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Effectiveness of sanitation regime in a milking parlour to control microbial contamination of teats and surfaces teat cups'

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Abstract

Introduction and Objective. The major sources of bacterial contamination of raw milk are post-harvest manipulation; therefore the disinfection of teat and teat cups which decrease the bacterial load has a positive impact on minimizing new infection rates. The aim of the study was determination of the incidence of pathogens on investigated surfaces, evaluation of the effectiveness of sanitation regime in the reduction of surface microbial load, and determination of the effectiveness of mechanical cleaning of teats in a milking parlour for dairy cows.

Materials and method. Samples from surfaces were taken by microbiological swabs using a sterile cotton swab from area of 5×2 cm^2. Sanitation regime was evaluated based on the effectiveness of active substances – lactic acid and sodium hypochlorite.

Results. From a total of 105 swab, 44 samples were found positive for *Staphylococcus aureus*, 16 samples for *E. coli,* 15 samples for *Micrococcus* spp., 8 samples for *Staphylococcus xylosus,* 9 samples for *Staphylococcus cohni urealyticum*, 1 sample for *Enterococcus faecalis*. Among isolates, *S. aureus* was the predominat species from teats – 19/45, teat cups, 15/45 and from wiping cloths 10/15. Sanitation regime was confirmed by a decrease in the number of coliform bacteria (CB) determined on teat and teat cups from 2.33–0.95 Log_{10} CFU/cm^2 ($p<0.001$) and 0.90–0.62 Log_{10} CFU/cm^2 ($p<0.001$), respectively, and in the number of total bacteria count (TBC) determined on teat and teat cups from 4.36–0.99 Log_{10} CFU/cm^2 ($p<0.001$), and 1.85–0.77 Log_{10} CFU/cm^2 ($p<0.001$), respectively. Incidence of CB (2.53 Log_{10} CFU/cm^2) and TBC (3.83 Log_{10} CFU/cm^2) on wiping cloths after mechanical cleaning of udders stress the importance of this step.

Conclusions. Results show that disinfectant with lactic acid as the main active ingredient is suitable for bacterial reduction. Post-milking disinfection of teat and teat cups reduces bacterial contamination and proves to be most effective against environmental bacteria.

Key words

disinfection, cleaning, microbiological swabs, dairy cows, milking parlor

INTRODUCTION

Milk from healthy animals is initially sterile, but post-harvest manipulation remains the major source of the bacterial contamination of raw milk. Milk, therefore, should be produced under hygienic conditions [1]. Cleaning regimes have an important role in the reduction of bacterial numbers in milk [2]. According to the National Mastitis Council (NMC) Recommended Mastitis Control Programme, routine application of pre- and post-milking teat disinfectants during each milking is highly recommended to prevent new intramammary infections [3].

Milking hygiene includes pre- and postmilking routines, as well as the cleanliness of the equipment used to milk the cows. Premilking procedures may consist of predipping, dry wiping, forestripping, and cleaning or drying of the teats and teat ends [4]. The use of effective preparations and means for the sanitary treatment of a cow's udder significantly reduces contamination of the skin of the udder teats, and reduces the overall bacterial contamination of milk [5]. A number of different types of disinfectants are used in teat dips, including iodine, chlorhexidine; acidified sodium chlorite, peroxides, organic acids (lactic acid, salicylic acid, capric acid, glycolic acid), quarternary ammonium chlorides, and others. Chlorinated compounds are used extensively as disinfectants to control both spoilage bacteria and pathogenic bacteria. Chlorine, whether in the form of chlorine gas (Cl_2) or as solid sodium hypochlorite (NaOCl), dissolves in water to form hypochlorous acid (HOCl) and Lactic acid is a non-chlorine-containing compound commonly used in sprays and washes for the control of pathogens [6]. Sanitary measures can reduce morbidity of inflammation in the mammary gland in cows in the herd by 50–70%, increase the level of hygienic cleanliness of the udde, and reduce infection with pathogens of mastitis [5].

