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Strain diversity and infection durations of *Staphylococcus* spp. and *Streptococcus* spp. causing intramammary infections in dairy cows

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ABSTRACT

To effectively prevent and control bovine mastitis, farmers and their advisors need to take infection pathways and durations into account. Still, studies exploring both aspects through molecular epidemiology with sampling of entire dairy cow herds over longer periods are scarce. Therefore, quarter foremilk samples were collected at 14-d intervals from all lactating dairy cows (n = 263) over 18 wk in one commercial dairy herd. Quarters were considered infected with *Staphylococcus aureus*, *Streptococcus uberis*, or *Streptococcus dysgalactiae* when ≥100 cfu/mL of the respective pathogen was detected, or with *Staphylococcus epidermidis* or *Staphylococcus haemolyticus* when ≥500 cfu/mL of the respective pathogen was detected. All isolates of the mentioned species underwent randomly amplified polymorphic DNA (RAPD)-PCR to explore strain diversity and to distinguish ongoing from new infections. Survival analysis was used to estimate infection durations. Five different strains of *Staph. aureus* were isolated, and the most prevalent strain caused more than 80% of all *Staph. aureus* infections (n = 46). In contrast, 46 *Staph. epidermidis* and 69 *Staph. haemolyticus* strains were isolated, and none of these caused infections in more than 2 different quarters. The 3 most dominant strains of *Strep. dysgalactiae* (7 strains) and *Strep. uberis* (18 strains) caused 81% of 33 and 49% of 37 infections in total, respectively. The estimated median infection duration for *Staph. aureus* was 80 d, and that for *Staph. epidermidis* and *Staph. haemolyticus* was 28 and 22 d, respectively. The probability of remaining infected with *Strep. dysgalactiae* or *Strep. uberis* for more than 84 and 70 d was 58.7 and 53.5%, respectively. *Staphylococcus epidermidis* and *Staph. haemolyticus* were not transmitted contagiously and the average infection durations were short, which brings into question whether antimicrobial treatment of intramammary infections with these organisms is justified. In contrast, infections with the other 3 pathogens lasted longer and largely originated from contagious transmission.

Key words: staphylococci, streptococci, non-*aureus* staphylococci (NAS), subclinical mastitis, microbiological cure

INTRODUCTION

Intramammary infections cause subclinical and clinical mastitis in dairy cows and are an important economic challenge in dairy farming (Ruegg, 2017). In addition to the direct costs associated with milk yield losses and treatments, the continuous control and prevention of mastitis cases is costly. Thus, the most promising target-oriented management practices should be chosen according to each farm's conditions. Furthermore, clinical mastitis cases can impair animal wellbeing, and udder health problems are among the most important reasons for premature culling of cows (Fogsgaard et al., 2015; Rilanto et al., 2020).

Worldwide, staphylococci and streptococci are among the most prevalent mastitis-causing microorganisms (Piepers et al., 2007; Ericsson Unnerstad et al., 2009; Sampimon et al., 2009; Vakkamäki et al., 2017). Due to its persistence, resistance to treatment, strong impact on SCC, and negative effect on milk production, *Staphylococcus aureus* is often considered to be one of the most important mastitis pathogens (Heikkilä et al., 2018; Rainard et al., 2018). Also, NAS (e.g., *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*) have received increasing attention in recent years. They are often isolated from milk of clinical mastitis cases and

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have become the most frequently isolated group of bacteria from subclinical cases in many regions (Piepers et al., 2007; Sampimon et al., 2009). Furthermore, NAS can be associated with milk production losses (Heikkilä et al., 2018). In most regions with modern dairy farming, *Streptococcus uberis* and *Streptococcus dysgalactiae* are currently the most relevant streptococci (Piepers et al., 2007; Ericsson Unnerstad et al., 2009; Sampimon et al., 2009; Vakkamäki et al., 2017). Both can cause considerable milk losses, and long-lasting infections have been reported for *Strep. uberis* (Zadoks et al., 2001a; Heikkilä et al., 2018).

Microbiological analyses of milk samples are usually carried out to determine the most prevalent pathogen(s) when udder health problems occur in a farm. Consequently, prevention and control plans are created based on the traditional categorization of mastitis causing organisms into contagious (e.g., *Staph. aureus*) and environmental pathogens (e.g., *Strep. uberis*; Ruegg, 2017). It is postulated that pathogens designated as being contagious are spread primarily during the milking process via fomites (e.g., milking liners) from infected to uninfected quarters. Therefore, preventive measures against these pathogens focus on reducing the transmission of pathogens via an improved milking hygiene (e.g., use of individual paper towels for teat cleaning). For those pathogens categorized as environmental, it is assumed that they have reservoirs in the housing environment or are inhabitants of other ecological niches (e.g., skin or intestinal tract of cows) and are, under favorable conditions, causing IMI. The improvement of general hygiene is therefore usually advised to lower the risk of infection.

The strain diversity of a pathogen in a herd is indicative of its mode of transmission. Low diversity indicates pathogen spread from infected to uninfected quarters (contagious transmission) or via an environmental hotspot (Zadoks and Schukken, 2006). In contrast, high strain diversity indicates that infections occur due to unconnected infection events. Some studies that used strain-typing techniques indicate that the traditional categorization of mastitis-causing organisms might be partly misleading. For example, it was demonstrated that *Strep. uberis* can behave contagiously in some herds (Wente et al., 2019; Leelahapongsathon et al., 2020). For *Strep. dysgalactiae*, contrasting assignments can be found in the literature, sometimes designating it as an "intermediate" organism (Wente and Krömker, 2020). However, recent literature indicates characteristics of contagious spread in some herds (Wente and Krömker, 2020; Smistad et al., 2022).

The duration of infections is also important for the design of cost-effective prevention and control plans and when considering the justification of antimicrobial treatment of infections. As strain typing is expensive and time consuming, it has only rarely been carried out in studies investigating infection durations. Nonetheless, it is a necessary tool to discriminate ongoing infections with the same strain from new infections with a different strain, and is useful to better approximate the actual durations of IMI (Oliver et al., 1998). In addition, many studies exploring the strain diversity of mastitis pathogens or infection durations have focused on herds with ongoing mastitis problems, and little is known for both aspects in herds currently not experiencing udder health issues (Sommerhäuser et al., 2003; Haveri et al., 2008; Leelahapongsathon et al., 2020).

Therefore, we carried out a longitudinal study over 18 wk in one commercial dairy herd with an unremarkable udder health situation, as assessed by the farmer and advising veterinarian, to explore the strain diversity and durations of infections of selected *Staphylococcus* and *Streptococcus* spp. causing IMI.

MATERIALS AND METHODS

The study was conducted in accordance with the ethical guidelines published by the International Society of Applied Ethology (Sherwin et al., 2003). The authors declare that according to the Swedish Animal Welfare Act, no ethical approval is needed for this type of study, so the research was not submitted to an animal ethics committee.

Study Herd

From June to October 2020, a conventional Swedish dairy herd was visited 10 times at 14-d intervals. The herd was chosen because of the availability of technical facilities needed for other parts of the overall project, which focused on social interactions and disease transmission in dairy cows (i.e., a real-time location system and automatic recording of cow positions during milking). The herd had approximately 250 milking cows, housed in 2 groups in a freestall barn. The raised cubicles with rubber mats were covered with sawdust. Group 1 consisted of lactating cows up to approximately 150 DIM, and group 2 comprised lactating cows over 150 DIM. Dry cows were housed separately. Cows in the dry period were, depending on the weather conditions, kept either on pasture or in pens consisting of deep bedding with straw. The herd did not participate in DHI testing. Thus, the average daily milk production of 33.5 L/cow was calculated based on data from the milk recording of the milking parlor (2 × 12 GEA Euro class 800 with Dematron 75,

GEA Farm Technologies). The milking routine during the entire study period was as follows. All cows were milked twice daily in a herringbone milking parlor, and group 1 was always milked first. Before milking, the teats of all cows were cleaned with shaved wood fibers (also known as wood wool or excelsior) and all quarters were pre-milked. After milking, an iodine-based post-dip was applied to all teats. Between milking of individual cows, clusters were flushed (water only) when a cow with signs of clinical mastitis in the withdrawal period had been milked.

Data on calving, drying off and culling events, the farmer's documentation of animal health and treatments, as well as the bulk milk SCC reported by the dairy were collected throughout the study period.

Sample Collection

During each visit, quarter foremilk samples were collected aseptically from all lactating cows during the afternoon milking according to the German Veterinary Association guidelines (GVA, 2018). First, teat ends were disinfected with disposable wipes soaked with ethanol (70%); then, 3 milk streams were discarded before the milk was sampled into a sterile 13-mL tube containing a boric acid–based preserving agent (Ly-20; Heeschen et al., 1969). The Ly-20 was prepared using 50.0 g of boric acid (Carl Roth), 0.75 g of potassium sorbate (SigmaAldrich), 10 g of water-free glycerin (Carl Roth), 0.5 g of methylene blue (1%, SigmaAldrich), and 1,000.0 mL of distilled water. Immediately after milking, samples were transported cooled to the laboratory (University of Applied Sciences and Arts, Hannover, Germany), and microbiological analysis commenced within 18 h after sampling.

