Antimicrobial Factors Effects on Biofilm Formation in *Staphylococcus aureus*

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Abstract

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We determined the disinfectant effects of benzalkonium chloride (BC) and Savo (SV), a chlorine compound, on the biofilm and planktonic cells in 23 strains *S. aureus* mainly food isolates. The biofilm formation was performed in a model system using microtiter polystyrene plates COSTAR 3797 in trypton-soy broth with 1% glucose at 30°C. Benzalkonium chloride (BC) at 125 mg/l, applied directly on 24 h old biofilm, was able to remove the biofilm matrix in 21 strains, and to stop the reproduction of the biofilm cells in 23 strains. BC at the concentration of 125 mg/l was lethal to planktonic cells, coincubated for 24 h or treated for 10 minutes. None of the strains studied was able to grow in SV at 1X recommended concentration, while the safety lethal concentration for planktonic cells treated for 10 min was 4X. The application of 4X concentration SV into the 24 h old suspension removed the biofilm matrix in all strains and devitalised the biofilm cells in 10 strains and inhibited the viability in 13 strains by 70%.

Keywords: *Staphylococcus aureus*; biofilm; planktonic cells; disinfectants; benzalkonium chloride; Savo

Staphylococcus aureus is a Gram-positive facultatively anaerobic bacterium, recognised as foodborne and clinical pathogen, inhabiting the skin, skin glands and mucous membranes of humans, other mammals and birds. This organism is a common part of human and animal microflora, found in healthy state without pathological manifestations on skin or mucous membranes. *S. aureus* is one of the most biochemically active bacteria and produces a family of virulence factors such as adhesion proteins, enterotoxins, superantigens, pore-forming hemolysins, ADP-ribosylating toxins, and proteases (Bhunia 2008). Under certain circumstances (the organism enfeeblement, wound, skin barrier disruption, and high-level dose of virulent strain) can these factors cause invasive or toxic disease. For food industry and food safety the main topic of interest is its ability to produce a wide spectrum of thermostable enterotoxins, which cause acute gastroenteritis after food consumption (Bednář *et al.* 1999; Bhunia 2008).

Biofilm is a consortium of microorganisms surrounded as slime by extracellular polymeric substances (EPS), attached to either inert or living surfaces (Poulsen 1999). EPS are biopolymers, secreted or released by biofilming cells, consisting largely of polysaccharides, a wide variety of proteins, glycoproteins, glycolipids, and eventually extracellular DNA (Flemming *et al.* 2007). In contrast to the biofilm cells the planktonic cells are

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freely and individually living in liquids (Poulsen 1999). The biofilm formation starts by attaching cells onto an acceptable surface. While with many bacteria flagella and fimbrie play a significant role in this process (Van Houdt & Michiels 2010), with *S. aureus* it proceeds by the so-called MSCRAMMs – microbial surface components recognising adhesive matrix molecules (Otto 2008). Some of them such as autolysins (Heilmann *et al.* 2003) have the capacity not only to bind specifically on human matrix proteins, but also to bind non-specifically on hydrophobic surfaces. During the biofilm maturation, the cells divide and aggregate through EPS secretion to form a specific three-dimensional structure, described to consist of "towers" or "mushrooms" with porins and canals (Blaschek *et al.* 2007). In most staphylococci, the EPS is composed mainly of the intercellular polysaccharide adhesin (PIA; Mack *et al.* 1996) and other polymers like teichoic acids and proteins (Otto 2008). In aging, the biofilm structure weakens and single cells or larger cells clusters are detached. This can be caused by mechanical forces or by destroying the biofilm matrix by enzymes or surfactants or by cessation of EPS production (Otto 2008). This process is crucial for the dissemination of bacteria to other colonisation sites (Otto 2008). Most of the genes involved in the biofilm formation are regulated by quorum sensing systems in the direct relation to the cell density. For the expression of the quorum-sensing regulated genes, a certain threshold level is needed of signal molecules produced by cells, called autoinductors (Miller & Bassler 2001). *S. aureus* uses two quorum-sensing systems *agr* and *lux*S (Kong *et al.* 2006). The biofilm cells exhibit significant differences in gene expression and physiology. In staphylococci, a low oxygen concentration in biofilms leads to a switch to fermentative processes such as acetoin metabolism (Beenken *et al.* 2004) while the physiological status is characterised by a down-regulation of active cell processes as the protein, DNA and cell wall biosynthesis, different however, from those of planktonic cells in the stationary growth phase (Otto 2008). Also, specific resistance mechanisms were found to be upregulated in staphylococcal biofilms (Yao *et al.* 2005) and the spatial arrangement promotes more often the horizontal gene transfer (Hausner & Wuertz 1999). The commonly known fact of the dramatically increased biofilm cells resistance to antibiotics, disinfectants, and innate host defense or physical treatment in comparison to planktonic cells can be attributed to two main mechanisms (Blaschek *et al.* 2007; Otto 2008). The biofilm architecture prevents antibacterial substance from reaching its target by electrostatic repulsion or sequestration by surface polymers (Otto 2008) or slows down this proces, e.g. by limited diffusion or repulsion (Xu *et al.* 1996). This biofilm arrangement also allows microorganisms to persist in the environments and to resist physical factors such as UV, dessication in hydratated biofilm matrix (Flemming *et al.* 2007), etc. The specific physiology of the biofilm cells then limits the efficacy of antibiotics, mainly of those target active cell, and may also include specific subpopulations of resistant cells ("persisters") (Keren *et al.* 2004). While the biofilm matrix is a network providing sufficient mechanical stability to maintain the spatial arrangement for microconsortia over a prolonged period (Flemming *et al.* 2007) protected from physical and chemical influences, it can be difficult to control biofilms in food processes. A variety of direct and indirect experimental procedures have been developed for studying the bacterial attachment and colonisation (Lindsay & von Holy 1997; Poulsen 1999). A microtiter plate procedure belongs to indirect methods for the estimation of the amount of bacteria *in situ* and can be modified for various biofilm formation assays. In this study, the procedure using microtiter plates according to Djordjevic *et al.* (2002) was employed using 0.1% crystal violet solution for staining the biofilm cells. The published studies, dealing with the effects of disinfectants on staphylococcal biofilm, are often restricted to studying only a single well-characterised collection strain, mainly *S. epidermidis* (as CIP53124 by Houari & Di Martino 2007; NCTC 11047 by Eginton *et al.* 1998), less often *S. aureus* (as ATCC 6538 by Toté *et al.* 2010), and focused only on some aspects: the effects of disinfectants on the biofilm formation by Houari and Di Martino (2007); differences between the biofilm and planktonic cells in susceptibility to disinfectants by Eginton *et al.* (1998); disinfectant matrix effect by Toté *et al.* (2010); biofilm formation and planktonic cells resistance to disinfectants in 86 *S. aureus* isolates by Marino *et al.* (2010). For this reason, the objective of this study was to examine the collection of 23 strains of *S. aureus* (22 food isolates, 1 clinical isolate) from the Czech Republic from different points of view. The objective of the experiments