Insufficient hygiene practices, such as poor mechanical cleaning, use of unsuitable disinfectant and poor cleanness of equipment, microbial load of the surrounding air in the milking parlour, and other environmental factors including water supply and housing conditions have an important effect on the contamination of raw milk [7]. Typical microflora of milking equipment present bacteria such as *Escherichia coli*, *S.*

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aureus, *Listeria monocytogenes*, *Salmonella* spp., *Micrococcus* spp., *Campylobacter jejuni*, *Enterococcus faecalis*, *Citrobacter freundii* [8]. Some studies have shown that premilking teat disinfection is beneficial [9, 10].

Teat disinfection and disinfection of teat cups reduce the bacterial load on teat skin [11], and also reduce the risk of bacterial contamination of milk [12, 13]. In some studies, the concentration of microorganisms, such as *Staphylococcus aureus* obtained by teat skin swabbing, was lower after dipping of teats into disinfectant solution after milking compared to untreated teats [14, 15]; therefore, reducing the bacterial load on teat skin can have a positive impact on minimizing new infection rates [16].

The microbial load of raw milk is influenced by microorganisms present in the teat canal and on the surface of teat skin [17] which has been identified as the greatest contributor to raw milk microbiota, followed by faeces [18]. This is consistent with a study by Verdier et al. [19], which suggested that the teat skin was a source of microbial populations in raw milk. Teat skin of cows represent the source of bacterial populations found in raw milk, with the rate of mastitis and intramammary infections (IMIs) having previously been shown to increase with increasing bacterial numbers on the teat skin [20]. Many bacterial strains have been associated with mastitis, with the main strains being identified as *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*, *Staphylococcus aureus*, and *Escherichia coli* [21]. The variation in naturally present microbial levels on the teat skin is caused by environmental factors and sanitation regime which can affect the level of occurrence of bacterial contamination of the teat skin surface [19].

An increase in the microbial load of raw milk may also occur during long milk storage at an insufficient temperature [22]. Contamination of the environment of the milking parlour is a potential source of food-borne pathogens and spoilage bacteria, which affect the milk quality and represent a risk for public health [23, 24, 25].

Bacteria attach to the surface of teat skin as well as on milking equipment surfaces, either as single cells or in binary biofilms, which may become difficult to remove. Bacteria that remain on surfaces after unsufficient cleaning and disinfection have the potential to proliferate and cause health problems. Therefore, the hygiene of teat skin and equipment surfaces definitely affects the safety of raw milk [26].

OBJECTIVE

The aim of the study was to determine the incidence of pathogens on investigated surfaces, evaluation of the effectiveness of the sanitation regime in reduction of surface microbial load on teats and teat cups, as well as determination of the effectiveness of mechanical cleaning of teats by using wiping cloths in the milking parlour for dairy cows.

MATERIALS AND METHOD

Sanitation regime. At the commencement of milking, Prefoam$^+$ (Hypred S.A., Dinard, France), a preparation intended for predipping teats, was used. Prefoam$^+$ with active biocidal ingredient 5% <= L-(+)-lactic acid < 10% (CAS number 79–33–4), 1% <= Sodium p-cumenesulphonate < 5% (CAS number 15763–76–5) and 1% <= Glycerol < 5% (CAS number 56–81–5) was used for udder hygiene before milking and applied as foam. After foaming, the teats were mechanically cleaned with UdderClean wet wipes which are intended for cleaning the entire udder and fore-stripping. The UdderClean wipes have four advantages in their use: udder cleaning, stimulation of milk production, shortening of milking time, cleaning of the milker‘s hands.

After milking, the teats are treated with Filmadine (Hypred S.A., Dinard, France), which is used for cleaning and disinfection. Filmadine contains a high proportion of plasticizers and thus protects the teat from cracking, and it is an anti-inflammatory mixture containing the active ingredient of 5% <= acid (+)-L-lactic < 10% (CAS No. 79–33–4); 1% <= glycerol < 10% (CAS number 56–81–5), and other ingredients such as moisturizers, emollients, softeners and surfactants to promote the beneficial effect of lactic acid on the skin of teats.

