Comparative Immunology, Microbiology and Infectious Diseases

Coagulase negative staphylococci from ovine bulk-tank milk: effects of the exposure to sub-inhibitory concentrations of disinfectants for teat-dipping

--Manuscript Draft--

Manuscript Number:	
Article Type:	Full Length Article
Keywords:	Coagulase-negative staphylococci; teat dips; ovine milk; sub-inhibitory stress; resistance induction; biofilm
Corresponding Author:	Barbara Turchi Universita degli studi di Pisa Dipartimento di Scienze Veterinarie Pisa, ITALY
First Author:	Filippo Marzoli
Order of Authors:	Filippo Marzoli
	Barbara Turchi
	Francesca Pedonese
	Beatrice Torracca
	Fabrizio Bertelloni
	Giovanni Cilia
	Domenico Cerri
	Filippo Fratini
Abstract:	Teat-dipping is one of the most effective methods to prevent mammary infections in ruminants, including sub-clinical mastitis caused by coagulase-negative staphylococci (CoNS). Improper disinfectant application could expose microorganisms to sub-inhibitory concentrations leading to phenotypic variations. In this study, 12 chlorhexidine-digluconate (CHDG)-tolerant (of which 4 qac positive) and 12 benzalkonium chloride (BC)-tolerant (of which 7 qac-positive) CoNS isolates from ovine milk were exposed to sub-inhibitory concentrations of CHDG and BC, respectively. Changes in disinfectant susceptibility against BC and CHDG, antibiotic resistance against 12 antibiotics and biofilm production were then assessed for both groups. After CHDG stress, 67% and 83% of the CHDG-stressed isolates doubled their MICs for BC and CHDG, respectively and 2 qac-negative isolates showed a four-fold increase of their MBCs for CHDG. After BC stress, MICs for BC and CHDG doubled in 58% and 83% of the BC-stressed isolates, respectively, while one qac-positive isolate increased four-fold the MIC for BC. Cross-resistance to antibiotics was assessed by disc diffusion method. Some qac-positive isolates varied their resistance profile, while a blaZ-positive isolate showed a resistant phenotype against ampicillin only after the exposure to the disinfectant. As for qac-positive isolates, one CHDG-stressed and 2 BC-stressed showed a new antibiotic resistance to kanamycin and cefoxitin, respectively. The Congo Red Agar test was carried out to assess the invitro slime production: all isolates were negative after stress. In conclusion, sub-inhibitory exposure to disinfectants may affect especially disinfectant and antibiotic susceptibility, the latter in particular for qac-positive isolates and those hosting unexpressed antibiotic resistance genes.
Suggested Reviewers:	Marianne Sunde marianne.sunde@vetinst.no Expert in the field of disinfectant resistance in staphylococci
	Eugenia Agnese Cannas agnese.cannas@izs-sardegna.it Expert in the field of ovine milk and mastitis in sheep
	Ayman Elbehiry aymanella2007@yahoo.com Carried out studies on sub-lethal stress on Staphylococcus aureus from milk

	Katheryne Benini Martins katheryne_bm@yahoo.com.br Expert in the field of antimicrobial resistance in staphylococci from sheep

Department of Veterinary Science
University of Pisa
Viale delle Piagge 2, 56124 Pisa
Italy

April 4th, 2020

Dear Editor,

please find enclosed a manuscript entitled "Coagulase negative staphylococci from ovine bulk-tank milk: effects of the exposure to sub-inhibitory concentrations of disinfectants for teat-dipping" by Filippo Marzoli, Barbara Turchi, Francesca Pedonese, Beatrice Torracca, Fabrizio Bertelloni, Giovanni Cilia, Domenico Cerri, Filippo Fratini to be submitted as Original Research Paper to Comparative Immunology, Microbiology & Infectious Diseases.

The aim of the study was to gain information concerning the effects of the exposure of CoNS from ovine bulk-tank milk to sub-inhibitory concentrations of disinfectants employed in dipping routine. Isolates characterized in a previous work for their antibiotic and disinfectant susceptibility, and biofilm production, were exposed to sub-inhibitory concentrations of two disinfectants, then modifications in their phenotypic characteristic were assessed. Our results showed that isolates could develop a tolerant phenotype for disinfectants despite their resistant genotype. Also, the antibiotic susceptibility profile was affected, especially in those isolates hosting *qac* genes or non-expressed antibiotic resistance genes, while biofilm production seemed to be not affected. This is the first report evaluating the effect of sub-inhibitory exposure to disinfectants commonly employed in the dipping routine in staphylococcal isolates from ovine milk. Thus, this study could provide useful information for the implementation of correct dipping plans and for bacterial resistance to disinfectants in general, with the objective to determine reference cut-off values to discriminate disinfectants-resistant and susceptible strains.

This manuscript has not been published previously in any language and is not under consideration by another journal.

All authors have approved the manuscript and agree with its submission to Comparative Immunology, Microbiology & Infectious Diseases.

Please address all correspondence concerning this manuscript to the e-mail address barbara.turchi@unipi.it.

Thank you for considering our work.

Your Sincerely

Barbara Turchi

- Sub-inhibitory exposure to disinfectants increased disinfectant resistance in CoNS.
- Disinfectant resistance increase was not dependent on *qac* gene presence.
- Antibiotic resistance was affected by sub-inhibitory exposure to disinfectants.
- Unexpressed antibiotic resistance genes or *qac* genes varied antibiotic resistance.
- Biofilm production was not affected by sub-inhibitory exposure to disinfectants.

