J. Dairy Sci. 106:7893–7907
https://doi.org/10.3168/jds.2022-22995

Intramammary infections and risk factors in freshly calved heifers in Swedish dairy herds

K. Persson Waller,[1]* Å. Lundberg,[2] S. Börjesson,[3] and A.-K. Nyman[2,4]
[1]Department of Animal Health and Antimicrobial Strategies, National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden
[2]Växa, SE-104 25 Stockholm, Sweden
[3]School of Health Science, Örebro University, Örebro, SE-701 82, Sweden
[4]Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), SE-750 07 Uppsala, Sweden

ABSTRACT

Subclinical mastitis can be common among freshly calved heifers (FCH), but the prevalence differs between herds, possibly due to variation in risk factors. The aims of this observational study were to identify differences in occurrence of intramammary infection (IMI) in FCH between herds with documented good or poorer first-parity udder health based on cow somatic cell count (CSCC) in early lactation, and to study herd differences in animal factors important for udder health, such as udder and hock skin lesions and animal cleanliness. Three groups of herds were included: those with high proportions of FCH with low CSCC (≤75,000 cells/mL) at the first 2 milk recordings after calving (LL), herds with high proportions of FCH with high CSCC (>100,000 cells/mL) at the first and low CSCC at the second recording (HL), and herds with high proportions of FCH with high CSCC at both recordings (HH). Thirty-nine herds (13 LL, 11 HL, 15 HH) were visited 3 times during a 12-mo period for observation of cleanliness and hock lesions, and sampling of udder and teat skin using swab cloths of milk-fed calves, early-pregnant heifers, and late-pregnant heifers. In 25 (9 LL, 9 HL, 7 HH) udder quarter samples from colostrum and milk on d 3 to 4 after calving were taken by the farmers from FCH during one year. The farmers also provided information on calving (individual or group), use of restraint and oxytocin at milking, and presence of teat and udder skin lesions. Bacterial growth in swab samples and quarter samples was investigated by culturing, and a selection of isolates was genotyped using whole-genome sequencing. Cleanliness, hock and udder skin lesions other than udder-thigh dermatitis, and growth of bacteria in swab samples did not differ between herd groups. It was more common that FCH from LL herds, compared with FCH in HH and HL herds, calved in a group of animals. Use of restraint at milking was more common in LL herds than in HH herds, whereas presence of udder-thigh dermatitis was lowest in LL herds. Specific infection was found in 14% of 5,593 quarter samples from 722 FCH. The most common IMI was *Staphylococcus chromogenes*. Growth of *Staphylococcus simulans* was more common in HH than in LL and HL herds. In colostrum samples, *Staphylococcus haemolyticus* was more common in HL and HH than in LL herds. The proportion of quarters with the same specific infection at both samplings was higher in HH than in LL herds and tended to be higher in HH than in HL herds. The proportion of quarters with *Staph. chromogenes* IMI at both samplings tended to differ between herd groups and was highest in HH herds. Whole-genome sequencing found the same sequence type of *Staph. chromogenes* and *Staphylococcus aureus* in both samples in almost all quarters with the same infection at both samplings. The differences in IMI between herd groups were in line with the higher somatic cell count in HH herds. The reasons for the predominance of *Staph. chromogenes* IMI in FCH need further studies.

Key words: mastitis, cleanliness, skin lesions, whole-genome sequencing, *Staphylococcus chromogenes*

INTRODUCTION

In a previous study on udder health in early lactation, we found that many dairy cows have bacterial IMI already at calving or within a few days of calving (Lundberg et al., 2016). Furthermore, such infections were associated with subclinical mastitis, diagnosed by increased cow SCC in early lactation, but that study did not focus on first-parity cows. In a recent study, however, we found that elevated cow SCC was common in early lactation in Swedish first-parity cows and that the incidence of mastitis varied between herds (Persson Waller et al., 2020). The elevated SCC was probably due to IMI at or close to calving, but specific studies on presence of IMI at or around calving in first-parity cows have not previously been performed in Sweden. Publications from other countries, however, show that NAS

Received November 7, 2022.
Accepted April 7, 2023.
*Corresponding author: karin.persson-waller@sva.se

are common IMI in freshly calved heifers (De Vliegher et al., 2012; De Buck et al., 2021).

Identification of IMI at calving may give an indication of the distribution of mastitis-associated pathogens in a herd. Moreover, IMI caused by certain pathogens, especially those considered contagious, may also explain why some herds have poorer udder health among freshly calved heifers than others. In addition, the teat and udder skin can be a source of mastitis-associated pathogens such as staphylococci and streptococci (e.g., Svennesen et al., 2019; De Buck et al., 2021), and poor hygiene in the close environment of dairy cows can be an important risk factor for IMI (reviewed by McDougall et al., 2009; De Vliegher et al., 2012). However, the importance of cleanliness among calves and heifers for the risk of IMI and poor udder health of freshly calved heifers is less well understood.

Other factors that have been associated with poor udder health or skin colonization of mastitis-associated pathogens in cows are, for example, udder edema, skin lesions on teats, udders, and hocks, and having nonfunctional udder quarters (Capurro et al., 2010; De Vliegher et al., 2012; Persson Waller et al., 2014). Moreover, different types of stress, for example due to management, may also have negative effects on udder health (Svensson et al., 2006; Ivemeyer et al., 2018).

As already mentioned, we identified herd differences in early lactation udder health of first-parity cows (Persson Waller et al., 2020). Based on those findings we performed a study comparing dairy herds with documented good or poorer first-parity udder health based on cow SCC, and identified several success and risk factors in calf and heifer management that are important for udder health in early-lactation first-parity cows (Persson Waller et al., 2021). In association with that study we also performed an in-depth investigation of freshly calved heifers (**FCH**) in a subset of the herds. The main aims of that investigation, presented in the present paper, were to identify differences in occurrence and bacterial etiology of IMI in FCH between herds that have good or poorer udder health in this group of cows based on the cow SCC (**CSCC**) in early lactation, and to study differences between these herds in animal factors of importance for udder health, such as presence of udder and hock skin lesions and animal cleanliness.

MATERIALS AND METHODS

Herd Selection

This observational study was part of the same project as a previously published study on the risk and success factors associated with udder health of FCH (Persson Waller et al., 2021). The study was performed in commercial farms and, according to Swedish laws, did not require ethical approval. In the current study, a subgroup of the herds was selected for a one-year study involving farmer-performed milk sampling of FCH, and animal registrations and samplings, as described below, performed by specially trained technicians at 2 additional herd visits.

As described in detail by Persson Waller et al. (2021) a selection of herds was made in October and November 2017. All herds affiliated to the Swedish Official Milk Recording Scheme (SOMRS) in 2014 to 2016, having at least 60 cows/yr, production data from all 3 years, and at least 10 first-parity cows per year with their first test milking 5 to 35 d after calving and their second test milking 20 to 40 d after the first test milking for each of the 3 years, were eligible. In those herds, all first-parity cows were categorized based on their SCC at the first 2 test milkings after calving as low-low (**LL**), low-high, high-high (**HH**), high-low (**HL**), or inconclusive (i.e., a CSCC in between low and high in one or both of the 2 test milkings). For example, LL means low SCC at both test milkings, and HH means high SCC at both test milkings. Low CSCC and high CSCC were defined as $\leq$75,000 cells/mL and >100,000 cells/mL, respectively (Persson Waller et al., 2020). After categorization of the primiparous cows, the proportions of LL, HL, and HH cows within each herd were calculated. Herds having above-median proportion of LL, HL, and HH cows during year 1 of herd selection and above the third quartile proportion of LL, HL, and HH cows during years 2 and 3 of selection of LL, HL, or HH cows were considered to be LL, HL, or HH herds (Persson Waller et al., 2021). Such herds were contacted by telephone until a convenient sample of a maximum of 30 herds per LL, HL, and HH category had accepted to participate in the previous study, in which specially trained field technicians visited each herd (herd visit 1) once in January to March or April 2018. During the telephone conversation, farmers having between 100 and 250 cows/herd were also asked to participate in a study during a 12-mo period, involving aseptic quarter milk sampling of FCH at 2 occasions (colostrum and d 3–4 after calving). Moreover, these herds would receive 2 additional herd visits by the technicians, in late spring (herd visit 2) and late autumn (herd visit 3), for animal registrations and skin swab samplings of the udders for bacteriological culturing. The aim was to include 15 herds from each herd group (LL, HL, HH) in this part of the study.

Questionnaires

A preformed questionnaire on housing and management was produced as described previously (Persson

Waller et al., 2021), and this questionnaire was used at the first herd visit. In addition, a milk sample protocol and a second questionnaire used for the additional herd visits were produced specifically for the present study.

