Efficacy of some commercial sanitizers for devitalization and removal of bacterial biofilms

JANKA KOREŇOVÁ – KATARÍNA URDOVÁ – KATARÍNA ORAVCOVÁ

Summary

The devitalization and removal of biofilms formed by bacteria isolated from meat and ewes' milk processing plants (*Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus* sp., *Stenotrophomonas maltophilia*), using eight industrial sanitizers based on benzalkonium chloride, hypochlorite, peroxides and aldehydes, were studied. Experiments were performed in polystyrene microplates. The devitalization of the biofilm was followed by the fluorescein diacetate assay, removal of the biofilm was followed by the crystal violet assay. None of the tested sanitizers was sufficiently effective with all the tested strains, either at the devitalization of the biofilm or at its removal, when used at a concentration recommended by the manufacturer and at a duration of the exposure of 10 min. Neither a prolonged exposure of 30 min, nor a three-fold increase in the concentration led to a significant improvement in the effeciency of the sanitizers. Best results at the removal of bacterial biofilm were achieved applying Ban-Kaz (Banchem, Dunajská Streda, Slovakia) or a peroxide-based sanitizer Desana-Max (Thonhauser, Perchtoldsdorf, Austria) in an ultrasonic bath, eventually with scrubbing. The results underline the need for performing mechanical cleaning together with the application of sanitizers, in order to achieve good hygiene in food processing plants.

Keywords

biofilm; sanitizer; *Staphylococcus* spp.; *Pseudomonas* spp.

Presence of spoilage or pathogenic microflora in food processing plants is an important source of contamination of food products. Levels of spoilage or pathogenic microorganisms in the food processing environment are most effectively controlled by regular cleaning and disinfection. However, certain strains are able to form biofilm, which may facilitate their extensive survival on food contact surfaces due to the increased resistance to sanitizers and biocides [1–6]. The most commonly occuring bacteria in biofilms of food processing environments are pseudomonads, staphylococci and enterobacteria [4, 7–11].

The most effective sanitizers suitable for use in food industry are based on quarternary ammonium compounds, hypochlorite, peroxides and aldehydes as the active components [12, 13]. However, these sanitizers may not always be as efficient on the devitalization of biofilms as on planctonic cells. The effectiveness of devitalization of biofilms may be improved by using special formulations of sanitizing agents, their increased concentration and increased exposure time, respectively [8, 14]. Although the biofilm is devitalized, the effectiveness of devitalization is not proportional to the removal of biomass from the surface of biofilm [15, 16]. Moreover, excessive concentration of a sanitizer may induce the change in microstructure of the surface and foulness attached and thus promotes consequent binding of impurities on the surface material and obstructs their removal [10, 17, 18]. Accordingly, the removal of biofilm should be considered along with the disinfection process optimization, taking into account general factors as intensive cleaning, selection of suitable sanitizers and their application at optimized concentrations, selection of appropriate application temperature of mechanical action such as scrubbing or pulse flows, and/or modification of surfaces [12, 14, 17].

Janka Koreňová, Katarína Urdová, Katarína Oravcová, Department of Microbiology, Molecular Biology and Biotechnology, VÚP Food Research Institute, Priemyselná 4, P. O. Box 25, SK – 82475 Bratislava 26, Slovakia.

Correspondence author:
Janka Koreňová, e-mail: korenova@vup.sk

In this study, we investigated the devitalization and removal of biofilms formed by bacteria isolated from meat and ewes' milk processing plants, using eight industrial sanitizers based on benzalkonium chloride, hypochlorite, peroxides and aldehydes. Experiments were performed in polystyrene microplates. The devitalization of the biofilm was followed by the fluorescein diacetate assay, while the removal of the biofilm was followed by the crystal violet assay. The application of selected sanitizers at increased concentration, increased temperature, at additional mechanical and ultrasonic treatment, as well as sequential use of two sanitizers were attempted to improve the effectiveness of the devitalization and removal of bacterial biofilms.