Cyto-Microbiological Analysis

Ten microliters of each milk sample was streaked onto esculin blood agar (Oxoid Inc.) and agar plates were incubated aerobically at 37°C. Microbial growth was examined after 24 and 48 h. Preliminary identification was conducted using the cell morphology, Gram status, catalase test (3% H_2O_2, Merck), clumping factor test (DiaMondiaL Staph Plus Kit, Sekisui Virotech), hemolysis patterns, ability to hydrolyze esculin, and Lancefield serotyping (DiaMondiaL Streptococcal Extraction Kit, Sekisui Virotech). Also, catalase-negative, gram-positive cocci hydrolyzing esculin were subcultured on modified Rambach agar for 24 h to differentiate *Strep. uberis* from other streptococci (Watts et al., 1993). Furthermore, the number of colony-forming units per milliliter was recorded in a semiquantitative way (for *Staph. aureus*, *Strep. uberis*, and *Strep. dysgalactiae*: 1–9, 10–49, and ≥50 cfu/mL; for all other species: 5–9, 10–49, and ≥50 cfu/mL).

After preliminary identification, individual colonies were picked and transferred onto a new esculin blood agar plate and incubated for another 24 h. Then, colony material from pure cultures was directly smeared onto a MALDI-TOF MS steel target according to the manufacturer's instructions (BrukerBiotyper, Bruker Daltonik). A MALDI score of ≥1.7 was considered to identify a genus securely and a score of ≥2.0 to identify a species securely (Nonnemann et al., 2019). Isolates with a score <1.7 were considered "not identified." From the pure cultures used for MALDI-TOF MS, one colony was picked, dissolved in a solution containing 80% brain heart infusion and 20% glycerol, and stored at −80°C.

Additionally, the SCC of milk samples was measured by flow cytometry (SomaScope Smart, Delta Instruments B.V.).

Randomly Amplified Polymorphic DNA PCR

Due to financial restrictions, strain typing was carried out for only a selection of species (n = 5). Based on the frequency of isolation and the SCC in samples growing as pure cultures (Figure 1), we decided to conduct strain typing for the following 4 species: *Staph. aureus*, *Staph. epidermidis*, *Strep. dysgalactiae*, and *Strep. uberis*. Additionally, an increase in the prevalence of *Staph. haemolyticus* was observed throughout the study (Table 1) and this organism was therefore included in the selection for strain typing.

For all isolates of the 5 selected species, strain typing was carried out. This was done to enable the distinction of ongoing infections with the same strain from new infections with a different strain of the same species. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Random amplified polymorphic DNA (**RAPD**)-PCR was carried out in 25-μL reaction volumes containing 12.5 μL of ReadyMix Taq PCR Reaction Mix (SigmaAldrich), 20 pmol of primer OPE 04, ERIC 1R, or Primer C (Table 2), 5 μL of template, and water to make up the volume. Amplification was performed in an Mx3005 P qPCR System (Agilent Technologies), using previously published methods as described in Table 2. The RAPD-PCR products were stained with MIDORIGreen Direct (Nippon Genetics Europe GmbH) and separated on a 2% agarose gel. Gel pictures were taken with the InGenius LHR-system (Syngene), and GeneTools software (Syngene) was used to analyze banding patterns. Isolates were considered

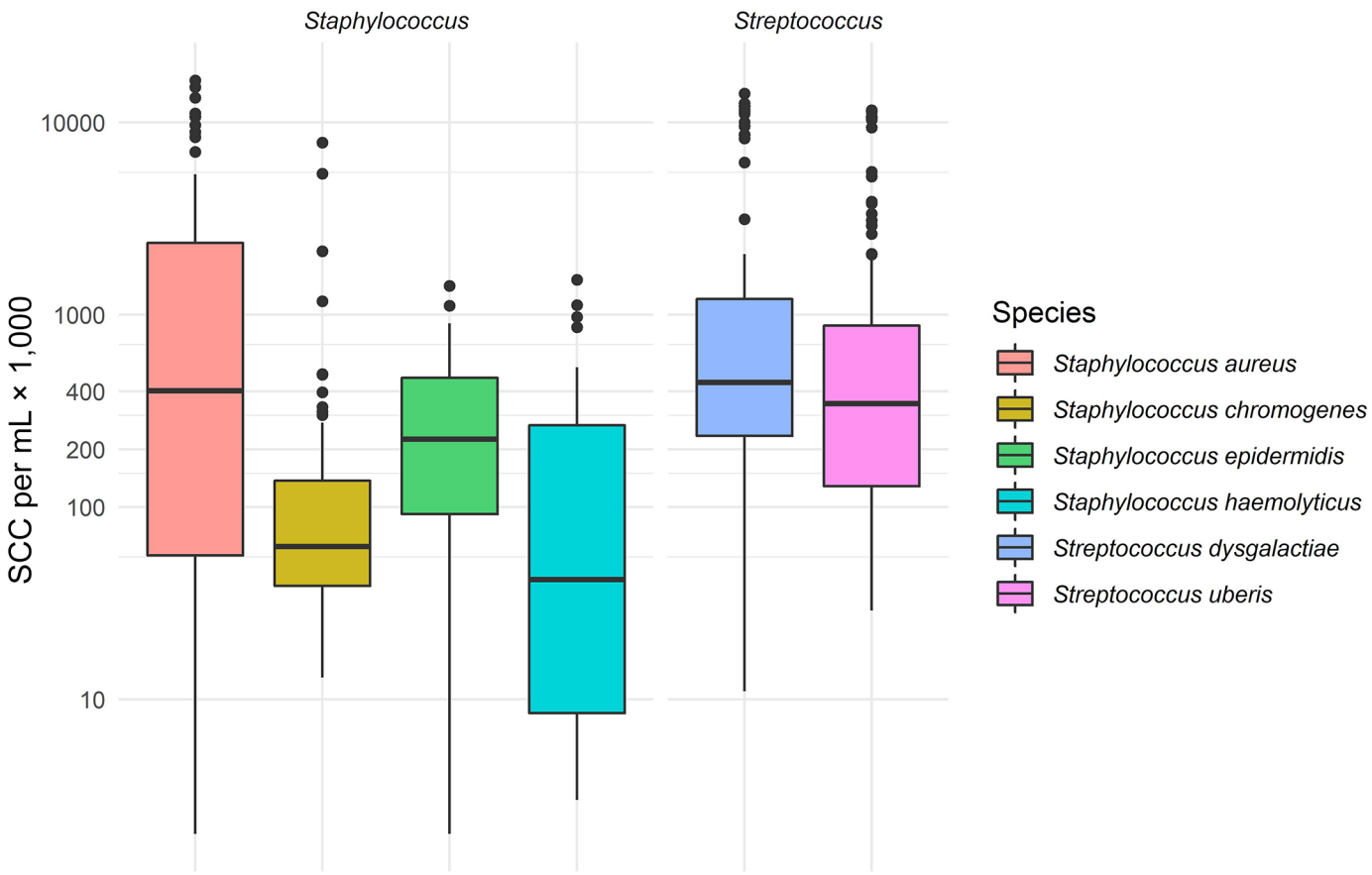

Figure 1. Somatic cell count measurements for milk samples with pure cultures collected from cows during lactation in one Swedish dairy herd: *Staphylococcus aureus* (n = 62), *Staphylococcus chromogenes* (n = 128), *Staphylococcus epidermidis* (n = 69), *Staphylococcus haemolyticus* (n = 35), *Streptococcus dysgalactiae* (n = 93), or *Streptococcus uberis* (n = 98). The observed data are displayed (i.e., quarters that were repeatedly positive for the same species contributed with several SCC measurements). The upper and lower hinges of the box represent the 25% and 75% quantiles. The bold line within the boxes corresponds to the median SCC. The whiskers extend to the highest and lowest values, but not more than 1.5 times the interquartile range from the box limits. All observations beyond this range are marked as outliers (dots).

to be the same RAPD type if they had identical banding patterns (number and size of bands; Wuytack et al., 2020). The PCR products of isolates with identical banding patterns were run a second time next to each other on 2% agarose gels to confirm that the banding patterns were the same.

Table 1. Number and proportion of lactating quarters positive for *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* at each sampling during the study period[1]

Date (mm-dd-yyyy)	No. of quarters sampled	*Staph. aureus*		*Staph. epidermidis*		*Staph. haemolyticus*		*Strep. dysgalactiae*		*Strep. uberis*	
		n	%	n	%	n	%	n	%	n	%
06-18-2020	805	8	0.99	8	0.99	3	0.37	14	1.74	10	1.24
07-02-2020	826	3	0.36	8	0.97	5	0.61	13	1.57	7	0.85
07-16-2020	835	6	0.72	8	0.97	4	0.48	9	1.08	13	1.56
07-30-2020	834	6	0.72	10	1.20	6	0.72	12	1.44	12	1.44
08-13-2020	825	15	1.82	10	1.21	7	0.85	7	0.85	15	1.82
08-27-2020	826	12	1.45	11	1.33	8	0.97	10	1.21	14	1.69
09-10-2020	819	8	0.98	11	1.34	8	0.98	10	1.22	14	1.71
09-24-2020	781	4	0.51	12	1.54	11	1.41	14	1.79	15	1.92
10-08-2020	757	10	1.32	7	0.92	10	1.32	8	1.06	7	0.92
10-22-2020	748	14	1.87	11	1.47	9	1.20	10	1.34	8	1.07

[1]n = number of positive quarters; % = proportion of quarters positive by total number of sampled quarters.