was to study the effects of two disinfectants (Savo – mixture of natrium hypochlorite and natrium hydroxide, benzalkonium chloride – quarternary ammonium salt) on the planktonic cells viability and biofilm formation and on biofilm cells viability, biofilm matrix removing or the induction of biofilm formation under different conditions of application (directly on biofilm or in the suspension modelling different plant situations) at various concentrations.

MATERIAL AND METHODS

Culture preparation. 23 strains of *Staphylococcus aureus*: 22 strains isolated from different food matrices and one clinical strain (SA 720), obtained from the National Institute of Public Health (NIPH), were used in this study (Table 1). Stock cultures were stored at –80°C in brain-heart infusion (BHI; Merck, Darmstadt, Germany) and 15% glycerol. The working cultures were maintained on Baird-Parker agar plates (BPA; Merck, Darmstadt, Germany) at 4°C for 30 days. Prior to each experiment, one colony from BPA was grown in 6 ml of BHI at 37°C for 24 hours.

Microtiter plate biofilm production assay. Microtiter polystyrene plates COSTAR 3797 (Corning Incorporated, Lowell, USA) were chosen as standard tools in all experiments. Biofilm formation was proceeded in tryptone-soya broth with 1% of glucose – TSBG (TSB; Merck, Darmstadt, Germany) at 30°C, the conditions which were previously found to be the optimal out of eleven media at four temperatures (8°C, 25°C, 30°C, and 37°C). Overnight cultures grown in BHI were diluted in TSBG and equilibrated at 0.5 McFarland value (app. 10^8 CFU/ml). Microtiter plates wells, washed with 200 µl of 70% ethanol and air dried, were filled with 100 µl of individual strain culture dilutions at 0.5 McFarland density scale, incubated at 30°C for 24 h after which the antimicrobial treatment was performed. The ability to grow was taken as the difference between the absorbances measured before and after incubation at 620 nm by Tecan-Spectra 9440012 (Tecan Austria GmbH, Grödig, Austria).

Disinfectants/antibiotics treatment assay. If tested: **A. the direct disinfectants treatment effects on biofilm cells** 24 h grown suspension was removed from the wells and the microtiter plate wells were washed six times with 350 µl of sterile distilled water to remove the loosely associated bacteria and were left to dry. The tested disinfectants in the volume of 100 µl were added directly onto the biofilm for 10 min treatment. Then it was washed six times with 350 µl sterile distilled water and 100 µl of fresh TSBG was added to each well. If tested **B. the indirect disinfectants treatment effects on biofilm cells** 100 µl of each of the tested disinfectants was added directly into 24 h grown suspension in the wells and after 10 min, the plates were also washed and fresh TSBG was added as described in case B. But before washing, 10 µl of the treated suspension was transferred into a new plate with 90 µl of TSBG for testing **C. the disinfectants treatment effects for 10 min on planktonic cells**. In all cases (A, B, C), the plates were incubated at 30°C for 24 h, after which the biofilm staining was performed. The difference between A_{620} measured before and after incubation was taken as the level of growing. If tested **D. biofilm formation in the disinfectants presence** 100 µl of the disinfectant

Table 1. List of tested strains obtained from National Institute of Public Health, Brno, Czech Republic