MATERIALS AND METHODS

Bacterial strains

Staphylococcus saprophyticus subsp. *saprophyticus* 10/1 was isolated from food contact stainless steel surface of meat processing plant I, *Proteus mirabilis* 3/1 was isolated from a traditional meat product produced in meat processing plant II, *Staph. saprophyticus* subsp. *saprophyticus* 17/5 was isolated from the surface of the cheese produced in ewes' milk processing plant I, *Staph. warneri* 18/2 was isolated from cheese, *Stenotrophomonas maltophilia* 2/1 and *Pseudomonas aeruginosa* 2/13 were isolated from plastic surfaces from ewes' milk processing plant II. The biofilm forming ability of the strains has been previously determined [11]. Biochemical identification of the strains based on fermentation tests with extensive numbers of substrates were done in the Czech Collection of Microorganisms, Brno, Czech Republic and were stored freeze-dried. *Staph. aureus* CCM 2022 and *Ps. aeruginosa* CCM 1961 were used as reference strains with a high rate of biofilm formation (both obtained from Czech Collection of Microorganisms).

Formation of the biofilm

A modified version of a previously described method for quantification of biofilm formation in microtitre plates [15, 19, 20] was used. Cultures of individual strains were grown on tryptone soy agar (Merck, Darmstadt, Germany) for 20–24 h at 37 °C. One colony was transferred to 10 ml of tryptone soy broth (TSB; Merck) and incubated for 20–24 h at 37 °C. Then, the culture was centrifuged at 10 000 *g* for 10 min and the sediment was resuspended in TSB to absorbance at 570 nm A_{570} = 0.5, which corresponded to 10^8 CFU·ml^{-1}. Concentration of bacterial cells was determined by plate counting on tryptone soya agar (Merck). A volume of 100 μl of the culture was pipetted in a well of a 96-well polystyrene microtitre plate – colourless for experiments with spectrophotometric reading (Sarstedt, Nümbrecht, Germany) and white for experiments with fluorimetric reading (Nunc, Roskilde, Denmark). The culture was incubated for 18–20 h at 20 °C.

Quantification of the biofilm

Total biomass of the biofilm was quantified using a modified version of a previously described method utilizing staining with crystal violet [21]. The biofilm formed in the well of the microtitre plate was washed a defined number of times with 200 μl of phosphate buffered saline solution (PBS) [22] and dried for 30 min at 37 °C in an inverted position. A volume of 50 μl of a 1% (w/v) solution of crystal violet (Merck) in ethanol was added and incubated for 15 min at 25 °C. The dye solution was aspirated away and the well was washed with 5 × 400 μl of distilled water. After removing water and drying for 10 min at 25 °C, 200 μl of the mixture ethanol : acetone (80 : 20, v/v) were added. The absorbance at 570 nm of the dye solutions was measured in ELx808IU microtitre plate spectrophotometer (BioTek, Winooski, Vermont, USA).

For quantification of the viable biomass of the biofilm, a slightly modified version of a previously described method with fluorescein diacetate [19] was used. The biofilm formed in the well of the microtitre plate was washed a defined number of times with 200 μl of a buffer containing, per liter, 20.9 g 3-(*N*-morpholino)propanesulfonic acid (MOPS; Sigma-Aldrich, Steinheim, Germany) and 5.6 g NaCl. Afterwards, a volume of 100 μl of MOPS buffer and 100 μl of freshly prepared fluorescein diacetate working solution was added. Fluorescein diacetate working solution was prepared by 1 : 50 dilution in MOPS buffer of the stock solution containing 10 mg·ml^{-1} fluorescein diacetate (Sigma-Aldrich) in acetone; the stock solution was stored at –18 °C. The microtitre plate was incubated at 37 °C for 60 min in the dark. Fluorescence was measured in a Genios microtitre plate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength λ_{ex} = 492 nm and an emission wavelength λ_{em} = 520 nm at 10 flashes per measurement, integration time 40 μs, in an optimal gain mode.

Sanitizers

Eight industrial sanitizers were tested, being selected based on the variety of active agents and

on the high frequency of use in food processing enterprises in Slovakia.