The teat cups were disinfected by preparations Savo Original (Unilever Slovensko, Bratislava, Slovakia), used in in liquid form at 10% concentration, applicated by spraying without heating. Savo Original is a cleaning and disinfecting agent which contains active substance sodium hypochlorite ≥ 1 < 5% (CAS No. 7681–52–9), sodium hydroxide ≥ 0.5 < 2% (CAS No. 1310–73–2) and < 5% anionic surfactant. The disinfectant is used against a broad-spectrum of vegetative bacteria.

Dairy farm, sampling from teats, surfaces and sample preparation. The study was carried out on a dairy farm located in Eastern Slovakia, with conventional farming, herd size 230 dairy cows of Slovak spotted cattle breed were used. Dairy cows were kept in a free housing system with straw bedding and were allowed ad libitum access to water. Annual milk yield (305 d) was 8.405 kg. The cows were milked twice daily in a herring bone milking parlour (DeLaval, Sweden) and the milk transported once a day to a milk processing plant, and subsequently pasteurized).

Samples from surfaces (n = 105) were taken from 15 cows. From each cow, three samples were obtained – from teat, teat cup, and from the wiping cloth for the udder. Samples were taken three times: from teat – before milking, after mechanical cleaning and after dipping; from teat cup – before milking, after milking, after disinfection; from the wiping cloth for the udder – after mechanical cleaning. Samples were taken from the same place in three repetitions, transferred to the laboratory and processed.

Microbiological swabs from surfaces for collecting microorganisms were performed using a sterile cotton swab pre-wetted in physiological solution from area of 5 × 2 cm^2 in three replicates. The replicates were from the same surface at randomly selected different places. Swabs were taken by rotating the cotton swab in contact with the monitored surfaces before and after a sanitation regime. The swabs were placed in a sterile tube containing 10 ml of sterile saline solution (8.5 g/1.000 ml) and shaken using a vortex for 2 minutes to dislodge the bacteria.

Microbial analysis. For total bacteria count (TBC) and for coliform bacteria (CB), swabbed samples were serially diluted in sterile saline solution [27]. The dilutions (volume 0.1 ml) were then plated using the pour plate method on the

selective diagnostic medium Endo agar (EA, HiMedia, India) and selective diagnostic medium Nutrient agar No. 2 (NA, HiMedia, India). Detection of TBC was performed according to ISO 18593:2004 [28] and detection of the number of CB was performed according to ISO 4832:2006 [29]. The results from the Endo agar and Nutrient agar was obtained after 24 hours of incubation at 37 °C.

Staphylococci were isolated according to the instructions of STN ISO 6888–1:1999 [30]. From the first two consecutive dilutions, a 0.1 ml spread was inoculated onto the surface of Baird-Parker selective diagnostic medium (Hi-Media, India). The inoculated samples were incubated at 37 °C for 24–48 hours. Based on their characteristic appearance, typical staphylococcal colonies collected from the Petri dishes were used for further investigation. For the identification of species of the genus *Staphylococcus,* biochemical identificationwas achieved by using the STAPHYtest 24 (Erba Lachema, Brno, Czech Republic), and evaluated according to the manufacturer's instructions by the software TNW Pro 7.0 (Erba-Lachema, Brno, Czech Republic).

Suspected colonies of *Micrococcus spp*., were isolated on blood agar (Columbia Blood Agar Base with 5% of defibrinated blood), cultivated at 37 °C for 24 h and identified biochemically using the ENTEROtest, using the software TNW Pro 7.0 (Erba-Lachema, Brno, Czech Republic), according to the manufacturer's instructions.

