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Chlorhexidine dihydrochloride's effect on clinical, veterinary and food-origin *Staphylococcus aureus*

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Abstract: Chlorhexidine (CHX) is a bactericidal agent used as a common disinfectant since the 1950s. However, its effectiveness may have diminished over the time due to the rise of microbial resistance even among nonantibiotics. In this study, we evaluate the response of 46 *Staphylococcus aureus* isolates to CHXdihydrochloride according to their origin and phenotype (haemolysis induction, coagulase production, methicillin resistance and biofilm formation). Following classification, the influence of seven CHX concentrations (10.00–0.50 mg·L^{-1}) on planktonic cell growth and biofilm formation was evaluated spectrophotometrically at 620 nm and 595 nm (24 h). Even though the effect of CHX was strain-specific irrespective of origin or phenotypic profile, concentrations above 2.50 mg·L^{-1} were almost uniformly determined as bactericidal. Although the non-bactericidal concentrations did not indicate any statistically significant differences, they did promote biofilm formation in some cases. Overall, our results suggest that CHX is still an effective disinfectant and an antimicrobial agent against *S. aureus*.

Keywords: staphylococci; disinfectant; chlorhexidine; antibacterial effect; biofilm formation

For decades, biocides (such as antiseptics or disinfectants) in various forms have been applied in clinical, veterinary and foodprocessing sectors to prevent bacterial colonisation of surfaces or patients. Nowadays, they are used empirically in high concentrations to fight resistant or protected bacteria in biofilms, organic matter and/or limiting environments (Ortega Morente et al. 2013).

One of the common biocides is chlorhexidine (CHX). It is a synthetic cationic biguanide used since the 1950s which is effective against Gram-positive and Gram-negative bacteria, some microfungi and even viruses (Gilbert and Moore 2005; Milstone et al. 2008). As CHX is not readily water-soluble, three different salts (dihydrochloride, digluconate, and acetate) are frequently used in the form of an alcohol solution, a gel, or a dusting powder. CHX is mainly used in medical care for washing patients, their controlled decolonisation, oral healthcare or catheter impregnation (Horner et al. 2012). Currently, it is also added to electrospun nanofibrous materials (Lencova et al. 2021b). An added benefit of CHX is its persistent activity and efficacy against methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant enterococci (Milstone et al. 2008).

Over the whole period of use, little data has been collected regarding microbial resistance to CHX (Gilbert and Moore 2005). This phenomenon may be linked to the means of action of CHX because it binds covalently to the cell wall and cytoplasmic membrane. In low concentrations, this cross-linking affects os-

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moregulation and metabolic activity of enzymes, making the biocide bacteriostatic. However, at higher concentrations, the membrane crystallises so the cell structural integrity is lost and CHX becomes bactericidal (Gilbert and Moore 2005).

Staphylococci, and mainly *S. aureus* as one of the most prevalent opportunistic pathogenic bacteria in hospitals and foodstuffs, can adapt to stressful conditions and gain resistance relatively quickly. Nevertheless, they have so far only become less susceptible to CHX mainly due to plasmid-encoded efflux pumps which can also lead to increased resistance to other antibiotics like tetracycline or imipenem (Kampf 2019; Van den Poel et al. 2022). The effect of CHX may also diminish by the presence of body fluids, pus or phosphate ions. Minimal inhibitory concentration (MIC) values of CHX for *S. aureus* range from 0.125 mg·L^{-1} to 60 mg·L^{-1} with the epidemiological cut-off at 8 mg·L^{-1}. MRSA strains are proven to be more tolerant to CHX than methicillin-susceptible strains of *S. aureus* (MSSA) (Horner et al. 2012; Bock et al. 2018). Although *S. aureus* strains which are tolerant or resistant to CHX have already been described and *S. aureus* susceptibility to CHX differs based on the geographic area of origin, very little conclusive data concerning phenotypic features and CHX susceptibility has been collected in general (Van den Poel et al. 2022).

Despite the long-term use of CHX in practice and the associated risks of microbial resistance, no current studies confirming the overall antimicrobial effect of CHX have been published. Therefore, our study is the first to monitor the effect of CHX on a defined set of strains of *S. aureus* obtained mainly from the Czech Republic. We analysed relationships between CHX susceptibility and the origin or phenotype of current or collection strains of *S. aureus*. Our presented data may further accelerate the development of nanomaterials functionalised with appropriate CHX concentrations, applicable in the food industry and medicine.

