Minimum inhibitory concentrations of chlorhexidine- and lactic acid-based teat disinfectants: An intervention trial assessing bacterial selection and susceptibility

J. A. Schwenker,1* U. Schotte,2 and C. S. Hözel1
1Department for Animal Hygiene and Animal Health, Institute of Animal Breeding and Husbandry, Christian-Albrechts-University Kiel, 24098 Kiel, Germany
2Department A-Veterinary Medicine, Central Institute of the Bundeswehr Medical Service Kiel, 24119 Kronshagen, Germany

ABSTRACT

Teat disinfection is a recommended preventive tool to improve udder health and to prevent new intramammary infections. However, side effects are discussed, such as bacterial selection of less-susceptible bacteria with the application of certain teat disinfectants. The objective of this study was to assess the species composition and bacterial in vitro susceptibility by means of an interventive trial. For this purpose, 3 different postmilking teat treatments (disinfection with 0.215% chlorhexidine or 3.5% lactic acid, or control group with no dipping) were applied to 28 cows in a 6-d intervention approach using a split-udder design. Milk samples were taken before and after intervention. Bacteria were cultured and differentiated to species or genus level by MALDI-TOF mass spectrometry. Minimum inhibitory concentrations (MIC) were determined, and MIC changes over time were recorded. Susceptibilities to chlorhexidine and lactic acid were compared between species of the genera Staphylococcus, Streptococcus, Corynebacterium, and others. Species composition changed during the intervention. Under the treatment of chlorhexidine and lactic acid, the proportion of coagulase-negative staphylococci (CNS) decreased. An increased proportion of species belonging to the genus Corynebacterium was observed especially under the application of lactic acid. An increased proportion of species of the genera Staphylococcus aureus, and Corynebacterium spp. showed significantly lower absolute MIC values for chlorhexidine. Compared with other species, Corynebacterium spp. showed the lowest susceptibility for chlorhexidine as well as for lactic acid. A significant increase in MIC values after 6 d of intervention was observed with the lactic acid treatment in all isolates, as well as in CNS. This increase can be interpreted as either adaptation of isolates or displacement of more-susceptible species by less-susceptible species. Further studies using long-term intervention might reveal more pronounced effects on MIC values and species composition.

Key words: chlorhexidine, lactic acid, mastitis pathogens, MIC, teat disinfection

INTRODUCTION

Mastitis, an inflammation of the mammary gland, is a widespread factorial disease in dairy herds and causes high economic losses due to premature culling, antibiotic treatment, and decreased milk yield, as well as negative effects such as pain and discomfort for the cow (Bradley, 2002; Oviedo-Boyso et al., 2007; Leslie and Petersson-Wolfe, 2012). Especially in the early-lactation phase around parturition, the risk of intramammary infections is increased (Cremonesi et al., 2018), because the udder tissue undergoes a rapid increase in productivity and vulnerability due to histological changes during this period (Sordillo, 2005). In addition to optimal milking and housing hygiene, teat disinfection is a way to prevent mastitis and might thereby increase animal welfare and reduce antibiotic use as well as economic losses (Bradley, 2002; Cheng and Han, 2020). The application of a teat disinfectant reduces the bacterial load on the teat apex and can thus prevent new intramammary infections (Kumar et al., 2012). In particular, post-milking teat disinfection helps reduce mastitis caused by environmental bacteria such as Streptococcus uberis, Escherichia coli, and coagulase-negative staphylococci (Oliver and Mitchell, 1984; Galton, 2004; Quirk et al., 2012). Because the teat canal remains open for up to several hours after milking, a post-milking teat disinfectant protects the orifice from invasion of the previously mentioned bac-

Received June 4, 2021.
Accepted September 9, 2021.
*Corresponding author: jschwenker@tierzucht.uni-kiel.de
teria (Pankey et al., 1984). Intramammary infection caused by contagious bacteria such as Staphylococcus aureus and Streptococcus agalactiae, which can be transmitted in particular by milking personnel or by milking machines, can be reduced by applying a post-milking teat disinfectant (Pankey et al., 1985; Barkema et al., 2009; Breen, 2016). Species of the genus Corynebacterium, minor pathogens that often trigger subclinical mastitis, have also been demonstrably reduced in this way (Williamson and Lacy-Hulbert, 2013; Haune et al., 2018). Numerous post-milking teat disinfectants may be used, such as iodophors, quaternary ammonium compounds, chlorhexidines, chlorine dioxide, and organic acids such as lactic acid (Fitzpatrick et al., 2021). Several publications have already demonstrated their effectiveness in preventing intramammary infections in various experimental designs (Hogan et al., 1995; Oliver et al., 1999; Quirk et al., 2012; Williamson and Lacy-Hulbert, 2013; Enger et al., 2016). The use of a lactic acid-based teat disinfectant reduced the bacterial load on the teat skin and decreased the prevalence of mastitis due to coliforms such as E. coli (Chotigarpa et al., 2019). In addition, Boddie and Nickerson (1992) reported a reduction of 70.5% in intramammary infections caused by Staph. aureus and of 40.4% in those caused by Strept. agalactiae. The use of a chlorhexidine teat disinfectant also showed effectiveness against numerous minor and major pathogens, such a reduction of Staphylococcus species infections and Corynebacterium bovis infections by 49% and 65.2%, respectively (Hicks et al., 1981; Oliver et al., 1990; Drechsler et al., 1993; Hogan et al., 1995).