The milk sample protocol, to be used by the farmers and herd personnel, contained questions on cow identification number, calving date, sampling dates (colostrum or milk), clots in milk (yes or no), presence of nonlactating quarters (yes or no), antibiotic treatment during the latest 14 d (yes or no), type of calving box (single or group), presence of warts or wounds on teats (yes or no), presence of udder edema (yes or no), presence of udder-thigh dermatitis (yes or no), presence of udder cleft dermatitis (yes or no), use of oxytocin before milking (yes or no), and use of restraints at milking (yes or no), as well as type of restraint method if used. The milk sample protocol also contained detailed instructions on how to perform aseptic milk sampling with a link to an online video. Pictures of udder edema, udder-thigh dermatitis, and udder cleft dermatitis were provided. The protocol, video link, and pictures can be provided by the first author upon request.

The questionnaire for herd visits 2 and 3, performed by the technicians, included the same questions related to group registrations of group-housed milk-fed calves (cleanliness), early-pregnant heifers (first trimester; cleanliness), and late-pregnant heifers (last 2 mo; cleanliness, hock lesions), as in the questionnaire used at herd visit 1 (Persson Waller et al., 2021). The questionnaire for herd visits 2 and 3 also included questions on individual cleanliness and hock lesions of late-pregnancy heifers. The questionnaire was tested by the technicians involved before use in the herds. The questionnaire (in Swedish), containing 18 questions of which 8 were included in this study, can be provided by the first author.

Herd Visits

The technicians and farmers were not informed about which herds belonged to which herd group. At herd visit 1, technicians gave instructions on aseptic milk sampling and delivered material for the samplings. Herd visits 2 and 3 were performed by the same technicians as herd visit 1. All technicians had been trained in the second questionnaire and in skin sampling for bacteriological examination. Herd visit 2 was performed in late spring, before animals were let out on pasture, whereas herd visit 3 was performed in late autumn, when all animals were housed. According to Swedish law, all dairy heifers and cows have to be let out on pasture during a specified period from April to October. The specific period varies, however, depending on the part of the country (North, Middle, South).

At herd visits 2 and 3 group-housed milk-fed calves, early-pregnant heifers, and late-pregnant heifers were observed at group level as described previously (Persson Waller et al., 2021). In short, the cleanliness of the animals and their close environment was scored (1–3) in all age groups, and presence of hock hair loss or hock skin wounds was scored (1–3) in late-pregnant heifers. In addition, individual cleanliness scores (1–3) and presence of hock hair loss (yes or no) or hock skin wounds (yes or no) were registered for up to 9 late-pregnant heifers (last 2 mo of pregnancy) in each herd. The cleanliness of individual heifers was evaluated by observing the rear of the animal above the hocks and was scored as follows: 1 = manure on more than one-third (in total) of udder, rear, and flanks; 2 = at least 3 areas contaminated with dry manure >10 cm in diameter or area of udder, rear, and flanks; 3 = the animal is clean or has only flecks of manure on udder, rear, and flanks.

At herd visits 2 and 3, 3 calves, 3 early-pregnant heifers, and 6 late-pregnant heifers with visibly clean udders were chosen, based on convenience and availability, for swab sampling of the udder skin. For each animal, the technicians put on new clean gloves before sampling. Animals were restrained by the farmer or farm personnel, and a dry paper towel was used to remove dust and dry bedding from the udder skin. Sampling was performed by using a premoistened sterile swab cloth in a ready-to-use surface sampling kit (SodiBox) for each animal. The cloth was removed from the bag and unfolded, and all 4 teats, especially the teat ends, were wiped with the towel. When sampling calves, both teat and udder skin were sampled. The cloth was folded and returned to the plastic bag of the kit, which was sealed carefully. At all times, care was taken to avoid contaminating the cloth. The sample bags were labeled with animal identification number, age group, and herd number. The sample bags from each age group were placed in a clean plastic bag. After finishing the questionnaire and skin sampling, the technicians reminded the farmer about taking milk samples and collected the frozen milk samples that had been taken at the herd. Those milk samples were packed with freezer packs in an insulated box together with the swab samples (care was taken to avoid direct contact between these samples and the freezer packs). Then, the samples were sent to the Swedish National Veterinary Institute (**SVA**; Uppsala, Sweden) by overnight courier, and the questionnaire was sent to the project leader via postal mail.

Milk Sampling, Milk Sample Protocol, and Transportation of Milk Samples

Farmers were instructed by the technicians on how to perform aseptic milk sampling at herd visit 1. The farmers were asked to take quarter milk samples from all lactating quarters of at least 50% of the first-parity cows calving evenly spread over a 12-mo period. From each FCH, quarter milk samples were taken twice, once within 24 h of calving and once 3 to 4 d after calving. The milk samples were frozen at the farm as quickly as possible after collection. For each sampled animal, the farmer recorded information in the milk sample protocol described above.

Milk samples were stored frozen at the farm until herd visits 2 and 3, when the technicians collected the milk samples and protocols and sent them to SVA as described above. When needed, and at the end of the 12-mo sampling period, each farmer was contacted by the first author, and an insulated box with freezer packs and instructions was sent to the farmer for overnight transportation of milk samples to SVA.

At arrival at SVA, the condition of the milk samples (i.e., room temperature, cold but thawed, frozen) was registered, and the samples were frozen at −20°C until bacteriological examination.

Bacteriological Examination of Skin Swab Samples

After arrival at SVA, the swab samples were stored at 4°C until further processing within 4 h. Thirty milliliters of sterile saline (SVA) was added to each sample bag. Then, each bag was placed in a stomacher for 30 s at 230 rpm. Thereafter, the swab cloth in the bag was squeezed to free as much saline as possible from the swab cloth. Approximately 10 mL of the saline were transferred to a sterile glass tube. For each herd and sampling occasion, the samples from calves were pooled into one sample by transferring 2 mL from each sample to a new test tube. The same procedure was performed for samples from heifers in early pregnancy. The samples from heifers in late pregnancy were pooled into 2 new test tubes (maximum 3 samples/tube). After thorough mixing of the pooled samples, each sample was diluted 1:10 (0.5 mL sample and 4.5 mL saline) 4 times. After thorough mixing of the tubes, 100 μL from each dilution step was spread on 5% bovine blood agar containing 0.05% esculin (SVA), and the plates were incubated at 37°C for 48 h. Growth was evaluated by selecting an agar plate (i.e., dilution step) suitable for counting of colony-forming units. The total number of colony types and the total number of colony-forming units per plate was counted, and the concentration of colony-forming units per milliliter of the original saline solution was calculated. If growth of staphylococci or streptococci was suspected based on colony morphology, bacterial material from such colonies was spread on 5% bovine blood agar containing 0.05% esculin (SVA), and the plates were incubated for 24 to 48 h. If growth of colonies in pure culture was detected, the colonies were identified using MALDI-TOF on a MALDI Biotyper (Bruker Daltonics). Isolates of staphylococci and streptococci were frozen in broth with glycerol at −70°C.

Bacteriological Examination of Milk Samples

The milk samples were thawed at 4°C overnight, placed in room temperature for 1 h, and mixed thoroughly. Then, 10 μL of milk was spread on 5% bovine blood agar plates containing 0.05% esculin (SVA). The agar plates were incubated at 37°C for a total of 36 h. Bacterial growth was evaluated after 16 and 36 h of incubation. After 16-h incubation, the number of colony types was recorded. Moreover, the number of different types of colonies, the number of colonies per suspected udder pathogen (if more than 50 cfu/pathogen, the number was given as >50 cfu), and the total number of other colonies (if more than 50 cfu, the number was given as >50 cfu) were recorded after 36-h incubation. At the same occasion, colonies of suspected udder pathogens were selected and cultured on 5% bovine blood agar plates containing 0.05% esculin. The agar plates were incubated at 37°C for a total of 36 h. Colonies of potential udder pathogens were investigated using MALDI-TOF. Isolates were frozen in trypticase soy broth with 15% glycerol at −70°C. A milk sample was considered positive if at least 1 cfu of *Staphylococcus aureus*, *Streptococcus dysgalactiae*, or *Streptococcus uberis* was detected. For other bacteria, a positive classification required ≥3 cfu. Growth of 1 bacterial agent was evaluated as pure culture, growth of 2 types of colonies as mixed infection, and growth of 3 or more colony types as contamination. In line with NMC (2017) guidelines, a sample was also identified as being positive for a specific pathogen if a few colonies of different types were found together with numerous colonies of the specific pathogen.