MATERIAL AND METHODS

Bacterial isolates and culture conditions. Forty-six *S. aureus* isolates were tested in this study. Clinical isolates of *S. aureus* were obtained from two Czech hospitals (isolates C1–C5), the BIOCEV centre (Prague, Czech Republic, isolates C6–C8), the Czech Collection of Microorganisms (CCM; Brno, Czech Republic, strains C9 and C10) and the collection at the University of Chemistry and Technology Prague (UCT Prague; Prague, Czech Republic, strain C11). Veterinary isolates were obtained from the State Veterinary Institute Prague (SVI Prague; Czech Republic, isolates V1–V12) and UCT Prague (isolate V13). Food-related isolates were obtained from the Faculty of Medicine, the Masaryk University (MUNI; Brno, Czech Republic, isolates F1–F19), and the UCT Prague (isolates F20–F22). Non-collection *S. aureus* isolates were obtained in the years 2015 and 2019–2022. The bacteria were stored in Tryptone Soy Broth (TSB; Oxoid, Great Britain) supplemented with glycerol (25%; Penta, Czech Republic) in a freezer at –80 °C. For recultivation, bacterial cultures were inoculated into sterile TSB (3 mL) and incubated for 24 h at 37 °C.

Genotypic and phenotypic characterisation of isolates. To observe the genotype of isolates, their total genomic DNA was obtained via thermal lysis. It was used to:

i) identify all isolates as *S. aureus* using duplex PCR with the targets of 16S rRNA (specific for the genus *Staphylococcus*) and SA442 (specific for *S. aureus*) as described in Alibayov et al. (2014),

ii) assess methicillin resistance by the *mecA* gene (ditto), and

iii) determine the spa type of isolates. For spa typing, we used primers Spa1113f (5'-TAA AGA CGA TCC TCC GGT GAG C-3') and Spa1514r (5'-CAG CAG TAG TGC CGT TTG CTT-3') in KAPA HiFi HotStart ReadyMix (2X). DNA was amplified using the following settings: initial denaturation (5 min at 95 °C), 34 cycles of annealing (20 s at 98 °C, 15 s at 65 °C and 30 s at 72 °C), and final extension (5 min at 72 °C) in T-Gradient Thermocycler Biometra (Whatman, Germany). The obtained PCR products were cleaned by Monarch PCR & DNA Cleanup Kit (New England Biolabs Inc., USA) and sent for Sanger sequencing to SEQme s.r.o. (Czech Republic). Sequences were then analysed by spaTYPER (https://spatyper.fortinbras.us/) using Ridom nomenclature.

Phenotypic characteristics included the ability to grow on Baird-Parker agar (BP agar; Merck, Germany), MRSA*Select* II agar (Bio-Rad, USA) and Columbia agar with 5% of sheep blood (Viamar International, Czech Republic) after 24 h at 37 °C. Typical growth of *S. aureus* on BP agar is in shiny black colonies with a clear halo zone around them (ISO 6881-1:2021). On MRSA*Select* II agar, MSSA isolates should not grow, and MRSA isolates should grow in pinkish colonies. MRSA isolates were verified by 30-µg cefoxitin discs on Mueller-Hinton agar (Merck, Germany) with a diameter smaller than 21 mm (Hernandez et al. 2016). Haemolysis was differentiated accord-

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ing to the presence or absence of a zone around the colonies on Columbia agar as α-haemolysis (greenish zone), β-haemolysis (clear zone) and γ-haemolysis (no zone). Coagulase production was tested using the Bactident™ Coagulase kit (Merck, Germany) on 24 h bacterial cultures in BrainHeart Infusion broth (Oxoid, Great Britain) according to the producer's manual. To assess biofilm production, isolates were cultivated statically in TSB for 24 h at 37 °C in a sterile 96-well microtiter plate (GAMA GROUP, Czech Republic). The groups of biofilm producers were inspired by Lencova et al. (2021a) and decided as follows:

i) weak biofilm producers (absorbance of 0.10–0.25),
ii) medium biofilm producers (absorbance of 0.26–0.40),
iii) strong biofilm producers (absorbance of 0.41–1.00),
iv) very strong biofilm producers (> 1.01).