Coagulase negative staphylococci from ovine bulk-tank milk: effects of the exposure to sub-inhibitory concentrations of disinfectants for teat-dipping

Filippo Marzoli, BarbaraTurchi*, Francesca Pedonese, Beatrice Torracca, Fabrizio Bertelloni, Giovanni Cilia, Domenico Cerri, Filippo Fratini

Department of Veterinary Science, University of Pisa, Viale delle Piagge 2, Pisa, Italy

*Corresponding author: barbara.turchi@unipi.it, Viale delle Piagge 2, 56124 (Pisa), Italy, phone: +39 050 2216958, fax: +39 050 2216941

Authors e-mail addresses:

Marzoli F.: filippo.marzoli@vet.unipi.it

Pedonese F.: francesca.pedonese@unipi.it

Torracca B.: beatrice.torracca@unipi.it

Bertelloni F.: fabrizio.bertelloni@unipi.it

Cilia G.: giovannicilia23@gmail.com

Cerri D.: domenico.cerri@unipi.it

Fratini F.: filippo.fratini@unipi.it

Abstract

Teat-dipping is one of the most effective methods to prevent mammary infections in ruminants, including sub-clinical mastitis caused by coagulase-negative staphylococci (CoNS). Improper disinfectant application could expose microorganisms to sub-inhibitory concentrations leading to phenotypic variations. In this study, 12 chlorhexidine-digluconate (CHDG)-tolerant (of which 4 *qac* positive) and 12 benzalkonium chloride (BC)-tolerant (of which 7 *qac*-positive) CoNS isolates from ovine milk were exposed to sub-inhibitory concentrations of CHDG and BC, respectively. Changes in disinfectant susceptibility against BC and CHDG, antibiotic resistance against 12 antibiotics and biofilm production were then assessed for both groups. After CHDG stress, 67% and 83% of the CHDG-stressed isolates doubled their MICs for BC and CHDG, respectively and 2 *qac*-negative isolates showed a four-fold increase of their MBCs for CHDG. After BC stress, MICs for BC and CHDG doubled in 58% and 83% of the BC-stressed isolates, respectively, while one *qac*-positive isolate increased four-fold the MIC for BC. Cross-resistance to antibiotics was assessed by disc diffusion method. Some *qac*-positive isolates varied their resistance profile, while a *blaZ*-positive isolate showed a resistant phenotype against ampicillin only after the exposure to the disinfectant. As for *qac*-positive isolates, one CHDG-stressed and 2 BC-stressed showed a new antibiotic resistance to kanamycin and cefoxitin, respectively. The Congo Red Agar test was carried out to assess the invitro slime production: all isolates were negative after stress. In conclusion, sub-inhibitory exposure to disinfectants may affect especially disinfectant and antibiotic susceptibility, the latter in particular for *qac*-positive isolates and those hosting unexpressed antibiotic resistance genes.

Keywords: Coagulase-negative staphylococci; teat dips; ovine milk; sub-inhibitory stress; resistance induction; biofilm

1. Introduction

Coagulase-negative staphylococci (CoNS) are classified as opportunistic pathogens, they represent the main etiological agents causing ruminant subclinical mastitis [1–4] and reservoir of genes

encoding several pathogenicity factors [5]. To prevent intramammary infections (IMI), teat-dipping is considered one of the most effective methods, since it allows to decrease mammary infection onset [6]. Also, IMI control is ensured by appropriate shed hygiene and milking machine disinfection [7]. Disinfectants are widely used in ruminant management, and improper applications and dilutions, as well as the possible presence of residues in the environment, can occur and result in the formation of biocide concentration gradients. As a consequence, microorganisms can be frequently exposed to non-lethal (i.e., sub-inhibitory, non-inhibitory, over-inhibitory) concentrations of biocides [8]. Various authors reported that exposure to sub-inhibitory biocide concentrations facilitates the evolution of resistance to the biocide, and may also lead to co-resistance and cross-resistance to other antimicrobials agents such as antibiotics [9–14]. In presence of disinfectant sub-minimum inhibitory concentrations (sub-MICs) levels, bacteria might apply multiple strategies to improve their disinfectant resistance, mostly related to the increase of efflux pump activity [15]. Even though such efflux pumps could have a positive effect on raising antimicrobial resistance, their contribution has recently been questioned [14]. Mechanisms other than efflux pumps may be involved and can co-develop during sub-inhibitory exposure to disinfectants, such as modification of cell membrane structure and composition, enhanced biofilm formation, genetic transfer of resistance determinants and biodegradation of the disinfectant itself [16]. As for biofilm, although several studies show an increase in its production in *Staphylococcus* species exposed to sub-lethal concentration of biocides [17–22], some authors, on the contrary, reported a reduction [22].

To obtain a better understanding of antimicrobial sub-inhibitory stress, the present study aimed to assess the effect of benzalkonium chloride (BC) and chlorhexidine-digluconate (CHDG) exposure on CoNS antimicrobial susceptibility, including not only the evaluation of disinfectant resistance but also cross-resistance to 12 clinically important antibiotics. The biofilm production capacity of the selected isolates was also screened.

2. Materials and Methods

70 *2.1 Bacterial isolates*

71 In a previous work [23], CoNS from bulk-tank ovine milk were identified and characterized in regard
72 to their antibiotic and disinfectant susceptibility, and biofilm production. In particular, 20 isolates
73 belonging to different species (*S. epidermidis*, *S. simulans*, *S. arlettae*, *S. haemolyticus*, *S. xylosus*, *S.*
74 *chromogenes* and *S. caprae*), were tested for BC (ZEUS, Bozen, Italy) and CHDG (Sigma-Aldrich,
75 Saint Louis, USA) susceptibility. Twelve BC tested isolates and 12 CHDG tested isolates showed a
76 MIC $\geq$ 2 μg/ml, suggesting a reduced susceptibility [24–28]. For this study, both CHDG- and BC-
77 tolerant isolates were exposed to a sub-inhibitory concentration, each group with the respective
78 disinfectant. Supplementary table (S1) shows genotypic and phenotypic features of the selected
79 isolates concerning antibiotic resistance and biofilm production as assessed by Turchi et al. [23]. As
80 for disinfectant resistance, characteristics are included in tables provided in the results section, with
81 the purpose to compare data before and after sub-inhibitory disinfectant stress.