Whole-Genome Sequencing of Bacterial Isolates

If at least 3 milk isolates of a bacterial species were found within a herd, those isolates were eligible for whole-genome sequencing. Moreover, if at least 3 skin isolates of one of the species found in milk were also found in those herds, these isolates were also eligible. In addition, isolates from udder quarters classified as having persistent infection (see section on Statistical Analyses) were also eligible for whole-genome sequenc-

ing. Based on these criteria, 175 isolates from milk samples (18 *Staphylococcus aureus*, 133 *Staphylococcus chromogenes*, 24 *Staphylococcus haemolyticus*) and 25 isolates from skin swab samples (4 *Staph. chromogenes*, 21 *Staph. haemolyticus*) were selected. The isolates were thawed and cultured on 5% bovine blood agar containing 0.05% esculin (SVA). DNA was extracted by suspending one loopful of colonies in 500 μL of nuclease-free water, and the bacterial cells were disrupted by bead-beating with 0.1-mm zirconia-silica beads in a FastPrep24 homogenizer (MP Biomedicals) for 6 min at 6.5 m/s. The DNA was subsequently extracted from the suspension using an Indimag Pathogen Kit (Indical Bioscience) together with a Maelstrom-9600 (TANBead Inc.) and eluted with nuclease-free water.

Sample DNA concentrations were determined using a Qubit HS DNA Kit (Life Technologies), and the samples were then submitted to SciLifeLab Clinical Genomics (Stockholm, Sweden) for sequencing by Illumina-based technology. To check for potential contamination of isolates and confirmation of bacterial species the reads were checked with Kraken using the MiniKraken 8 GB database (October 19, 2017; https://ccb.jhu.edu/software/kraken/MANUAL.html). The raw reads were then trimmed using Trimmomatic 0.36 (Bolger et al., 2014), and genome assembly was performed using SPAdes v.3.11.1 (Prjibelski et al., 2020) with "—careful" parameter, followed by Pilon v.1.22 (Walker et al., 2014). Seven-loci multilocus sequence typing (**MLST**; *Staph. aureus* and *Staph. chromogenes* schemes available from https://pubmlst.org/) and whole-genome MLST (**wgMLST**) were performed and visualized in SeqSphere + software (version 8.3; Ridom GmbH). Because both *Staph. chromogenes* and *Staph. haemolyticus* lack defined core genome MLST schemes, ad hoc core genome MLST schemes were created in SeqSphere+ with default settings using the genomes NZ_CP031274.1 and NZ_CP035291.1, respectively, as seed genomes. This resulted in a scheme of 1,710 genes and 437 accessory genes for *Staph. chromogenes* and a scheme of 1,847 genes and 415 accessory genes for *Staph. haemolyticus*.

Data Handling and Editing

The implementation of the herd visits and the milk sampling were monitored by the first author, and reminders were sent when needed. All data from the questionnaires, milk sample protocols, and laboratory analyses were transferred to Excel files by the first author. The data were then transferred to Stata (release 15.1; StataCorp LLC), which was used for data editing and analyses.

Two of the original 41 herds were excluded because they opted to withdraw. The remaining 39 herds had 3 visits each. For each age group, scores on cleanliness and hock lesions from these herds (13 LL, 11 HL, 15 HH) were summarized within each herd group (LL, HL, HH). Likewise, total colony-forming units per milliliter and number of colony types from skin swab samples from each age group in these herds were summarized per herd group. The number of herds with swab samples from 2 samplings varied somewhat between the age groups (milk-fed calves = 39; heifers in early pregnancy = 35, heifers in late pregnancy = 38).

Before analyzing differences between herd groups in bacterial results of milk sample bacteriology and in cow factors registered in the milk sample protocol, the number of first-parity cows sampled in each herd was compared with the total number of first-parity cows that had calved in the herd over the year of sampling. Based on this data, 14 out of 39 herds were excluded because they had sampled too few (<35%) of these cows. Thus, 25 herds (9 LL, 9 HL, 7 HH) remained for statistical analyses of bacterial findings and cow factors.

Statistical Analyses

All statistical analyses were performed using Stata (release 15.1; StataCorp LLC). Descriptive statistics were used to summarize the results from the questionnaires, protocols, and laboratory analyses. When categories for a question had few or no observations, one or more categories were merged when possible.

To investigate associations between registrations of cleanliness and hock lesions (hair loss or skin wounds on hocks) registered by the technicians at all 3 herd visits (the visits being the observational level), and being a LL, HL, or HH herd (outcome), we used univariable multinomial logistic regression models with the standard errors adjusted for clustering within herd (mlogit command in Stata, with "cluster" specified to be used as variance estimator). The clustering was used due to repeated observations (observations made at all the 3 visits) within herd of the explanatory variables (cleanliness, hair loss, or skin wounds on hock).

To investigate associations between bacterial growth in udder skin swab samples (bacterial findings on skin, log cfu, and diversity), taken by the technicians at all 3 herd visits (visits being the observational level), and being a LL, HL, or HH herd (outcome), we used univariable multinomial logistic regression models with the standard errors adjusted for clustering within herd (mlogit command in Stata, with "cluster" specified to be used as variance estimator). The clustering was used due to repeated samplings (samplings made at all the 3

visits) within herd of the explanatory variables (bacterial findings on skin, log cfu, and diversity).

To investigate associations between cow variables registered in the milk sample protocol (presence of nonfunctional quarters, whether the cow had been treated with antibiotics during the 14 d before sampling, type of calving box, presence of teat warts, presence of teat wounds, presence of udder edema, presence of udder-thigh dermatitis, presence of udder cleft dermatitis, use of oxytocin before milking, and use of restraint at milking), registered for each sampled cow (cow being the observational level), and being a LL, HL, or HH herd (outcome), we used univariable multinomial logistic regression models with the standard errors adjusted for clustering within herd (mlogit command in Stata, with "cluster" specified to be used as variance estimator). The clustering was used to adjust for cows being more similar within herd than between herds.

To investigate associations between bacterial growth in colostrum and milk samples at quarter level and being a LL, HL, or HH herd (outcome), we used multivariable multinomial logistic regression models including day of sampling (at calving or 3–4 d after calving) as a fix factor. The bacterial findings were dichotomized as presence or absence of bacterial finding in total [bacterial finding: yes (isolation of a specific bacteria) or no (no growth)] and for each bacterial finding (e.g., finding of *Staph. aureus*: yes or no). Contaminated samples were not included in the analyses. The same regression analysis method, with being a LL, HL, or HH herd as outcome, but univariable, was also performed separately for samples taken at calving and samples taken at d 3 to 4 after calving.

To investigate associations between clots in milk (explanatory variable) and bacterial findings (outcome) in quarter milk samples, a univariable mixed-effect logistic regression analysis, with herd and cow as random factors to adjust for cows within a herd being more similar than cows between herds, and quarters within a cow being more similar than quarters between cows, were used with an independent variance-covariance structure of the random effects.

To investigate associations between bacterial findings in quarters at calving and bacterial findings on d 3 to 4, where findings at d 3 to 4 was the outcome variable and findings at calving was the explanatory variable, univariable mixed-effect logistic regression analyses, with herd and cow as random factors to adjust for cows within a herd being more similar than cows between herds, and quarters within a cow being more similar than quarters between cows, were used with an independent variance-covariance structure of the random effects.

Table 1. Numbers of group or individual observations with cleanliness scores 1 to 3 in milk-fed calves, heifers in early pregnancy, and heifers in late pregnancy in herds with a large proportion of first-parity cows with low SCC at first and second milk recording after calving (LL herds, n = 13), a large proportion of first-parity cows with high SCC at first milk recording and low SCC at the second milk recording (HL herds, n = 11), or a large proportion of first-parity cows with high SCC at first and second milk recording (HH herds, n = 15) after calving[1]

Score[2]	LL	HL	HH	Total (%)	*P*-value
Milk-fed calves (group)[3]					0.87
1	0	0	0	0 (0)	
2	6	8	11	25 (9)	
3	87	70	98	255 (91)	
Heifers in early pregnancy (group)[3]					0.57
1	0	0	2	2 (1)	
2	19	13	18	50 (30)	
3	36	35	41	112 (68)	
Heifers in late pregnancy (group)[3]					0.61
1	1	0	3	4 (2)	
2	13	10	19	42 (25)	
3	39	36	45	120 (72)	
Heifers in late pregnancy (individual)[4]					0.30
1	9	2	15	26 (6)	
2	22	19	33	74 (17)	
3	114	95	114	323 (76)	

[1]*P*-values are from univariable multinomial logistic regression analyses of associations between type of herd and each variable.

[2]Score 1 = very dirty, score 2 = dirty, score 3 = clean. For details on scoring of groups of heifers and individual heifers, see Materials and Methods.

[3]Summary of 3 herd visits (winter, late spring, late autumn).

[4]Summary of 2 herd visits (late spring, late autumn).

To investigate associations between udder quarters being healthy (culture-negative d 0 or d 3–4), infected at both samplings (the same bacterial species in the quarter d 0 and d 3–4), or newly infected (culture-negative d 0 and specific infection d 3–4) and being a LL, HL, or HH herd, univariable multinomial logistic regression models, with the standard errors adjusted for clustering within herd, were used. Only culture-negative quarters or quarters with specific infection at d 0 and 3 to 4 were included. Quarters where milk samples were considered contaminated at one or both samplings were not included in the analyses.