Chlorhexidine solutions. Stock solution (1 000 mg·L^{-1}) was prepared from powdered chlorhexidine-dihydrochloride (CHX, Sigma-Aldrich, USA) in sterile distilled water. First, the antimicrobial effect of 100.00, 50.00, 10.00, 5.00, 1.00 and 0.50 mg·L^{-1} of CHX in TSB was tested on *S. aureus* C9 strain. Based on the results, we chose final concentrations of 10.00, 7.50, 50, 2.50, 1.00, 0.75 and 0.50 mg·L^{-1} (CHX in TSB) for the follow-up experiments performed in a ratio of 1:1 [1 portion of cell culture (see below) and 1 portion of 2 × CHX solution].

Minimal inhibitory concentration (MIC) and minimal inhibitory biofilm formation concentration (MICB). Bacterial suspensions cultivated overnight in TSB were adjusted to the optical density of 0.5 McFarland. These inocula (100 µL) were put into sterile 96-well microtiter plates and mixed with 100 µL of sterile distilled water (positive control) or 100 µL of CHX solution. Sterile TSB (100 µL) served as the negative control. The plates were cultivated for 24 h at 37 °C and absorbance at 620 nm was measured spectrophotometrically both before and after the cultivation to obtain the differences in planktonic cell growth in each well. MIC values were determined as the lowest concentration of CHX suppressing bacterial growth after the cultivation.

Biofilm formation was determined after the removal of planktonic cells using 0.1% Crystal Violet (CV; Sigma Aldrich, USA) according to (Lencova et al. 2021a). Final ethanol solutions were measured spectrophotometrically at 595 nm (Tecan, Switzerland), and the lowest concentration to suppress biofilm formation (MICB) was determined.

Inhibitory rates. The inhibitory rate (IR) was calculated for every isolate for bacterial growth and biofilm formation according to Equation (1).

$$IR = 1 - \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad [\%] \tag{1}$$

where: $A_{control}$ – the mean absorbance of untreated wells; A_{sample} – the mean absorbance of treated wells.

Data analysis. All biofilm formation tests were performed in technical and biological triplicates and results in graphs are expressed as means and standard deviations. To determine multiple comparisons of respective data groups, one-way analysis of variance (ANOVA) was applied, and statistically significant differences were assumed to be at $P \leq 0.05$. The obtained data were plotted using RStudio (program R, version 4.3.1) into box-and-whisker plots where the middle line marks the median, the whiskers show the minimum and the maximum, and the outliers are marked as individual points beyond the whiskers. We hypothesised that there would not be any significant differences in CHX susceptibility related to the *S. aureus* phenotype, however some differences could still be found.

RESULTS AND DISCUSSION

Characterisation of strains. *S. aureus* is an abundant opportunistic pathogen in various environments, with the most risks associated with foodstuffs and hospitals. This bacterium possesses a wide range of enzymes and other virulence factors, making its phenotype diverse and easily adjustable regarding outer conditions (Tong et al. 2015). Therefore, there could also be differences in response to CHX.

Firstly, all isolates were positively assessed as *S. aureus* species by qPCR (241 bp for 16S rRNA and 108 bp for SA442, see Supplementary Figure 1). Secondly, the spa type of each isolate was determined, and 35 unique types were found, with six types being found multiple times (5× t2873, 3× t091, 3× t267, 2× t008, 2× t021 and 2× t2932). Only spa type t2873 was exclusive for veterinary isolates. Then the isolates were classified by their origin as either clinical (23.9%), veterinary (28.3%), food–meat (21.7%) or food–dairy (26.1%). Lastly, the isolates were characterised in terms of *i*) growth on BP agar, *ii*) methicillin resistance (selective medium, cefoxitin disc and *mecA* gene), *iii*) haemolysis, *iv*) coagulation and *v*) rate of biofilm formation (see Table 1).

On BP agar, 33 isolates (71.7%) formed shiny black colonies with a cleared halo zone around them and, although the remaining 13 isolates (nine veterinary and four foodborne) grew black, they did not create the

