Intramammary infections and teat canal colonization with coagulase-negative staphylococci after postmilking teat disinfection: Species-specific responses

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ABSTRACT

Coagulase-negative staphylococci (CNS) are the most common pathogens associated with intramammary infections (IMI) in dairy cows. We hypothesized that postmilking teat disinfection would reduce microbial colonization of the teat canal and thus reduce the prevalence of IMI caused by certain CNS species. The efficacy of iodine postmilking teat dip was tested against CNS colonization of the teat canal, and incidence of IMI was measured. Using an udder-half model, 43 Holstein cows at the Washington State University Dairy were enrolled in the trial; postmilking teat dip was applied to one udder-half, treatment (TX), and the remaining half was an undipped control (CX). Teat canal swabbing and mammary quarter milk samples were taken in duplicate once a week for 16 wk for microbial culture. Isolates from agar cultures were presumptively identified as CNS and then speciated using PCR-RFLP and agarose gel electrophoresis. Colonization of the teat canal and IMI by CNS were assessed. Thirty CNS IMI were diagnosed and the number of new IMI in CX quarters (21) was significantly greater than that in TX mammary quarters (9). The majority of CNS IMI were caused by *Staphylococcus chromogenes* (30%) and *Staphylococcus xylosus* (40%), and the latter were appreciably reduced by teat dip. Except for *S. xylosus*, an association was observed between teat canal colonization and IMI by all CNS species in this study, in which the majority of IMI were preceded by teat canal colonization. The total number of CNS IMI was greater for CX group cows compared with TX group cows. However, the effect of disinfection on IMI did not appear to be the same for all CNS species.

Key words: coagulase-negative staphylococci, teat disinfection, teat canal colonization

INTRODUCTION

Coagulase-negative staphylococci are a heterogeneous group of opportunistic mastitis pathogens comprising 39 species and subspecies (Taponen and Pyörälä, 2009). In general, CNS IMI have untoward effects on dairy production. Coagulase-negative staphylococci cause subclinical and clinical mastitis, although clinical cases appear to be often mild in nature (Taponen et al., 2006). Intramammary infections by CNS result in an increase in milk SCC and contribute significantly to the overall herd SCC for those with low (<200,000 cells/mL) bulk tank milk counts (Schukken et al., 2009). Elevations in SCC induced by CNS IMI may have an effect on milk quality (Pyörälä and Taponen, 2009), and milk production is negatively affected by IMI with CNS (Thorberg et al., 2009).

Common CNS species that cause IMI in dairy cows are *Staphylococcus chromogenes*, *Staphylococcus simulans*, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, *Staphylococcus hyicus*, and *Staphylococcus haemolyticus* (Thorberg et al., 2009; Park et al., 2011). It appears that the effects on mammary gland health with CNS IMI are species dependent. The *Staphylococcus* species *chromogenes*, *simulans*, and *xylosus* are the most persistent in terms of mean duration of IMI, and *S. chromogenes* had the greatest ratio of persistent to nonpersistent IMI (Supré et al., 2011). The differences in quarter milk SCC associated with IMI by different CNS species are not uniform (Supré et al., 2011). The SCC associated with noninfected mammary quarters and those with *S. chromogenes* IMI were significantly different, but SCC associated with *S. cohnii* IMI were not significantly greater than SCC associated with noninfected mammary quarters, as an example (Supré et al., 2011). Work of Piessens and coworkers (2011) indicates that distribution of CNS species associations with the cow and her dairy environment is herd dependent. Control and prevention of CNS IMI seem difficult given the diversity in the species and the herd effects that influence the disease process.

Studies have indicated that CNS are the most common colonizers of the teat skin and teat ends (Taponen

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et al., 2008; Krömker and Friedrich, 2009). A relationship between colonization of the teat epidermis and IMI is seen (Taponen et al., 2006). Thus, teat disinfection would seem to be an effective method to control CNS IMI.

In an earlier study, postmilking teat disinfection was found to reduce CNS; however, speciation was not done and thus the species most affected by disinfection was not determined (Hogan et al., 1995). Postmilking teat disinfection is a widely adopted practice with more than 94% of herds using this mastitis control technique (USDA, 2007). However, such treatment has not eradicated CNS mastitis, as CNS appear to be the most prevalent pathogens associated with IMI (Thorberg et al., 2009). Although several species cause IMI, some species are much more common agents of mastitis than others and thus may be more resistant to teat disinfection. Thus, we hypothesized that postmilking teat disinfection would reduce teat canal colonization and reduce IMI by some *Staphylococcus* species.