Detection of the number of bacteria of the family *Enterobacteriaceae* was performed according to ISO 21528–1:2017 [31] using the selective diagnostic medium Violet Red Bile Agar (VRBL; HiMedia, India) and incubated at 37 °C for 24 hours. Strains of the *Enterobacteriaceae* family were biochemically identified at species level using the ENTEROtest 24 (Erba Lachema, Brno, Czech Republic), and evaluated according to the manufacturer's instructions by the software TNW Pro 7.0 (Erba-Lachema, Brno, Czech Republic) with a probability of correct designations of the species above 90%.

Species identification of bacteria was subsequently provided with the aid of MALDI – TOF MS, according to the standard sample preparation protocol of manual Bruker Daltonics. The Bruker MALDI-TOF MS system compares the mass spectra of the test strain with a database of mass spectra of different bacteria, and calculates a score value that reflects the similarity between the obtained spectrum and the spectrum from the database (i.e., the quality of the match. A score value of 2.00 and above indicates species identification [32]. For isolates in which the score value was < 2.00, there was no reliable species identification, and for a more accurate identification of the given isolates, they were inoculated onto the surface of blood agar (Columbia Blood Agar Base), which ensured a higher purity of the isolates. Subsequently, MALDI-TOF MS identification was repeated. Analysis of the results was performed in an Ultraflex III device (Bruker, Billerica (MA) USA). The obtained results were processed using Flex Analysis software, version 3.0 and evaluated using BioTyper software, version 1.1 (Bruker, Billerica (MA) USA).

Statistical analysis. Counts were converted to decimal Logarithmic values (Log_{10} CFU/cm^2) to almost match the assumption of a normal distribution. Counts obtained for adhesion, detachment and biofilm formation and counts obtained for the effect of the sanitizers (before and after the application) on the biofilm matrix were submitted to Analysis of Variance (ANOVA). Data were analyzed using the software Graph Pad Prism, and probability value $p<0.05$ was accepted as a indicating significant difference.

RESULTS

By microbiological culture examination of samples (n = 105) and subsequent species identification by MALDI-TOF MS method, six bacterial species were identified: *S. aureus* 44/105 (42%), *E. coli* 16/105 (15.2%), *Micrococcus* spp 15/105 (14.3%), *Staphylococcus xylosus* 8/105 (17.7%), *Staphylococcus cohni urealyticum* 9/105 (8.6%), and *Enterococcus faecalis* 1/105 (0.95%) (Tab. 1). The most common pathogen from the investigated surfaces was *S. aureus* 44/105 (42%), identified from teats, teat cups, and from wiping cloths for the udder. Score value was in the range 1.65 – 1.98. In some cases – *E. coli* / wiping cloths – 1.98, *Staphylococcus aureus* / wiping cloths – 1.65, *S. aureus* / teat cup – 1.95, *S. aureus* / teat – 1.98, *Staphylococcus xylosus* / teat cup – 1.78 and *Micrococcus* spp. / teat cup – 1.90 – the score was lower than 2.00 (Tab. 1).

Table 1. Sample sources and pathogens from monitored surfaces

Sample source	n	Isolated bacteria	Score value	n (%)
Wiping cloths	15 (14.3%)	*E. coli*	2.03	2 (8.8%)
		Enterococcus faecalis	2.02	1 (8.8%)
		Micrococcus spp.	2.20	2 (6.6%)
		Staphylococcus aureus	2.00	10 (48.8%)
Teat cup	45 (42.8%)	*Staphylococcus xylosus*	2.05	8 (17.7%)
		Micrococcus spp.	2.04	13 (28.8%)
		Staphylococcus aureus	2.07	15 (33.3%)
		other bacteria	not identify	9 (20.0%)
Teat	45 (42.8%)	*Staphylococcus aureus*	2.18	19 (42.2%)
		E. coli	2.26	14 (31.1%)
		Staphylococcus cohni urealyticum	2.20	9 (20.0%)
		other bacteria	not identified	3 (6.6%)
Total	105 (100%)			

The effect of the sanitation regime – mechanical cleaning, disinfection and decrease in the microbial load reduction from evaluated surfaces is shown in Table 2. The study revealed significant differences ($p<0.0001$) among the states before milking, after mechanical cleaning, and after dipping of teats for the numbers of TBC and CB; the same