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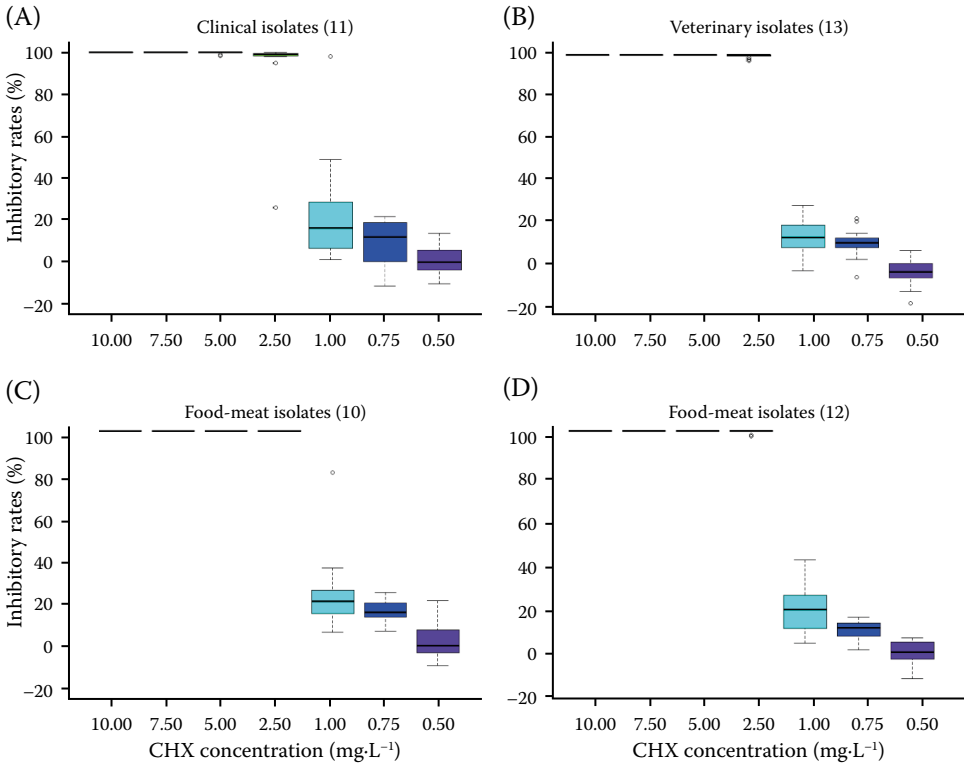

Figure 1. Inhibitory rates of *S. aureus* planktonic cells cultivated for 24 h in different CHX concentrations marked as box-and-whisker plots with outliers: (A) clinical isolates, (B) veterinary isolates, (C) foodborne isolates from meat products, and (D) foodborne isolates from dairy products

CHX – chlorhexidine

zone. In Brazil, Da Silva et al. (2000) have proven that 40% of *S. aureus* strains, mainly from milk, can grow on BP agar atypically due to a missing enzymatic apparatus. Furthermore, according to the BP agar manual of use, non-lipolytic strains of *S. aureus* isolated from food and dairy products do not produce halo zones (ISO 6881-1:2021). It supports our study as all our veterinary isolates were isolated from cows' milk and three out of four food isolates were from dairy products.

The MRSA phenotype was detected in eight isolates (17.4%) using MRSA*Select* II agar: three of them were clinical, three veterinary, and two foodborne from meat. However, exposure to cefoxitin discs narrowed this number to three isolates (6.5%) only: two clinical and one veterinary. Genetic screening confirmed that these three isolates carry the *mecA* gene. Hernandez et al. (2016) have compared the MRSA*Select* II agar to the standardised method and found 90.5% agreement. The study also showed that direct plating on MRSA*Select* II agar results in lower agreement of 76%. However, in our study, we observed an even lower agreement of only 37.5%, with five out of eight falsely positive samples. Obtaining fewer MRSA isolates is proven possible by studies like Alibayov et al. (2014) in the case of foodborne strains with a ratio of 10:83, Jaradat et al. (2021) in the case of hospital and environmental strains with a ratio of 158:222, and Preziuso et al. (2024) in the case of veterinary strains with a ratio of 19:144.

Coagulase production was positively confirmed by the bound coagulase test in all isolates, even when they did not produce the zone on BP agar, but four strains were identified as weak (2+). Weak coagulation has also been observed in 17.1% of strains obtained by Da Silva et al. (2000) from mastitis (similar to our veterinary strains) but not in strains obtained from humans or the environment. Most of their strains also presented a typical zone on the BP agar, which is in contrast with our study. However, comparison with other studies like Alibayov et al. (2014) is difficult because there are more ways to interpret data from plasma coagulation. Sometimes, only 4+ is considered positive, sometimes it is 4+ and 3+, and sometimes even 1+ strains may be accepted as coagulase-positive *S. aureus* following other tests (Bennett et al. 2013).