Strain	Sample	Genes coding SEs
SA 672	patisserie	A, H
SA 673	patisserie	D, I, G
SA 711	Balcan salt cheese	H
SA 719	chicken tetrazzini	E
SA 720	feces	A, C, E
SA 740	pork ham	C
SA 816	sea fish	A, B
SA 817	spinach	C
SA 921	cow's raw milk	D, I, G, J
SA 940	meat-product mass	A
SA 992	pork ham	B
SA 1003	long-life salam	B, D, J
SA 1041	cow's raw milk	D, J
SA 1106	patisserie	B
SA 1117	patisserie	C, I, G
SA 1141	sausages	C
SA 1173	pickled Hermelin (Camembert)	A, B, D, J
SA 1176	chopped raw meat	D, J
SA 1185	sea fish	A
SA 1238	cow's raw milk	–
SA 1241	cow's raw milk	–
SA 1247	poultry salame	A, C, I, G
SA 1249	cow's raw milk	B, I, G

Enterotoxin encoding genes were detected by PCR method

at the concentration twice the final one desired was added to 100 µl of the starting culture 0.5 McFarland described above and the biofilm staining and the growth level were determinated after incubation at 30°C for 24 hours.

Biofilm quantification by crystal violet staining. For biofilm quantification, crystal violet staining was used. The plates were washed six times with 350 µl of distilled water, air dried for 45 min and each well was stained with 150 µl of 0.1% crystal violet solution in water for 45 minutes. After staining, the plates were washed again with 350 µl of distilled water six times. Quantitative analysis of the biofilm production was performed by adding 200 µl of 95% ethanol to destain the wells. After 45 min of destaining, 100 µl from each well was transferred to a new microtiter plate and the level of crystal violet present in the destaining solution was measured at 620 nm using Tecan-Spectra 9440012.

Disinfectants. Two different disinfectants were tested: benzalkonium chloride (BC) – quartenary ammonium salt, QUAT group (Fluka Analytical, St. Louis, USA), approved for food industry, and Savo (SV) – supplied in the original concentration of max. 5% NaClO and 2% NaOH (Penta, Prague, Czech Republic), generally used in the food industry and household. The tested concentrations of BC were 125 mg/l; 62.5 mg/l; 31.25 mg/l, the range of its concentration used in commercial products being from 0.5 g/l (Desam Extra; Biochemie, Bohumín, Czech Republic) to 40 g/l (Microbac Forte; BODE Chemie GmbH, Hamburg, Germany; Hexaquart Forte; B. Braun AG, Melsungen, Switzerland). As to SV, the original concentration is equal to 10X concentration. The concentrations 1X (1:10 in sterile distilled water), recommended by the producer, and then 2X and 4X were tested.

Statistics. Each concentration and positive control (without disinfectants treatment) was measured in four parallel wells using two independent assays for each strain and disinfectant. The negative control was TSBG without cells treated with disinfectants in the same manner. All measured values were statistically evaluated using the software Statistica 8 (StatSoft, Inc., Tulsa, USA). The insufficient washing out of the unbound crystal violet was assumed to be a possible source of errors, therefore only the values lying in the 70% lower percentile were taken in account for the determination of the average and standard deviations, while those in 70% upper percentile were omitted as outline values. The interval determined by the average and double standard deviations consists of 95% of the considered values.

RESULTS AND DISCUSSION

The disinfectants treatment of planktonic cells can cause not only the cell density reduction, but at sublethal concentrations can also increase the biofilm formation (Chaieb *et al.* 2011). Hence, the planktonic cells were tested for their ability to grow and form biofilm in the presence of various disinfectants concentrations for 24 hours. Simultaneously, the same concentrations of BC and SV were applied for 10 min on 24 h grown cells in microtitre plates. The disinfectants could devitalise the biofilm cells embedded in matrix and/or remove this matrix or increase biofilm formation by the same way as with planktonic cells. In previous experiments, it was found that direct staining of biofilm with crystal violet after the treatment with disinfectants is not optimal because the cells surface is damaged. The dead cells bind crystal violet more readily (Purkrtová *et al.* 2010).

Benzalkonium chloride

BC proved to possess a highly lethal effect on the planktonic cells at all concentrations tested (31.25 mg/l; 62.5 mg/l; 125 mg/l). When planktonic cells were incubated in TSBG for 24 h (Figure 1) BC at 125 mg/l was lethal for all strains, while the lower concentrations also manifested lethal (31.25 mg/l – 11 strains, 62.5 mg/l – 20 strains) or highly inhibiting effects (ΔA_{620} app. 0.1 = 95% reduction). The treatment of planktonic cells with BC for 10 min was sufficient for the same lethal effect as that at 125 mg/l in all strains (Figure 2). Only one strain (SA 672) proved to be resistant to BC at 62.5 mg/l, while for the other strains the lower concentrations were again lethal (31.25 mg/l – 19 strains, 62.5 mg/l – 3 strains) or highly inhibiting the growth (ΔA_{620} app. 0.1 = 95% reduction). Because of the low growth level, no significant biofilm formation was observed (data not shown). Marino *et al.* (2010) examined 86 *S. aureus* strains isolated from food and the MIC of planktonic cells for BC ranged between 1.25 mg/l to 2.5 mg/l. The concentration of BC applied directly on the biofilm cells (31.25 mg/l for 10 min) was sufficient