82

83 *2.2 Exposure to sub-inhibitory concentrations of disinfectants*

84 Isolates were exposed to a sub-inhibitory concentration (MIC/2) of BC and CHDG in Trypticase Soy
85 broth (TSB) (Thermo Fisher Scientific, Milan, Italy) at 37°C for 24 h. For the subsequent 3 days,
86 cultures were daily sub-cultured in TSB containing the same disinfectant sub-inhibitory
87 concentration. Then, each broth culture was added with glycerol and stored at -20°C until use. At the
88 same time, they were seeded on a Trypticase Soy agar (TSA) (Thermo Fisher Scientific, Milan, Italy)
89 plate to check their purity.

90

91 *2.3 Evaluation of disinfectants susceptibility*

92 After the exposure to disinfectants, MIC values for BC and CHDG were determined by the broth
93 microdilution method in 96-well microtiter plates. Both groups of isolates stressed with BC or CHDG
94 were tested for both disinfectants (BC and CHDG). Isolates were revitalized on TSB at 37°C for 24
95 h. Serial dilutions of disinfectants (128-0.125 μg/ml) in Mueller Hinton Broth (Thermo Fisher

Scientific, Milan, Italy) were prepared and inoculated with standardized bacterial suspensions (approximately10^8cells/ml, OD_{550} 0.125 - 0.200). The plates were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of the disinfectant able to inhibit the growth of microorganisms. To determine Minimum Bactericidal Concentration (MBC) values, a loopful of broth culture from each well containing a disinfectant concentration ≥ MIC was seeded on TSA. Plates were incubated at 37°C for 24 h. The MBC was defined as the lowest concentration of the disinfectant able to completely prevent microbial growth on agar plates. Each experiment was carried out twice.

2.4 Evaluation of antibiotic susceptibility

After the exposure to disinfectants, antibiotic susceptibility testing was performed by agar disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (EUCAST Disk Diffusion Method for Antimicrobial Susceptibility Testing- Version 6.0, January 2017, www.eucast.org). Isolates were streaked on TSA and colonies were suspended in sterile saline water adjusting the turbidity to 0.5 MacFarland. Tested antibiotics were: ampicillin (AM, 10 μg), cefoxitin (FOX, 30 μg), cephalothin (KF, 30 μg), cefotaxime (CTX, 30 μg), chloramphenicol (C, 30 μg), clindamycin (DA, 2 μg), tetracycline (TE, 30 μg), trimethoprim-sulfamethoxazole (SXT, 19:1;25 μg), enrofloxacin (ENR, 10 μg), kanamycin (K, 30 μg), gentamicin (CN, 10 μg), and rifampin (RA, 5 μg). *Staphylococcus aureus* ATCC 25923 was used as quality control strain. Inhibition zone diameters were interpreted into susceptibility categories according to breakpoint tables provided by the EUCAST (2018), CLSI (2015), and the Société Française de Microbiologie (2017).

2.5 Evaluation of biofilm production

After the exposure to disinfectants, enhancement in biofilm production was evaluated through a phenotypic test on Congo Red Agar (CRA) according to Freeman et al. [29]. Results were compared

to those obtained before the stress [23], according to which none of the isolates resulted positive. *Staphylococcus aureus* ATCC 35556, strong biofilm producer, and *S. aureus* 1261, from the strains collection of the Department of Veterinary Science (University of Pisa), were included as positive and negative controls for biofilm formation, respectively. After incubation, slime-producing strains form black colonies, whereas non-producing strains develop red colonies [30].

3. Results

3.1 Susceptibility to disinfectants in CHDG-tolerant isolates (CHDG sub-inhibitory stress)

After sub-inhibitory exposure to CHDG, MICs values for BC and CHDG increased two-fold for 8 (67%) and 10 (83%) out of the 12 tested isolates, respectively (Table1). All isolates harboring *smr-qacC'* gene doubled their MIC values. Five and 7 *qac*-negative isolates tested for BC and CHDG, respectively, also doubled their MIC values. Despite the presence of *qacH* gene, isolate 70A did not show any increase in MIC value.

As for MBCs values, most of them remained unvaried. Indeed, the highest number of isolates showed unchanged values for BC (11) and CHDG (5) (Table1). Two *qac*-negative isolates (30B, 42A) showed a 4-fold increase of their MBC value for CHDG, while, despite the presence of *smr-qacC'* gene, the isolate 32B showed a halved MBC value for CHDG.

3.2 Susceptibility to disinfectants in BC-tolerant isolates (BC sub-inhibitory stress)

After sub-inhibitory exposure to BC, MICs values for BC and CHDG increased two-fold in 7 (58%) and 10 (83%) of the 12 tested isolates, respectively (Table 2). The *qac*-positive isolate 40B (*S. simulans*) increased four times its MIC value for BC. Seven out of 8 (88%) isolates which increased MICs values for BC hosted *qac* genes. In contrast to what observed after sub-inhibitory stress with CHDG, the isolate harboring the *qacH* gene (70A) doubled its MIC for both disinfectants.

As for MBCs values for CHDG, they doubled in 5 out of 12 isolates (42%) and remained unchanged in 5 (42%) out of 12 isolates as well. MBCs values for BC remained unchanged in the majority of the