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Table 1. Evaluation of phenotypic properties of the studied *S. aureus* strains

Isolate	Type of isolate /spa type	Selective media		Cefoxitin resistance[a]	Coagulase test[a]	Haemolysis	Biofilm formation[b]
		BP agar	MRSA*Select* II agar[a]				
C1	clinical/t148	black with zone	–	–	+	β	++
C2	clinical/t593	black with zone	–	–	+	γ	+++
C3	clinical/t1533	black with zone	–	–	+	α	+++
C4	clinical/t267	black with zone	+	+	+ (weak)	β	++
C5	clinical/t571	black with zone	–	–	+	β	++
C6	clinical/t10133	black with zone	–	–	+	γ	+++
C7	clinical/t002	black with zone	–	–	+	β	+++
C8	clinical/t019	black with zone	+	–	+	γ	++
C9	clinical/t021	black with zone	–	–	+	β	++++
C10	clinical/t3297	black with zone	–	–	+	α	+++
C11	clinical/t128	black with zone	+	+	+	β	++++
V1	veterinary/t1403	black without zone	–	–	+ (weak)	α	++++
V2	veterinary/t2873	black without zone	–	–	+	β	+
V3	veterinary/t2873	black without zone	–	–	+ (weak)	β	+
V4	veterinary/t2873	black without zone	–	–	+	β	+
V5	veterinary/t2873	black without zone	–	–	+	β	+
V6	veterinary/t2873	black without zone	–	–	+	α	+
V7	veterinary/t527	black with zone	–	–	+	α	+++
V8	veterinary/t18853	black without zone	–	–	+	α	+++
V9	veterinary/t1793	black without zone	+	+	+ (weak)	β	+++
V10	veterinary/t091	black with zone	+	–	+	β	++++
V11	veterinary/t034	black without zone	–	–	+	α	+++
V12	veterinary/t416	black with zone	+	–	+	β	+
V13	veterinary/t024	black with zone	–	–	+	β	+++
F1	food–meat/t008	black with zone	–	–	+	α	+++
F2	food–dairy/t1137	black with zone	–	–	+	α	+++
F3	food–meat/t156	black with zone	–	–	+	β	+++
F4	food–dairy/t065	black without zone	–	–	+	α	+++
F5	food–dairy/t021	black with zone	–	–	+	γ	++
F6	food–dairy/t1312	black with zone	–	–	+	β	+++
F7	food–dairy/t2932	black with zone	–	–	+	α	+++
F8	food–dairy/t267	black with zone	–	–	+	α	+++
F9	food–dairy/t095	black with zone	–	–	+	β	+++
F10	food–dairy/t084	black with zone	–	–	+	β	+++
F11	food–dairy/t2932	black with zone	–	–	+	α	+++
F12	food–meat/t1255	black with zone	–	–	+	β	+++
F13	food–meat/t267	black with zone	+	–	+	β	++++
F14	food–meat/t008	black with zone	–	–	+	β	++++
F15	food–meat/t091	black without zone	–	–	+	β	+++
F16	food–dairy/t4044	black with zone	–	–	+	α	+++
F17	food–meat/t1823	black with zone	–	–	+	β	++++
F18	food–meat/t693	black with zone	+	–	+	α	+++
F19	food–meat/t091	black with zone	–	–	+	α	+++
F20	food–dairy/t12029	black without zone	–	–	+	β	+++
F21	food–meat/t163	black with zone	–	–	+	α	++++
F22	food–dairy/t16278	black without zone	–	–	+	α	++++

[a] Positivity is expressed as '+' and negativity is expressed as '–', [b] strength of biofilm formation: + = weak formation (0.10–0.25), ++ = medium formation (0.26–0.40), +++ = strong formation (0.41–1.00), and ++++ = very strong formation (> 1.01); BP – Baird-Parker; MRSA – methicillin-resistant *Staphylococcus aureus*

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Based on the colony growth on Columbia agar, 18 isolates (39.1%) were α-haemolytic, 24 isolates (52.2%) were β-haemolytic, and the remaining four isolates (8.7%) were γ-haemolytic. The abundance of single haemolytic types was in general agreement with the already published study of foodborne strains by Alibayov et al. (2014). In their study, 28% of strains were β-haemolytic and 18.2% of them were α-haemolytic. Although Wang et al. (2020) have already proven that there is no correlation between MRSA phenotype and the type of haemolysis they produce, all MRSA strains in our study showed β-haemolysis on blood agar. Interestingly, Tang et al. (2013) suggest that haemolysis can be linked to the source of *S. aureus* isolates, but it has not been confirmed in our study. Nevertheless, they found the highest percentage of γ-haemolytic strains (56.3%) while in the other mentioned studies, such strains were a minority.