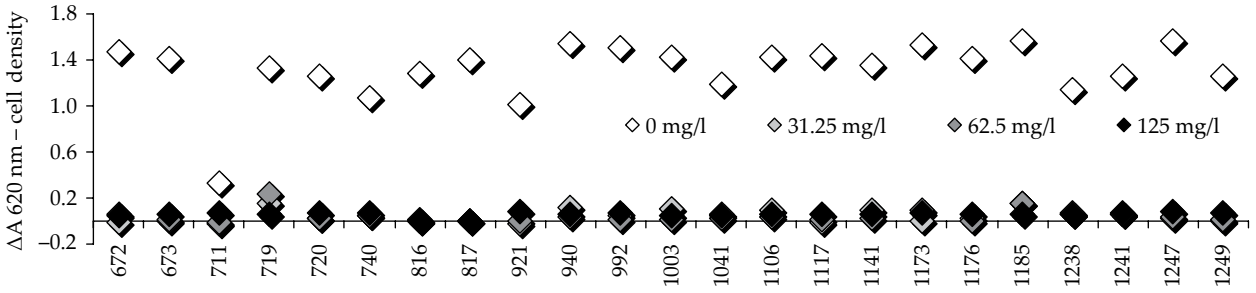

Figure 1. The ability of planktonic cells to grow in TSBG + BC for 24 h at 30°C. The average standard deviation: 0 mg/l – 0.03, 31.25 mg/l – 0.01, 62.5 mg/l – 0.01, 125 mg/l – 0.01

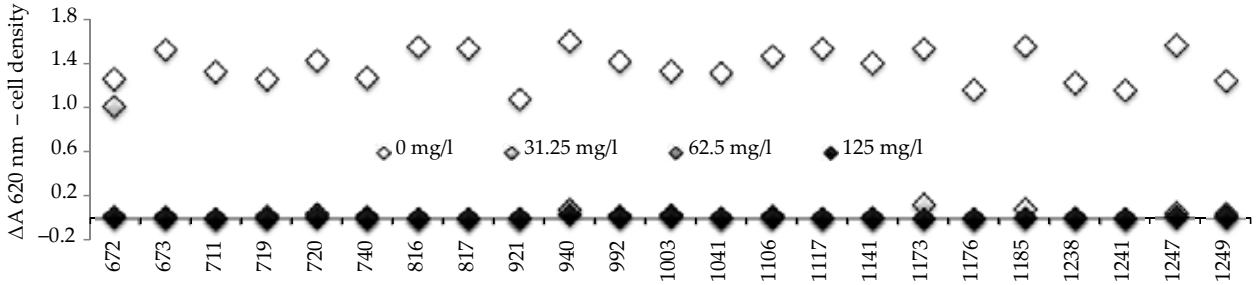

Figure 2. The ability of planktonic cells treated 10 mins by BC to grow in TSBG for 24 h at 30°C. The average standard deviation: 0 mg/l – 0.05, 31.25 mg/l – 0.04, 62.5 mg/l – 0.01, 125 mg/l – 0.01

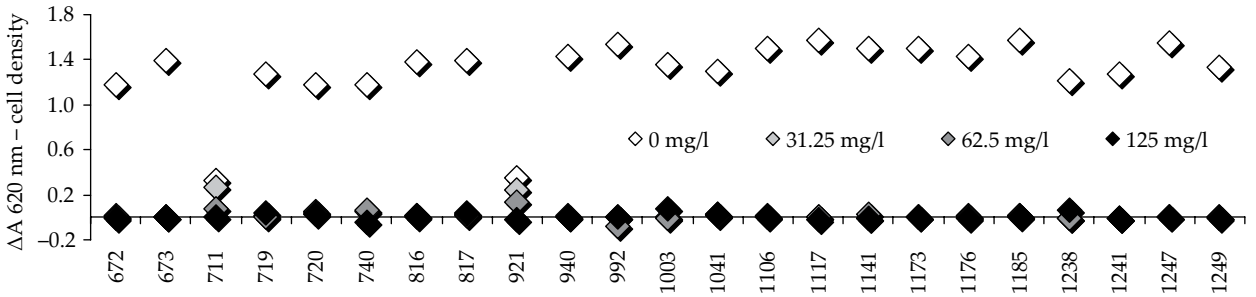

Figure 3. The viability of biofilm cells treated 10 min by BC directly on measured as the cell density after 24 h cultivation in TSBG at 30°C. The average standard deviation: 0 mg/l – 0.06, 31.25 mg/l – 0.07, 62.5 mg/l – 0.02 and 125 mg/l – 0.05