Table 2. Primers used for randomly amplified polymorphic DNA (RAPD) PCR

Species	Primer	Sequence 5′-3′	Reference
Staphylococcus aureus	Primer C	CGGGGGACTGTTGGGCGCCATCT	Damiani et al. (1996)
Staphylococcus epidermidis	ERIC 1R	ATGTAAGCTCCTGGGGATTCAC	Vogel et al. (1999)
Staphylococcus haemolyticus	Primer C	CGGGGGACTGTTGGGCGCCATCT	Damiani et al. (1996)
Streptococcus dysgalactiae	OPE 04	GTGACATGCC	Gillespie et al. (1998)
Streptococcus uberis	OPE 04	GTGACATGCC	Gillespie et al. (1998)

Definition of Infections and Data Analysis

A quarter was defined as being infected with a pathogen when ≥100 cfu/mL (or for *Staph. epidermidis* and *Staph. haemolyticus*, ≥500 cfu/mL) was detected in a milk sample (Sampimon et al., 2009). An infection episode started when a strain was detected for the first time in a quarter and ended when the respective strain was not detected in samples from the respective quarter until the end of the sampling period. If negative samples occurred between 2 samples that were positive for the same strain, we assumed false-negative results due to imperfect test sensitivity or potential intermittent shedding (Sears et al., 1990).

Strain diversity for each bacterial species was described by calculating the proportion of infection episodes caused by each strain out of all infection episodes caused by the respective bacterial species.

To calculate the duration of infection episodes (hereafter called "duration of infections"), the midpoint estimation method was used to define starting and ending time points for infections (Zadoks et al., 2003). Quarters that were negative before the dry period and positive for the first time after a dry period were considered newly infected since 7 d before the respective sampling (equaling the period assumed by the midpoint estimation used for continuously followed quarters). Likewise, when a heifer had a positive test result after first calving, we considered the quarter to be newly infected since 7 d before the respective sampling. All quarters that were positive at the first sampling time point or had a delayed entry to the study because of being in a dry period at the first sampling time point were considered left-censored. All quarters of cows that left the study (culled, dry period, or end of the sampling period) and were infected at their last sampling point were considered right-censored.

The durations of infections were estimated using the life table method with SPSS version 28.0 (IBM Corp.; Lücken et al., 2022). Estimations were conducted for all observations (left- and right-censored data were treated as censored observations) and for a subset of all observations only containing new infections (i.e., excluding left-censored observations). Additionally, durations of infections were estimated for the same 2 data sets with Kaplan-Meier survival analysis using the survival package (version 3.2–11) in R (https://www.r-project.org/), also taking censoring of data into account as described above. Quarters that were treated with antimicrobials during the study period and bacteriologically cured after treatment were not considered (*Staph. aureus* n = 2, *Staph. epidermidis* n = 0, *Staph. haemolyticus* n = 0, *Strep. uberis* n = 2, *Strep. dysgalactiae* n = 2) for any of the analyses on durations of infections. Two quarters that were treated with simple penicillin during the study period but did not cure bacteriologically after treatment (1 infection with *Staph. epidermidis* and 1 with *Staph. aureus*) were considered ongoing infected and therefore included for the estimation of infection durations.

RESULTS

Udder Health Situation

During the entire study period, 14 cows had at least one episode of clinical mastitis. One of these cows experienced 2 clinical mastitis episodes in the same quarter, and 1 cow had 1 episode of clinical mastitis in 2 different quarters. This corresponds to a clinical mastitis incidence for the whole cow herd (lactating and dry cows) of 1.6 cases/100 cows per month during the study period. Of all recorded clinical mastitis cases, 3 cases each could be linked to infections with *Staph. aureus*, *Strep. dysgalactiae*, or *Strep. uberis* (using the most recent milk sample results from the sampling visit before or after a case record). The geometric mean of the bulk milk SCC over the study period was 195,000 cells/mL of milk.

Cyto-Microbiological Results of Milk Samples

In total, 8,056 quarter foremilk samples from 263 cows were collected. Table 3 displays the number of milk samples positive for *Staphylococcus* or *Streptococcus* spp. The most frequently detected *Staphylococcus* spp. was *Staph. chromogenes* (n = 133), followed by *Staph. epidermidis* (n = 96), *Staph. aureus* (n = 86), and *Staph. haemolyticus* (n = 71; Table 3). In addition, 341 *Staphylococcus* isolates were only identified to the genus level. *Streptococcus dysgalactiae* (n = 107) and *Strep. uberis* (n = 115) were the most frequently

detected *Streptococcus* species. A further 35 *Streptococcus* isolates could be identified to the species level only. The quarter-level prevalence of IMI with each of the named pathogens was <2% over the entire study period (Table 3). The SCC in 1,000 cells/mL of milk of samples positive only for *Staph. aureus* was (median) 403 [interquartile ratio (**IQR**): 56–2,360], for *Staph. chromogenes* 63 (IQR: 39–137), for *Staph. epidermidis* 226 (IQR: 92–470), for *Staph. haemolyticus* 42 (IQR: 9–260), for *Strep. dysgalactiae* 446 (IQR: 235–1,206), and for *Strep. uberis* 341 (IQR: 130–893; Figure 1).

Table 3. *Staphylococcus* and *Streptococcus* species found in quarter foremilk samples from cows during lactation in one Swedish dairy herd (n = 8,056)

Genus and species	No. of positive milk samples	% of all milk samples
Staphylococcus		
Staph. aureus	86	1.07
Staph. capitis	6	0.07
Staph. chromogenes	133	1.65
Staph. cohnii	1	0.01
Staph. epidermidis	96	1.19
Staph. gallinarum	1	0.01
Staph. haemolyticus	71	0.88
Staph. hominis	1	0.01
Staph. hyicus	6	0.07
Staph. saprophyticus	2	0.02
Staph. sciuri	3	0.04
Staph. simulans	26	0.32
Staph. succinus	1	0.01
Staph. xylosus	1	0.01
Staphylococcus spp.[1]	341	4.23
Streptococcus		
Strep. canis	4	0.05
Strep. dysgalactiae	107	1.33
Strep. gallolyticus	2	0.02
Strep. uberis	115	1.43
Streptococcus spp.[1]	35	0.43

[1]MALDI-TOF MS score 1.70–1.99; therefore, identification to genus-level only.

Strain Diversity of Bacteria Isolated from Milk Samples

Five strains of *Staph. aureus* were detected by RAPD-PCR. The same strain (type A) caused 83% of all (n = 46) observed infections, whereas strain types B and E were both only isolated from 1 infected quarter each (Table 4). Strain types C and D caused each 7% of all infections. One quarter was first positive for strain type D, then had 1 sample without *Staph. aureus* detection, and then was positive for type B in the next 5 samples (Figure 2). Seven of 38 positive cows were infected with the same *Staph. aureus* strain in 2 different quarters during the study.

The diversity of *Staph. epidermidis* strains was much greater, with 46 different strains. No *Staph. epidermidis* strain was cultured from samples from more than 2 quarters, and only strains A, H, and O were isolated from more than 1 cow (Table 4). Twenty-three of the *Staph. epidermidis*–positive quarters (n = 33) were positive for 1 strain type only throughout the whole study period. However, 10 of the quarters with *Staph. epidermidis* detection were first positive for one strain type and at later time points for other strains (2 different strains: n = 5; 3 different strains: n = 2; 4 different strains: n = 2; 5 different strains: n = 1; Figure 3).

The 71 *Staph. haemolyticus* isolates corresponded to 69 different strains. Strain A was isolated from 1 quarter each in 2 different cows and strain B from 2 different quarters of 1 cow. All other strains were only isolated at 1 sampling from 1 quarter (Table 4). Still, 9 quarters were repeatedly positive for *Staph. haemolyticus* (Figure 4).

Seven different strains of *Strep. dysgalactiae* were isolated from 107 positive samples (Table 5). The 3 most frequent strains, A, B, and D, caused 48, 21, and 12% of all infections, respectively. Of the 4 infections with type D, 3 were infections of different quarters of the same cow (Figure 5). Only 2 of the *Strep. dysgalactiae* strains caused only 1 IMI. No more than one *Strep. dysgalactiae* strain was isolated throughout the study from any positive quarter (n = 33).

Eighteen different strains of *Strep. uberis* were isolated from 115 samples (Table 5). Of these, 3 each caused more than 10% of the observed infections: type A (24%), type B (14%), and type D (11%). Three additional strains affected 2 or 3 quarters, and 12 strains (67%) were isolated from samples of 1 quarter only (Table 5). Throughout the study, 23 quarters were positive for 1 strain of *Strep. uberis* only, 2 different strains were isolated from 5 quarters, and 1 quarter was, over time, positive for 4 different strains (Figure 6).