RESULTS

Scoring of Animal Cleanliness and Hock Lesions at Herd Visits

A summary of the cleanliness scores of group observations of animals at 3 herd visits in the 3 age groups and of individual heifers in late pregnancy is presented in Table 1. The results did not differ significantly between the herd groups. Most of the animals were considered clean (score 3) in all age groups. The proportion of

Table 2. Numbers of group or individual observations with hock lesions (hair loss or skin wounds) in heifers in late pregnancy in herds with a large proportion of first-parity cows with low SCC at first and second milk recording after calving (LL herds, n = 13), a large proportion of first-parity cows with high SCC at first milk recording and low SCC at the second milk recording (HL herds, n = 11), or a large proportion of first-parity cows with high SCC at first and second milk recording (HH herds, n = 15) after calving[1]

Score	LL	HL	HH	Total (%)	*P*-value
Hair loss on hocks of heifers in late pregnancy (group)[2]					0.52[3]
1	0	4	4	8 (5)	
2	5	5	10	20 (12)	
3	47	37	52	136 (83)	
Skin wounds on hocks of heifers in late pregnancy (group)[2]					0.37[3]
1	0	1	0	1 (0.6)	
2	2	5	5	12 (7)	
3	50	40	61	151 (92)	
Hair loss on hocks of heifers in late pregnancy (individual)[4]					0.87
Yes	4	5	6	15 (4)	
No	141	111	147	399 (96)	
Skin wounds on hocks of heifers in late pregnancy (individual)[4]					NA[5]
Yes	0	4	0	4 (1)	
No	145	112	153	410 (99)	

[1]*P*-values are from univariable multinomial logistic regression analyses of associations between type of herd and each variable.

[2]Summary of 3 herd visits (winter, late spring, late autumn). Score 1: >25% with hair loss/skin wounds; score 2: 1% to 25% with hair loss or skin wounds; score 3: 0% with hair loss or skin wounds.

[3]Scores 1 and 2 were combined.

[4]Summary of 2 herd visits (late spring, late autumn).

[5]NA = not applicable.

clean animals was numerically highest in the milk-fed calves group (Table 1).

The results from the group and individual observations of hock lesions in heifers in late pregnancy are shown in Table 2. Most of the animals did not have hair loss and very few had skin wounds on their hocks, and the results did not differ significantly between herd groups.

Bacterial Growth in Udder Skin Swab Samples Taken at Herd Visits

Growth of streptococci was not detected in any of the samples, but a total of 17 species of staphylococci were identified. Including all skin samples (n = 283) taken at the herd visits, the most common species was *Staph. haemolyticus*, which was found in 35% of the samples. The other species were each found in 4 to 6% of the samples (*Staphylococcus capitis*, *Staph. chromogenes*, *Staphylococcus hominis*, *Staphylococcus equorum*) or in ≤2% of the samples (*Staph. aureus*, *Staphylococcus auricularis*, *Staphylococcus cohnii*, *Staphylococcus devriesei*, *Staphylococcus epidermidis*, *Staphylococcus hyicus*, *Staphylococcus pettenkoferi*, *Staphylococcus sciuri*, *Staphylococcus simulans*, *Staphylococcus vitulinus*, *Staphylococcus warneri*, *Staphylococcus xylosus*). Many species in the latter group were found in only 1 or 2 samples. Overall, the proportion of samples with growth of staphylococci was 41% (32 out of 78) for milk-fed calves, 66% (48 out of 73) for heifers in early pregnancy, and 51% (67 out of 132) for heifers in late pregnancy.

The culture results of swab samples taken from milk-fed calves, heifers in early pregnancy, and heifers in late pregnancy in herds where the animals were sampled twice (spring and autumn) are given in Table 3. The numerically lowest concentrations of colony-forming units were found in samples from milk-fed calves. No significant differences in the concentration of colony-forming units or number of colony types were found between herd groups. The distribution of staphylococcal species did not differ significantly between herd groups (milk-fed calves $P = 0.80$; early pregnancy $P = 0.66$; late pregnancy $P = 0.51$; data not shown).

Comparisons of Cow Variables Between Herd Groups

Cow data registered by farmers in the milk sample protocol on d 3 to 4 after calving are summarized in Table 4. Overall, nonfunctional quarters, udder edema, udder-thigh dermatitis, udder cleft dermatitis, or teat warts or wounds were found in 1 to 4% of the animals. A total of 2% of the FCH had been treated with antibiotics. Restraint (most often a steel anti-kicking bow) or oxytocin had been used at least once at milking of 14% and 7%, respectively, of the animals. Overall, calving in single box or group box was equally common. It was

Table 3. Total cfu/mL [mean (SD)] and number of samples with different numbers of colony types (CT) in udder skin swab samples in milk-fed calves, heifers in early pregnancy, and heifers in late pregnancy in herds with a large proportion of first-parity cows with low SCC at first and second milk recording after calving (LL herds, n = 13), a large proportion of first-parity cows with high SCC at first milk recording and low SCC at the second milk recording (HL herds, n = 11), or a large proportion of first-parity cows with high SCC at first and second milk recording (HH herds, n = 15) after calving[1]

Variable	LL	HL	HH	Total	*P*-value
Milk-fed calves					
Samples[2] (n)	26	22	30	78	—
Log_{10} cfu/mL	5.91 (1.12)	5.47 (0.87)	5.73 (1.04)	5.72 (1.02)	0.31
Samples per CT class (n)					
≤4	2	2	5	9	0.27
5	8	5	9	22	
6	11	10	4	25	
≥7	5	5	12	22	
Heifers in early pregnancy					
Samples[2] (n)	23	22	28	73	—
Log_{10} cfu/mL	6.07 (1.09)	5.75 (0.79)	5.96 (0.94)	5.93 (0.94)	0.46
Samples per CT class (n)					
≤4	7	6	8	21	0.94
5	6	5	8	19	
6	5	4	7	16	
≥7	5	7	5	17	
Heifers in late pregnancy					
Samples[2] (n)	44	34	54	132	—
Log_{10} cfu/mL	6.01 (0.90)	5.81 (0.74)	6.11 (0.84)	6.00 (0.84)	0.46
Samples per CT class (n)					
≤4	6	7	10	26	0.13
5	12	6	14	32	
6	16	14	11	41	
≥7	7	7	19	33	

[1]*P*-values are from univariable multinomial logistic regression analyses of associations between type of herd and each variable.
[2]Summary of herds that had samples taken at 2 herd visits (late spring, late autumn).

more common that heifers from LL herds, compared with heifers in HH ($P = 0.01$) and HL ($P = 0.054$) herds, calved in a group box. Use of restraint at milking and occurrence of udder-thigh dermatitis tended ($P = 0.06$ and $P = 0.08$, respectively) to differ between herd groups, where use of restraint was more common in LL herds than in HH herds (pairwise comparison, $P = 0.02$), and presence of udder-thigh dermatitis was numerically lowest in LL herds, but the differences between herd group were not statistically significant in any pairwise comparison. We found no other significant differences between herd groups in the variables registered.

Bacterial Growth in Colostrum and Milk Samples

Detailed culturing results for all the samples, and for colostrum and milk from d 3 to 4 after calving separately, are presented in Supplemental Table S1 (https://doi.org/10.5281/zenodo.7794752; Persson Waller, 2023). In total, 5,593 udder quarter samples from 722 FCH in 25 herds were examined. Overall, a third of the samples were contaminated. In 10% of those samples, abundant growth (>5,000 cfu/mL) was found, whereas the remaining 90% had sparse (<1,000 cfu/mL) or moderate (1,000–5,000 cfu/mL) growth. Specific infection was found in 14% (n = 408) of the samples. The prevalence of specific infections was significantly ($P < 0.001$) higher in colostrum samples (16%) than in milk samples (12%). Overall, more than 20 bacterial species were identified. The most common specific infection was *Staph. chromogenes* (51% of 408 samples), followed by *Staph. aureus* (13%), *Staph. haemolyticus* (7%), *Staph. simulans* (6%), *Strep. dysgalactiae* (4%), and *Staph. hyicus* (4%). The other species were each found in ≤3% of the samples with specific infection.

Presence of clots was registered by the farmers in 1.1% (32 out of 2,822) of all colostrum samples and in 1.1% (31 out of 2,744) of all milk samples. The likelihood of finding clots was significantly higher ($P = 0.004$) in quarter samples with specific infection than in samples with no growth.

