S. hominis* in their gastrointestinal tract. Wuytack et al. (2020b) suggest that some NAS species originating from RF can cause IMI. However, our tested *S. hominis* strains from RF were unable to significantly use lactoferrin for growth recovery, making this niche an unlikely source of IMI, a hypothesis supported by the fact we did not find the same strains in the 3 different habitats.

Overall, these observations indicate that *S. hominis* is a species of lower virulence that is less likely to colonize the mammary gland compared with *S. chromogenes*; moreover, our results explain why the growth of *S. aureus* appeared to be unencumbered in different media. Further research on characterizing the genes responsible for iron uptake in different NAS species and

strains should be explored to elucidate their relevance and virulence capacity in causing IMI.

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