Durations of Infections

When the duration of infection was estimated using the life table method, taking censoring into account, the median duration was 80 d for *Staph. aureus*, 28 d for *Staph. epidermidis*, and 22 d for *Staph. haemolyticus* (Supplemental Tables S1 to S3, https://doi.org/10.5281/zenodo.7785231, Woudstra et al., 2023; Figure 7). For the 2 streptococci, only the lower quartile could be estimated (<28 d). Still, for *Strep. dysgalactiae*–infected quarters, the probability of remaining infected for more than 84 d was 58.7% (Table S4; https://doi.org/10.5281/zenodo.7785231). For *Strep. uberis*, the probability of remaining infected for more than 70 d was 53.5% (Table S5; https://doi.org/10.5281/zenodo

Table 4. Strain typing results for *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Staphylococcus haemolyticus* isolates cultivated from milk samples from cows during lactation in one Swedish dairy herd

Species and strain	Positive samples, n	Positive cows, n (%)	Observed infection episodes, n (%)
Staph. aureus			
A	67	32 (82)	38 (83)
B	5	1 (3)	1 (2)
C	8	2 (5)	3 (7)
D	5	3 (8)	3 (7)
E	1	1 (3)	1 (2)
Total	86	38 (100)	46 (100)
Staph. epidermidis			
A	12	2 (8)	2 (4)
B	2	1 (4)	1 (2)
C	5	1 (4)	1 (2)
D	2	1 (4)	2 (4)
E	5	1 (4)	1 (2)
F	2	1 (4)	1 (2)
G	2	1 (4)	2 (4)
H	2	2 (8)	2 (4)
I	3	1 (4)	1 (2)
J	3	1 (4)	2 (4)
K	8	1 (4)	1 (2)
L	2	1 (4)	1 (2)
M	3	1 (4)	1 (2)
N	4	1 (4)	1 (2)
O	2	2 (4)	2 (4)
P	2	1 (4)	1 (2)
Q	2	1 (4)	1 (2)
R	3	1 (4)	1 (2)
S	2	1 (4)	1 (2)
T	2	1 (4)	2 (4)
U	3	1 (4)	1 (2)
V	2	1 (4)	1 (2)
W–AS[1]	1	1 (4)	1 (2)
Total	96	26 (100)	52 (100)
Staph. haemolyticus			
A	2 (3)	2 (3)	2 (3)
B	2 (3)	1 (1)	2 (3)
C–BQ[2]	1 (1)	1 (1)	1 (1)
Total	71 (100)	70 (100)	71 (100)

[1]Including 23 strains: W, X, Y, Z, AA, AB, AC, AD, AE, AF, AG, AH, AI, AJ, AK, AL, AM, AN, AO, AP, AQ, AR, AS.

[2]Including 67 strains: C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X, Y, Z, AA, AB, AC, AD, AE, AF, AG, AH, AI, AJ, AK, AL, AM, AN, AO, AP, AQ, AR, AS, AT, AU, AV, AW, AX, AY, AZ, BA, BC, BD, BE, BF, BG, BH, BI, BJ, BK, BL, BM, BN, BO, BP, BQ.

.7785231). Estimations for durations of infections based on the Kaplan-Meier method and for new infections only can be found in Supplemental Tables S1 to S5 (https://doi.org/10.5281/zenodo.7785231, Woudstra et al., 2023).

DISCUSSION

Although several research articles have described the strain diversity of important mastitis pathogens within dairy cow herds and some have explored the duration of IMI at the species level, only a few studies have linked a longitudinal study design with strain typing to discriminate ongoing infections with the same strain from new infections with a different strain (Oliver et al., 1998; Pullinger et al., 2007). Furthermore, many studies on strain diversity of mastitis pathogens focused on herds experiencing ongoing udder health problems or only studied isolates from clinical mastitis cases (Zadoks et al., 2003; Lundberg et al., 2014; Wente and Krömker, 2020). Hence, further studies exploring strain diversity within the entire herd over longer periods of time and investigating infection durations on strain level were necessary to improve our understanding of infection dynamics on the herd level. Therefore, this longitudinal study was conducted to explore the diversity of *Staphylococcus* spp. and *Streptococcus* spp. isolated from milk samples and the strain-specific durations of IMI with these pathogens in a herd that was not experiencing udder health problems.

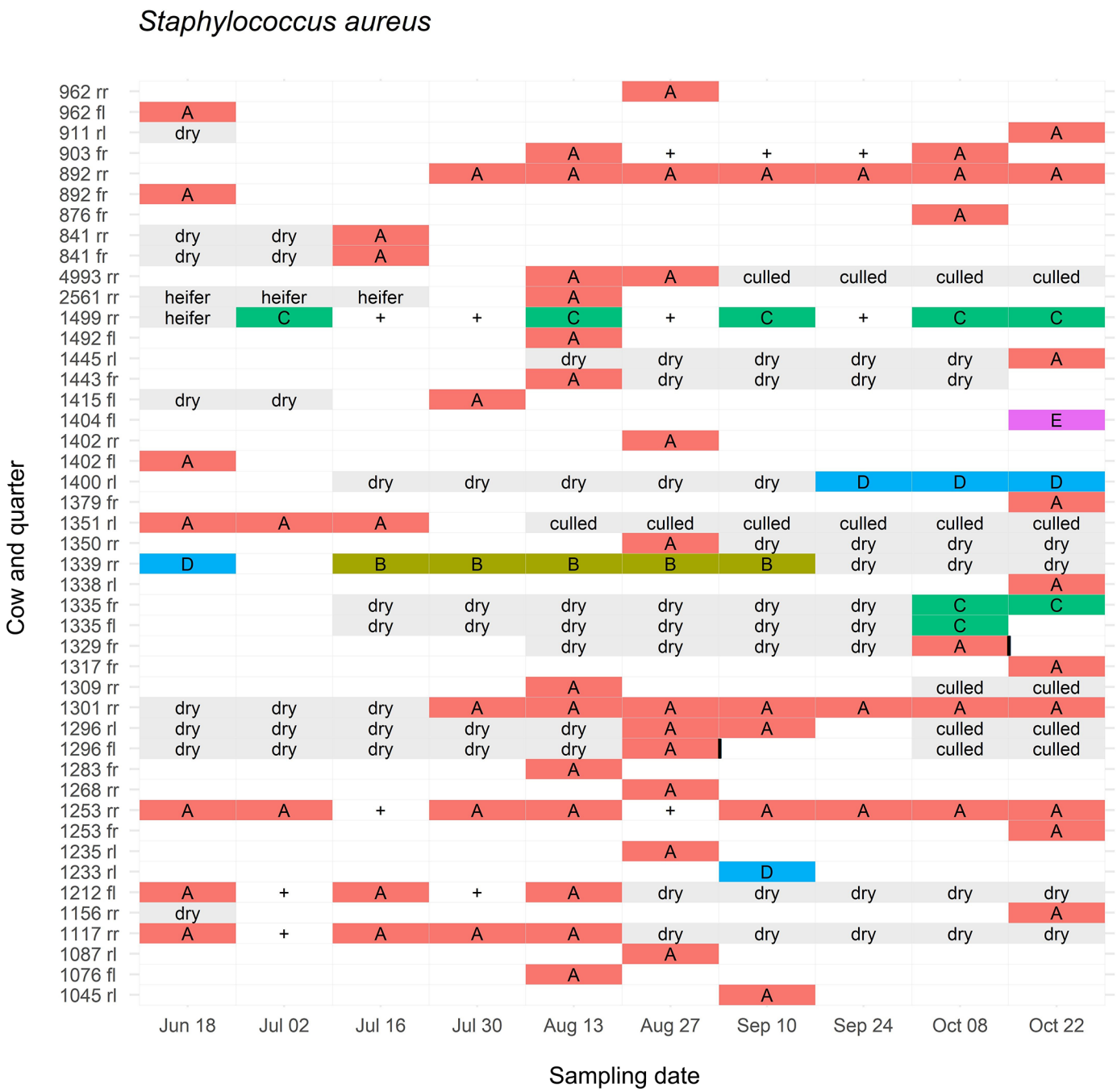

Figure 2. Observed IMI with *Staphylococcus aureus* during the study period. Cow and quarter: number = cow ID, fr = front right, fl = front left, rr = rear right, rl = rear left quarters; A–E = strain type detected at the respective sampling; dry = cow in dry period; culled = cow was culled; heifer = cow before first calving; + = presumably ongoing infected; | = infection end associated with antibiotic treatment (n = 2).

Strain Diversity of Bacteria Isolated from Milk Samples

Staph. aureus. The lowest strain diversity among all studied species was observed for *Staph. aureus*: 1 strain caused >80% of all infections. This indicates that most *Staph. aureus* IMI were connected either through contagious spread, from infected quarters to uninfected quarters, or via an environmental hotspot within the herd (Zadoks and Schukken, 2006). In contrast, a study by Sommerhäuser et al. (2003), using a combination of different typing techniques, found a dominating strain in only 4 of 7 herds. Another study that collected quarter milk samples from 91