148 isolates (58%), while the remaining 5 isolates (42%) doubled their MBC. Isolate 10B, belonging to
149 *S. simulans* and *smr-qacC'* positive, showed an increased BC MBC value of 32 μg/ml. A reduction
150 of MBC values for the two disinfectants was observed in some *qac*-positive isolates after BC exposure.
151 In particular, for BC, the isolate 15A halved its value, while for CHDG, 10B and 40B halved their
152 value and 32B even presented a four-fold reduction.
153
154 *3.3 Antibiotic susceptibility profile after sub-inhibitory exposure to disinfectants*
155 Tables 3a and 3b show the variations observed in the antibiotic susceptibility profiles after CHDG
156 and BC sub-inhibitory stress, respectively. The majority of the isolates were not affected by the
157 treatments, however, differences in antibiotic susceptibility profile were recorded in 3 (25%) and 4
158 (33%) isolates after CHDG and BC sub-inhibitory stress, respectively.
159 After the exposure to sub-inhibitory concentrations of CHDG, 2 isolates (17%) (32B and 71A), one
160 of them (32B) hosting *qac* gene, were defined as resistant to K and AM, respectively. At the same
161 time, isolates 32B and 37A, both *qac*-positive, were categorized susceptible to at least one antibiotic
162 for which they were previously resistant.
163 After BC stress, one *qac*-positive isolate (8%) (32B) was redefined as susceptible to AM and DA;
164 while 3 isolates (25%) (10B, 25D, and 71A), 2 of them *qac*-positive, changed their phenotype from
165 susceptible to resistant for one antibiotic: 10B and 25D were defined resistant against FOX, and 71A
166 against AM. Two *qac*-positive isolates (17%) (32B and 25D) changed their profile from susceptible
167 to intermediate.
168 Concerning the specific antibiotic, differences in susceptibility were recorded for 3 (AM, DA, K) and
169 4 (AM, FOX, CTX, DA) antibiotics after CHDG and BC sub-inhibitory stress, respectively. Two
170 isolates (17%), after both CHDG and BC stresses, showed the same phenotypic variation for AM: the
171 isolate 32B (*qac*-positive) was redefined as susceptible, while the isolate 71A (*qac*-negative and *blaZ*
172 positive) as resistant. As concerns FOX, a variation in the profile from susceptible to resistant was
173 observed only after BC stress for 2 isolates (17%) hosting *qac* genes (10B and 25D). The same

disinfectant led to an intermediate resistance acquisition against CTX for 2 *qac*-positive isolates. Two and 1 *qac*-positive isolates lost their resistance to the antibiotic DA after CHDG and BC stress, respectively.

3.4 Variation in biofilm production

After sub-inhibitory stress with both disinfectants, none of the isolates was positive for biofilm production after streaking onto Congo Red Agar phenotypic test. Therefore, no difference was found with the previously reported biofilm production before sub-inhibitory stress [23].

4. Discussion

Pre- and post-dipping with disinfectants are effective management tools for the reduction of IMI rate and to limit the presence of pathogens in dairy animals; however, incorrect practices could lead to microorganisms exposure to low biocide concentrations. Hence the importance of studying how sub-inhibitory concentrations of disinfectants could affect some virulence traits of bacteria. Indeed, several studies suggest that the exposure to sub-inhibitory concentrations of biocides promotes the emergence of resistance or the development of co-resistance and cross-resistance to other antimicrobials, such as antibiotics or biocides [8,9]. Based on our knowledge, this is the first report evaluating the effect of sub-inhibitory exposure to disinfectants commonly employed in the dipping routine (BC and CHDG) in staphylococcal isolates from ovine milk. Indeed, previous studies were conducted on staphylococcal isolates from bovine milk [13], food [14,22] and clinical samples [31–33]. Phenotypic variations in antimicrobial susceptibility profiles as well as biofilm production were assessed in the present study.

Exposure to a sub-inhibitory concentration of CHDG allowed both *qac*-positive and *qac*-negative isolates to increase their tolerance to CHDG and BC. Similarly, after the sub-inhibitory stress with BC, there was a widespread increase in MICs and MBCs values, especially against BC, for which the isolate 10B (*S. simulans*) showed an MBC value of 32 μg/ml, near to the MBC resistance level (> 32

200 μg/ml) established by Morrissey et al. [34] for *S. aureus*. According to these authors, to define an
201 isolate as BC resistant, it should be able to grow in presence of a BC concentration higher than 32
202 μg/ml. However, considering that, in our experiments, 64 μg/ml was the immediately higher tested
203 disinfectant concentration after 32 μg/ml, we can not rule out that 10B isolate could have been able
204 to grow in presence of a BC concentration between 32 and 64 μg/ml. To verify this occurrence, further
205 tests should be carried out.
206 After sub-inhibitory stress, *qac*-positive isolates did not show a clear and evident increase in MIC
207 and MBC values compared to *qac*-negative isolates. This was also the case in the study by Furi et al.
208 [31] for MBC values in *S. aureus* from human samples. Indeed, resistance mechanisms other than
209 *qac* efflux pumps can be triggered after disinfectant exposure, conferring advantages also to *qac*-
210 negative isolates. Supporting this hypothesis, by inhibiting efflux pumps in Gram-positive isolates
211 from food, Gadea et al. [14] have proven that disinfectant-adapted strains showed similar tolerance
212 to biocides and antibiotics compared to wild-type strains, suggesting that these efflux proteins do not
213 play an essential role in the acquisition of tolerance to biocides and/or antibiotics after step-wise
214 exposure to quaternary ammonium compounds (QACs).
215 While for chlorhexidine the resistance systems implemented by bacteria are mainly related to *qacA/B*
216 genes [35], adaptation to BC and in general to QACs, can be due to many other systems, including
217 modification of bacteria outer membranes and recombinational events [36].
218 After disinfectant exposure, antibiotic resistance profile also varied in some isolates. Indeed, sub-
219 inhibitory disinfectant concentrations seemed to play a role in activating the expression of the *blaZ*
220 gene in a *qac*-negative isolate (71A) belonging to *S. epidermidis*, which modified its AM phenotypic
221 profile from susceptible to resistant. Similar variations of antibiotic resistance profile were observed
222 only for some of the CoNS hosting *qac* genes. In fact, except for isolate 32B (*S. arlettae*), which
223 showed a particular behavior either by losing or by acquiring resistance to different antibiotics, after
224 BC stress the *qac*-positive isolates 10B and 25D, identified as *S. simulans* and *S. caprae,* respectively,
225 were reclassified as resistant against FOX (β-lactam), although they were negative for the main genes