to devitalise the biofilm cells in all strains tested except SA 711, where 62.5 mg/l was necessary, and SA 921, where 125 mg/l was needful (Figure 3). This concentration (31.25 mg/l) also partly removed the biofilm matrix (Figure 4). The biofilm matrices of strains SA 711, SA 720, SA 921 were more resistant to reduction by 62.5 mg/l of BC. For strains SA 1238 and SA 1241 the concentration necessary to remove the biofilm matrix was 125 mg/l. Strains SA 711 and SA 921 displayed the same sensitivity of the biofilm cells and matrix to BC. When BC was applied into suspension, its ability to devitalise the biofilm cells and destroy biofilm was much more attenuated. BC at 125 mg/l was lethal to the biofilm cells in 14 strains only (Figure 5), causing biofilm removing (Figure 6) in 16 trains (except SA 672, SA 673, SA 711, SA 921, SA 1041, SA 1238, SA 1241 – in none of them were the biofilm cells devitalised). BC is cationic antiseptics, acting by general perturbation of the lipid bilayer membranes (Gilbert & Moore 2005). If BC is applied into suspension, it can be firmly bound to the exposed anionic sites on the cell membranes (Houari & Di Martino 2007) of the planktonic cells, thus its lethal effect on the exposed biofilm cells is lower. Some strains

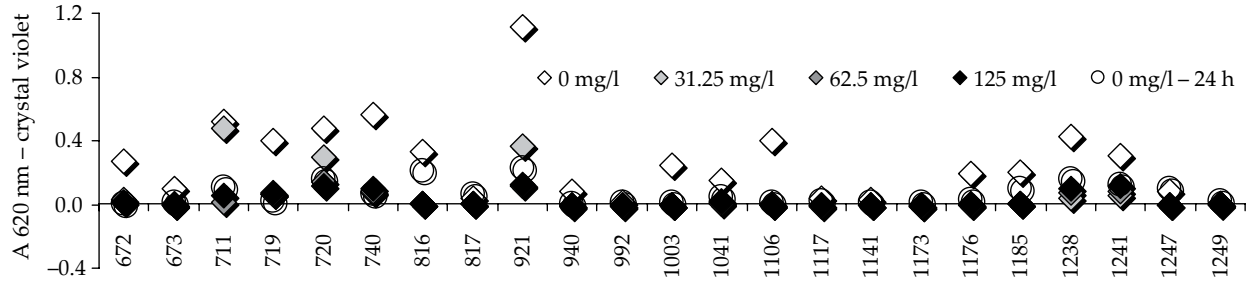

Figure 4. Staining of biofilm cells treated 10 min by BC directly on measured after 24 h cultivation in TSBG at 30°C. The average standard deviation: 0 mg/l – 0.07, 31.25 mg/l – 0.04, 62.5 mg/l – 0.01, 125 mg/l – 0.01, 0 mg/l – 24 h – 0.03

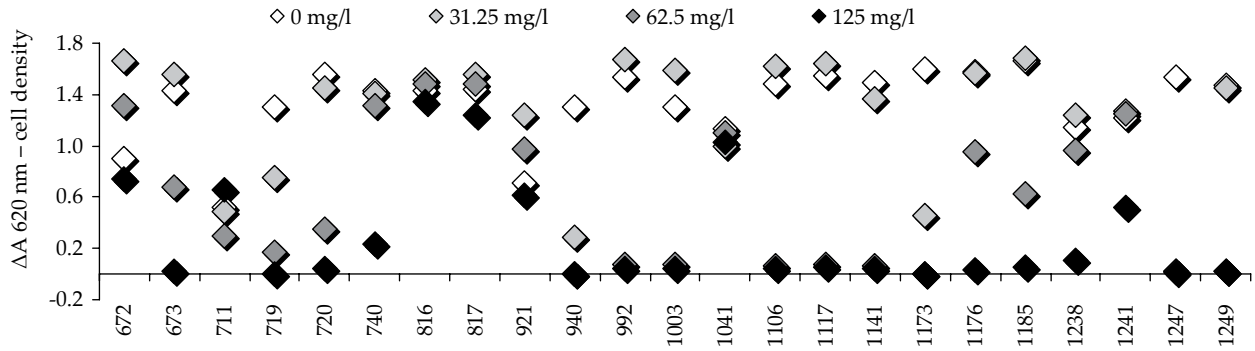

Figure 5. The viability of biofilm cells treated 10 min by BC into the suspension measured as the cell density after 24 h cultivation in TSBG 30°C. The average standard deviation: 0 mg/l – 0.06, 31.25 mg/l – 0.07, 62.5 mg/l – 0.09, 125 mg/l – 0.05