Summarizing the literature, teat disinfectants are reported to be very effective, and applied concentrations in vitro do not face bacterial resistance (Langsrud et al., 2003). However, bacteria that attach to surfaces generally appear to be more resistant to disinfectants (LeChevallier et al., 1988). Bacteria can also form extracellular polymers that protect against the attack of disinfectants (Brown and Gilbert, 1993). Thus, in vitro susceptibility does not exclude bacterial selection due to teat disinfection.

In general, disinfectants can select bacteria irrespective of acquired resistance, as the natural (intrinsic) susceptibility of bacteria to disinfectants differs widely (Schwaiger et al., 2014), particularly depending on cell wall structures (Poole, 2002). Thus, not every bacterial genus or species is reduced equally well by disinfectants (Eberhart et al., 1983; Quirk et al., 2012). Compared with gram-negative bacteria, gram-positive bacteria are more susceptible to disinfectants (Köljalg et al., 2002; Langsrud et al., 2003) and coccoid forms less than rod-shaped bacteria (Gao and Liu, 2014). Considering acquired resistance, some strains, such as Staphylococcus aureus, also form variants with lower susceptibility through mutation or horizontal gene transfer, as seen in bacteria developing antibiotic resistance (Langsrud et al., 2003). Particularly, methicillin-resistant Staph. aureus (MRSA) strains often tolerate higher disinfectant concentrations than methicillin-sensitive strains (Wootton et al., 2009). In several studies, in response to application of a chlorhexidine teat disinfectant (CH), bacterial species showed a significant reduction in susceptibility to CH (Horner et al., 2012; do Vale et al., 2019; Yeon and Young, 2019). Kampf (2016) links the widespread use of chlorhexidine to an increase in bacterial resistance of gram-negative bacteria such as Pseudomonas aeruginosa, Serratia marcescens, and Klebsiella pneumoniae. Cross-resistance between chlorhexidine and several antibiotics has also been reported (Köljalg et al., 2002). To our knowledge, studies on reduced susceptibility to lactic acid teat disinfectants have not been published yet; only resistance mechanisms of lactic acid bacteria coping with acid stress have been observed (Wang et al., 2018).

Any differences in disinfectant susceptibility between bacterial strains and bacterial species may result in the selection of certain bacterial species that are less susceptible to disinfectants (Khan et al., 2017). In addition, a shift in the udder microbiota is discussed (Cassir et al., 2015), which may lead to a reduction of certain mastitis pathogens but not to a lower incidence of new intramammary infection overall (Schukken et al., 1989).

Most studies rely on laboratory investigations or infection trials, and prospective observational studies using interventional approaches are rare. Thus, we determined species composition and MIC values before and after using teat disinfectants based on a 6-d intervention approach on 28 cows. Two different teat disinfectants (chlorhexidine- and lactic acid-based) were included, as well as an undipped control group. We hypothesized (1) that teat disinfection would lead to selection of less-susceptible bacteria, resulting in shifts in species composition as well as adaptation processes, leading to increased MIC values, and (2) that these effects differ between chemically unrelated disinfectants and are more pronounced if MIC values of bacteria are closer to applied concentrations.

MATERIALS AND METHODS

Experimental Design

The study was conducted on a research dairy farm of Kiel University in Schleswig-Holstein, Germany. The average somatic cell count of 180 lactating cows was 190,000 cells/mL of milk at the start of the experiment, with the proportion of cows below 100,000 cells
being 70%, between 100,000 and 200,000 cells 15%, and above 200,000 cells 15%. The herd was milked twice a day in a rotary milking parlor (28 stalls, GEA Group) with an automatic takeoff and no intermediate disinfection of the cluster. During the study none of the cows (n = 28) were involved in invasive trials or antibiotic treatments. Approxi-mately 8 wk before the expected calving date, all cows were intramammarily instilled once with an antibiotic teat sealant (Orbenin Extra, cloxacillin-benzathine, Zoetis). The last routine post-milking teat disinfection was performed 11 wk before the expected calving date. Two weeks after calving, a 6-d intervention in split-udder design began. The animals were divided into 6 groups, balanced by lacta-tion number and somatic cell count before intervention (average of 2 sampling dates before drying-off; Supplemental Table S1, https://zenodo.org/record/5564627#.XYAPZRxOliG; Schwenker, 2021). Heifers were not included in the trial. Cows were assigned to respective treatment groups with the help of colored leg bands. A partial block design with 3 different disinfectant treat-ments per group was chosen. Quarters were divided into controls and trials. Control quarters did not receive disinfection. To account for different infection risks in fore and hind quarters, we duplicated 1 application of 1 teat disinfectant in every cow, so that in one-third of the cows, 2 quarters were treated with chlorhexidine gluconate and 1 quarter with lactic acid; then in the second third of the cows, 2 quarters were treated with lactic acid and 1 quarter with chlorhexidine gluconate. In the last third of the cows, controls were doubled, so that controls were also balanced between fore and hind quarters (Table 1). The treatments were a lactic acid-based teat disinfectant [LA; 3.5%, wt/wt; relative density of 1.06 g/mL ± 0.02; LactiFence, DeLaval] and a chlorhexidine gluconate-based teat disinfectant (CH; 2,150 mg/kg; Iophile, Hypred GmbH). Chlorhexidine was chosen because it is a commonly used teat disinfec-tant in this region, and lactic acid was chosen because it is chemically unrelated and increasingly used in organic farms. Chlorhexidine had previously been applied in the study cows but was stopped 13 wk before starting the intervention. No lactic acid-based disinfectant had been applied on the study farm before.