For each udder quarter, the bacteriological findings in milk samples from d 3 to 4 after calving were compared with the findings in the colostrum samples (Supplemental Table S2; https://doi.org/10.5281/zenodo.7794752; Persson Waller, 2023). It was significantly more common that udder quarters with any type of specific growth or specific growth of *Staph. chromogenes*, *Staph. aureus*, *Staph. haemolyticus*, *Staph. simulans*, or *Staph. hyicus* in colostrum samples also had the same type of bacterial growth in the milk samples than udder

Table 4. Numbers (%) of animal registrations d 3 to 4 after calving in 685 freshly calved heifers in herds with a large proportion of first-parity cows with low SCC at first and second milk recording after calving (LL herds, n = 9), a large proportion of first-parity cows with high SCC at first milk recording and low SCC at the second milk recording (HL herds, n = 9), or a large proportion of first-parity cows with high SCC at first and second milk recording (HH herds, n = 7) after calving[1]

Item	LL	HL	HH	Total	*P*-value
Presence of nonfunctional udder quarter or quarters					0.70
Yes	9 (4)	13 (5)	8 (4)	30 (4)	
No	241 (96)	236 (95)	174 (96)	651 (96)	
Treated with antibiotics 14 d before calving					0.14
Yes	7 (3)	3 (1)	1 (1)	11 (2)	
No	243 (97)	244 (99)	178 (99)	665 (98)	
Use of restraint at milking					0.06
Yes	42 (17)	39 (16)	10 (6)	91 (14)	
No	207 (83)	207 (84)	169 (94)	583 (86)	
Use of oxytocin at milking					0.40
Yes	23 (10)	14 (6)	7 (4)	45 (7)	
No	225 (90)	232 (94)	172 (96)	629 (93)	
Presence of udder edema					0.15
Yes	3 (1)	10 (4)	13 (7)	26 (4)	
No	247 (99)	237 (96)	165 (93)	649 (96)	
Presence of udder-thigh dermatitis					0.08
Yes	5 (2)	15 (6)	10 (6)	30 (4)	
No	245 (98)	232 (94)	168 (94)	645 (94)	
Presence of udder cleft dermatitis					NA[2]
Yes	0 (0)	4 (2)	4 (2)	8 (1)	
No	250 (100)	243 (98)	174 (98)	667 (99)	
Presence of teat warts or wounds					0.77
Yes	8 (3)	6 (2)	3 (2)	17 (3)	
No	242 (97)	241 (98)	175 (97)	658 (97)	
Location of heifer calving					0.04
Single box	52 (21)	147 (60)	120 (69)	319 (48)	
Group box	197 (79)	97 (40)	53 (31)	347 (52)	

[1]*P*-values are from univariable multinomial logistic regression analyses of associations between type of herd and each variable.

[2]NA = not applicable.

quarters with no growth in colostrum samples. Overall, 146 udder quarters had the same specific infection in both samples. Among those, *Staph. chromogenes* was most common (53%; 78 out of 146), followed by *Staph. aureus* (21%), *Staph. simulans* (10%), *Staph. haemolyticus* (4%), *Staph. epidermidis* (3%), *Strep. dysgalactiae* (3%), *Staph. hyicus* (3%), *Strep. uberis* (1%), and *Arcanobacterium pluranimalium* (1%).

Table 5 presents comparisons between herd groups regarding bacterial growth in all colostrum and milk samples. A significantly larger proportion of quarter samples in HH herds, compared with LL and HL herds, had growth of *Staph. simulans* in the samples. This difference was also observed when analyzing the findings in the colostrum samples ($P = 0.001$) and in milk samples from d 3 to 4 ($P = 0.01$) separately (data not shown). When analyzing the colostrum samples separately, growth of *Staph. haemolyticus* was significantly ($P = 0.001$) more common in HL (4%) and HH (3%) herds than in LL (1%) herds (data not shown).

The results from the comparison between herd groups in the proportion of udder quarters categorized as healthy, cured, the same infection at both samplings, or newly infected are given in Table 6. The proportion of quarters with the same infection at both samplings was higher in HH than in LL herds ($P = 0.006$) and tended to be higher ($P = 0.06$) in HH than in HL herds. The proportion of quarters with *Staph. chromogenes* IMI at both samplings tended to differ between herd groups and was numerically highest in HH herds.

Genotypic Comparisons of Isolates from Milk Samples

In total, genotyping data were generated for 132 of 133 milk isolates of *Staph. chromogenes* from 13 herds, and 40 sequence types (**ST**; including 17 new ST, ST130–ST146) were found by 7-loci MLST (Supplemental Table S3; https://doi.org/10.5281/zenodo.7794752; Persson Waller, 2023). The number of isolates per herd varied between 4 and 15, and the number of ST per herd between 3 and 7. Including all isolates, ST6 was most common (n = 23) followed by ST109, ST1, ST8, and ST127 each having 8 to 9 isolates. A total of 11 ST had 3 to 5 isolates each, and 25 ST had 1 to 2 isolates. We found ST1, ST6, and ST109 in 5 herds each, ST19

Table 5. Numbers (%) of bacterial findings in colostrum and milk udder quarter samples collected from 722 freshly calved heifers in herds with a large proportion of first-parity cows with low SCC at first and second milk recording after calving (LL herds, n = 9), a large proportion of first-parity cows with high SCC at first milk recording and low SCC at the second milk recording (HL herds, n = 9), or a large proportion of first-parity cows with high SCC at first and second milk recording (HH herds, n = 7) after calving[1]

Item	LL	HL	HH	Total	*P*-value
No vs. specific growth of bacteria					0.14
No	1,189 (81)	1,087 (80)	716 (74)	2,992 (79)	
Specific	275 (19)	271 (20)	258 (26)	804 (21)	
No or specific growth vs. contamination					0.77
No/specific	1,464 (69)	1,358 (67)	974 (67)	3,796 (68)	
Contamination	644 (31)	664 (33)	489 (33)	1,797 (32)	
Growth of *Staphylococcus chromogenes*					0.20
Yes	163 (12)	117 (8)	128 (15)	408 (12)	
No	1,189 (88)	1,087 (90)	716 (85)	2,992 (88)	
Growth of *Staphylococcus aureus*					0.41
Yes	30 (2)	33 (3)	41 (5)	104 (3)	
No	1,189 (98)	1,087 (90)	716 (85)	2,992 (88)	
Growth of *Staphylococcus haemolyticus*					0.11
Yes	14 (1)	29 (3)	12 (2)	55 (2)	
No	1,189 (99)	1,087 (97)	716 (98)	2,992 (98)	
Growth of *Staphylococcus simulans*					<0.001
Yes	11 (1)	14 (1)	27 (4)	52 (2)	
No	1,189 (99)	1,087 (99)	716 (96)	2,992 (98)	
Growth of *Streptococcus dysgalactiae*					0.49
Yes	9 (0.4)	14 (1)	10 (1)	33 (1)	
No	2,099 (99.6)	1,008 (99)	1,453 (99)	5,560 (99)	
Growth of *Staphylococcus epidermidis*					0.11
Yes	4 (0.2)	17 (1)	7 (0.5)	28 (0.5)	
No	2,104 (99.8)	2005 (99)	1,456 (99.5)	5,565 (99.5)	
Growth of *Staphylococcus hyicus*					0.15
Yes	17 (0.8)	8 (0.4)	5 (0.3)	30 (0.5)	
No	2,091 (99)	2014 (99.6)	1,458 (99.7)	5,563 (99.5)	
Growth of other NAS					0.45
Yes	8 (0.4)	7 (0.4)	3 (0.2)	18 (0.3)	
No	2,100 (99.6)	2015 (99.6)	1,460 (99.8)	5,575 (99.7)	

[1]*P*-values are from multivariable multinomial logistic regression analyses of associations between type of herd and each variable including time of sampling in all models.

in 4 herds, and ST102 and ST127 in 3 herds each. The remaining ST were found in 1 or 2 herds. In 38 (93%) of 41 quarters or cows with infection at both samplings, the same ST was found in both colostrum and milk samples from d 3 to 4 after calving. The distribution of these quarters or cows among ST and herds is given in Supplemental Table S3. The core and accessory wgMLST analyses gave further insight into the high genetic variation among herds (Supplemental Figure S1; https://doi.org/10.5281/zenodo.7794752; Persson Waller, 2023).

A total of 18 milk isolates of *Staph. aureus* from 3 herds were genotyped, and 4 ST (ST705, ST3140, ST5835, ST5979) were found. Each ST was only found in one of the herds. One herd had 2 ST (ST5835: 3 cows, 6 isolates; ST705: 1 cow, 2 isolates) whereas the other 2 herds had 1 ST each (ST5979: 3 cows, 6 isolates; ST3140, 3 cows, 6 isolates). The same ST was found in both colostrum and milk samples in all quarters or cows (n = 9) with infection at both samplings. The ST results were confirmed by the wgMLST results (Supplemental Figure S2; https://doi.org/10.5281/zenodo.7794752; Persson Waller, 2023).

A total of 24 milk isolates of *Staph. haemolyticus* from 5 herds were investigated. According to the wgMLST results, each isolate was of a separate genotype (Supplemental Figure S3; https://doi.org/10.5281/zenodo.7794752; Persson Waller, 2023).