The last observed phenotypic feature was biofilm formation. All isolates formed biofilm, and the majority of 35 strains (76.1%) were strong or very strong biofilm producers. The remaining strains were either weak (six isolates, 13.0%) or medium (five isolates, 10.9%) biofilm producers. Veterinary isolates were generally among the weaker biofilm producers, while food-borne isolates belonged among the stronger ones. Preziuso et al. (2024) have found 79.1% of strong biofilm producers among their *S. aureus* strains, so it can be deduced that *S. aureus* commonly forms biofilm strongly. Also, all their MRSA strains were strong biofilm producers which was not the case in our study. In Bosnia and Herzegovina, 77 *S. aureus* strains (clinical or veterinary) were studied. The highest number of strains were medium and weak biofilm producers (54.5% and 29.9% respectively), and the rest were either strong or non-producers. Similarly to our study, most veterinary strains were among weak producers (Šmitran et al. 2023).

Susceptibility of the strains to CHX. Collection strain C9 (CCM) was used for the initial testing of the CHX effect on *S. aureus*, and concentrations higher than 10 mg·L^{-1} were determined as bactericidal because we observed no followup growth on agar plates (see Supplementary Table 1). As 8 mg·L^{-1} is considered the sensitivity threshold for *S. aureus* and MIC is 4 mg·L^{-1} (Morrissey et al. 2014), 10 mg L^{-1} was set as the highest concentration for the follow-up experiments. Nonetheless, we did not find any resistant or tolerant isolates and support the study of Morrissey et al. (2014) which proved that CHX-resistant subpopulations are uncommon among wild types of bacteria.

The effect of CHX was studied both on planktonic cell growth and biofilm mass formation after 24-h exposure. Then MIC and MIC-B were determined (Table 2), and only four isolates (three clinical and one veterinary) differed from the whole tested

Table 2. Chlorhexidine minimal inhibitory concentrations (mg·L^{-1}) on planktonic cells (MIC) and biofilm formation (MIC-B)

Strain	MIC	MIC-B
C1	2.5	1.0
C2	2.5	2.5
C3	2.5	2.5
C4	2.5	2.5
C5	2.5	2.5
C6	2.5	2.5
C7	**5.0**	**5.0**
C8	2.5	2.5
C9	2.5	2.5
C10	2.5	2.5
C11	**5.0**	2.5
V1	2.5	2.5
V2	2.5	2.5
V3	2.5	2.5
V4	2.5	2.5
V5	2.5	2.5
V6	2.5	**1.0**
V7	2.5	2.5
V8	2.5	2.5
V9	2.5	2.5
V10	2.5	2.5
V11	2.5	2.5
V12	2.5	2.5
V13	2.5	2.5
F1	2.5	2.5
F2	2.5	2.5
F3	2.5	2.5
F4	2.5	2.5
F5	2.5	2.5
F6	2.5	2.5
F7	2.5	2.5
F8	2.5	2.5
F9	2.5	2.5
F10	2.5	2.5
F11	2.5	2.5
F12	2.5	2.5
F13	2.5	2.5
F14	2.5	2.5
F15	2.5	2.5
F16	2.5	2.5
F17	2.5	2.5
F18	2.5	2.5
F19	2.5	2.5
F20	2.5	2.5
F21	2.5	2.5
F22	2.5	2.5

Isolates with a different susceptibility are in bold

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group by having higher or lower MIC or MIC-B than 2.5 mg·L^{-1} (in bold). Their MIC-B was either lower than MIC (three strains), or the same as MIC (one strain). Furthermore, MIC values for strain C9 range from 2.5 mg·L^{-1} (Anand et al. 2015) to < 2 mg·L^{-1} (Grare et al. 2010). In our study, the obtained MIC was 2.5 mg·L^{-1} and is comparable to Anand et al. (2015). Strain C10 has been reported to have a MIC of 1 mg·L^{-1} while our study found the MIC at 2.5 mg·L^{-1}. This difference may be caused by the tested CHX salt because Bock et al. (2018) used CHX-digluconate.