The teat disinfectants were applied using conventional teat cups without back flush; they did not require prior mixing and were thus ready to use. Each cow was disinfected once a day after the milking process with a separate teat cup, which was cleaned after each application.

### Laboratory Procedures

**Sample Collection and Culturing.** Quarter fore-milk samples were taken before (d 0) and at the end of the intervention (d 5 and 6), immediately before evening milking. Sampling was performed according to standard recommendations by the National Mastitis Council’s Laboratory Handbook on Bovine Mastitis (NMC, 2017), thus preventing contamination. The teats were forestripped and thoroughly disinfected in 70% ethanol-drenched cell material. First milk streams were discarded, and 10 mL of quarter foremilk were collected into sterile 15-mL plastic tubes (Cellstar, Greiner Bio-One). Quarter milk samples were transported on ice (but prevented from freezing) and were processed immediately in the laboratory. After sufficient homog-enization by mixing thoroughly at 2,500 rpm for 5 min using a Vortex-Genie 2 (Scientific Industries), 1 mL of milk was diluted 1:10 and 1:100 in 1/4 Ringer’s solution. Both dilutions and the undiluted milk (0.1 mL each) were plated on Columbia blood agar with 5% sheep blood (Thermo Fisher Scientific). After incubation at 37°C for 18 to 24 h, colony morphology was observed and colonies were counted (cfu/mL of milk). Isolates were subcultured, and pure cultures were further investigat-ed with basic biochemical tests (catalase, oxidase, 3% potassium hydroxide) and Gram staining according to National Mastitis Council Guidelines (NMC, 2017), to choose diverse isolates for subsequent MALDI-TOF identification. Sample collection and cultivation were performed from May to October 2019.

**MALDI-TOF Identification.** Further identification at genus or species level was performed by MAL-DI-TOF MS using an Autoflex 3 Smartbeam (Bruker Daltonics GmbH). For each application, the instrument was calibrated with the *E. coli* DH5α bacterial test standard (Bruker Daltonics) according to the manufac-turer’s instructions. Isolates were prepared for MALDI-TOF MS according to the on-plate preparation method described by Schmitt et al. (2013). Briefly, a sterile wooden applicator (Carl Roth GmbH & Co. KG) was used to directly apply some colony material onto a polished steel target plate in duplicate (MSP 96-well plate; Bruker Daltonics). Each well was covered with 1 µL of

### Table 1. Overview over the different groups and the treatments

<table>
<thead>
<tr>
<th>Quarter</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>0 CH</td>
<td>LA CH</td>
<td>CH CH</td>
<td>CH LA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RF</td>
<td>LA 0</td>
<td>CH LA</td>
<td>0 CH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LR</td>
<td>0 LA</td>
<td>0 LA</td>
<td>0 CH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR</td>
<td>CH 0</td>
<td>0 LA</td>
<td>LA CH</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1LA = lactic acid teat disinfectant; CH = chlorhexidine teat disinfectant; 0 = control (no teat disinfection).
2LF = left front; RF = right front; LR = left rear; RR = right rear.
70% formic acid (Carl Roth GmbH & Co. KG) and air-dried at room temperature. To allow co-crystallization with the sample, wells were overlaid with 1 µL of matrix solution, a α-cyano-4-hydroxycinnamic acid (Bruker Daltonics) dissolved in a standard solvent containing of 50% acetonitrile, 2.5% trifluoroacetic acid, and 47.02% water (Sigma-Aldrich Chemie GmbH). Mass spectra were obtained within the mass range of 2 to 20 kDa and using flexControl 3.4 (Bruker Daltonics). Spectra were checked for their quality using flexAnalysis 3.4 to enable further differentiation in the Biotyping software system 3.1 (Bruker Daltonics), which is equipped with a library of 6,228 reference spectra (BDAL-7854 database; Bruker Daltonics). To ensure adequate identification at species and genus level, score cutoff values of ≥2.0 (species) and ≥1.7 (genus) were chosen.