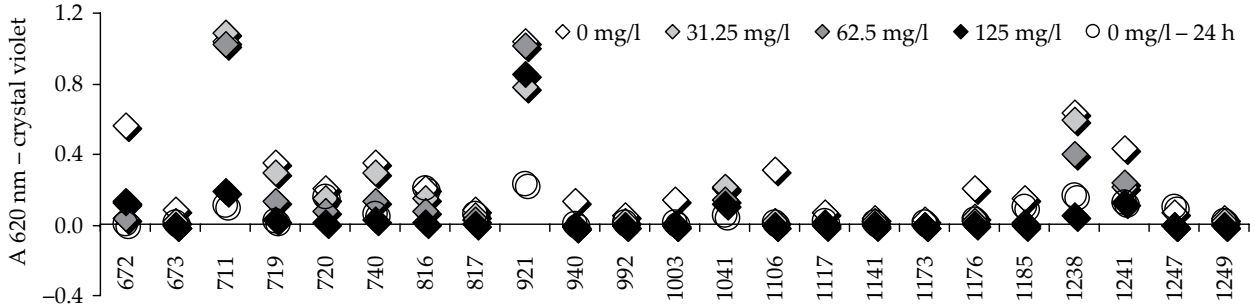

Figure 6. Staining of biofilm cells treated 10 min by BC into the suspension measured after 24 h cultivation in TSBG at 30°C. The average standard deviation: 0 mg/l – 0.07, 31.25 mg/l – 0.05, 62.5 mg/l – 0.04, 125 mg/l – 0.04, 0 mg/l – 24 h – 0.03

seem also to be more resistant to BC (SA 817 in Figures 5 and 6). The increased resistance of the biofilm cells in contrast to the biofilm matrix removal can be attributed to the most firmly attached cells located deep within the biofilm (Eginton *et al.* 1998). The high viability, almost unaffected by the treatment of these cells, can be caused by the failure of the biocide to penetrate the biofilm matrix (Huang *et al.* 1995), by the maturation in the attachment process (Das *et al.* 1998), also in the cells adhered in very slimy layer on the well surface or as the population of biofilm persisters cells (Keren *et al.* 2004). As a detergent, BC is able to disrupt the adhesive forces in biofilm, but only up to a certain level. BC at 125 mg/l was not able to remove completely the most abundant biofilm. It could mean that the more mature and abundant the biofilm is, the lower is the ability of BC to remove it. For example Toté *et al.* (2010) observed in experiments with the biofilming by the clinical isolate *S. aureus* (ATCC 6538), that the treatment 72-h-old biofilm formed in TSBG at 37°C with 0.1% BC for 60 min revealed no removing effect, while reduction close to 2 log of viable

biofilm cells occurred. In comparison to Houari and Di Martino (2007) no effect was observed of BC on the biofilm inhibition due to a decrease in membrane fluidity was observed.

SAVO

Savo (SV) at 1X concentration inhibited the growth of all strains after incubation in its presence for 24 h (Figure 7). With 24 h old planktonic cells treated for 10 min, 1X and 2X SV concentrations caused 80–100% reduction of the growth, while the 4X concentration was completely lethal for all strains (Figure 8). On direct SV treatment of biofilm cells, the assayed strains significantly differed in their behaviour. Toté *et al.* (2001) proved that 1% solution of natrium hypochlorite causes a 5 log reduction in the viability of *S. aureus* (ATCC 6538) planktonic cells after 1 min treatment. The 4X concentration of SV proved to be efficient for the biofilm matrix removal effect in all strains except SA 921, SA 1106, SA 1185 and SA 1238. The same concentration devitalised the biofilm

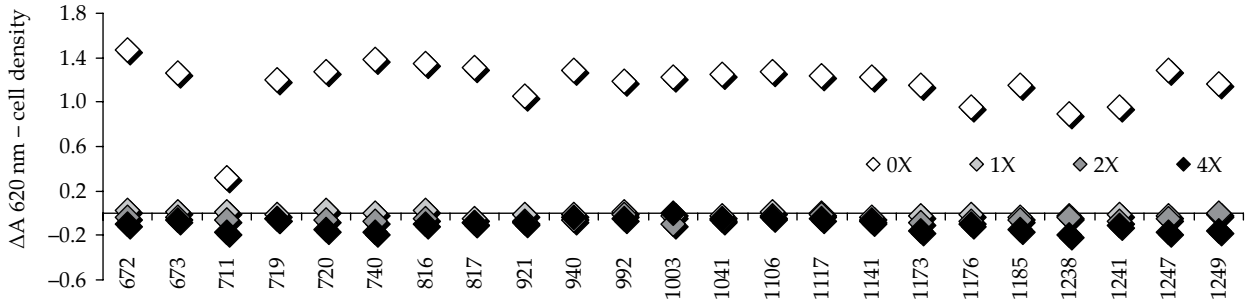

Figure 7. The ability of planktonic cells to grow in TSBG + SV for 24 h at 30°C. The average standard deviation: 0X – 0.01, 1X – 0.02, 2X – 0.02, 4X – 0.01

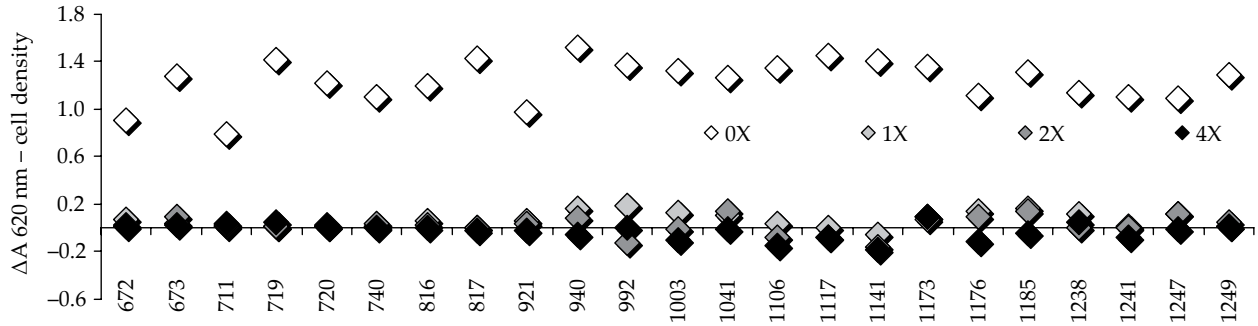

Figure 8. The ability of planktonic cells treated 10 min by SV to grow in TSBG for 24 h at 30°C. The average standard deviation: 0X– 0.04, 1X – 0.06, 2X mg/l – 0.06, 4X – 0.06