MATERIALS AND METHODS

Cows

Holstein dairy cows ($n = 139$) from the Washington State University Dairy Center herd (Pullman) were used for this trial in accordance with the Institutional Animal Care and Use Committee's regulations. Cows were milked twice daily in a double-5 herringbone parlor that included automatic milking unit detachments. Before initiation of treatments, teat ends and teat skin of cows in the herd were examined and milk was expressed from functional mammary quarters. All cows enrolled ($n = 43$) met the following inclusion criteria before enrollment: lactating with 4 functional teats, free of IMI, teat-end score <3 , and teat skin score <4 . A teat end score of 1 indicated a smooth teat end devoid of a raised ring at the orifice, and a score of 4 signified a severely eroded teat end. A teat skin score of 1 indicated soft and pliable teat skin devoid of scales, and a teat end score of 4 was evidenced by rough skin and marked with multiple lesions (Zecconi et al., 2005). The treatment period lasted 16 wk, from September 22, 2009, to January 5, 2010.

Milking Procedures and Treatments

Standard milking time hygiene procedures [0.1% iodine premilking teat disinfection, foremilk stripping, cleaning the teats with a single-use cloth towel, and application of 1% iodine postmilking teat disinfectant] were altered with the initiation of the trial. During a 5-d pretreatment period, premilking preparation

included foremilk stripping and cleaning the teats of dirt and debris with a damp, single-service cloth towel; extra towels and water were used if necessary, and no disinfectant was applied. Use of postmilking teat disinfection was also suspended. The pretreatment washout period was initiated on September 17, 2009, and was used to ensure that previous teat disinfection would not influence response to treatment. Results from a pilot study indicated that the day-to-day variation in total bacteria counts from teat skin swabbing samples had stabilized by 5 d after cessation of pre- and postmilking teat disinfection.

Mammary gland halves, right versus left, were randomly allocated to treatment (TX) and control (CX) groups with the aid of a random number table such that within-cow comparisons could be made, with each cow having 2 teats in a TX udder half and 2 in a CX udder half. Teats in the TX udder half received postmilking teat disinfection with a 1% iodine commercial product (Teat-Kote, GEA Farm Technologies, Naperville, IL) immediately after milking unit detachment. Teats in the CX udder halves did not receive postmilking teat disinfection. Premilking preparation during the treatment period included foremilk stripping and cleaning the teats of dirt and debris with a damp, single-service cloth towel; extra towels and water were used if necessary, and no disinfectant was applied.

Sample Collection

To obtain teat canal swab samples, all teat ends were scrubbed with 70% isopropyl alcohol immediately before a daily milking. A calcium alginate, fiber-tipped, ultrafine aluminum applicator swab (Fisher Scientific, Tustin, CA) was inserted 2 to 3 mm into the distal end of the teat canal and rotated. The swab was then placed into a tube containing 1 mL of 0.2% sodium thiosulfate solution. This process of disinfecting the teat, collecting a swab sample, and storing that sample was repeated to obtain a second, duplicate sample. The thiosulfate solution acts as a neutralizer of the iodine that may have remained on the teat and could interfere with bacterial culture (Rendos et al., 1975; Fox et al., 1992). The teat ends were disinfected a third time before milk samples were collected. Duplicate milk samples were collected aseptically into sterile containers from all mammary quarters after the first stream of milk was discarded (Hogan et al., 1999). Duplicate milk samples were aseptically collected at the start of the pretreatment period. Throughout the treatment period, milk and teat canal swabs were collected at weekly intervals and were placed on ice, transported to the laboratory, stored at 5°C, and cultured within 24 h.