**MIC Determination of Teat Disinfectants In Vitro.** A disinfectant susceptibility test was performed with all isolates that could be identified at species level. The following reference strains were also included in the test: *Bacillus licheniformis* (DSM 13), *C. bovis* (DSM 20582), *E. coli* (DSM 1103), *Staph. aureus* (DSM 1104), *Staphylococcus sciuri* (DSM 20345), *Staphylococcus xylosus* (DSM 6179), *Streptococcus dysgalactiae* (DSM 20662), and *Strep. uberis* (DSM 20569), where DSM indicates type strains from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The test was performed using broth macrodilution. The procedure was carried out in a row dilution test following the method of the German Veterinary Medical Society (DVG, 2017). The test disinfectants (LA and CH) in graduated dilutions were 1:1 mixed with double-concentrated broth and inoculated with a small amount of a diluted bacterial test suspension of the isolate being tested. All microbiological work was performed under a laminar flow workbench. For diluting, water of standardized hardness was produced, according to the specifications of DVG (2017). To prepare 1,000 mL of water of standardized hardness, 6 mL of solution A (19.84 g of magnesium chloride and 46.24 g of calcium chloride, dissolved in 1,000 mL of deionized water and autoclaved) and 8 mL of solution B (35.02 g of sodium hydrogen carbonate, dissolved in 1,000 mL of deionized water and sterile filtered through a 0.22-µm membrane) were mixed in 700 mL of deionized water and filled up to 1,000 mL. The pH (7.0 ± 0.2) was adjusted with 1 mol/L sodium hydroxide. The test disinfectants were diluted in graduated concentrations with the water of standardized hardness, and 5 mL of each was added to 15-mL glass reagent tubes. Then, 5 mL of a double-concentrated tryptic soy broth (Carl Roth GmbH & Co. KG) was added. Following Watts and Rossbach (2000), a supplement of 1% Tween 80 (VWR International GmbH) was added to the tryptic soy broth when testing fastidious species of the genus *Corynebacterium*. Previously, we confirmed that 1% Tween 80 did not change the CH-MIC value of non-fastidious reference strains (*Staph. scwuri* DSM 20345, *Staph. xylosus* DSM 6179 in triplicate). The LA-MIC values of the tested reference strains increased by a factor of 10 with the addition of 1% Tween 80. However, the supplement had to be used in the MIC tests of *Corynebacterium* spp. despite this observation, because it was required to obtain valid results (sufficient growth) as tested in a pretrial (data not shown). The bacterial test suspension was prepared according to CLSI (2015). For this purpose, fresh overnight cultures were used, which were inoculated at 37°C for 18 ± 2 h on blood agar. For the cultivation of fastidious species of the genus *Corynebacterium*, 1% Tween 80 was previously spread on the agar plate. Using a sterile swab (ROTILABO, Carl Roth GmbH & Co. KG), some colony material was collected, and a turbidity equivalent to 0.5 McFarland standard (Carl Roth GmbH & Co. KG) with approximately 1.5 × 10^8 cfu/mL was adjusted in sodium chloride. A 1:10 dilution in tryptone sodium chloride was prepared, and 100 µL of a final bacterial concentration of 1.5 × 10^7 cfu/mL was added to each test tube.

All MIC values were recorded in 2 different ways: for comparing MIC values before and after the intervention, we simply recorded the concentration (wt/wt) of the respective disinfectant (active ingredient) in the lowest concentrated test suspension impeding growth. For comparing MIC values between disinfectants, we calculated the relative inhibitory concentration (relative efficiency) by setting the recommended applicable concentration of each test disinfectant (0.215% for chlorhexidine gluconate, 3.5% for lactic acid) as 100%. The relative inhibitory concentration was then given as percentage of this recommended concentration to enable comparisons of MIC shifts between differently active chemicals. First, a pre-test was performed for each isolate and both teat disinfectants in triplicate with the following concentrations: undiluted, 1:10, 1:100, 1:1,000, and 1:10,000. The test tubes were incubated at 37°C for 72 h and regularly mixed every 24 h using a Vortex-Genie 2 (Scientific Industries). After a successful pre-test, the isolates were retested with lower gradation steps (smaller intervals) of disinfectant concentrations. For this purpose, the test disinfectant concentration determined in the pre-test was then fine-scaled in relative concentrations from 0.49% to 0.37%, 0.24%, 0.12%, 0.049%, 0.037%, 0.024%, 0.012%, 0.0049%, 0.0037%, 0.0024%, 0.0012%, and 0.00049% of the recommended concentrations. The chosen range in this fine-scaled test depended on the pre-test-results of the bacterial species. The fine-scaled tests were made in single approaches
and were also incubated at 37°C for 72 h and regularly mixed once a day. Dilution series of teat disinfectants without inoculation of bacterial test suspension served as a negative control. In addition, a growth control of each isolate (without a teat disinfectant added) was included as a positive control. The MIC value, defined as the lowest concentration of disinfectant without visible bacterial growth (clear tube), was evaluated after 72 h of incubation. To check for purity and, at the MIC level, absence of growth, 100 µL of the test tubes were spread on blood agar and incubated again at 37°C for 24 h. In total, 322 isolates of Bacillus spp., Brevibacterium spp., Corynebacterium spp., Staphylococcus spp., Streptococcus spp., and others were tested.