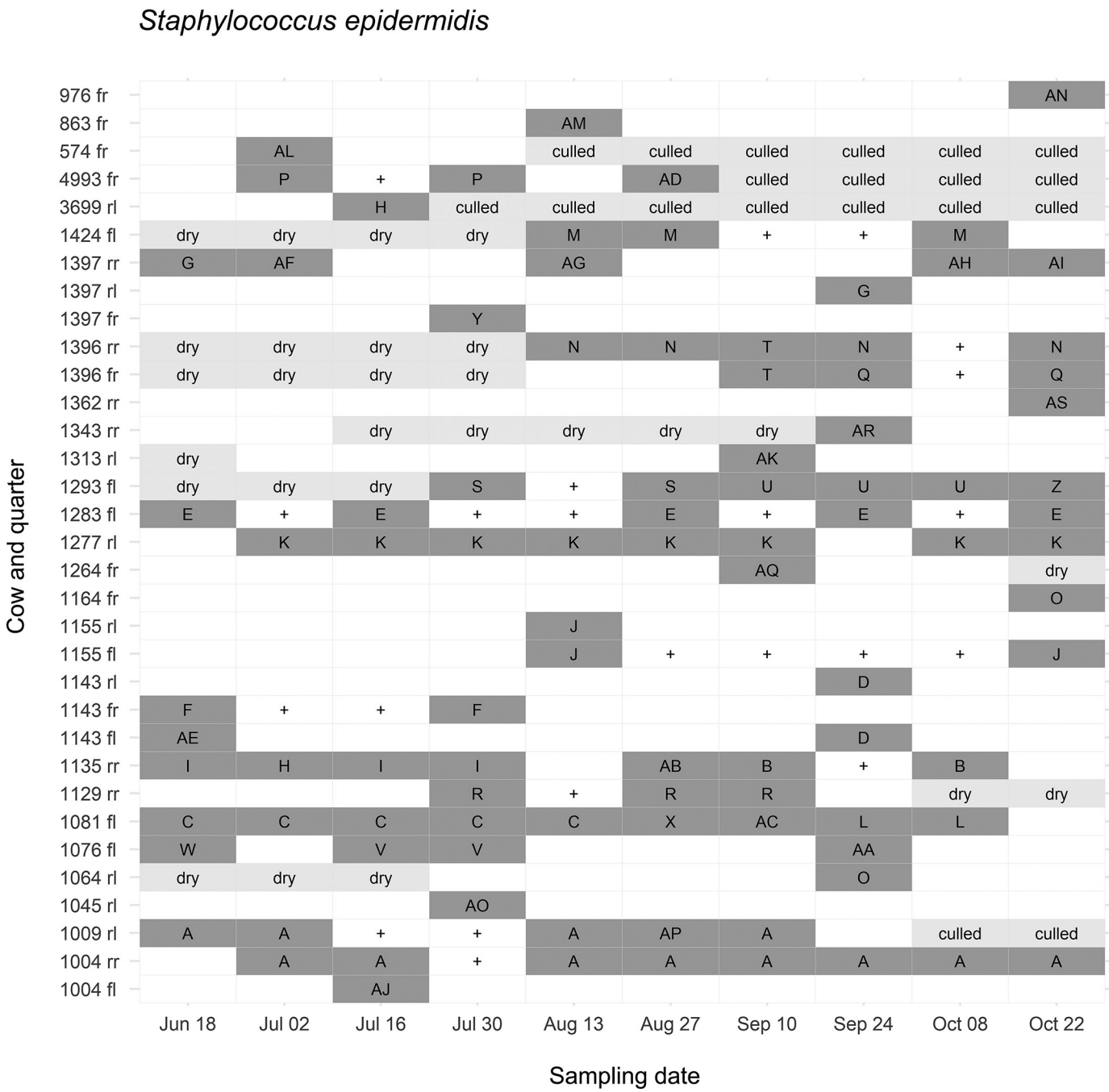

Figure 3. Observed IMI with *Staphylococcus epidermidis* during the study period. Cow and quarter: number = cow ID, fr = front right, fl = front left, rr = rear right, rl = rear left quarters; A–AS = strain type detected at the respective sampling; dry = cow in dry period; culled = cow was culled; heifer = cow before first calving; + = presumably ongoing infected; | = infection end associated with antibiotic treatment (n = 0).

dairy herds and investigated the genetic diversity of *Staph. aureus* found 22 different *spa* types among the studied isolates (Pichette-Jolette et al., 2019). No information about diversity within individual farms was given. On the farm level, research often demonstrates a low diversity of *Staph. aureus* strains isolated from milk samples (Larsen et al., 2000; Haveri et al., 2008). The results of this study indicate that the common recommendations for the prevention of *Staph. aureus* IMI, which are targeted at reducing transmission from infected to uninfected quarters via improved milking hygiene, seem to be most important, including in herds currently not experiencing *Staph. aureus* outbreaks.

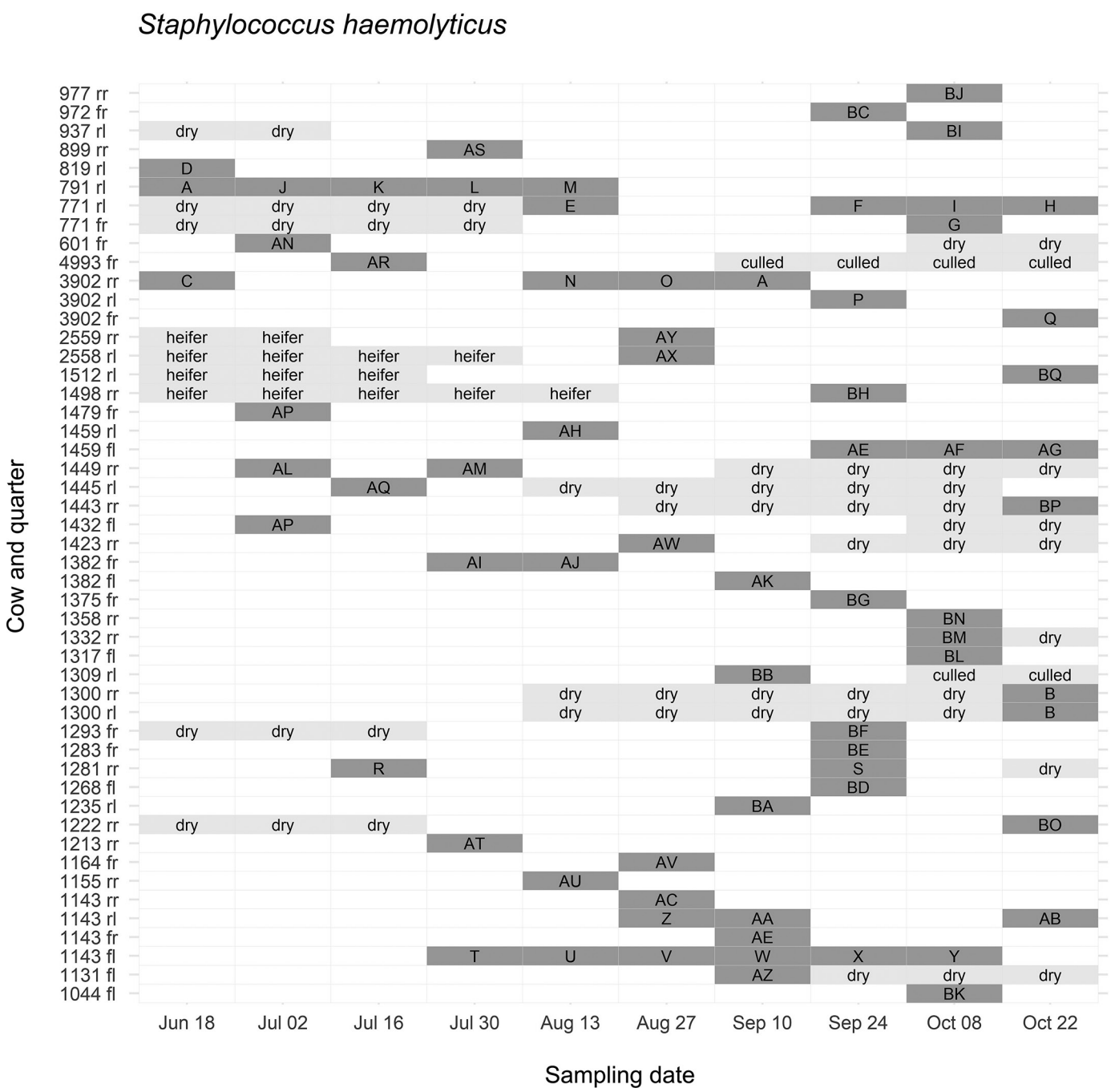

Figure 4. Observed IMI with *Staphylococcus haemolyticus* during the study period. Cow and quarter: number = cow ID, fr = front right, fl = front left, rr = rear right, rl = rear left quarters; A–BQ = strain type detected at the respective sampling; dry = cow in dry period; culled = cow was culled; heifer = cow before first calving; + = presumably ongoing infected; | = infection end associated with antibiotic treatment (n = 0).

Staph. epidermidis and Staph. haemolyticus. The most frequently isolated NAS species in this study were *Staph. chromogenes*, *Staph. epidermidis*, and *Staph. haemolyticus*. This finding is similar to that of other studies that detected, in varying order, *Staph. chromogenes*, *Staph. epidermidis*, *Staph. haemolyticus*, and *Staph. simulans* most frequently in milk samples (Piessens et al., 2011; Nyman et al., 2018; Valckenier et al., 2021). This study conducted strain typing only for 2 NAS species due to financial limitations. We selected *Staph. epidermidis* isolates, as these were associated with the highest average SCC of all isolated NAS species, and *Staph. haemolyticus*, for which we observed an increase in the proportion of quarters that tested

Table 5. Strain typing results for *Streptococcus dysgalactiae* and *Streptococcus uberis* isolates cultivated from milk samples from cows during lactation in one Swedish dairy herd

Species and strain	Positive samples, n	Positive cows, n (%)	Observed infection episodes, n (%)
Strep. dysgalactiae			
A	58	15 (52)	16 (48)
B	28	6 (21)	7 (21)
C	10	2 (7)	2 (6)
D	6	2 (7)	4 (12)
E	1	1 (3)	1 (3)
F	2	2 (7)	2 (6)
G	2	1 (3)	1 (3)
Total	106	28 (100)	33 (100)
Strep. uberis			
A	28	7 (22)	9 (24)
B	22	4 (13)	5 (14)
C	6	1 (3)	1 (3)
D	10	4 (13)	4 (11)
E	1	1 (3)	1 (3)
F	1	1 (3)	1 (3)
G	1	1 (3)	1 (3)
H	6	1 (3)	1 (3)
I	1	1 (3)	1 (3)
J	1	1 (3)	1 (3)
K	1	1 (3)	1 (3)
L	10	2 (6)	3 (8)
M	1	1 (3)	1 (3)
N	6	2 (6)	2 (5)
O	14	1 (3)	2 (5)
P	5	1 (3)	1 (3)
Q	1	1 (3)	1 (3)
R	1	1 (3)	1 (3)
Total	115[1]	21 (100)	37 (100)

[1]From 1 sample, 2 different strains of *Strep. uberis* were isolated (A and K).

positive over the study period, which could have been indicative of contagious transmission.