encoding such resistance (*mecA* and *mecC*). This may suggest that exposure to BC has led to a *qac* gene up-regulation allowing to gain a non-*mec* gene-dependent resistance against some β-lactams since the exposure to the QAC can increase the efflux of disinfectants, effect directly related to increased antibiotic resistance [37]. Moreover, BC sub-inhibitory stress may have also determined single mutations which may have either a non-specific effect on the uptake of the antibiotic or activate multi-drug efflux pumps [38]. It would be necessary to evaluate whether this variation in the antibiotic resistance profiles is determined either by a temporary and reversible phenotypic adaptation, related to cell wall changes [39], efflux pumps over-expression [31], stress-responses induction [40], or by a selection of mutants for which the reduction of susceptibility is stable over time [41]. Based on our results, the use of BC seemed to promote resistance to antibiotics. In fact, as already reported by other authors [14,37], long-term exposure of microbial communities to QACs can increase the risk of selecting antibiotic-resistant bacteria. This occurs especially when antibiotics and disinfectants have a common cellular target or when the disinfectant efflux induction is enough to confer also an antibiotic resistance profile [42].

A reduction in resistance for disinfectants and some antibiotics was observed after disinfectant sub-inhibitory exposure. In particular, this happened for one isolate (8%) after CHDG exposure in MBC for CHDG, and one and two isolates after BC exposure in MBC for BC and CHDG, respectively. As for antibiotic resistance, two CHDG-exposed isolates (17%) and one BC-exposed isolate (8%) lost their resistance for some antibiotics. Therefore, either adapting or stressing isolates with a disinfectant does not necessarily imply an increase in biocide or antibiotic resistance, as stated by other studies [14,43].

Although some of the tested isolates were found positive for biofilm production associated genes, they did not phenotypically show this trait before sub-stress [23]. We observed the same phenotype after sub-inhibitory stress. The negative CRA test results observed in our study could also be due to the limitations of this test, which is known to generate false-negative results [44]. However, it has been reported that sub MICs exposure to disinfectants such as BC could reduce the ability to produce

biofilm in staphylococci [22]. Nevertheless, the risk that biofilm production may increase after sub-inhibitory stress remains high. In fact, isolates positive for biofilm production genes should be considered as potential biofilm producers [44] and other studies have shown how the sub-inhibitory exposure to disinfectants BC and chlorhexidine can lead to a significant biofilm production increase in *S. epidermidis* [17].

5. Conclusion

The use of disinfectants in pre- and post-dipping in milking is essential to prevent IMI, however, the inappropriate use of these products, especially QAC, could create a sub-inhibitory stress condition for bacteria affecting their susceptibility to both biocides and antibiotics. In this study, after sub-inhibitory stress, we did not observe a correlation between the presence of *qac* genes and an increase in disinfectant tolerance, suggesting that CoNS could develop a tolerant phenotype despite their *qac* genotype. Nonetheless, the antibiotic susceptibility profile was affected, especially in those isolates hosting *qac* genes or non-expressed antibiotic resistance genes. It would be therefore essential to implement disinfectant control plans, like those in place for antibiotics, and constantly monitor *qac* gene distribution to gain a better understanding of their role in antimicrobial resistance. Moreover, it would be useful to increase the number of studies on bacterial resistance to disinfectants, leading to the determination of reference cut-off values to discriminate susceptible and resistant strains.

Conflict of interest statement

Authors have no competing interests to declare.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

278 **References**

279 [1] G. Marogna, S. Rolesu, S. Lollai, S. Tola, G. Leori, Clinical findings in sheep farms affected
280 by recurrent bacterial mastitis, Small Rumin. Res. 88 (2010) 119–125.
281 https://doi.org/10.1016/j.smallrumres.2009.12.019.

282 [2] T. Onni, A. Vidili, E. Bandino, G. Marogna, S. Schianchi, S. Tola, Identification of coagulase-
283 negative staphylococci isolated from caprine milk samples by PCR-RFLP of *groEL* gene,
284 Small Rumin. Res. 104 (2012) 185–190. https://doi.org/10.1016/j.smallrumres.2011.10.004.

285 [3] W. Vanderhaeghen, S. Piepers, F. Leroy, E. Van Coillie, F. Haesebrouck, S. De Vliegher,
286 Identification, typing, ecology and epidemiology of coagulase negative staphylococci
287 associated with ruminants, Vet. J. 203 (2015) 44–51. https://doi.org/10.1016/j.tvjl.2014.11.001.

288 [4] K.B. Martins, P.Y. Faccioli, M.F. Bonesso, S. Fernandes, A.A. Oliveira, A. Dantas, L.F. Zafalon,
289 M. de L.R.S. Cunha, Characteristics of resistance and virulence factors in different species of
290 coagulase-negative staphylococci isolated from milk of healthy sheep and animals with
291 subclinical mastitis, J. Dairy Sci. 100 (2017) 2184–2195. https://doi.org/10.3168/jds.2016-
292 11583.

293 [5] F. Bertelloni, F. Fratini, V.V. Ebani, A. Galiero, B. Turchi, D. Cerri, Detection of genes
294 encoding for enterotoxins, TSST-1, and biofilm production in coagulase-negative
295 staphylococci from bovine bulk tank milk, Dairy Sci. Technol. 95 (2015) 341–352.
296 https://doi.org/10.1007/s13594-015-0214-9.

297 [6] A. Contreras, D. Sierra, A. Sánchez, J.C. Corrales, J.C. Marco, M.J. Paape, C. Gonzalo,
298 Mastitis in small ruminants., Small Rumin. Res. 68 (2007) 145–153.
299 https://doi.org/10.1016/j.smallrumres.2006.09.011.

300 [7] D. Bergonier, R. de Crémoux, R. Rupp, G. Lagriffoul, X. Berthelot, Mastitis of dairy small
301 ruminants, Vet. Res. 34 (2003) 689–716. https://doi.org/10.1051/vetres:2003030.