Table 2. Effect of sanitation regime on microbial load of monitored surfaces

Teat	Before milking (mean Log_{10} CFU/cm^2)		After mechanical cleaning (mean Log_{10} CFU/cm^2)		After dipping (mean Log_{10} CFU/cm^2)	
	TBC	CB	TBC	CB	TBC	CB
Mean ± SE	4.36	2.33	3.18	1.78	0.99	0.95
Teat cup	Before milking (mean Log_{10} CFU/cm^2)		After milking (mean Log_{10} CFU/cm^2)		After disinfection (mean Log_{10} CFU/cm^2)	
	TBC	CB	TBC	CB	TBC	CB
Mean ± SE	1.85	0.90	2.09	1.22	0.77	0.62

TBC – total bacteria count; CB – coliform bacteria; CFU colony forming units.

result (p<0.0001) was recorded for the numbers of TBC and CB from teat cups before milking, after milking, and after disinfection.

On comparing the state before milking and after dipping of teat, a decrease was recorded in the number of TBC, from 4.36–0.99 Log_{10} CFU/cm^2, which represents 77.29% microbial load reduction. The numbers of CB from teats decreased from 2.33 Log_{10} CFU/cm^2 before milking to 0.95 Log_{10} CFU/cm^2 after dipping (59.22% microbial load reduction) (Tab. 2). Before milking, teat cups had a mean microbial residual load of 1.85 Log_{10} CFU/cm^2 of TBC and 0.90 Log_{10} CFU/cm^2 of CB. After disinfection, this was 0.77 and 0.62 Log_{10} CFU/cm^2 of TBC and CB, respectively (p<0.0001).

According to the percentage of microbial load reduction, it was found that the effectiveness of the evaluated disinfectants was high on teats by 77.29 and 59.22% for TBC and CB, respectively; and on teat cups by 58.37 and 31.11% for TBC and CB, respectively (Tab. 3).

Table 3. Comparison of microbial reduction of monitored surfaces

	Initial microbial load	Final microbial load	Change in microbial load		% Microbial load reduction	
	TBC CB	TBC CB	TBC	CB	TBC	CB
Teat	4.36 2.33	0.99 0.95	3.37	1.38	77.29	59.22
Teat cup	1.85 0.90	0.77 0.62	1.08	0.28	58.37	31.11

TBC – total bacteria count; CB – coliform bacteria.

Microbial load of the wiping cloth after mechanical cleaning of the udder is shown in Table 4. The numbers of TBC (3.83 Log_{10} CFU/cm^2) and CB (2.53 Log_{10} CFU/cm^2) from wiping cloths used for mechanical cleaning of udder confirm the necessity for mechanical cleaning because it decreases the microbial load of the udder.

Table 4. Microbial load of wiping cloth after mechanical cleaning of udder.

wiping cloth for udder	after mechanical cleaning (mean Log10 CFU/cm2)	
	TBC	CB
	3.83	2.53

TBC – total bacteria count; CB – coliform bacteria

DISCUSSION

The disinfection of teats and and teat cups decreases the microbial load on the teat skin surface [33] has become the most important part of the milking routine [34]. The current study revealed that the sanitation regime which includes mechanical cleaning, dipping and disinfection, had a significant effect (p<0.0001) in the reduction of microbial load (TBC and CB), and therefore the effective procedures of cleaning and disinfection had an impact on the effectiveness of the sanitation process. Studies by many authors have shown that pre-milking teat disinfection reduces the bacterial numbers on teat skin [35, 36]. The efficacy of the the sanitation is evaluated by the reduction of microbial load on a surfaces before and after the process of cleaning and disinfection, and the one with the highest death rate or highest percent reduction of microbial load, is considered to be the most highly efficiency [37].