Genotypic Comparisons of Isolates from Skin Swab Samples and Milk Samples

One herd had at least 3 isolates of *Staph. chromogenes* from both skin and milk samples, and 5 herds had at least 3 isolates of *Staph. haemolyticus* from both skin and milk samples. Within herd, all skin isolates of *Staph. chromogenes* were of different genotypes, and they did not match any of the milk isolates (Supplemental Figure S1). Likewise, the skin isolates of *Staph. haemolyticus* were of different genotypes and did not match any of the milk isolates within herd (Supplemental Figure S3).

Table 6. Numbers (%) of udder quarters considered healthy, cured, the same infection at both samplings, or newly infected based on bacterial findings in colostrum and in milk d 3 to 4 after calving collected from 722 freshly calved heifers in herds with a large proportion of first-parity cows with low SCC at first and second milk recording after calving (LL herds, n = 9), a large proportion of first-parity cows with high SCC at first milk recording and low SCC at the second milk recording (HL herds, n = 9), or a large proportion of first-parity cows with high SCC at first and second milk recording (HH herds, n = 7) after calving[1]

Item	LL	HL	HH	Total	*P*-value
Healthy (no growth in colostrum and d 3–4)					0.32
Yes	390 (76)	344 (75)	228 (67)	962 (73)	
No	125 (24)	117 (25)	110 (33)	352 (27)	
Cured (specific infection in colostrum and no growth d 3–4)					0.99
Healthy	390 (87)	344 (86)	228 (86)	962 (87)	
Cured	59 (13)	54 (14)	36 (14)	149 (13)	
Same specific infection at both samplings					0.05
Healthy	390 (91)	344 (88)	228 (79)	962 (88)	
Persistent	40 (9)	46 (12)	60 (21)	146 (13)	
Infected with *Staphylococcus chromogenes* at both samplings					0.09
Healthy	390 (94)	344 (94)	228 (88)	962 (92)	
Persistent	26 (6)	22 (6)	30 (12)	78 (8)	
Infected with *Staphylococcus aureus* at both samplings					0.61
Healthy	390 (98)	344 (97)	228 (95)	962 (97)	
Persistent	8 (2)	10 (3)	13 (5)	31 (3)	
New infection (no growth in colostrum and specific infection d 3–4)					0.70
Healthy	390 (94)	344 (95)	228 (94)	962 (94)	
New infection	26 (6)	17 (5)	14 (6)	57 (6)	
New infection with *Staphylococcus chromogenes*					0.34
Healthy	390 (96)	344 (98)	228 (98)	962 (97)	
New infection	16 (4)	7 (2)	5 (2)	28 (3)	
New infection with *Staphylococcus aureus*					0.82
Healthy	390 (99)	344 (99)	228 (99)	962 (99)	
New infection	2 (1)	3 (1)	2 (1)	7 (1)	

[1]*P*-values are from univariable multinomial logistic regression analyses of associations between type of herd and each variable.

DISCUSSION

In the present study, we found some differences in occurrence of IMI between the herd groups, which could at least partly explain why the udder health in FCH differs between these groups. For example, finding a larger prevalence of udder quarters with the same IMI at both samplings (i.e., in both colostrum and milk samples taken 3–4 d after calving) in HH herds is in line with the higher milk SCC in FCH in these herds. The presence of the same IMI at both samplings, although close in time, most likely increases the likelihood of a long-lasting increase in SCC. This is in line with our previous studies, although the SCC may vary depending on type of IMI (Lundberg et al., 2016; Nyman et al., 2018). Among quarters with persistent IMI, *Staph. chromogenes* was the most common finding, which agrees with previous studies (De Vliegher et al., 2012; Nyman et al., 2018; De Buck et al., 2021). However, in those studies, genotyping of the isolates was not performed, but in the present study we found the same genotype at both samplings in almost all udder quarters with 2 isolates of *Staph. chromogenes*, supporting classifying these IMI as true infections likely to persist for a longer time. The same was the case for quarters with *Staph. aureus* IMI at both samplings. In the present study, *Staph. chromogenes* accounted for around half and *Staph. aureus* and *Staph. simulans* for around 30% of the quarters having the same infection at both samplings. In line with a previous study (Nyman et al., 2018), the likelihood of an IMI at calving remaining 3 to 4 d later varied between species. In the present study this was most common among *Staph. simulans* (83%) and *Staph. aureus* (76%). In comparison, the proportions for *Staph. chromogenes* and *Staph. haemolyticus* were 53% and 25%, respectively. Differences in persistence between species may be due to variation in virulence factors (Naushad et al., 2019).

Overall, *Staph. chromogenes* was the dominating IMI in both colostrum and milk samples, which is in line with other studies on FCH (De Vliegher et al., 2012; De Buck et al., 2021). Among the isolates sequenced in the present study, ST6 was most common, followed by ST109, ST1, ST8, and ST127. Isolates belonging to ST6, ST109, ST1, and ST127 were also among the most prevalent ST in another data set of 105 isolates from subclinical cases of mastitis in Swedish dairy cows (Persson Waller et al., 2023). In a study of *Staph. chromogenes* isolates from the United States and Belgium, ST1 was most common followed by ST28, but a few

of the isolates belonged to ST6 (Huebner et al., 2021). These findings indicate both similarities and differences in genotypes from different countries or regions.

The reasons for the predominance of *Staph. chromogenes* among FCH are not known but indicate spread between animals or a common source of infection in the environment. The fact that *Staph. chromogenes* IMI was common already at calving indicates that the infection had already taken place before calving, which is in line with what has been observed previously (Aarestrup and Jensen, 1997; Krömker and Friedrich, 2009). Given that many of these IMI were also present 3 to 4 d after calving, and considering the relationship with a high SCC, this emphasizes the need to prevent such IMI. In previous publications *Staph. chromogenes* has mainly been considered as a host-adapted udder pathogen, but some studies indicate environmental spread (reviewed by De Buck et al., 2021). In support for host-adapted pathogenesis, *Staph. chromogenes* has been isolated from udder and teat skin as well as from the teat canal (reviewed by De Buck et al., 2021). Based on the theory that the teat and udder skin can also be a reservoir before calving, we collected udder skin swab samples from animals in different age groups. However, *Staph. chromogenes* was not a common finding in those samples. Moreover, different genotypes of *Staph. chromogenes* were found in skin and milk samples in the herd, having multiple isolates from both skin and milk. Taken together, these findings did not support the theory. Although based on a limited number of farms and isolates, our findings of a marked diversity in genotypes between and within herds, as well as of the same genotype in more than one animal within herd, support the hypothesis that *Staph. chromogenes* can act as both a host-adapted contagious and an environmental pathogen.

We also found that *Staph. simulans* IMI was more common in HH herds, and that *Staph. haemolyticus* IMI was more common in colostrum samples from HH and HL herds than in LL herds. Although these species accounted for only a small part of all IMI, the results indicate that such IMI may have a negative effect on the SCC of FCH, which is in line with our previous study (Nyman et al., 2018). Both species have been found in association with mastitis in several parts of the world (De Buck et al., 2021). *Staphylococcus simulans* is often considered as a host-adapted contagious pathogen and *Staph. haemolyticus* as a contaminant or an environmental pathogen (Jenkins et al., 2019; Hamel et al., 2020). In the present study *Staph. haemolyticus* was the most common finding in skin samples of all 3 age groups, but the genotypes found among the skin isolates differed from the quarter isolates. Differences in *Staph. haemolyticus* IMI between herd groups may therefore indicate differences in udder cleanliness, but in the present study we found no differences between herd groups in any of the hygiene variables investigated. In general, late pregnancy is a risk period for IMI, as teat canal closure is compromised during this period (Krömker and Friedrich, 2009). Thus, management routines to keep the environment clean are even more important during late pregnancy. In the present study only 76% of the heifers in late pregnancy scored as clean, which indicates room for improvement. The present recommendation is to let heifers calve in single boxes rather than in group boxes, as the latter system is considered more difficult to keep clean. It was therefore somewhat surprising to find that heifer calving in group box was more common in LL herds than in HH and HL herds. In our previous study, including a larger number of herds, the site for heifer calving did not differ between herd groups (Persson Waller et al., 2021). However, HH herds let the cows stay longer in the calving area after calving, probably increasing the risk for IMI due to problems keeping the area dry and clean. It may be hypothesized that heifers might experience more stress if housed separately, especially if they have previously been housed in groups with other heifers.