Culture Procedures

Fifty microliters of duplicate milk and teat canal solutions were spread evenly and cultured on mannitol salt agar plates (Difco Laboratories Inc., Detroit, MI) and incubated at 37°C and 5% CO₂ for 48 h. Plates were considered contaminated if cultures grew 3 or more dissimilar colonies. All unique colony culture types were identified by gross morphological characteristics, and a representative colony was re-cultured on blood agar plates (Hardy Diagnostics, Santa Maria, CA) with incubation as before. Cultures from blood agar were characterized by their catalase reaction, Gram stain, colony morphology, and the coagulase test (Hogan et al., 1999). The Slidex Staph Plus (bioMerieux Inc., Durham, NC) was used to further differentiate coagulase-positive cultures from *Staphylococcus aureus*. All presumptively identified CNS cultures were stored in sterile glycerin solution (60%) at -80°C until speciation.

Speciation of CNS Isolates

The CNS isolates were speciated using a PCR-RFLP as previously described (Park et al., 2011). Once an isolate was identified as CNS by presumptive identification, DNA was extracted as described by Pitcher et al. (1989) and the presence of the 931 bp of the *gap* gene was amplified using *Taq* polymerase with PCR. The PCR products of the *gap* gene were digested using the *AluI* restriction enzyme and analyzed by agarose gel electrophoresis. The banding patterns from the PCR-RFLP of *gap* gene were compared with known sequences from the NCBI GenBank database (NCBI; <http://www.ncbi.nlm.nih.gov/genbank/>), using the NEBcutter program (version 2.0; Vincze et al., 2003) to determine the species of CNS isolates.

Determination of IMI and Teat Canal Colonization

Mammary quarters were identified as having an IMI when the same CNS species was isolated from 2 of 3 consecutively collected milk samples in duplicate where the number of colony-forming units per milliliter milk of the same CNS species >120. A milk threshold of 120 cfu/mL of CNS is a count considered to have possible IMI significance (Barnes-Pallesen et al., 1987). Both duplicate samples had to result in cultures of 120 cfu/mL or more to be deemed to be from a quarter with a CNS infection.

A cured IMI was determined when all samples were free of the pathogen from 3 consecutive collection periods. A mammary quarter with a cured IMI was eligible for a new IMI at a subsequent sampling. Recovery of the same species of CNS from the duplicate swab samples

of the teat canal classified colonization of the canal for each sampling period. The threshold for determination of the teat canal-colonizing pathogen was 40 cfu/mL of solution, similar to the 50 cfu/mL threshold used by Trinidad et al. (1990) to classify teat canal colonization. Moreover, in the current study, duplicate samples were collected to guard against a contaminant falsely classified as a colonizing agent. If more than one CNS species was identified from teat canal samples, then the teat canal was deemed colonized by multiple species. A 2 × 2 table was created in which groups (rows) considered were either TX or CX, and outcomes (columns) were new CNS IMI or no new CNS IMI for each mammary quarter. With this table, IMI by group were contrasted, both in total and by individual species, using Fisher's exact test (Shoukri and Pause, 1998). Similarly, the number of teat canals colonized by CNS between treatments was contrasted by Fisher's exact test.

RESULTS

The mean parity of enrolled cows was 2.1, with a range of 1 to 5 and a median parity of 2. The mean DIM was 210.1, with a range of 39 to 467 and a median of 175. Thirty CNS IMI were diagnosed in 30 mammary quarters over the 16-wk treatment period in 21 cows (Table 1). These IMI included 8 species of CNS: *S. chromogenes*, *S. cohnii*, *S. epidermidis*, *S. equorum*, *S. hemolyticus*, *S. hyicus*, *S. simulans*, and *S. xylosus*. All identified species, except *S. equorum*, caused IMI in at least one CX mammary quarter. However, in TX mammary quarters, IMI were caused by only 3 species: *S. chromogenes*, *S. equorum*, and *S. xylosus*. A significant difference ($P = 0.003$) was observed between the occurrence of IMI in CX and TX quarters by CNS (21 vs. 9). The ecology of CNS causing IMI, by species, was affected by TX. *Staphylococcus xylosus* and *S. chromogenes* accounted for 40 and 30% of the CNS IMI, respectively. An appreciable difference ($P = 0.065$) was observed in *S. xylosus* IMI between TX (3) and CX (9) mammary quarters. Overall, the CX quarters harbored significantly more IMI caused by more CNS species than TX ($P < 0.003$). *Staphylococcus chromogenes* was the most prevalent CNS species causing IMI in the TX quarters, with 5 of 9 mammary quarters infected with this species. *Staphylococcus xylosus* caused a larger proportion of IMI in the CX mammary quarters; 9 of 21 had IMI with this pathogen compared with 3 of 9 in TX mammary quarters.