1. Ban-Kaz (Banchem, Dunajská Streda, Slovakia) contained benzalkonium chloride (5.0%), didecyl-dimethylammonium chloride (2.25%), hexamethylenediamine hydrochloride (1.5%) and non-ionic surfactants. It was used in a 0.5%, as recommended by the manufacturer, and in a 1.5% solution, respectively.
2. The sanitizer 5P Plus (Banchem) contained benzalkonium chloride (3.8%) and non-ionic surfactants. It was used in a 2.0%, as recommended by the manufacturer, and in a 6.0% solution, respectively.
3. Savo Prim (Bochemie, Praha, Czech Republic) contained sodium hypochlorite (5%) and anionic surfactants. It was used in a 1.0% solution, as recommended by the manufacturer.
4. Xinchlor (Xintex, Prešov, Slovakia) contained sodium hypochlorite. It was used in a 3.0% solution; this concentration was used to be comparable with Savo Prim, although the manufacturer recommended its use at a 17% concentration.
5. Desana-Cid (Thonhauser, Perchtoldsdorf, Austria) contained sulfamic acid (as a source of active oxygene). It was used in a 1.0% solution, as recommended by the manufacturer, and in a 3.0% solution, respectively.
6. Desana-Max (Thonhauser) contained disodium peroxodisulphate and sodium hydroxide. It was used in a 1.0% as recommended by the manufacturer, and in a 3.0% solution, respectively.
7. Forten (Banchem) contained benzalkonium chloride (5%), glutaraldehyde (4.6%), 2-bromo-2-nitro-1,3-propanediol (0.3%) and non-ionic surfactants. It was used in a 1.0% solution, as recommended by the manufacturer.
8. Incidur SP (Ecolab, Düsseldorf, Germany) contained glyoxal (8.8%) and glutaraldehyde (4.5%). It was used in a 0.5% solution, as recommended by the manufacturer.

The solutions of sanitizers were prepared in distilled water. Volumes of 100 μl of solutions were used to treat the biofilm formed in polystyrene microtitre plates at 20 °C during the contact time of 10 min and 30 min, respectively. Additionaly, selected sanitizers were used at an increased temperature (of 45 °C), or with scrubbing using interdental brushes (diameter, 0.8 mm; TePe, Malmö, Sweden), or by immersing the microtitre plate in the solution of the sanitizer in an ultrasonic bath (UC 002 BM1; Tesla, Vráble, Slovakia) operating at 25 kHz at a power of 160 W.

Absorbance or fluorescence were measured in all wells i. e. blank, treated with sanitizers, or treated with destilled water. Each raw absorbance or fluorescence value was adjusted by subtracting the mean of absorbance or fluorescence readings for the blank.

For absorbance or fluorescence of the control, the biofilm was grown in the wells of the plate, then treated with distilled water under the same conditions like wells treated with sanitizers.

A measure of efficacy of sanitizers for removal (crystal violet assay) of biofilm (percentage of remainder) was calculated from the absorbance of the blank (B), control (C) and wells treated with sanitizer (T):

$$\text{Percentage of remainder} = \frac{T-B}{C-B}\times 100 \qquad (1)$$

A measure of efficacy of sanitizers for devitalization (fluorescein diacetate assay) of biofilm (percentage of survival) was calculated from the fluorescence of the blank (B), control (C) and wells treated with sanitizer (T).

$$\text{Percentage of survival} = \frac{T-B}{C-B}\times 100 \qquad (2)$$

Statistical analysis

All measurements were carried out in six replicates, the results were tested for single outliers by Dixon's Q-test [23] and mean values were calculated. One-way ANOVA and Tukey's multiply comparison post test (GraphPad Prism 4.0, GraphPad Software, San Diego, California, USA) were used to compare data. The p-value of < 0.05 were considered significant.

RESULTS AND DISCUSSION

Devitalization and removal of bacterial biofilms in food processing industry is a challenge. One of the reasons is that bacterial cells in the biofilm are much more resistant to antimicrobial agents than cells in a suspension, planktonic cells [12, 14, 15]. In this study, devitalization and removal of bacterial biofilms formed by strains isolated from food processing environments, using a range of sanitizers based on different active components was studied.