Using RAPD-PCR, we found very high strain diversity among *Staph. epidermidis* and *Staph. haemolyticus* isolates, and no dominating strains were found (Figures 3 and 4). This indicates that quarters likely became infected from different environmental sources (including animal-related niches). On a much smaller number of isolates, Piessens et al. (2012) also found high strain diversity for *Staph. haemolyticus* in samples from 6 herds, whereas for *Staph. epidermidis*, they found a dominant strain in 1 of 4 herds.

Non-*aureus* staphylococci can be regularly isolated from animal-related niches such as teat skin or rectal fecal samples (Wuytack et al., 2020). Also, the teat canal was previously reported to be colonized by different NAS species (Traversari et al., 2019). We could argue, therefore, that the observed high diversity came from teat canal colonization rather than IMI. However, 75% of the samples positive for *Staph. epidermidis* only (n = 69) had an SCC of ≥92,000 cells/mL, and the median SCC was 226,000 cells/mL. Therefore, most of the milk samples positive for *Staph. epidermidis* had elevated SCC, which demonstrates the presence of subclinical mastitis. *Staphylococcus haemolyticus*, on the other hand, was isolated from samples with a median SCC of 42,000 cells/mL of milk. Hiitiö et al. (2016) compared a sampling technique circumventing the teat canal for the collection of milk samples and the traditional aseptic collection technique (used in this study) and found that the detection of NAS was significantly higher in samples collected with the traditional technique. Thus, we cannot rule out that the cultured *Staph. haemolyticus* were teat canal inhabitants rather than the cause of IMI.

Strep. dysgalactiae. Approximately half of all infections with *Strep. dysgalactiae* were caused by the same strain. Another 33% were caused by either strain type B or D. Therefore, *Strep. dysgalactiae* seems to have mostly spread contagiously within the herd. Only a few studies on the strain diversity of *Strep. dysgalactiae* from bovine milk samples have been published to date. A recent study investigating strain diversity in milk samples from clinical mastitis cases from 16 farms found a very low strain diversity in several herds. However, in those herds with the largest number of investigated isolates, up to 7 different strains were found, comparable to the results of the present study (Wente and Krömker, 2020). Another study, where milk samples were collected in 7 Norwegian herds from cows with high SCC, found the same multilocus sequence type in several cows of the same herd (Smistad et al., 2022). Additionally, in several different farms, the same sequence types could be found. Furthermore, a Swedish study on isolates from clinical mastitis cases found the same pulsed-field gel electrophoresis (PFGE) pulso-types in up to 13 different herds (Lundberg et al., 2014). To the best of our knowledge, this is the first study to investigate the strain diversity of *Strep. dysgalactiae* on a larger number of samples from subclinical mastitis cases, as well as over time in a longitudinal design. Still, it is only a study of one herd. Thus, to better understand the epidemiology of *Strep. dysgalactiae* infections, further research on its transmission pathways within and across dairy herds using strain-typing techniques is needed.

Strep. uberis. Among the *Strep. uberis* isolates, dominating strains were identified. However, they each comprised a much smaller proportion (24, 14, and 11%) of all IMI caused by this species compared with the proportion of the dominating strains of *Staph. aureus* or *Strep. dysgalactiae*. This indicates that contagious transmission from cow to cow seems to have played a role in the development of new *Strep. uberis* IMI, whereas environmental hotspots cannot be ruled out. Zadoks et al. (2003) reported a dominating strain in each of 2 herds with high *Strep. uberis* IMI incidences.

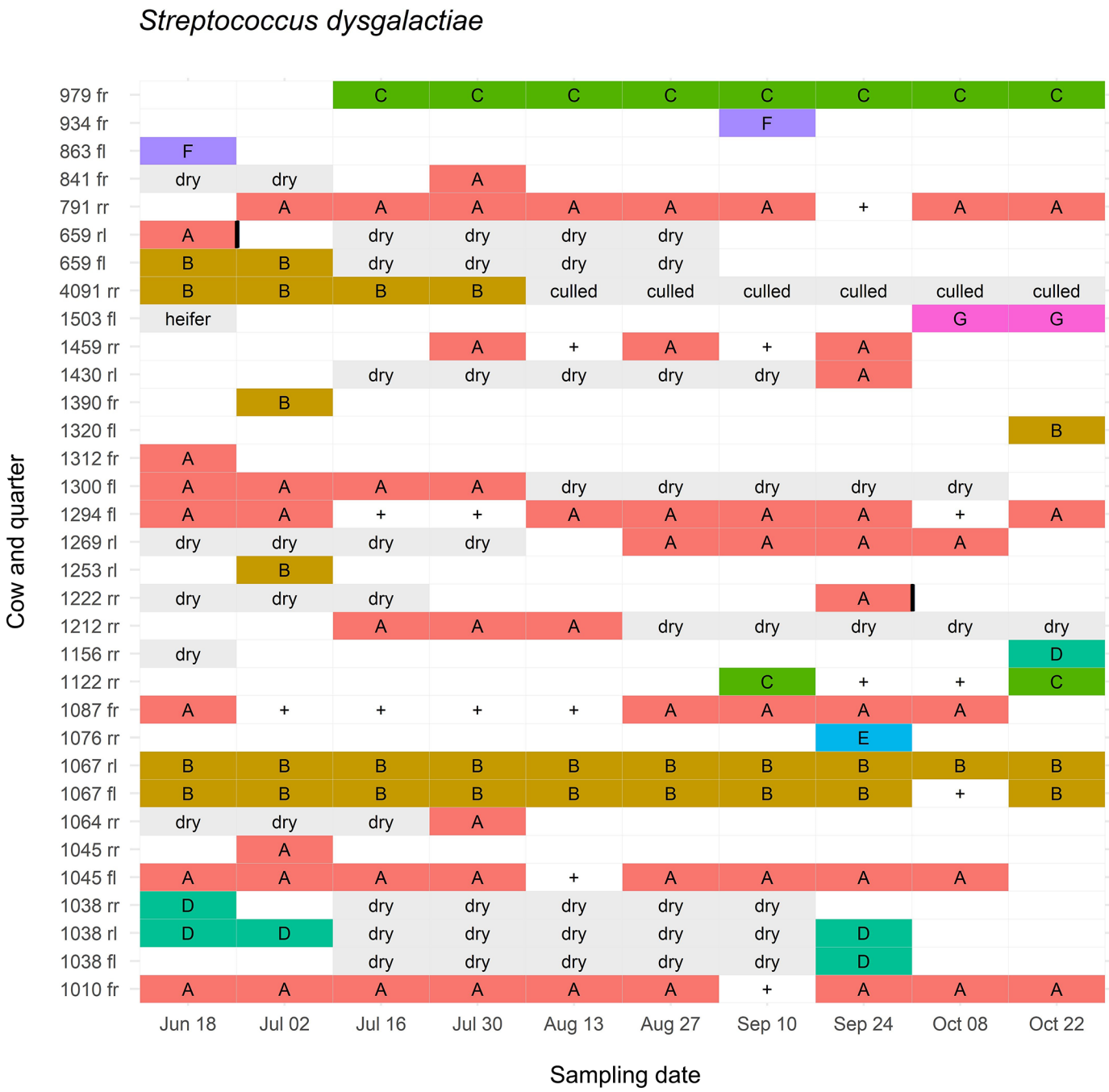

Figure 5. Observed IMI with *Streptococcus dysgalactiae* during the study period. Cow and quarter: number = cow ID, fr = front right, fl = front left, rr = rear right, rl = rear left quarters; A–G = strain type detected at the respective sampling; dry = cow in dry period; culled = cow was culled; heifer = cow before first calving; + = presumably ongoing infected; | = infection end associated with antibiotic treatment (n = 2).

Furthermore, in a more recent study, 86% of all *Strep. uberis*–positive samples collected from clinical cases in 1 herd were positive for the same strain (Fenske et al., 2022). Still, in the literature, *Strep. uberis* is often referred to as the model environmental pathogen, implying that it resides in the environment over longer periods and only infects individual quarters under favorable conditions. The results of the current study and other studies indicate that this attribution is questionable. In practice, this can mislead farmers and advisors toward a focus on environmental hygiene only. However, following research, a possible conta-