Thirty CNS IMI were diagnosed, and at the time of diagnosis, 15 of the quarters' teat canals were colonized by the same CNS species that were causing the IMI (Table 2). Mammary quarters infected by *S. chromogenes* had teat canals colonized by *S. chromogenes* in 7

Table 1. Number of mammary quarters with IMI by treatment and by *Staphylococcus* species as identified by weekly milk sampling during a 16-wk study period¹

Species	No. of IMI	Control	Treatment	P-value ²
<i>S. chromogenes</i>	9	4	5	0.500
<i>S. cohnii</i>	2	2	0	0.248
<i>S. epidermidis</i>	1	1	0	0.500
<i>S. equorum</i>	1	0	1	0.500
<i>S. hemolyticus</i>	3	3	0	0.128
<i>S. hyicus</i>	1	1	0	0.500
<i>S. simulans</i>	1	1	0	0.500
<i>S. xylosus</i>	12	9 ^a	3 ^b	0.065
Total	30	21 ^a	9 ^b	0.003

^{a,b}Values with different superscripts are significantly different.

¹Control mammary quarters received no postmilking teat disinfection; treated mammary quarters received postmilking teat disinfection with 1% iodophor solution.

²Contrast of the number of IMI between treatment and control groups; P-values were generated from Fisher's exact test calculations.

of 9 cases at the time of IMI diagnosis. In only 3 of 12 cases of *S. xylosus* did teat canal colonization occur at the time of diagnosis of IMI. *Staphylococcus cohnii* was the only CNS that did not concurrently cause IMI and colonize the teat canal. Of the 8 teat canals colonized by *S. hyicus*, only 1 was associated with an *S. hyicus* IMI and the remainder were associated with IMI by 3 other CNS. Overall, *S. chromogenes* was the most common CNS species found colonizing teat canals. Three teat canals were colonized by more than one species of CNS. One mammary quarter with *S. chromogenes* IMI was colonized by both *S. chromogenes* and *S. hemolyticus*, and 2 *S. hemolyticus* IMI were colonized by both *S. hemolyticus* and *S. hyicus*. *Staphylococcus equorum* and *epidermidis* IMI were not associated with teat canal colonization.

Most teat canal colonizations occurred 1 to 2 wk before IMI diagnosis with the same species. Of the 15 mammary quarters that had teat canal colonization before IMI, 3 occurred at least 3 wk before IMI, 7 occurred 2 wk prior, and 5 occurred 1 wk prior. Most (5/7)

teat canal colonizations with *S. chromogenes* preceded diagnosis of IMI with this agent at least 2 wk before IMI was diagnosed. However, the majority (9/12) of *S. xylosus* teat canal colonizations were detected after an IMI diagnosis.

The summary of all teat canal colonizations independent of IMI is given in Table 3. Six species of CNS were isolated from CX teat canals, whereas only 3 species of CNS were isolated from the TX quarters. The majority of CNS isolates from teat canals were found in CX quarters, and the predominant species were *S. chromogenes* and *S. hyicus*. *Staphylococcus haemolyticus* and *S. hyicus* were both isolated from more than half the colonized teat canals of CX quarters, and *S. hyicus* colonized only one teat canal from TX quarters. Significantly more cases of colonization of the teat canal were found in CX than TX teats in total ($P < 0.001$), and for colonization by *S. hemolyticus*, *S. hyicus*, and *S. xylosus* ($P < 0.01$). *Staphylococcus haemolyticus*, *S. hyicus*, *S. xylosus*, and *S. chromogenes* were associated with 96% (66/69) of teat canal colonizations.