First, devitalization of bacterial cells in the biofilm and removal of the biofilm were studied at concentrations recommended by the manufacturers, at room temperature. The devitalization of the biofilm was determined by fluorescein di-

Tab. 1. Percentage of survival of bacterial cells in the biofilm after exposure to sanitizers at concentrations recommended by the manufacturer at 20 °C during the indicated period.

Strain	Fluorescence of the control (log F_{rfu})	Percentage of survival [%]															
		Ban-Kaz 0.5%		5P plus 2%		Savo 1%		Xinchlor 3%		Desana-Cid 1%		Desana-Max 1%		Forten 1%		Incidur 0.5%	
		10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min
Staph. aureus CCM 2022	3.659 ± 0.343	16 ± 4	14 ± 10	28 ± 9	2 ± 2	33 ± 9	3 ± 1	40 ± 7	25 ± 8	47 ± 13	41 ± 14	80 ± 31	40 ± 10	83 ± 18	63 ± 21	49 ± 7	48 ± 12
Staph. saprophyticus 10/1	3.362 ± 0.263	10 ± 3	7 ± 1	9 ± 4	1 ± 1	47 ± 10	7 ± 2	54 ± 13	49 ± 16	22 ± 7	12 ± 3	56 ± 12	33 ± 8	9 ± 2	11 ± 5	25 ± 4	21 ± 13
Staph. saprophyticus 17/5	3.071 ± 0.240	4 ± 3	0 ± 1	25 ± 16	0 ± 1	0 ± 1	2 ± 1	46 ± 22	11 ± 6	8 ± 5	0 ± 1	31 ± 13	20 ± 11	6 ± 4	7 ± 2	34 ± 5	15 ± 7
Staph. warneri 18/2	2.869 ± 0.567	7 ± 3	4 ± 2	20 ± 8	0 ± 1	36 ± 10	7 ± 2	10 ± 2	12 ± 4	8 ± 3	4 ± 2	54 ± 12	54 ± 11	15 ± 4	8 ± 1	2 ± 1	0 ± 0
Sten. maltophilia 2/1	4.523 ± 0.960	76 ± 11	44 ± 8	71 ± 19	69 ± 8	66 ± 4	73 ± 22	84 ± 8	72 ± 11	18 ± 2	14 ± 5	27 ± 3	20 ± 4	63 ± 5	62 ± 8	54 ± 2	50 ± 4
Prot. mirabilis 3/1	2.449 ± 0.364	96 ± 8	80 ± 14	90 ± 3	78 ± 9	36 ± 7	42 ± 4	81 ± 9	89 ± 2	3 ± 1	3 ± 1	32 ± 6	25 ± 2	57 ± 11	57 ± 19	78 ± 18	70 ± 22
Ps. aeruginosa CCM 1961	4.271 ± 0.235	79 ± 22	64 ± 24	76 ± 10	79 ± 6	32 ± 16	25 ± 12	83 ± 46	38 ± 18	12 ± 1	3 ± 1	37 ± 4	28 ± 4	56 ± 9	35 ± 7	19 ± 5	15 ± 4
Ps. aeruginosa 2/13	3.597 ± 0.197	75 ± 17	80 ± 14	80 ± 11	74 ± 7	0 ± 0	0 ± 0	84 ± 11	34 ± 4	0 ± 0	0 ± 0	25 ± 11	29 ± 9	87 ± 14	62 ± 21	45 ± 19	35 ± 15

F_{rfu} – fluorescence at 492/520 nm, output of the fluorescein diacetate assay. Controls were represented by the untreated biofilm. Values represent mean ± standard deviation.

Tab. 2. Percentage of remainder of the bacterial biofilm biomass after exposure to sanitizers at concentrations recommended by the manufacturer at 20 °C during the indicated period.