**Antimicrobial Susceptibility Test.** Staphylococcus aureus isolates were tested for cefoxitin resistance to detect MRSA. In addition to the MALDI-TOF-identified Staph. aureus isolates (n = 15), a negative control (Staph. aureus DSM 1104) and a positive control (ATCC 29213; Smith et al., 2008) were included. The test was performed following EUCAST instructions (EUCAST, 2021), using the disk diffusion method according to Bauer et al. (1966). The strains were suspended in 0.9% sodium chloride and adjusted to a density equivalent of 0.5 McFarland standard. Using sterile swabs (ROTILABO, Carl Roth GmbH & Co. KG), material from the suspension was applied in dense lines in 2 rectangular and 1 diagonal layer on Mueller-Hinton agar plates (Bio-Rad Laboratories Inc.). Subsequently, the antibiotic disks (Thermo Fisher Scientific) containing cefoxitin (30 µg) were loaded onto the plates using sterile forceps and incubated at 37°C for 18 ± 2 h. Then, the inhibition zone diameters (in mm) were measured, interpreted based on the EUCAST breakpoints for Staphylococcus spp., and reported as susceptible, intermediate, or resistant.

**Assessing Persistent and New Infections.** In the case of Corynebacterium spp., we assessed persistent and newly occurring udder infections during the trial. Udder infections with Corynebacterium spp. were defined as SCC >200,000 cells/mL and detection of Corynebacterium spp. in the absence of major pathogens. New infections were absent on d 0 but present on d 5 or 6. Persistent infections were present on d 0 and d 5 or 6.

**Statistics**

Graphical processing and statistical analysis of MIC values were performed in GraphPad Prism (version 9.0.2; GraphPad Software). To compare MIC values between the teat disinfectants and the difference before and after the intervention, suitable nonparametric tests (paired and unpaired Mann-Whitney U-test) were performed. Spearman rank correlation coefficients were calculated to determine the associations between CH and LA before and after the intervention. For this purpose, the Spearman rank rho value (ρ) was used.

**RESULTS**

In total, 336 quarter milk samples of 28 cows were investigated, representing d 0, 5, and 6 of the intervention. Collected samples were cultured, and up to 6 isolates per time point and quarter, which did not resemble each other in morphology and pretest results, were chosen for MALDI-TOF analysis. This resulted in 445 isolates, of which 358 isolates were successfully identified to species level and 24 to genus level. Remaining isolates (n = 63) could not be successfully analyzed by MALDI-TOF, either because they did not regrow on subculture or because score cutoff values were below 1.7, resulting in uncertain identification. The most frequently identified isolates were Staphylococcus spp. and Corynebacterium spp. Species or genera that were prevalent both before and after the intervention were selected and tested by macrodilution with the 2 used teat disinfectants. In the pre-test, 35 different species, including 8 reference strains, were tested in triplicate to obtain more precise concentration levels with lower gradation steps in the subsequent MIC test. In the next step, 287 isolates were successfully tested in the fine-scaled MIC test (Supplementary Table S2, https://zenodo.org/record/5564627#.YXAPZRxOlhG; Schwenker, 2021). With 148 isolates, species of CNS were the most tested, ahead of Corynebacterium spp. (n = 55) and Aerococcus viridans (n = 36). The species Staph. aureus and Strep. uberis are represented with lower numbers of 15 and 12 isolates, respectively, along with Brevibacterium spp. (n = 13) and Bacillus spp. (n = 8).

**Correlation Between MIC Values of Chlorhexidine and Lactic Acid Before and After Intervention**

Significant correlations were found between MIC values of CH and LA before as well as after the intervention. Spearman rank correlation coefficients (ρ) between CH and LH were 0.44 before (d 0, P < 0.001) and 0.64 after the intervention (d 5 + 6, P < 0.001), when determined independently of genera (P < 0.001). The correlation was mainly due to species effects, as the correlation of MIC values between CH and LA was insignificant when compared within CNS, Corynebacterium spp., and Staph. aureus. Aerococcus viridans isolates had significantly correlated MIC values for CH and LA before the intervention (ρ = 0.42; P = 0.024) but not afterward (ρ = 0.30; P = 0.33). In CNS, cor-
Species Composition Before and After Intervention

The composition of the tested species or genera before and after the intervention can be seen in Figure 1. To check whether the composition before intervention differed substantially in the grouped quarters, their composition was examined in advance. The grouped quarters had a similar composition before intervention: in all 3 groups, CNS were the species with the highest proportion. After the 6-d intervention, the composition of the quarters using CH and LA differed from the undipped control group. Treatment using CH and LA reduced the proportion of CNS from 64.3% to 51% and from 58.6% to 39.6%, respectively. In the CH and undipped control groups, the proportion of Corynebacterium spp. (percent of isolates) remained relatively constant, whereas in the LA group, the proportion of Corynebacterium spp. increased after intervention. The percentage of quarters with persistent infections by Corynebacterium spp. within the 6-d intervention occurred in 5.4% of quarters in the CH treatment, in 13.5% in the LA treatment, and in 5.2% of quarters with no disinfection. In the undipped control group, isolates of the species Strep. uberis and Staph. aureus were only found before the intervention, not afterward, whereas, in the CH and LA groups, Staph. aureus and Strep. uberis isolates were detected before and after intervention. The percentage of quarters with Staph. aureus decreased after the intervention with LA and CH treatment, as was the case for Strep. uberis after intervention in the CH group. However, an insignificant increase in Strep. uberis prevalence was seen in the LA group after the intervention.