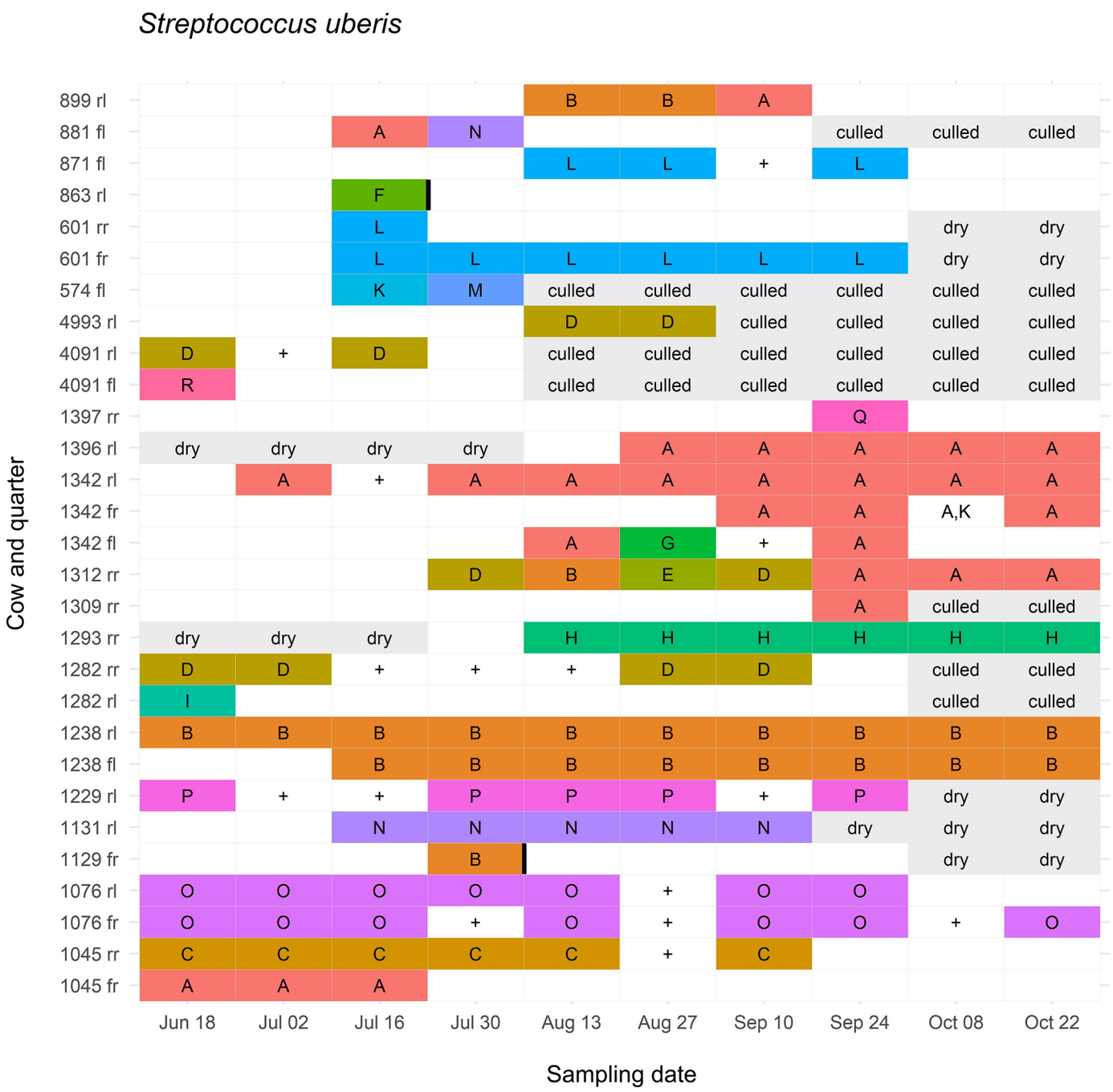

Figure 6. Observed IMI with *Streptococcus uberis* during the study period. Cow and quarter: number = cow ID, fr = front right, fl = front left, rr = rear right, rl = rear left quarters; A–U = strain type detected at the respective sampling, A,K = strains A and K detected; dry = cow in dry period; culled = cow was culled; heifer = cow before first calving; + = presumably ongoing infected; | = infection end associated with antibiotic treatment (n = 2).

gious component should be considered when setting up a prevention and control program for *Strep. uberis* infections. Strain typing should probably be used more often in practice for the workup of herd health problems with *Strep. uberis*. It could be a useful tool to identify the most important infection pathway and consequently the most effective preventive measures.

Durations of Infections

Staph. aureus. The probability of a quarter remaining infected with *Staph. aureus* for at least 84 d was 48% in this study. This finding is similar to the observed median duration of infections of 64 and 91 d in 2 Danish herds with average *Staph. aureus* prevalences of 2.6 and

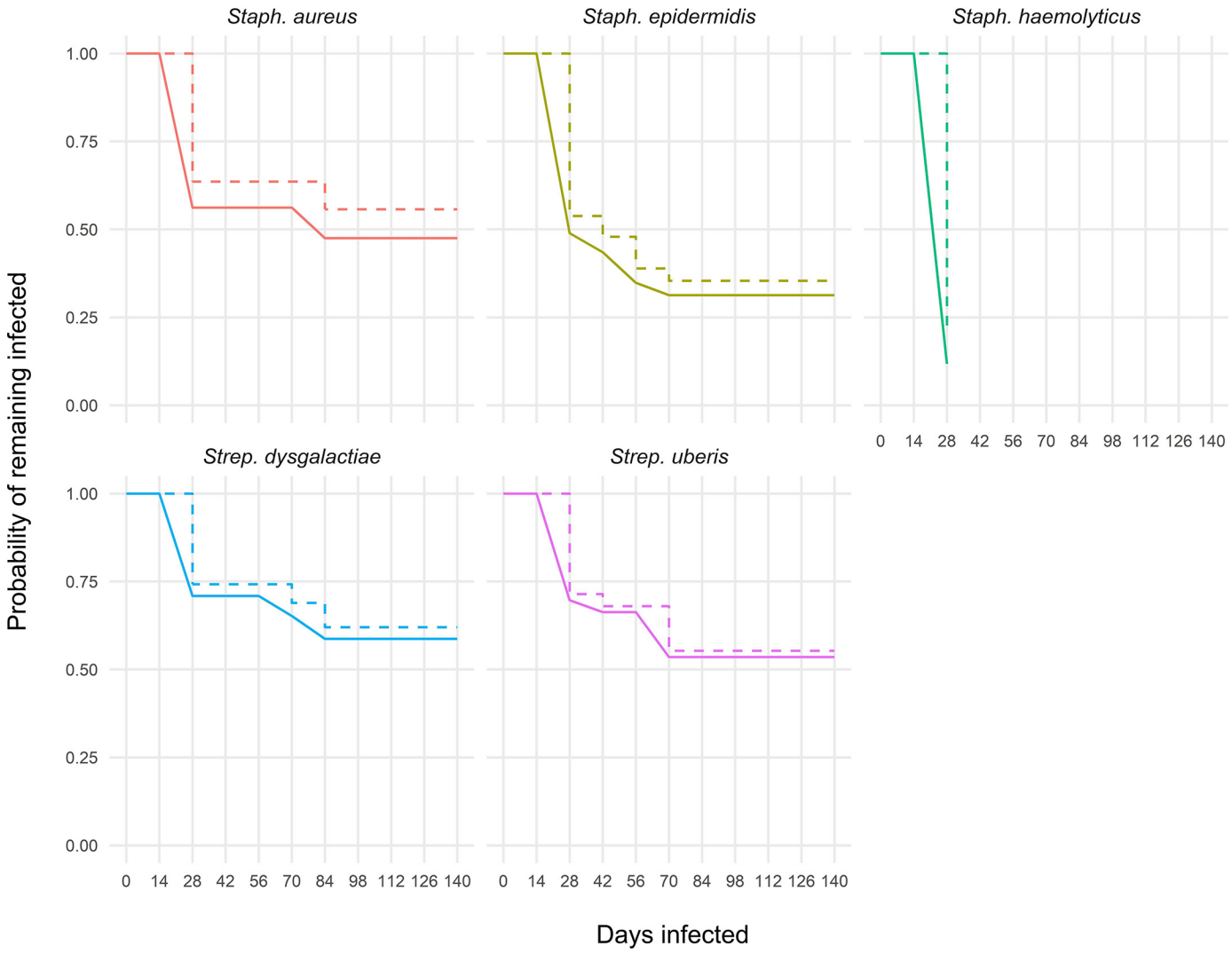

Figure 7. Estimated durations of IMI for selected *Staphylococcus* spp. and *Streptococcus* spp. isolated from milk samples in one Swedish dairy cow herd. Displayed is the cumulative probability of remaining infected estimated by the life table method (solid line) and the Kaplan-Meier method (dashed line). Data from treated quarters were excluded if these were bacteriologically cured after treatment (*Staph. aureus* n = 2, *Staph. epidermidis* n = 0, *Staph. haemolyticus* n = 0, *Strep. uberis* n = 2, *Strep. dysgalatiae* n = 1). Left- and right-censored data were treated as censored observations.

34%, respectively (Kirkeby et al., 2019). Other studies estimated the mean duration of *Staph. aureus* IMI in different herds at 64, 84, 136, and 192 d, where the 2 longest reported infection durations were observed in herds with much higher prevalences than in the present study (Lam et al., 1996; Zadoks et al., 2002).

Of all *Staph. aureus* infections, 44% were estimated to last no longer than 28 d. Also in Kirkeby et al. (2019), more than 20% of all *Staph. aureus* infections could be detected at only one sampling point (monthly sampling intervals were used). The results from both studies indicate that a considerable number of *Staph. aureus* infections, at least in herds with relatively low prevalences, are short. This needs to be taken into account when designing control measures like culling or separation of infected animals, especially because, in practice, these are often based on microbiological test results generated from one-time sampling only.

Staph. epidermidis and Staph. haemolyticus. We observed short durations of infections for both NAS species (estimated median: 22 d for *Staph. haemolyticus* and 28 d for *Staph. epidermidis*), and only 1 clinical case of mastitis due to *Staph. epidermidis* throughout the study period. In contrast, previous research on *Staph. epidermidis* has shown that individual quarters can be positive for the same strain over several months (Gillespie et al., 2009). Still, our findings lead us to question whether antibiotic treatment of quarters prov-

en to be infected with one of these species is justified. In most European countries, subclinical cases are not treated during lactation, but the detection of a pathogen (including NAS) at the end of lactation can be used as a justification for an antibiotic dry cow treatment. None of the NAS-infected quarters in this study could be observed before and after the dry period and, to the best of our knowledge, no other study has investigated the self-cure rate from NAS IMI over the dry period. Further research is therefore needed to explore whether untreated NAS infections remain throughout the dry period or if the self-cure rate is high and NAS infected quarters should not be treated with antimicrobial substances at dry off.