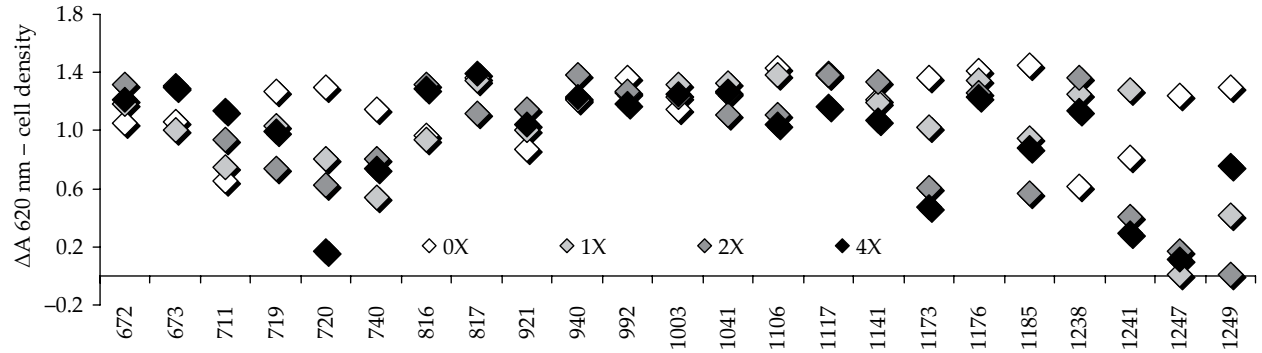

Figure 9. The viability of biofilm cells treated 10 min by SV directly on measured as the cell density after 24 h cultivation in TSBG at 30°C. The average standard deviation: 0X – 0.07, 1X – 0.08, 2X – 0.07, 4X – 0.06

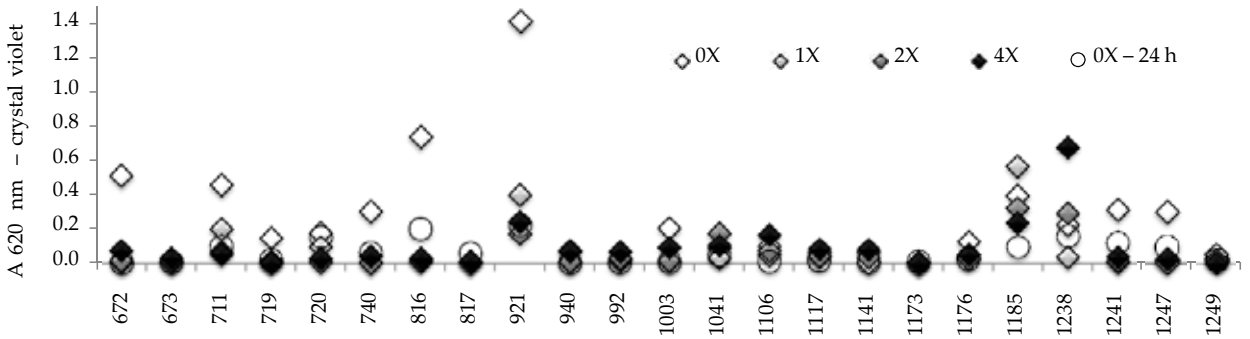

Figure 10. Staining of biofilm cells treated 10 min by BC directly on measured after 24 h cultivation in TSBG at 30°C. The average standard deviation: 0X – 0.06, 1X – 0.03, 2X – 0.03, 4X – 0.02, 0X – 24 h – 0.03