Comparison of MIC Values Between Teat Disinfectants

The relative effectiveness of both teat disinfectants was assessed in bacterial isolates taken before the start of treatment (d 0; Figure 2). Comparisons included MIC values of 14 isolates of the genus Corynebacterium, and isolates of the species A. viridans (n = 9), Strep. uberis (n = 6), Staph. aureus (n = 8), Staphylococcus haemolyticus (n = 25), Staphylococcus epidermidis (n
= 13), *Staphylococcus chromogenes* (n = 14), and other CNS (n = 15) such as *Staphylococcus arlettae*, *Staphylococcus hominis*, *Staphylococcus equorum*, *Staph. sciuri*, and *Staph. xylosus*. Higher MIC values and greatest dispersion were observed for all genera or species when using LA. Overall, the highest MIC were measured in *Corynebacterium* spp., being 0.0248% (CH) and 0.1238% (LA) of the recommended in praxi concentration. The lowest MIC of CH was recorded as 0.0001% in *A. viridans* and other CNS, and the lowest MIC of LA was recorded as 0.0012% in *Strep. uberis*. Median MIC (MIC50) were significantly closer to applied concentrations of LA, compared with CH, for most bacterial groups (*Corynebacterium* spp., *Staph. aureus*, *Staph. haemolyticus*, *Staph. chromogenes*, *Staph. epidermidis*, *A. viridans*, and other CNS, all P < 0.01). However, MIC values of *Strep. uberis* did not indicate a significant difference in the relative efficiency of LA and CH (LA-MIC50 = 0.0012%, CH-MIC50 = 0.0004%, P = 0.344, perhaps due to low statistical power with only 6 available isolates).

**Comparison of MIC Values Before and After Treatment**

The course of MIC values (mg of CH/L; mg of LA/L) over time was also tested. For this purpose, MIC values of CH and LA were assessed before intervention (d 0) and compared with MIC values under and after the respective intervention (d 5 + 6). Figure 3 shows an unpaired comparison of all species (different numbers of isolates before and after the intervention), to reflect the effect of intervention. The LA-MIC values were significantly higher after the 6-d intervention (d 0 MIC50 = 459 mg/L; d 5 + 6 MIC50 = 918 mg/L; P = 0.024). No significant differences in LA-MIC values over time were seen in quarters after CH treatment or in the undipped controls. The CH-MIC values showed
no significant differences in the isolates of quarters treated with CH for 6 d or the untreated control quarters. However, a significant difference was seen under LA treatment: species isolated after LA intervention evolved toward higher MIC values of CH (d 0 MIC50 = 0.8 mg/L; d 5 + 6 MIC50 = 1.06 mg/L; \( P = 0.03 \)) than those isolated before the intervention. Species composition differed in the compared groups; thus, a paired comparison (same species, same number of species before and after intervention) was performed to clarify whether higher MIC values were related to shifts in species composition. Paired comparisons of all prevalent CNS and, additionally, of only the species *Staph. haemolyticus* before and after intervention are shown in Figure 4. For the susceptibility tests of LA and CH, a total of 69 different CNS isolates were included: 25 from CH-dipped quarters, 16 from LA-dipped, and 28 from undipped quarters. No differences in LA-MIC values were observed under the influence of CH, LA, or in the undipped control group. Also, CH-MIC values of CNS species from untreated or CH-treated quarters did not differ significantly before and after the intervention. However, we observed a significant difference in CH-MIC values before and after LA treatment: CH-MIC values of CNS were significantly higher after LA intervention (d 0 MIC50 = 0.8 mg/L; d 5 + 6 MIC50 = 1.06 mg/L; \( P = 0.019 \)). No significant differences were seen in LA- and CH-MIC values under the influence of CH, LA, and in the undipped control when comparing only *Staph. haemolyticus* isolates.

A paired Mann-Whitney U-test was performed on CH- and LA-MIC values, comparing the same species from the treatment groups (CH, LA, control) after the intervention. No significant differences were present between the interventions, as shown in Figure 5.

**Antimicrobial Susceptibility**

The *Staph. aureus* isolates (n = 15) tested in the disk diffusion method showed MRSA as well as methicillin-susceptible *Staph. aureus* (MSSA) phenotypes with the indicator substance cefoxitin (breakpoint at 22 mm). Of the 15 isolates tested, 8 were isolated before and 7 after the intervention. Of the 8 *Staph. aureus* strains isolated before intervention, 4 had a MRSA phenotype. After the intervention, 7 isolates were divided into 4 MSSA and 3 MRSA strains. The determined inhibition zones of the tested *Staph. aureus* isolates and controls, as well as the MIC values of CH and LA before and after the intervention, are shown in Supplemental Table S3 (https://zenodo.org/record/5564627#.YXAPzRx0llG; Schwenker, 2021). Only 1 strain had an LA-MIC value above 459 mg/L (i.e., 918 mg/L); this strain had a MSSA phenotype. Five strains had CH-MIC values above the median. Of these strains, 3 had a MRSA phenotype.