Strep. dysgalactiae. *Streptococcus dysgalactiae* had the longest infection durations of all studied species. Although around 30% of the infections were shorter than 28 d, the probability of remaining infected up to 140 d was almost 60%. To our knowledge, only 2 other studies have investigated the infection durations of *Strep. dysgalactiae* IMI. In a study from 1985, conducted in 7 commercial herds, infection durations averaging 69 d for spontaneously cured infections and 131 d for infections persisting until the beginning of the dry period have been reported (Grommers et al., 1985). Another study, following 12 quarters over 2 lactations, found that several quarters remained infected for several months, and 1 quarter remained positive over the entire 2 lactations (Oliver et al., 1998). Interestingly, the observed infection durations in the current study were long, the strain diversity was low (indicating contagious spread or a hotspot), and 73% of all positive samples harbored ≥5,000 cfu/mL (data not shown). This indicates a high infectious pressure. Still, the dominant *Strep. dysgalactiae* strains caused only a limited number of new infections (n = 11; Figure 6) over the entire study period.

Strep. uberis. We estimated that 30% of all *Strep. uberis* infections lasted for a maximum of 28 d, and 46% of all infections lasted no longer than 70 d. Other studies, conducted at the species level only, have reported median infection durations of 16, 26, 46, and 100 d (Zadoks et al., 2003; McDougall et al., 2004; Leelahapongsathon et al., 2016). Many potential factors explain this large variation between study results. For example, durations of *Strep. uberis* infections might depend strongly on the individual farm management, cow characteristics, or strain properties. Additionally, all studies used slightly different methods (e.g., estimation only for new infections vs. inclusion of left-censored data; or different infection definitions and sampling intervals). Therefore, further research exploring the factors that are associated with the duration of *Strep. uberis* infections in a larger number of herds while using a standardized methodology could help improve the control of *Strep. uberis* IMI in the future.

Study Design and Methodology

Estimation of Infection Durations Based on Strain Typing. Only a few of the mentioned studies investigating the durations of infections identified isolates by strain typing. Therefore, we cannot exclude the possibility that infection durations have been overestimated in previous studies. This can happen when new infections with other strains of the same species are not recognized and infections are considered ongoing. In the present study, this was most relevant for the estimation of infection durations of the 2 studied NAS species. The durations of many infections with *Staph. epidermidis* (in 7 of 33 positive quarters) and *Staph. haemolyticus* (all quarters that were positive at more than one consecutive sampling, n = 7) would have been overestimated without using the strain typing results. The necessity of using strain typing to distinguish between new and ongoing infections has been emphasized previously (Oliver et al., 1998). In the present study, also for *Staph. aureus* infections, strain typing allowed us to identify 1 new infection with a different strain (Figure 3: cow 1339, rear right quarter). Other authors have described the isolation of a new strain after a quarter had been cured from a first strain (Sommerhäuser et al., 2003; Barlow et al., 2013), and it has been suggested that quarters previously infected with *Staph. aureus* are more likely to acquire a new infection with the same pathogen (Zadoks et al., 2001b).

Also, from milk samples of 4 quarters with *Strep. uberis* infections, we cultured more than 1 strain and therefore adjusted the assigned infection durations. Consecutive infections with different strains of *Strep. uberis* have been described previously (Oliver et al., 1998). In that study, the presence of several strains in 1 quarter at the same time was evaluated, and no indication of a parallel infection with different strains was found. In contrast, all *Strep. dysgalactiae*–infected quarters were positive throughout the present study for 1 strain type only. Oliver et al. (1998), in contrast, cultured up to 5 different strains of *Strep. dysgalactiae* from the same quarters over a period of 2 lactations.

Although strain typing allowed us to differentiate new infections with different strains from ongoing infections within the same quarter, we also used strain typing to correct for presumably false-negative results between positive samplings. In many studies, the occurrence of 1 negative sample between 2 positives is considered a false-negative outcome due to imperfect test sensitivity and corrected to positive (Kirkeby et al., 2019). A positive sample after 2 negative samples is

usually considered a new infection. We decided to consider infections ongoing as long as the same strain was detected in samples from the same quarter. Especially for rare strains (e.g., strain C in *Staph. aureus* or strain P in *Strep. uberis*), this approach likely improved the estimation of infection durations, because new infections with these strains are very unlikely. To be consistent in our methodology, we also used this approach for the dominating strains of *Staph. aureus* (type A) and *Strep. dysgalactiae* (type A) that each had a detection gap in 1 quarter over 3 and 4 samplings, respectively. For these rare cases, we cannot exclude the possibility that our correction to an ongoing infection was inappropriate and we assigned too-long infection durations by assuming an ongoing infection while a new IMI with the same strain might have occurred.

In summary, we adjusted the observed infection durations based on strain typing results for all 5 studied species because of identified new infections with different strains or presumably ongoing infections. For the reasons discussed above, we suggest that in future studies on infection durations of IMI, the inclusion of strain typing should become more widely used.

Estimation Methods for the Duration of Infections. We estimated the durations of infections using the life table method. This method is typically used when observations are only collected at predefined intervals. In contrast to the Kaplan-Meier method, it assumes a uniform distribution of risk and withdrawal within each sampling interval (Celentano et al., 2019). This is appropriate when the sampling intervals are short, as in the present study.

We additionally provide the estimates for the durations of infections using the Kaplan-Meier method because this has been used in several other publications on the duration of IMI (Zadoks et al., 2003; Leelahapongsathon et al., 2016). Furthermore, we decided to provide both the estimates for the durations of infections including data from left-censored observations and only of new infections. Many of the infection durations of particularly *Strep. dysgalactiae* and *Strep. uberis* were long and consequently censored. While excluding all these long infections from the analysis would have biased the results toward shorter infection durations, the inclusion of left-censored data may have led to an overestimation.

Sampling Interval and Duration. In contrast to most other studies exploring the duration of infection that sampled herds approximately once a month (Leelahapongsathon et al., 2016; Kirkeby et al., 2019), we used a sampling interval of only 14 d. This or an even shorter sampling interval seems necessary, especially when studying the duration of infections of NAS. Also, a considerable share of all infections with the other 3 pathogens were estimated (by the life table method) to last no longer than 28 d (*Staph. aureus* 44%, *Strep. dysgalactiae* 29%, *Strep. uberis* 30%). On the other hand, microbiological analyses of quarter foremilk samples of a whole herd (with, on average, 207 cows in this study) are labor intensive and costly. Therefore, we had to limit the study duration to a 18-wk period. This observation period was not long enough to estimate the median durations of infections with *Strep. uberis* and *Strep. dysgalactiae*. However, we estimated that 59% and 54% of all infections with *Strep. dysgalactiae* and *Strep. uberis* lasted longer than 84 and 70 d, respectively.

Study Herd. Until now, most studies investigating the strain diversity of mastitis pathogens focused on dairy cow herds in outbreak situations or those with ongoing udder health issues (Sommerhäuser et al., 2003; Haveri et al., 2008; Leelahapongsathon et al., 2020). These studies provide valuable information for problem herds, but in herds with better udder health, the strain diversity and infection durations of a pathogenic species might differ (potentially being factors for a better udder health situation or consequences of good management). To improve the udder health management strategies in farms currently not experiencing mastitis problems and to explore the cost-effectiveness of certain control and preventive measures for such farms in the future, more information on the strain diversity and duration of infections in herds with an acceptable udder health situation is necessary. The present study provides such data for a single herd. The strain diversity was similar to that observed in studies investigating herds with ongoing udder health issues (Sommerhäuser et al., 2003; Haveri et al., 2008; Leelahapongsathon et al., 2020). Especially for *Strep. uberis*, *Strep. dysgalactiae*, and *Staph. epidermidis*, the occurrence of dominating strains seems to generally vary between herds. Furthermore, the infection durations of *Staph. aureus*, *Strep. dysgalactiae*, and *Strep. uberis* were similar to that observed in previous studies, although data on infection durations are generally scarce. Nonetheless, for *Staph. aureus*, we observed much shorter infection durations (median: 80 d) than the mean infection duration estimated by other studies that have been conducted in herds with much higher prevalences (Lam et al., 1996; Zadoks et al., 2002). A limitation of this study is that it was carried out in one herd only and infection durations may differ between herds. Therefore, further studies should be carried out to generate data on infection durations for a larger number of herds with acceptable udder health status.

CONCLUSIONS

In a herd with low prevalence for all of the studied pathogens, infections with *Staph. epidermidis* and

Staph. haemolyticus mostly seem to have established through independent infection events. In contrast, dominating strains were found for *Staph. aureus*, *Strep. dysgalactiae*, and *Strep. uberis*. Farmers and their advisors need to consider transmission routes when designing prevention and control measures for their herds, and strain typing can be one tool to identify these. Furthermore, short infection durations were observed for *Staph. epidermidis* and *Staph. haemolyticus*. This raises the question of whether treatment of IMI with these pathogens with antimicrobial substances is justified. In contrast, infections with the other 3 pathogens were, on average, much longer, and many of these infections seem to have originated from contagious transmission within the herd. The outcomes of this study underline the importance of appropriate measures to prevent contagious transmission within dairy cow herds.

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