The main grouping of the *S. aureus* isolates was done based on their source, and the inhibitory rates are shown in Figure 1 and Figure 2. The data indicate that, for most isolates, concentrations lower than 2.5 mg·L^{-1} are not lethal, and bacteria can form biofilm. Moreover, the sub-lethal CHX concentration of 0.5 mg·L^{-1} allowed for more cells (up to 20%) to be in planktonic form than in pure TSB. In the case of biofilm, the concentration of 1 mg·L^{-1} began to enhance biofilm mass in food-meat isolates and concentrations of 0.75 mg·L^{-1} and 0.5 mg·L^{-1} enhanced the biofilm mass of at least one isolate in all categories, similar to the study of Ebrahimi et al. (2014). They studied *S. aureus* strains from mastitis cow milk cultivated with CHX-digluconate and discovered the sub-MIC concentrations induced biofilm formation in not only *S. aureus* but also *Escherichia coli*, *Salmonella* spp. or *Streptococcus agalactiae* which poses a serious risk in all areas of human activity (Ebrahimi et al. 2014).

Despite the individual variance in data (Figures 1 and 2), statistically significant differences within the same CHX concentration were observed in neither planktonic cell growth nor biofilm mass in relation to the main origin of the isolates ($P > 0.05$). On the other hand, differences in the effect of different CHX concentrations were significant for all origins in planktonic cell growth ($P < 0.01$) and biofilm formation of foodborne strains ($P < 0.05$).

Then we compared foodborne isolates to evaluate differences between meat and dairy products. All differences were statistically insignificant except for the

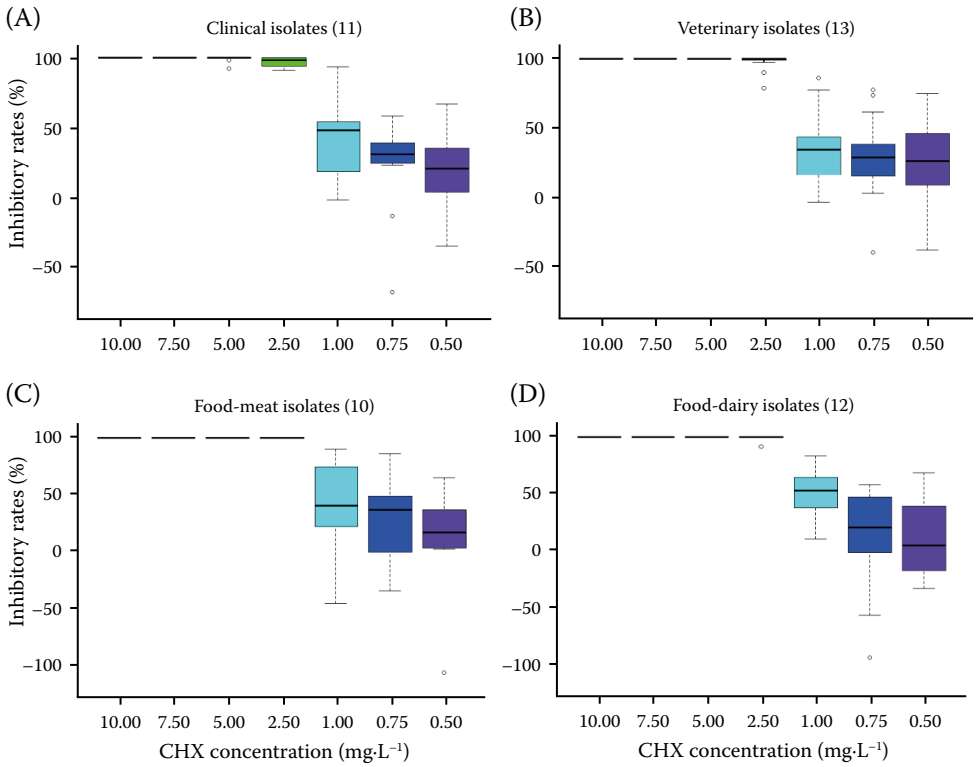

Figure 2. Inhibitory rates of *S. aureus* biofilm formation after cultivation for 24 h in different CHX concentrations marked as box-and-whisker plots with outliers: (A) clinical isolates, (B) veterinary isolates, (C) foodborne isolates from meat products, (D) foodborne isolates from dairy products

CHX – chlorhexidine

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concentration of 0.75 mg·L^{-1} CHX for planktonic cells ($P < 0.05$). No significant differences were also observed between the isolates that did and did not produce the halo zone on BP or between isolates with different types of haemolysis ($P > 0.05$).