302 [8] U. Tezel, S.G. Pavlostathis, Quaternary ammonium disinfectants: microbial adaptation,
303 degradation and ecology, (2015). https://doi.org/10.1016/j.copbio.2015.03.018.

[9] P.H. Mc Cay, A.A. Ocampo-Sosa, G.T.A.F. Correspondence, G.T.A. Fleming, Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of *Pseudomonas aeruginosa* grown in continuous culture, (2010). https://doi.org/10.1099/mic.0.029751-0.

[10] D. Elhanafi, V. Utta, S. Kathariou, Genetic characterization of plasmid-associated benzalkonium chloride resistance determinants in a *Listeria monocytogenes* train from the 1998 -1999 outbreak, Appl. Environ. Microbiol. 76 (2010) 8231–8238. https://doi.org/10.1128/AEM.02056-10.

[11] K. Hegstad, S. Langsrud, B.T. Lunestad, A.A. Scheie, M. Sunde, S.P. Yazdankhah, Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health?, Microb. Drug Resist. 16 (2010) 91–104. https://doi.org/10.1089/mdr.2009.0120.

[12] M. Rakic-Martinez, D.A. Drevets, V. Dutta, V. Katic, S. Kathariou, *Listeria monocytogenes* strains selected on ciprofloxacin or the disinfectant benzalkonium chloride exhibit reduced susceptibility to ciprofloxacin, gentamicin, benzalkonium chloride, and other toxic compounds, Appl. Environ. Microbiol. 77 (2011) 8714–8721. https://doi.org/10.1128/AEM.05941-11.

[13] A. El Behiry, G. Schlenker, I. Szabo, U. Roesler, In vitro susceptibility of *Staphylococcus aureus* strains isolated from cows with subclinical mastitis to different antimicrobial agents, J. Vet. Sci. 13 (2012) 153–161. https://doi.org/10.4142/jvs.2012.13.2.153.

[14] R. Gadea, M.Á. Fernández Fuentes, R. Pérez Pulido, A. Gálvez, E. Ortega, Effects of exposure to quaternary-ammonium-based biocides on antimicrobial susceptibility and tolerance to physical stresses in bacteria from organic foods, Food Microbiol. 63 (2017) 58–71. https://doi.org/10.1016/J.FM.2016.10.037.

[15] K. Poole, Efflux-mediated antimicrobial resistance, J. Antimicrob. Chemother. 56 (2005) 20–51. https://doi.org/10.1093/jac/dki171.

[16] B. Moen, K. Rudi, E. Bore, S. Langsrud, Subminimal Inhibitory Concentrations of the

Disinfectant Benzalkonium Chloride Select for a Tolerant Subpopulation of *Escherichia coli* with Inheritable Characteristics, Int. J. Mol. Sci. 13 (2012) 4101–4123. https://doi.org/10.3390/ijms13044101.

[17] A. Houari, P. Di Martino, Effect of chlorhexidine and benzalkonium chloride on bacterial biofilm formation, Lett. Appl. Microbiol. 45 (2007) 652–656. https://doi.org/10.1111/j.1472-765X.2007.02249.x.

[18] A. Kumar, Y.P. Ting, Effect of sub-inhibitory antibacterial stress on bacterial surface properties and biofilm formation, Colloids Surfaces B Biointerfaces. 111 (2013) 747–754. https://doi.org/10.1016/j.colsurfb.2013.07.011.

[19] S. Lee, K.H. Choi, Y. Yoon, Effect of NaCl on biofilm formation of the isolate from *Staphylococcus aureus* outbreak linked to ham, Korean J. Food Sci. Anim. Resour. 34 (2014) 257–261. https://doi.org/10.5851/kosfa.2014.34.2.257.

[20] M.K. Luther, S. Bilida, L.A. Mermel, K.L. LaPlante, Ethanol and Isopropyl Alcohol Exposure Increases Biofilm Formation in *Staphylococcus aureus* and *Staphylococcus epidermidis,* Infect. Dis. Ther. 4 (2015) 219–226. https://doi.org/10.1007/s40121-015-0065-y.

[21] C. V Redelman, C. Maduakolam, G.G. Anderson, Alcohol treatment enhances *Staphylococcus aureus* biofilm development, Immunol. Med. Microbiol. 66 (2012) 411–418. https://doi.org/10.1111/1574-695x.12005.

[22] L. Buzón-Durán, C. Alonso-Calleja, F. Riesco-Peláez, R. Capita, Effect of sub-inhibitory concentrations of biocides on the architecture and viability of MRSA biofilms, Food Microbiol. 65 (2017) 294–301. https://doi.org/10.1016/j.fm.2017.01.003.

[23] B. Turchi, F. Bertelloni, F. Marzoli, D. Cerri, S. Tola, E. Azara, C.M. Longheu, R. Tassi, M. Schiavo, G. Cilia, F. Fratini, Coagulase negative staphylococci from ovine milk: Genotypic and phenotypic characterization of susceptibility to antibiotics, disinfectants and biofilm production, Small Rumin. Res. 183 (2020). https://doi.org/10.1016/j.smallrumres.2019.106030.