**DISCUSSION**

Although all handling occurred under sterile conditions and the first sprays of milk were discarded, we were able to isolate a broad variety of bacteria. This is feasible, as fresh milk, even from healthy cows, is not considered to be sterile (Metzger et al., 2018). This is also supported by several studies dealing with the microbiome in milk (Lima et al., 2018; Andrews et al., 2019). In our interventive trial, we tried to assess intervention-related changes in species composition and MIC values. As we hypothesized, bacterial composition obviously changed after disinfectant intervention, particularly under treatment with LA. Thus, as we also hypothesized, shifts in bacterial composition were most pronounced in the LA group, where relative MIC values were much closer to applied concentrations, compared with the CH group. The proportion of *Corynebacterium* spp. increased after LA application, likely resulting from the fact that *Corynebacterium* spp. are less susceptible to organic acids, possibly because of adaptation, as also demonstrated by Jakob et al. (2007). As expected, the application of a teat disinfectant (LA or CH) reduced environmentally associated pathogens such as CNS. This is consistent with numerous studies (Hogan et al., 1995; Galton, 2004; Quirk et al., 2012; Cheng and Han, 2020; Fitzpatrick et al., 2021). Fitzpatrick et al. (2019) attributed this to the fact that the teat canal stays open for a time after milking, so that the layer of teat disinfectant in the teat canal replaces the physical barrier until the teat canal closes firmly again. However, differences were not statistically significant. The proportion of major pathogens (*Strep. uberis, Staph. aureus*) remained unchanged in the disinfected quarters, whereas these pathogens were no longer detected after the 6-d trial period in the undisinfected quarters. The absence in undisinfected quarters after intervention could be explained by spontaneous self-healing or by intermittent shedding (Owens and Nickerson 1990; Linder et al., 2013). The latter also speaks to the reduction of *Streptococcus uberis*, as this is also known to be intermittently shed (Field et al., 2003). Loss upon subculturing or MIC testing, as well as unsuccessful identification by MALDI-TOF, might have led to bias in the true bacterial composition, which, at genus level, might be better accessible by metagenomic molecular methods such as amplicon typing. However, this metagenomic method does not allow linkage of bacterial composition to MIC values.

Considering MIC testing, bacterial communities differed in their susceptibility to the applied disinfectants,
further substantiating the observations on shifts in bacterial composition before and after the intervention. *Corynebacterium* spp. had the highest MIC concentrations in the susceptibility test, compared with other isolates. This may be due to the unusual envelope of their cell wall (Puech et al., 2001). The genus *Corynebacterium* is related to *Mycobacterium* which, according to Russell (1999), is considered the genus most resistant to disinfectants. The lowest inhibitory concentration of CH was detected for *Strep. uberis*, which may be related to the fact that streptococci are comparatively susceptible to cationic disinfectants, as also shown by Kõljalg et al. (2002) for *Streptococcus pyogenes* and by Gehlen et al. (2000) for oral streptococci.

Focusing on the individual species or genus, we also found differences in efficacy between as well as within the tested bacterial species and genera, as shown by El Behiry et al. (2012) and Langsrud et al. (2003). This was why we adjusted the number of isolates to equal numbers of species in some of our analyses, in which we focused on adaptation more than on bacterial shifts.

The intervention revealed significant differences in some MIC values in the unpaired comparison of all species (community MIC) and in the paired compari-

---

**Figure 3.** Values of MIC of all tested isolates (n = 287) in response to lactic acid (a) or chlorhexidine (b) before (d 0) and after intervention (d 5 + 6) under the influence of the respective treatment. CH = chlorhexidine teat disinfectant; LA = lactic acid teat disinfectant; 0 = control (no teat disinfection). Unpaired Mann-Whitney U-tests: ns = not significant; *P = 0.05. Numbers and species compositions between isolates differ between d 0 and d 5 + 6. Upper and lower edges of the boxes correspond to the upper and lower quartile, midline = mean value, + = median value, whiskers = 5th to 95th percentile.
son of CNS species. Differences in MIC values in the unpaired comparison are mainly attributed to different species composition, as also seen in the correlation of MIC values between LA and CH (which is present if all species are mixed and absent when comparing isolates of the same species). As described earlier, species-related differences in MIC values are very well known and are the reason for using species-based comparisons whenever possible. However, in a more practical approach, with a factorial disease associated with a broad variety of minor pathogens, changes in community MIC are relevant findings as well, even if they simply result from changes in community composition. With our split-udder design, during a brief intervention, we hypothesize that changes in species proportions are not due to chance but are indeed effects of the intervention, as microbiome shifts—particularly with regard to the proportion of CNS and Corynebacterium spp.—were much more pronounced in the LA group compared with the control.

The macrodilution test of the 2 teat disinfectants showed that CH and LA were both effective against all tested bacterial isolates in vitro, as also shown by Boomsma et al. (2015) and Boddie and Nickerson (1992) for LA, and by Hogan et al. (1995), Oliver et al. (1990), and Drechsler et al. (1993) for CH. If comparing the chemically unrelated disinfectants in terms of their relative efficacy against the isolates tested, CH was clearly effective at much lower concentrations than LA, which agrees with the literature (Qiao et al., 2008; Kampf, 2016; Stanojević-Nikolić et al., 2016). In contrast to all other species, Corynebacterium spp. had to be tested with the addition of 1% Tween 80. Although we found no increase of CH-MIC values in non-fastidious species tested with or without Tween for comparison (Staph. sciuri, Staph. xylosus), LA-MIC
values increased by 1 to the power of 10 with Tween. Thus, we cannot exclude that Tween might interact with LA in a partly inactivating way, so the absolute LA-MIC values of \textit{Corynebacterium} spp. should be considered with reservation. However, a markedly higher effectiveness of LA, compared with CH, was also present in all species tested without Tween.