Table 2. Prevalence of *Staphylococcus* species¹ colonizing the teat canal at the time of IMI diagnosis

Species associated with IMI	Species associated with teat canals								Total
	<i>S. cohnii</i>	<i>S. simulans</i>	<i>S. hemolyticus</i>	<i>S. hyicus</i>	<i>S. xylosus</i>	<i>S. chromogenes</i>	<i>S. epidermidis</i>	<i>S. equorum</i>	
<i>S. cohnii</i>	0	0	0	1	0	0	0	0	1
<i>S. simulans</i>	0	1	0	0	0	0	0	0	1
<i>S. hemolyticus</i>	0	0	3	2	1	1	0	0	7
<i>S. hyicus</i>	0	0	0	1	0	0	0	0	1
<i>S. xylosus</i>	1	1	3	4	3	2	0	0	14
<i>S. chromogenes</i>	0	0	1	0	1	7	0	0	9
<i>S. epidermidis</i>	0	0	0	0	0	0	0	0	0
<i>S. equorum</i>	0	0	0	0	0	0	0	0	0
Total	1	2	7	8	5	10	0	0	33

¹Three teat canals of mammary quarters with IMI were colonized by more than one species. One mammary quarter with *S. chromogenes* IMI was colonized by both *S. chromogenes* and *S. hemolyticus*, and 2 *S. hemolyticus* IMI were colonized by both *S. hemolyticus* and *S. hyicus*.

Table 3. Prevalence of *Staphylococcus* species colonizing teat canals during the 16-wk treatment period¹

Species	No. of teat canals colonized	Control	Treatment	P-value ²
<i>S. chromogenes</i>	23	10	13	0.327
<i>S. epidermidis</i>	1	1	0	0.500
<i>S. equorum</i>	1	0	1	0.500
<i>S. hemolyticus</i>	10	10 ^a	0 ^b	0.007
<i>S. hyicus</i>	21	20 ^a	1 ^b	<0.001
<i>S. simulans</i>	1	1	0	0.500
<i>S. xylosus</i>	12	12 ^a	0 ^b	<0.001
Total	69	54 ^a	15 ^b	<0.001

^{a,b}Values with different superscripts are significantly different.

¹Control mammary quarters received no postmilking teat disinfection; treated mammary quarters received postmilking teat disinfection with 1% iodophor solution.

²Contrast of the number of IMI between treatment and control groups; P-values were generated from Fisher's exact test calculations.

DISCUSSION

The hypothesis of this study was that the disinfectant properties of the iodine teat dip would be associated with lower prevalence of IMI caused by CNS, and such a decrease in IMI would not be uniformly observed among all species. Use of 1% iodophor postmilking teat disinfectant was associated with significantly fewer CNS IMI and the number of CNS colonizing the teat canal compared with teats of CX mammary quarters not receiving postmilking disinfections. Only 3 different species caused IMI in TX mammary quarters compared with 8 different species causing CNS IMI in CX mammary quarters. In addition to the apparent reduction in the number of species causing IMI in TX compared with CX mammary quarters, we observed an apparent selective effect of treatment on IMI by species. The TX mammary quarters had a lower incidence of *S. xylosus* IMI than the CX quarters. *Staphylococcus chromogenes* had the next highest incidence of new CNS IMI, and the TX incidence of IMI by *S. chromogenes* was not significantly different from that for CX. The teat canals of mammary quarters with *S. chromogenes* infections were frequently colonized before IMI diagnosis, which suggests that colonization for this species precedes IMI. Postmilking teat disinfection is known to reduce IMI by CNS (Hogan et al., 1995). This study suggests that teat disinfection may have a selective effect in reducing IMI by some species and not others. In support, Hogan et al. (1987) reported that postmilking teat disinfection significantly altered the *Staphylococcus* spp. that caused IMI. In their study (Hogan et al., 1987), it was found that the percentage of mammary quarters with IMI by *S. hyicus* significantly increased and that by *S. epidermidis* significantly decreased when teats received postmilking teat disinfection.

Except for IMI caused by *S. xylosus*, most mammary quarters (12/18) had teat canal colonization before