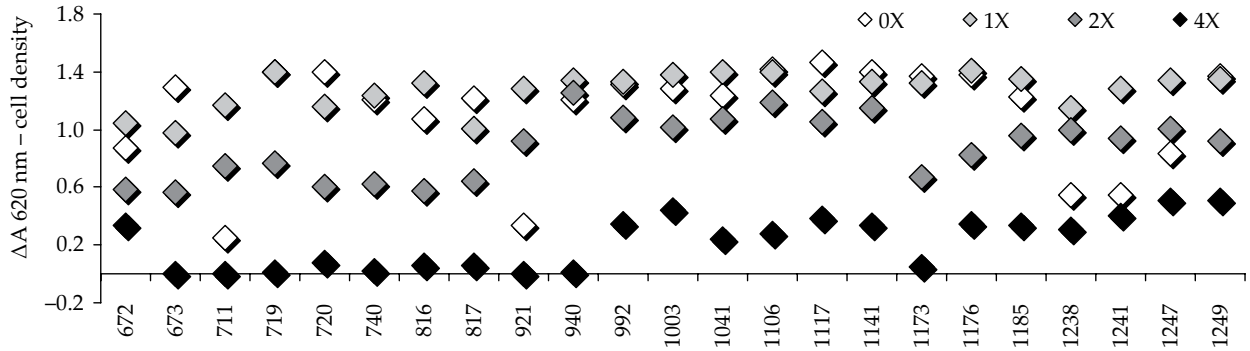

Figure 11. The viability of biofilm cells treated 10 min by SV into the suspension measured as the cell density after 24 h cultivation in TSBG at 30°C. The average standard deviation: 0X – 0.06, 1X – 0.03, 2X – 0.03, 4X – 0.02

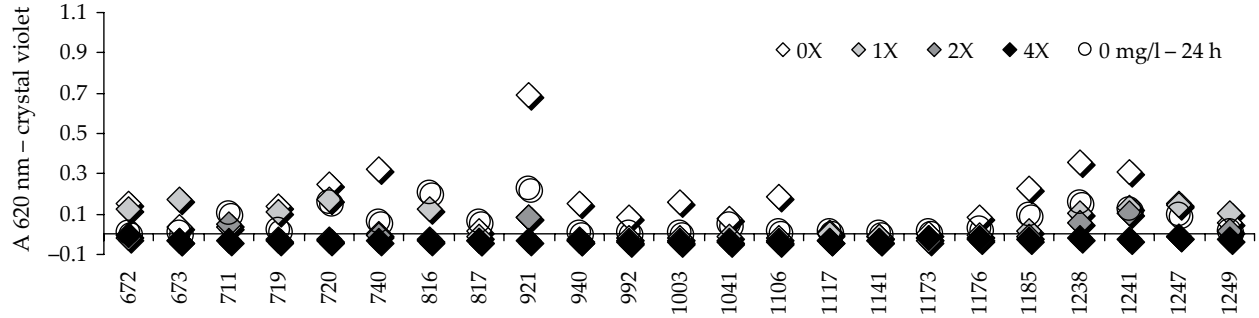

Figure 12. Staining of biofilm cells treated 10 min by SV into the suspension measured after 24 h cultivation in TSBG at 30°C. The average standard deviation: 0X – 0.05, 1X – 0.03, 2X – 0.01, 4X – 0.003, 0X – 24 h – 0.03

cells only in SA 720 and SA 1247. All the other strains biofilm cells exhibited the ability to survive 10 min treatment with SV in 4X concentration. For the same reason as discussed in BC (see Results and Discussion) it seems that although SV is efficient in removing EPS, its ability to act lethally during 10 min on the adhered or biofilm cells is lower. Eginton *et al.* (1998) proved the changes occurring in the strength of the attachment to the surfaces of the survivors of the disinfection treatment with sodium hypochlorite. In contrast for strain SA 1238 it is evident that the SV treatment is able to induce its biofilm formation. The application of SV into the suspension of 24 h old cells seems to be more efficient. Hypochlorite as a strong oxidiser can possibly react with the present bacterial suspension producing a more disinfectants efficient mixture (Eginton *et al.* 1998; Estrela *et al.* 2002). The concentration 4X is able not only to remove the biofilm matrix in all strains (Figure 12), but it also displayed lethality for the biofilm cells in 10 strains, while it

caused 70% inhibition in the others (Figure 11). In the experiments with 72 h old *S. aureus* biofilm (ATCC 6538) by Toté *et al.* (2010), it was showed that 1 min treatment with 1% sodium hypochlorite reduced the biofilm cells viability by 2 log and after 60 min 55% of the biofilm matrix was removed. In contrast, Stewart *et al.* (2001) observed in 6 day old biofilms of *P. aeruginosa* and *K. pneumoniae*, that although they had been treated with 1000 mg/l of sodium hypochlorite for 60 min, which penetrated them effectively, the viability of the biofilm cells was decreased by 0.4 log only.

The results presented proved the generally accepted fact of the decreased sensitivity of the biofilm cells to disinfectants in comparison to the planktonic cells. While biofilms are generally removed physically, for example by scraping them off, the combination with appropriate disinfectants treatment can help to remove them and also to inhibit their redevelopment. The crucial factors are the appliedd concentration and time of action and the frequency of the treatment. The efficiency of the disinfectants treatment decreases rapidly with the most mature and thick biofilms. Since strains differ in their readiness to form biofilms and in their properties, optimal conditions for an efficient disinfectants treatment must be tested specifically.

CONCLUSION

This study demonstrated that all 23 tested *S. aureus* strains (22 food isolates, 1 clinical isolate) in the Czech Republic were able to adhere and most of the them were able to produce biofilms at a significant level. Raw milk isolates showed the highest ability to form biofilms. The treatment with BC and SV at various concentrations and times of application displayed the different physiological properties of the strains studied. BC proved to be a more efficient disinfecting agent than SV. General recommendation for the disinfectant application ought to be based on the detailed knowledge of the dairy-plant persisting strains.

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