The teat orifice is a very important part of defence in protecting a dairy cow from the invasion of pathogens into the udder; consequently, the teat orifice can influence intramammary microbial colonization [38]. Microbiological load on teats prior to milking may be influenced by the pre-milking procedures. Various pre-milking cleaning regimes have been shown to reduce bacterial numbers on the teat skin surface [39]. Mechanical cleaning of teats was performed by using wiping cloths, and data obtained for TBC (3.83 Log_{10} CFU/cm^2) and CB (2.53 Log_{10} CFU/cm^2) show a high reduction in the microbial load. On Irish farms, pre-milking teat disinfection is generally applied directly to teats without prior cleaning, which may impact on the antimicrobial effectiveness of the disinfectant.

The total count of bacteria is used for the overview of microbial contamination, and count of the coliform bacteria is used for the evaluation of hygiene [40]. The concentration of TBC and CB obtained from teat skin by swabbing decreased to 0.99 Log_{10} CFU/cm^2 and 0.95 Log_{10} CFU/cm^2, respectively, when teats were dipped into disinfectant solution with active ingredient lactic acid. For disinfection of teat cups, disinfectatnt with the active substance sodium hypochlorite was used, which decreased the number of TBC from 1.85–0.77 Log_{10} CFU/cm^2 and the numbers of CB from 0.90–0.62 Log_{10} CFU/cm^2.

By microbiological culture examination of samples and subsequent species identification by the MALDI-TOF MS method, six bacterial species were identified; *S. aureus* 44/105 (42%), *E. coli* 16/105 (15.2%), *Micrococcus* spp. 15/105 (14.3%), *Staphylococcus xylosus* 8/105 (17.7%), *Staphylococcus cohni urealyticum* 9/105 (8.6%) and *Enterococcus faecalis* 1/105 (0.95%). In some cases, the score was lower than 2.00 (1.65–1.98). Although the manufacturer recommends using scores to determine species-level identification above 2.00, lower cut-off scores have been used in the past to identify individual Gram-positive cocci species [41]. In the current study, 44 samples from total samples were found positive for *S. aureus* (42%), which was the most common pathogen from the evaluated surfaces. *S. aureus* was identified from teats, teat cups, and from wiping cloths used for mechanical cleaning of the udder. *Staphylococcus aureus* represents a major food-borne and virulent pathogen that can increase the risk of mastitis in dairy ruminants [42]. In many countries, *Staphylococcus aureus* represent the main cause of mastitis [43], and every material occupied by *S. aureus* – in or on – can be a potential source of intramammary infection of lactating cows.

Teat disinfectants perform differently in reducing bacterial transfer between cows or from the cow's environment (i.e. surfaces, hands, bedding). Nowadays, a wide range of products are available for teat disinfection. In the current study, the disinfectant with active ingredient lactic acid, obtained a 77.29% reduction in the total count of bacteria, and 59.22% reduction in coliform bacteria of teats after dipping. Post-milking teat disinfection has been shown to be very effective at reducing udder bacterial contamination from the environment. These data are in agreement with results of authors [42, 44, 45]. According Fitzpatrick et al. [46], iodine combined with lactic acid and a lactic acid (2.4%) only product achieved a 73% and 79% reduction of naturally present staphylococcal isolates, respectively, on the teat skin, compared to 76% obtained by an iodine only product. Lactic acid 2% in combination with 0.1% salicylic acid product,

achieved a reduction of 63% against streptococcal isolates naturally present on the teat skin. Mišeikienė et al. [44] have demonstrated lactic acid to be effective also against streptococcal bacteria.

CONCLUSIONS

The results of the study explain the importance of cleaning and disinfecting of teats and teat cups, as well as using a wiping cloth for udders from the perspective of decreasing microbial load of these surfaces and subsequent decreasing of risk of contamination of milk. The obtained results show that the disinfectant with lactic acid as the main active ingredient is suitable for bacterial reduction. Post-milking teat disinfection and disinfection of teat cups has been shown to reduce bacterial numbers and to be most effective against environmental bacteria. Further studies are necessary to evaluate other products efficacy against microorganisms presented on teat skin.

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