356 [24] E. Heir, G. Sundheim, A.L. Holck, Resistance to quaternary ammonium compounds in
357 *Staphylococcus* spp. isolated from the food industry and nucleotide sequence of the resistance
358 plasmid pST827, J. Appl. Bacteriol. 79 (1995) 149–156. https://doi.org/10.1111/j.1365-
359 2672.1995.tb00928.x.
360 [25] E. Heir, G. Sundheim, A.L. Holck, Identification and characterization of quaternary
361 ammonium compound resistant staphylococci from the food industry, Int. J. Food Microbiol.
362 48 (1999) 211–219. https://doi.org/10.1016/S0168-1605(99)00044-6.
363 [26] J. Bjorland, M. Sunde, S. Waage, Plasmid-borne *smr* gene causes resistance to quaternary
364 ammonium compounds in bovine *Staphylococcus aureus*, J. Clin. Microbiol. 39 (2001) 3999–
365 4004. https://doi.org/10.1128/JCM.39.11.3999-4004.2001.
366 [27] M.S. Sidhu, E. Heir, H. Sørum, A. Holck, Genetic Linkage Between Resistance to Quaternary
367 Ammonium Compounds and β -Lactam Antibiotics in Food-Related *Staphylococcus* spp.,
368 Microb. Drug Resist. 7 (2001) 363–371. https://doi.org/10.1089/10766290152773374.
369 [28] N. Taheri, A. Ardebili, A. Amouzandeh-Nobaveh, E. Ghaznavi-Rad, Frequency of Antiseptic
370 Resistance Among *Staphylococcus aureus* and Coagulase-Negative Staphylococci Isolated
371 From a University Hospital in Central Iran., Oman Med. J. 31 (2016) 426–432.
372 https://doi.org/10.5001/omj.2016.86.
373 [29] D.J. Freeman, F.R. Falkiner, C.T. Keane, New method for detecting slime production by
374 coagulase negative staphylococci., J. Clin. Pathol. 42 (1989) 872–4.
375 https://doi.org/10.1136/JCP.42.8.872.
376 [30] C.R. Arciola, D. Campoccia, S. Gamberini, M. Cervellati, E. Donati, L. Montanaro, Detection
377 of slime production by means of an optimised Congo red agar plate test based on a
378 colourimetric scale in *Staphylococcus epidermidis* clinical isolates genotyped for *ica* locus,
379 Biomaterials. 23 (2002) 4233–4239. https://doi.org/10.1016/S0142-9612(02)00171-0.
380 [31] L. Furi, M. Laura Ciusa, D. Knight, V. Di Lorenzo, N. Tocci, D. Cirasola, L. Aragones, J.
381 Rosado Coelho, A. Teresa Freitas, E. Marchi, L. Moce, P. Visa, J. Blackman Northwood, C.

382 Viti, E. Borghi, G. Orefici, the BIOHYPO Consortium, I. Morrissey, M. Rinaldo Oggioni, E.
383 Biolab, Evaluation of Reduced Susceptibility to Quaternary Ammonium Compounds and
384 Bisbiguanides in Clinical Isolates and Laboratory-Generated Mutants of *Staphylococcus*
385 *aureus*, Antimicrob. Agents Chemother. 57 (2013) 3488–3497.
386 https://doi.org/10.1128/AAC.00498-13.
387 [32] C. Soumet, D. Méheust, C. Pissavin, P. Le Grandois, B. Frémaux, C. Feurer, A. Le Roux, M.
388 Denis, P. Maris, Reduced susceptibilities to biocides and resistance to antibiotics in food-
389 associated bacteria following exposure to quaternary ammonium compounds, J. Appl.
390 Microbiol. 121 (2016) 1275–1281. https://doi.org/10.1111/jam.13247.
391 [33] B. Yang, Z. Lei, Y. Zhao, S. Ahmed, C. Wang, S. Zhang, S. Fu, J. Cao, Y. Qiu, Combination
392 Susceptibility Testing of Common Antimicrobials in Vitro and the Effects of Sub-MIC of
393 Antimicrobials on *Staphylococcus aureus* Biofilm Formation, Front. Microbiol. 8 (2017) 2125.
394 https://doi.org/10.3389/fmicb.2017.02125.
395 [34] I. Morrissey, M.R. Oggioni, D. Knight, T. Curiao, T. Coque, A. Kalkanci, J.L. Martinez, the
396 B. Consortium, Evaluation of Epidemiological Cut-Off Values Indicates that Biocide Resistant
397 Subpopulations Are Uncommon in Natural Isolates of Clinically-Relevant Microorganisms,
398 PLoS One. 9 (2014) e86669. https://doi.org/10.1371/journal.pone.0086669.
399 [35] S.S. Costa, M. Viveiros, L. Amaral, I. Couto, Multidrug Efflux Pumps in *Staphylococcus*
400 *aureus*: an Update., Open Microbiol. J. 7 (2013) 59–71.
401 https://doi.org/10.2174/1874285801307010059.
402 [36] U. Tezel, S.G. Pavlostathis, Role of quaternary ammonium compounds on antimicrobial
403 resistance in the environment, in: A.R. in the Environment (Ed.), Keen PL, Montforts MHMM.
404 Wiley-Blackwell, 2012: pp. 349–387.
405 [37] W.H. Gaze, N. Abdouslam, P.M. Hawkey, E.M.H. Wellington, Incidence of Class 1 Integrons
406 in a Quaternary Ammonium Compound-Polluted Environment, Antimicrob. Agents
407 Chemother. 49 (2005) 1802–1807. https://doi.org/10.1128/AAC.49.5.1802-1807.2005.

[38] N. Akimitsu, H. Hamamoto, R. Inoue, M. Shoji, A. Akamine, K. Takemori, N. Hamasaki, K. Sekimizu, Increase in resistance of methicillin-resistant *Staphylococcus aureus* to beta-lactams caused by mutations conferring resistance to benzalkonium chloride, a disinfectant widely used in hospitals., Antimicrob. Agents Chemother. 43 (1999) 3042–3. https://doi.org/10.1128/AAC.43.12.3042.

[39] M. Braoudaki, A.C. Hilton, Mechanisms of resistance in *Salmonella enterica* adapted to erythromycin, benzalkonium chloride and triclosan, Int. J. Antimicrob. Agents. 25 (2005) 31–37. https://doi.org/10.1016/J.IJANTIMICAG.2004.07.016.

[40] M.A. Webber, N.G. Coldham, M.J. Woodward, L.J. V. Piddock, Proteomic analysis of triclosan resistance in *Salmonella enterica* serovar Typhimurium, J. Antimicrob. Chemother. 62 (2008) 92–97. https://doi.org/10.1093/jac/dkn138.