Despite in vitro effectiveness—MIC were below practically applied concentrations—we also observed that \textit{Corynebacterium} spp. displaced other bacteria in the LA group. Accordingly, in the LA- and CH-susceptibility test, significantly higher MIC values were recorded in the LA treatment of all species isolated from the LA treatment group after the 6-d intervention. In the CH group, by contrast, the selective pressure might have equally affected the \textit{Corynebacteriaceae}, due to the higher efficacy of CH. Our results indicate that \textit{Corynebacterium} spp. are relatively less susceptible to CH than other species. However, MIC values were still that far below recommended and applied concentrations, so that \textit{Corynebacterium} spp. are not expected to fill any gaps (they are killed themselves), and proportions remain unchanged. With regard to in vivo selection, despite in vitro efficiency, as seen for LA, Feßler et al. (2018) emphasize that in vitro MIC values are difficult to extrapolate into practical application, as disinfectants are mostly applied on surfaces. Additionally, organic material might impair the efficacy of the disinfectant, and milk residues can dilute the disinfectant (Best et al., 1990), resulting in subinhibitory concentrations that facilitate bacterial adaptation—which is even easier for bacteria when the margin between applied and inhibitory concentrations is smaller.

With regard to adaptation, species-related tests are more suitable to depict such individual processes of adaptation, whereas the unpaired test mainly depicts changes on a community level. We could not isolate species in a sufficient number to make meaningful com-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Comparisons of MIC values between treatments after intervention (d 5 + 6). CH = chlorhexidine teat disinfectant; LA = lactic acid teat disinfectant; 0 = control (no teat disinfection). Isolates \(n = 111\). Paired Mann-Whitney U-tests: ns = not significant, \(P > 0.05\). Groups were equal in number and species composition. Upper and lower edges of the boxes correspond to the upper and lower quartile, midline = mean value, + = median value, whiskers = 5th to 95th percentile.}
\end{figure}
parisons at species level, except for *Staph. haemolyticus*. Thus, we also made a paired test on (combined) species level for CNS species. In this test, we paired isolates of the same CNS species to equal numbers and tested them in a paired Mann-Whitney U-test. Interestingly, we detected significant signs of adaptation only for CH-MIC values in the LA treatment group. Cross-selection of increased CH-MIC values by LA application might be in line with the borderline-significant correlation between CH- and LA-MIC in CNS (*P* = 0.06), which was present only after intervention, not before, and might therefore indicate effects of untargeted adaptation, such as increased impermeability. Antimicrobial cross-resistance might also result from such untargeted adaptation to disinfectants, and vice versa. Thus, disinfectants are suspected to select MRSA strains (Wootton et al., 2009). The occurrence of MSSA and MRSA isolates before and after the intervention was balanced for CH-dipped quarters as well as for LA-dipped quarters. Disinfectant susceptibility of MSSA and MRSA did not differ systematically. The only strain with an increased LA-MIC value had a MSSA phenotype. Thus, we found no indications of MRSA selection associated with teat disinfection.

For practical reasons, interventive disinfection could be applied only once a day. Following best practice recommendations, teat disinfection should have been applied at each milking, thus twice a day. Selection might have been enhanced by this suboptimum praxis, as contact periods with sublethal concentrations were prolonged. By contrast, the rather short intervention period might not have been sufficient to cause full selective effects, so long-term intervention might result in more pronounced effects. In vitro trials have found the most pronounced adaptation to disinfectants within the first 6 d, but also a slight further increase of MIC within the following 5 to 7 wk (Schwaiger et al., 2014).

**CONCLUSIONS**

We conclude that both the CH and LA teat disinfectants were basically effective against the prevalent species and that, in case of such a pathogen spectrum, they are suitable for prevention of mastitis. However, most isolates were markedly more susceptible to the CH teat disinfectant, with a smaller safety margin for LA. Results point toward a selection of *Corynebacterium* spp. under LA treatment, possibly due to the fact that *Corynebacterium* spp. were generally less susceptible than all other species to both disinfectants. No selection occurred under CH treatment, likely due to the fact that MIC of CH were considerably below practically applied concentrations. With regard to the course of MIC values over time, significant differences were observed only under LA treatment. The unwanted selection of *Corynebacterium* spp. may have been preventable by best-practice application (twice-daily disinfection). Further research, including long-term intervention, is needed for a more accurate understanding of possible selection due to teat disinfectants, taking into account practical conditions and trends over time.

**ACKNOWLEDGMENTS**

The authors thank Evelyn Lass (Institute of Animal Breeding and Husbandry, Kiel, Germany) for technical assistance and Maike Sievers (Central Institute of the Bundeswehr Medical Service Kiel, Kronshagen, Germany) for valuable background assistance using the MALDI-TOF Biotyper. This project was financially supported by the H. W. Schaumann Foundation (Hamburg, Germany). The authors have not stated any conflicts of interest.

**REFERENCES**