Udder edema can be rather common in FCH and has been associated with mastitis (Waage et al., 2001; Morrison et al., 2018). In the present study, udder edema was registered for 4% of the heifers, but no difference was found between the herd groups. Likewise, a difference between herd groups was not found in our previous study at herd level, where approximately half of the 170 farmers stated that they had observed udder edema in at least one FCH during the last year (Persson Waller et al., 2021). Due to differences in study design, comparing occurrence of udder edema with other studies must be done with caution. For example, Morrison et al. (2018) and Fernandes et al. (2022) found udder edema in primiparous cows to be much more common (67% and 98%) than in the present study of cows during the first week after calving. In contrast, Melendez et al. (2006) diagnosed udder edema in 12% of the heifers just before calving. It is possible that the occurrence found in the present study is underestimating the true occurrence, as the registration of udder edema was performed by the farmers.

Skin lesions, such as hock lesions and different types of skin wounds on or close to the udder, can be a reservoir for bacteria, including mastitis-associated pathogens (Capurro et al., 2010; Persson Waller et al., 2014), and may therefore be a risk factor for mastitis. In the present study, however, we found no significant differences in prevalence of hock lesions, udder cleft dermatitis, udder-thigh dermatitis, or presence of warts or wounds on teats between the herd groups. Given

that the information was based on farmer observations, however, the data must be interpreted with caution, as some under-reporting is possible.

To our knowledge, information on prevalence of hock lesions in heifers is scarce. Our data indicate a very low prevalence of such lesions among heifers in late pregnancy, which was markedly different from the relatively high prevalence among Swedish dairy cows (Ekman et al., 2018a). The difference between heifers and cows is probably mainly due to differences in housing between the groups. Likewise, the present study indicates a much lower occurrence of udder cleft dermatitis among FCH than what has been found in dairy cows (Ekman et al., 2018b). This finding was, however, not surprising, as the prevalence in cows increased with lactation number and lactation month (Ekman et al., 2018b). However, in our previous study at herd level, including the herds from the present study as well as other herds, 12% of the farmers stated that they had observed udder cleft dermatitis at least once among FCH during the last year (Persson Waller et al., 2021).

The prevalence of udder-thigh dermatitis tended to differ between herd groups, with the numerically lowest occurrence in LL herds. Udder-thigh dermatitis occurs primarily among FCH and is often associated with udder edema (Sigmund et al., 1983). In the present study, udder-thigh dermatitis was reported in 4% of the FCH, which was slightly higher than in Sigmund et al. (1983) and much lower than in Roy et al. (2012), who reported 23 cases per 100 primiparous cows per year. In comparison, 25% of the farmers participating in our previous study at herd level stated that they had observed udder-thigh dermatitis at least once among FCH during the last year.

In the present study 4% of FCH had at least one nonfunctional quarter, but no difference was found between herd groups. According to the questionnaire in our previous study at herd level, around 80% of the farmers stated that they had had at least one such heifer during the last year (Persson Waller et al., 2021). We have not been able to identify many similar studies on this topic, but Duraes et al. (1982) and The Vet Group (2019) found nonfunctional quarters in similar proportions (3% and 2%) of heifers. Nonfunctional quarters may be due to teat sucking among calves or heifers, which was more common among HH herds (Persson Waller et al., 2021).

Milking the FCH can be stressful, and for some individuals this may result in inhibition of milk ejection (Van Reenen et al., 2002). Thus, restraining or injection of exogenous oxytocin, respectively, may be necessary for one or several milkings. In the present study we found that use of restraint at milking was more common in LL herds than in HH herds, which was in line with the findings from our herd-level study (Persson Waller et al., 2021). In contrast, use of restraint has also been associated with increased SCC (Svensson et al., 2006). It may be hypothesized that use of restraint measures may lead to a quicker acceptance of the milking procedure. The data of the present study indicate that restraint and use of oxytocin at milking are quite common on Swedish farms, as 14% of the sampled animals had been restrained and 7% had been treated with oxytocin. These results were in line with the results in our previous herd-level study, where approximately two-thirds of the farmers stated that they had used restraints and the same proportion stated that they had treated at least one FCH with oxytocin during the last year (Persson Waller et al., 2021).

As the number of herds per herd group included in the analyses of IMI was relatively small, the results must be interpreted with caution. Several herds had to be excluded from the analyses because they did not take milk samples from a sufficient proportion of the FCH. The reasons for not fulfilling the inclusion criteria are not known, but practical issues and lack of time were the most likely factors. Moreover, the herds were initially selected based on their categorization as LL, HL, or HH herds. Thus, the herds cannot be considered representative for all Swedish herds. However, the results may still be useful for herds other than those included in the study.

For practical and economic reasons, the farmers or their personnel took all milk samples in the present study. Despite careful instructions on aseptic milk sampling, one-third of the samples were considered contaminated. This made the identification of potential mastitis-associated pathogens more difficult.

Genotypic comparisons of bacterial isolates from milk and skin samples were only performed for *Staph. chromogenes* and *Staph. haemolyticus* from a few herds, due to a lack of sufficient numbers of isolates per herd and sample type. Moreover, the number of herds with sufficient numbers of milk isolates of *Staph. aureus* was also small. Thus, the results from these analyses must be interpreted with care.

The detection of few differences between herd groups could at least partly be due to the fact that the herds were too similar—that is, that the proportions of FCH with low CSCC or high CSCC at first and second test milking, respectively, were not sufficiently different when the number of herds was low. However, although the number of herds was low, the level of observation was cow or quarter, and for most of the variables investigated (27 of the 41 variables) the numbers of observations were quite large (approximately 600 to over 3,000 observations), thus having enough power to find a statistical difference if one existed. On the other hand,

for some of the variables (14 out of 41), the numbers of observations were quite small (approximately 70 to 400) due to lack of animals to observe. The risk of low numbers of observations is that the power to find a statistical difference is low; thus, a nonsignificant difference can be due to the small number of observations and not due to a true non-difference. Hence, for the 14 variables with few observations, we cannot say that the nonsignificance was due to a true non-difference or due to too few observations.

We chose to not perform any multivariable analyses in this study, as only a few variables were significant in the univariable analyses. However, because we only used univariable analyses, we could not consider potential confounding effects or interactions, although the possible confounding effect of age was adjusted for, as we stratified some of the analyses by age. Using a multivariable analysis could have shown which variables were more strongly associated with the outcomes and pinpointed whether any of the dependent variables had a confounding effect.

CONCLUSIONS

Some differences in IMI were found between the 3 herd groups that had good or poorer udder health among FCH. For example, quarters with the same IMI in both colostrum and milk sampled 3 to 4 d after calving were more common in HH herds than in HL and LL herds. Such infections were mainly due to *Staph. chromogenes*. In contrast, cleanliness and occurrence of most of the cow factors studied did not differ significantly between the herd groups. However, group calving and use of restraint at milking were most common in LL herds, and udder-thigh dermatitis was least common in LL herds. The reasons for the predominance of *Staph. chromogenes* IMI in FCH need further studies.

ACKNOWLEDGMENTS

The study was financed by the Swedish Farmers' Foundation for Agricultural Research (Stockholm, Sweden). The contributions of participating farmers, animal technicians, and veterinarians at Växa (Stockholm, Sweden) and of E. Jansson, T. Jinnerot, M. Myrenås, and M. Persson at the Swedish National Veterinary Institute (SVA; Uppsala, Sweden) are gratefully acknowledged. The authors have not stated any conflicts of interest.

REFERENCES

Aarestrup, F. M., and N. E. Jensen. 1997. Prevalence and duration of intramammary infection in Danish heifers during the peripartum period. J. Dairy Sci. 80:307–312. https://doi.org/10.3168/jds.S0022-0302(97)75939-3.

Bolger, A. M., M. Lohse, and B. Usadel. 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Capurro, A., A. Aspán, H. Ericsson Unnerstad, K. Persson Waller, and K. Artursson. 2010. Identification of potential sources of *Staphylococcus aureus* in herds with mastitis problems. J. Dairy Sci. 93:180–191. https://doi.org/10.3168/jds.2009-2471.

De Buck, J., V. Ha, S. Naushad, D. B. Nobrega, C. Luby, J. R. Middleton, S. De Vliegher, and H. W. Barkema. 2021. Non-*aureus* staphylococci and bovine udder health: Current understanding and knowledge gaps. Front. Vet. Sci. 8:658031. https://doi.org/10.3389/fvets.2021.658031.

De Vliegher, S., L. K. Fox, S. Piepers, S. McDougall, and H. W. Barkema. 2012. Invited review: Mastitis in dairy heifers: Nature of the disease, potential impact, prevention and control. J. Dairy Sci. 95:1025–1040. https://doi.org/10.3168/jds.2010-4074.

Duraes, M. C., C. J. Wilcox, H. H. Head, and H. H. van Horn. 1982. Frequency and effects on production of blind quarters in first lactation dairy cows. J. Dairy Sci. 65:1804–1807. https://doi.org/10.3168/jds.S0022-0302(82)82420-X.

Ekman, L., A.-K. Nyman, H. Landin, U. Magnusson, and K. Persson Waller. 2018b. Mild and severe udder cleft dermatitis—Prevalence and risk factors in Swedish dairy herds. J. Dairy Sci. 101:556–571. https://doi.org/10.3168/jds.2017-13133.