[41] L.M. McMurry, M. Oethinger, S.B. Levy, Overexpression of *marA, soxS* , or *acrAB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*, FEMS Microbiol. Lett. 166 (1998) 305–309. https://doi.org/10.1111/j.1574-6968.1998.tb13905.x.

[42] A.J. McBain, A.H. Rickard, P. Gilbert, Possible implications of biocide accumulation in the environment on the prevalence of bacterial antibiotic resistance, J. Ind. Microbiol. Biotechnol. 29 (2002) 326–330. https://doi.org/10.1038/sj.jim.7000324.

[43] J.A. Joynson, B. Forbes, R.J.W. Lambert, Adaptive resistance to benzalkonium chloride, amikacin and tobramycin: the effect on susceptibility to other antimicrobials, J. Appl. Microbiol. 93 (2002) 96–107. https://doi.org/10.1046/j.1365-2672.2002.01667.x.

[44] A. Oliveira, M. de L.R. Cunha, Comparison of methods for the detection of biofilm production in coagulase-negative staphylococci, BMC Res. Notes. 3 (2010) 260. https://doi.org/10.1186/1756-0500-3-260.

CHDG SUB-INHIBITORY STRESS										
ID isolate	Species	*qac* genes	MIC BC (μg/ml)		MBC BC (μg/ml)		MIC CHDG (μg/ml)		MBC CHDG (μg/ml)	
			PRE	POST	PRE	POST	PRE	POST	PRE	POST
10B	*S. simulans*	*smr-qaC'*	4	8	16	16	2	4	8	8
18B	*S. epidermidis*	-	2	4	8	4	2	4	2	4
28A	*S. simulans*	-	8	8	16	16	2	4	8	8
30B	*S. epidermidis*	-	1	2	8	8	2	4	2	8
32B	*S. arlettae*	*smr-qaC'*	4	8	16	16	2	4	16	8
37A	*S. haemolyticus*	*smr-qaC'*	2	4	2	4	2	4	2	4
42A	*S. epidermidis*	-	1	2	8	8	2	2	2	8
62A	*S. epidermidis*	-	2	2	8	8	2	4	4	4
70A	*S. xylosus*	*qacH*	2	2	4	4	2	2	4	4
71A	*S. epidermidis*	-	2	4	8	8	2	4	4	8
87A	*S. epidermidis*	-	1	2	8	8	2	4	2	4
95B	*S. epidermidis*	-	2	2	8	8	2	4	4	4

Table 1. Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) values after chlorhexidine-digluconate (CHDG) sub-inhibitory stress of 12 coagulase-negative staphylococci isolates (POST) with comparison to previously reported [23] pre-inhibitory ones (PRE).

BC SUB-INHIBITORY STRESS										
ID isolate	Species	*qac* genes	MIC BC (μg/ml)		MBC BC (μg/ml)		MIC CHDG (μg/ml)		MBC CHDG (μg/ml)	
			PRE	POST	PRE	POST	PRE	POST	PRE	POST
28A	*S. simulans*	-	8	8	16	16	2	4	8	8
10B	*S. simulans*	*smr-qacC'*	4	8	16	32	2	2	8	4
32B	*S. arlettae*	*smr-qacC'*	4	8	16	16	2	2	16	4
15A	*S. chromogenes*	*smr-qacC'*	2	2	8	4	1	2	2	4
18B	*S. epidermidis*	-	2	2	8	8	2	4	2	4
25D	*S. caprae*	*smr*	2	4	2	4	1	2	2	2
37A	*S. haemolyticus*	*smr-qacC'*	2	4	2	4	2	4	2	4
40B	*S. simulans*	*smr-qacC'*	2	8	8	16	1	2	8	4
62A	*S. epidermidis*	-	2	4	8	8	2	4	4	4
70A	*S. xylosus*	*qacH*	2	4	4	4	2	4	4	4
71A	*S. epidermidis*	-	2	2	8	8	2	4	4	8
95B	*S. epidermidis*	-	2	2	8	8	2	4	4	4

Table 2. Minimum inhibitory concentrations (MIC) and Minimum Bactericidal Concentration (MBC) values after benzalkonium chloride (BC) sub-inhibitory stress of 12 coagulase-negative staphylococci isolates (POST) with comparison to previously reported [23] pre-inhibitory ones (PRE). Isolates are listed in decreasing order of BC concentration employed for sub-stress (MIC/2).

a)

CHLORHEXIDINE-DIGLUCONATE SUB-INHIBITORY STRESS				
ID isolate	Species	Ab.-Resitance gene	*qac* gene	Ab.-resistance variation (from* > to)
32B	*S. arlettae*	*tetK*	*smr-qaC'*	AM: R > S ; DA: R > S ; K: S > R
37A	*S. haemolyticus*	*tetK*	*smr-qaC'*	DA: R > I
71A	*S. epidermidis*	*blaZ*	-	AM: S > R

b)

BENZALKONIUM CHLORIDE SUB-INHIBITORY STRESS				
ID isolate	Species	Ab.-Resitance gene	*qac* gene	Ab.-resistance variation (from* > to)
10B	*S. simulans*	*blaZ*	*smr-qaC'*	FOX: S > R
25D	*S. caprae*	*tetK*	*smr*	FOX: S > R; CTX: S > I
32B	*S. arlettae*	*tetK*	*smr-qaC'*	AM: R > S ; CTX: S > I ; DA: R > S
71A	*S. epidermidis*	*blaZ*	-	AM: S > R

Table 3. Antibiotic resistance variation in coagulase-negative staphylococci after sub-inhibitory exposure to chlorhexidine-digluconate (a) and benzalkonium chloride (b). Only isolates which showed resistance variation against antibiotics are listed. Resistance is given as follows: sensible (S), intermediate (I), resistant (R). AM: ampicillin; DA: clindamycin; K: kanamycin; FOX: cefoxitin; CTX: cefotaxim.

*Data previously obtained [23].

Conflict of interest statement

Authors have no competing interests to declare.