IMI diagnosis. This suggests that the teat canal colonization acts as a reservoir for IMI by the same CNS species. Nickerson and coworkers (1995) inferred that if a pathogen was able to survive in the teat canal, it could serve as a reservoir for IMI. Indeed, Zeconi and coworkers (2000) reported an increased risk for IMI by the same pathogen that colonized the teat canal. Half (15/30) of the mammary quarters with teat canal colonizations had IMI caused by the same CNS species as that which colonized their teat canal. These 15 mammary quarters were 21.7% (15/69) of quarters found to have CNS teat canal colonization at any time during the treatment period, indicating that a minority of mammary quarters with CNS teat canal colonization are associated with IMI. In the current study, we did not differentiate between a teat canal colonization and teat canal infection, although the distinction has previously been discussed (Watts et al., 1991). The CNS isolates deemed teat canal colonizing agents may have originated from IMI. In such cases, residual milk with the infectious agent could be in the teat canal. Isolation of this agent would then be classified as a colonizer of the teat canal in addition to the CNS causing IMI. This could be described as an IMI-dependent teat canal colonizing event. The experimental design used could not differentiate between an IMI-dependent colonizing event and an independent event. However, if IMI-dependent colonizing events occurred, they were less frequent than the independent events because the majority of IMI were preceded by the agent of infection that first colonized the teat canal. Four species of CNS (*hemolyticus*, *hyicus*, *xylosus*, and *chromogenes*) were associated with the majority (66/69) of teat canal colonization. Of these 4, *S. hyicus* accounted for 30.4% (21/69) of the teat canal colonization but only 3.3% (1/30) of the IMI. This would suggest that *S. hyicus* had a preferable niche within the teat canal, but that niche did not extend to the more dorsal parts

of the mammary gland. The data presented here may help explain why *S. chromogenes* is the most prevalent pathogen associated with CNS IMI, given the common practice of iodophor teat disinfection (USDA, 2007). In this study, considering both the reduction in relative IMI incidence and teat canal colonizations, *S. chromogenes* appeared to be less affected by teat disinfection, whereas other CNS appeared to be sensitive. Several studies have indicated that CNS commonly colonize the teat skin and teat ends (White et al., 1989; Trinidad et al., 1990; Taponen et al., 2008; Krömker and Friedrich, 2009), and it has been demonstrated that the most prevalent species colonizing the teat skin, teat canals, and causing IMI is *S. chromogenes* (White et al., 1989; Trinidad et al., 1990). Data from this study concur as this pathogen was the most prevalent teat canal colonizer and the second most prevalent pathogen associated with IMI. Extramammary colonization by specific pathogens, including CNS, appears to be related to IMI because of the similar distribution of predominant CNS species at both extramammary sites and those causing IMI (Taponen et al., 2008). However, it should be noted that others did not find a relationship between teat apex colonization and IMI by *S. chromogenes* (De Vliegher et al., 2003).

Previous studies have revealed that the use of different active ingredients in postmilking teat disinfectants was associated with different CNS species IMI (Hogan et al., 1987; Watts et al., 1991). Specifically, what is regarded as human-associated CNS species, primarily *S. epidermidis* (Zadoks and Watts, 2009), causes more IMI in quarters where teats received linear dodecyl benzene sulfonic acid (LDBSA) postmilking disinfection than those disinfected with iodophor (Hogan et al., 1987; Watts et al., 1991) and those disinfected with a chlorhexidine product (Hogan et al., 1987). In fact, it appeared that the percentage and type of IMI associated with quarters where teats received the LDBSA disinfectant was more similar to those IMI in quarters where teats were not disinfected postmilking than those receiving the iodophor and chlorhexidine disinfectants (Hogan et al., 1987). In the current study, an a priori power of the test calculation indicated that the experimental design included an adequate number of experimental units to determine whether CNS IMI would be reduced by 50% in TX mammary quarters. However, the number of experimental units might not have been adequate to determine if IMI by individual CNS species would be affected differently by postmilking teat disinfection. The tested dip had a significant effect on reducing the total number of CNS IMI ($P = 0.03$) and one-third the number of *S. xylosus* IMI (3) were found in TX compared with the number of IMI (9) in CX mammary quarters, and this was interpreted

as a difference approaching significance ($P = 0.065$). Watts and coworkers (1991) found significant differences in the prevalence of teat canal infections between teats receiving postmilking disinfectant with LDBSA compared with iodophor disinfectant. The prevalence of human-associated CNS species canal infections was significantly greater in teats receiving LDBSA. Additionally, *S. xylosus* teat canal infections were also greater with LDBSA disinfection. In the current study, treatment was associated with a significant reduction in teat canal colonization by *S. xylosus*, *S. hemolyticus*, and *S. hyicus* ($P < 0.01$), but not by *S. chromogenes*. Thus, it appears that postmilking teat disinfection did reduce teat canal colonization by some CNS, because *S. xylosus* changes in colonization appeared to be related to changes in IMI; and for *S. chromogenes* disinfection appeared to have no effect on teat canal colonization and IMI.

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