Ekman, L., A.-K. Nyman, H. Landin, and K. Persson Waller. 2018a. Hock lesions in dairy cows in freestall herds: A cross-sectional study of prevalence and risk factors. Acta Vet. Scand. 60:47. https://doi.org/10.1186/s13028-018-0401-9.

Fernandes, L., M. L. Celestino, P. R. Menta, T. H. Silva, D. Paiva, T. L. Ribeiro, L. S. Caixeta, N. R. Noyes, and V. S. Machado. 2022. Cow-related factors associated with intramammary infections during the 1st week postpartum in primiparous dairy cows in certified organic herds. Vet. J. 282:105822. https://doi.org/10.1016/j.tvjl.2022.105822.

Hamel, J., Y. Zhang, N. Wente, and V. Krömker. 2020. Non-*S. aureus* staphylococci (NAS) in milk samples: Infection or contamination? Vet. Microbiol. 242:108594. https://doi.org/10.1016/j.vetmic.2020.108594.

Huebner, R., R. Mugabi, G. Hetesy, L. Fox, S. De Vliegher, A. De Visscher, J. W. Barlow, and G. Sensabaugh. 2021. Characterization of genetic diversity and population structure within *Staphylococcus chromogenes* by multilocus sequence typing. PLoS One 16:e0243688. https://doi.org/10.1371/journal.pone.0243688.

Ivemeyer, S., C. Simantke, A. Ebinghaus, P. H. Poulsen, J. T. Sorensen, T. Rousing, R. Palme, and U. Knierim. 2018. Herd-level associations between human-animal relationship, management, fecal cortisol metabolites, and udder health of organic dairy cows. J. Dairy Sci. 101:7361–7374. https://doi.org/10.3168/jds.2017-13912.

Jenkins, S. N., E. Okello, P. V. Rossitto, T. W. Lehenbauer, J. Champagne, M. C. T. Penedo, A. G. Arruda, S. Godden, P. Rapnicki, P. J. Gorden, L. L. Timms, and S. S. Aly. 2019. Molecular epidemiology of coagulase-negative Staphylococcus species isolated at different lactation stages from dairy cattle in the United States. PeerJ 7:e6749. https://doi.org/10.7717/peerj.6749.

Krömker, V., and J. Friedrich. 2009. Teat canal closure in non-lactating heifers and its association with udder health in the consecutive lactation. Vet. Microbiol. 134:100–105. https://doi.org/10.1016/j.vetmic.2008.09.002.

Lundberg, Å., A.-K. Nyman, A. Aspán, S. Börjesson, H. Ericsson Unnerstad, and K. Persson Waller. 2016. Udder infections with *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* at calving in dairy herds with suboptimal udder health. J. Dairy Sci. 99:2102–2117. https://doi.org/10.3168/jds.2015-9487.

McDougall, S., K. I. Parker, C. Heuer, and C. W. R. Compton. 2009. A review of prevention and control of heifer mastitis via non-antibiotic strategies. Vet. Microbiol. 134:177–185. https://doi.org/10.1016/j.vetmic.2008.09.026.

Melendez, P., C. C. Hofer, and G. A. Donovan. 2006. Risk factors for udder edema and its association with lactation performance on

primiparous Holstein cows in a large Florida herd. Prev. Vet. Med. 76:211–221. https://doi.org/10.1016/j.prevetmed.2006.05.004.
Morrison, E. I., T. J. DeVries, and S. J. LeBlanc. 2018. Short communication: Associations of udder edema with health, milk yield, and reproduction in dairy cows in early lactation. J. Dairy Sci. 101:9521–9526. https://doi.org/10.3168/jds.2018-14539.
Naushad, S., S. A. Naqvi, D. Nobrega, C. Luby, J. P. Kastelic, H. W. Barkema, and J. De Buck. 2019. Comprehensive virulence gene profiling of bovine non-*aureus* staphylococci based on whole-genome sequencing data. mSystems 4:e00098-18. https://doi.org/10.1128/mSystems.00098-18.
NMC. 2017. Laboratory Handbook on Bovine Mastitis. 3rd ed. National Mastitis Council Inc.
Nyman, A.-K., C. Fasth, and K. Persson Waller. 2018. Intramammary infections with different non-*aureus* staphylococci in dairy cows. J. Dairy Sci. 101:1403–1418. https://doi.org/10.3168/jds.2017-13467.
Persson Waller, K. 2023. Intramammary infections and risk factors in freshly calved heifers in dairy herds. https://doi.org/10.5281/zenodo.7794752.
Persson Waller, K., M. Bengtsson, and A.-K. Nyman. 2014. Prevalence and risk factors for udder cleft dermatitis in dairy cattle. J. Dairy Sci. 97:310–318. https://doi.org/10.3168/jds.2013-7186.
Persson Waller, K., Å. Lundberg, and A.-K. Nyman. 2020. Udder health of early-lactation primiparous dairy cows based on somatic cell count categories. J. Dairy Sci. 103:9430–9445. https://doi.org/10.3168/jds.2020-18346.
Persson Waller, K., Å. Lundberg, and A.-K. Nyman. 2021. Risk and success factors for good udder health of early lactation primiparous dairy cows. J. Dairy Sci. 104:4858–4874. https://doi.org/10.3168/jds.2020-19683.
Persson Waller, K., M. Myrenås, S. Börjesson, H. Kim, M. Widerström, T. Monsen, A. K. Sigurðarson Sandholt, E. Östlund, and W. Cha. 2023. Genotypic characterization of *Staphylococcus chromogenes* and *Staphylococcus simulans* from Swedish cases of bovine subclinical mastitis. J. Dairy Sci. 106:7991–8004. https://doi.org/10.3168/jds.2023-23523.
Prjibelski, A., D. Antipov, D. Meleshko, A. Lapidus, and A. Korobeynikov. 2020. Using SPAdes de novo assembler. Curr. Protoc. Bioinformatics 70:e102. https://doi.org/10.1002/cpbi.102.
Roy, C., J.-L. Roque, P.-M. Francois, A. Ferrieres, and D. Raboisson. 2012. Investigation of the aetiology of udder-thigh dermatitis in French dairy cattle. Vet. J. 193:274–276. https://doi.org/10.1016/j.tvjl.2011.09.023.
Sigmund, H. M., W. Klee, and H. Schels. 1983. Udder-thigh dermatitis of cattle: Epidemiological, clinical and bacteriological investigations. Bov. Pract. 18:18–23. https://doi.org/10.21423/bovine-vol1983no18p18-23.
Svennesen, L., S. S. Nielsen, Y. S. Mahmmod, V. Krömker, K. Pedersen, and I. C. Klaas. 2019. Association between teat skin colonization and intramammary infection with *Staphylococcus aureus* and *Streptococcus agalactiae* in herds with automatic milking systems. J. Dairy Sci. 102:629–639. https://doi.org/10.3168/jds.2018-15330.
Svensson, C., A.-K. Nyman, K. Persson Waller, and U. Emanuelson. 2006. Effects of housing, management, and health of dairy heifers on first-lactation udder health in southwest Sweden. J. Dairy Sci. 89:1990–1999. https://doi.org/10.3168/jds.S0022-0302(06)72266-4.
The Vet Group. 2019. Heifer mastitis and blind quarters. Heifer mastitis and blind quarters. Accessed Jan. 24, 2023. https://www.thevetgroup.com.au/heifer-mastitis-and-blind-quarters/.
Van Reenen, C. G., J. T. N. Van der Werf, R. M. Bruckmaier, H. Hopster, B. Engel, J. P. T. M. Noordhuizen, and H. J. Blokhuis. 2002. Individual differences in behavioral and physiological responsiveness of primiparous dairy cows to machine milking. J. Dairy Sci. 85:2551–2561. https://doi.org/10.3168/jds.S0022-0302(02)74338-5.
Waage, S., S. A. Ødegaard, A. Lund, S. Brattgjerd, and T. Røthe. 2001. Case-control study of risk factors for clinical mastitis in postpartum dairy heifers. J. Dairy Sci. 84:392–399. https://doi.org/10.3168/jds.S0022-0302(01)74489-X.
Walker, B. J., T. Abeel, T. Shea, M. Priest, A. Abouelliel, S. Sakthikumar, C. A. Cuomo, Z. Qiandong, J. Wortman, S. K. Young, and A. M. Earl. 2014. Pilon: An integrated tool for comprehensive microbial variant detection and genome assembly improvement. PLoS One 9:e112963.

ORCIDS

K. Persson Waller https://orcid.org/0000-0002-8481-4313
Å. Lundberg https://orcid.org/0000-0003-0350-9892
S. Börjesson https://orcid.org/0000-0003-2219-2659
A.-K. Nyman https://orcid.org/0000-0002-6643-0404