The last comparison based on the methicillin resistance could not be properly assessed because the number of MRSA isolates was low. That is why the graphs of MRSA and MSSA isolates next to each other (Figure 3), do not show any statistically significant differences in all three sub-lethal concentrations of 1.00, 0.75 and 0.50 mg·L^{-1} CHX ($P > 0.05$). The inhibitory rates of planktonic cells follow a similar pattern of decrease, which cannot be observed in biofilm formation. Buxser's review statistically analysed published data about *S. aureus* susceptibility to CHX over time and found that MRSA strains exhibit slightly higher resistance to CHX than MSSA strains with a 1.6-fold difference. It also seems that current *S. aureus* strains have become about three times more susceptible to CHX than strains from fifty years ago (Buxser 2021), however, CHX susceptibility should remain relatively stable over time (Van den Poel et al. 2022).

CONCLUSION

We have characterised and tested 46 *S. aureus* isolates obtained from the Czech Republic for the effect of CHX to prove that all of them are CHX-susceptible. This way, we have successfully confirmed the absence of CHX-resistant or tolerant isolates in our tested group, which indicates the adequate use of CHX and the inability to create resistance in *S. aureus*. Through the phenotypic characteristics, the results cannot be generalised, however, susceptibility to CHX is not related to the origin of the isolate or its phenotype. The finding of the isolates with different MIC and MIC-B may provide a more than useful basis for further investigation of CHX tolerance in *S. aureus* in not only the Czech Republic but also other countries. Furthermore, our presented data may serve as a springboard for the development of antimicrobial materials with an adequate amount of CHX. These materials offer significant benefits in not only human medicine but also veterinary medicine or food packaging where the minimal effective CHX concentration is considered crucial.

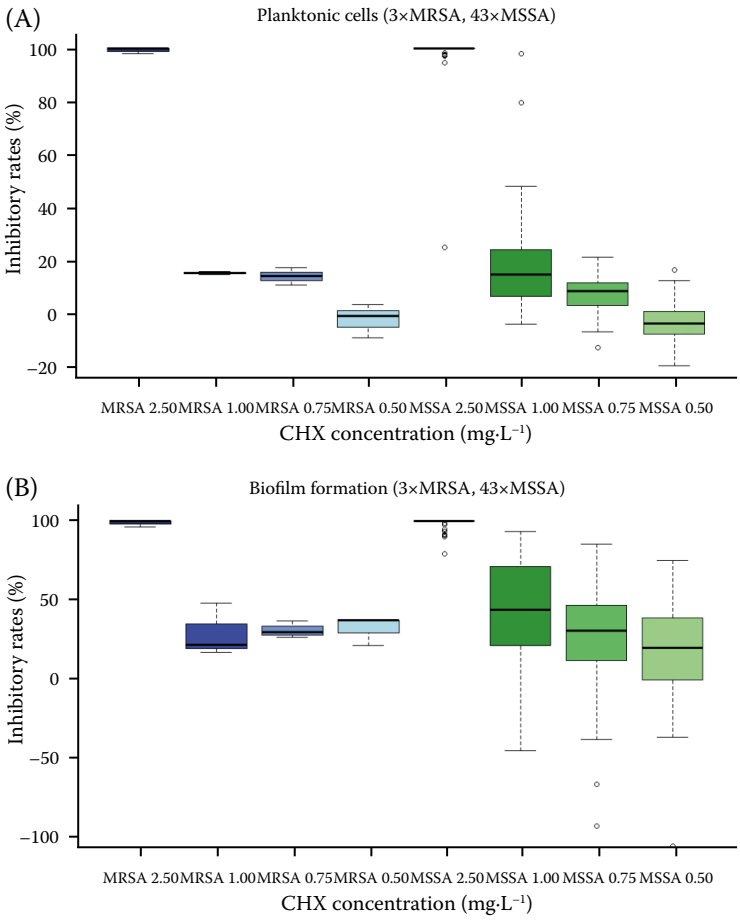

Figure 3. Inhibitory rates of planktonic cells: (A) and biofilm formation, (B) of MRSA (blue) and MSSA (green) strains after cultivation for 24 h in different CHX concentrations marked as box and whisker plots with outliers

CHX – chlorhexidine; MRSA – methicillin-resistant *Staphylococcus aureus*; MSSA – methicillin-susceptible strains of *S. aureus*

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