Strain	A_{570} of the control	Percentage of remainder [%]															
		Ban-Kaz 0.5%		5P plus 2%		Savo 1%		Xinchlor 3%		Desana-Cid 1%		Desana-Max 1%		Forten 1%		Incidur 0.5%	
		10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min
Staph. aureus CCM 2022	0.237 ± 0.085	39 ± 10	61 ± 30	143 ± 66	128 ± 60	124 ± 44	122 ± 63	118 ± 37	108 ± 27	100 ± 14	126 ± 21	22 ± 3	53 ± 8	120 ± 28	166 ± 29	144 ± 12	131 ± 29
Staph. saprophyticus 10/1	0.258 ± 0.083	31 ± 21	65 ± 18	98 ± 14	87 ± 25	63 ± 19	68 ± 12	84 ± 16	88 ± 31	127 ± 19	157 ± 18	78 ± 8	120 ± 22	175 ± 17	170 ± 19	71 ± 27	123 ± 21
Staph. saprophyticus 17/5	0.127 ± 0.036	53 ± 3	69 ± 30	106 ± 21	127 ± 25	62 ± 16	53 ± 26	171 ± 55	68 ± 21	147 ± 21	112 ± 27	50 ± 8	75 ± 13	161 ± 9	125 ± 18	102 ± 37	89 ± 33
Staph. warneri 18/2	0.204 ± 0.046	45 ± 9	66 ± 35	103 ± 23	133 ± 30	55 ± 5	67 ± 19	75 ± 9	65 ± 22	134 ± 13	108 ± 14	56 ± 14	72 ± 8	106 ± 6	136 ± 17	118 ± 21	79 ± 9
Sten. maltophilia 2/1	0.508 ± 0.059	55 ± 8	80 ± 16	69 ± 7	70 ± 2	17 ± 5	26 ± 12	29 ± 12	23 ± 4	9 ± 1	10 ± 2	12 ± 2	8 ± 4	104 ± 8	162 ± 9	50 ± 8	36 ± 6
Prot. mirabilis 3/1	0.213 ± 0.063	63 ± 7	72 ± 8	45 ± 6	76 ± 6	42 ± 21	37 ± 5	76 ± 30	43 ± 2	11 ± 7	13 ± 7	12 ± 2	13 ± 1	201 ± 9	156 ± 5	60 ± 25	72 ± 30
Ps. aeruginosa CCM 1961	1.063 ± 0.079	37 ± 11	40 ± 12	80 ± 9	111 ± 27	208 ± 18	100 ± 29	100 ± 29	199 ± 31	165 ± 25	114 ± 29	43 ± 7	60 ± 6	89 ± 19	85 ± 8	59 ± 19	61 ± 24
Ps. aeruginosa 2/13	0.714 ± 0.065	60 ± 16	60 ± 13	115 ± 11	96 ± 10	51 ± 22	36 ± 21	120 ± 33	82 ± 8	97 ± 10	108 ± 10	63 ± 6	73 ± 6	118 ± 6	96 ± 18	132 ± 42	98 ± 32

A_{570} – absorbance at 570 nm, output of the crystal violet staining assay. Controls were represented by the untreated biofilm. Values represent mean ± standard deviation.

acetate staining, and its removal was determined by crystal violet staining, using previously established methods [24]. Both of these parameters are important as long as the viable biofilm represents an acute safety problem and the devitalized but unremoved biofilm may be a source of nutrients and/or cause an increase in bacterial adhesion and subsequent formation of a new biofilm [3, 5, 10, 15, 25–27]. Experiments were done in polystyrene microtitre plates, taking advantage of the simplicity and precision of measurements in such a format, and supported with the existence of a definite correlation between the biofilm forming properties of bacterial strains on different surfaces [12]. Results are summarized in Tab. 1 and Tab. 2. None of the sanitizers was sufficiently effective with the tested strains, either at the devitalization of the biofilm or at its removal, when the duration of the exposure was 10 min, somewhat higher efficiencies being determined with Gram-negative bacteria. Percentage reductions were specific for individual microorganisms and antimicrobial agents. Disinfectant Desana Cid containing active oxygen was more efficient than disinfectants containing sodium hypochlorite. Similar results with *Listeria* spp. and *P. aeruginosa* biofilms were reported previously [28–30]. In another study [18], sodium chloride removed a significant amount of the *P. aeruginosa* biofilm, but scarcely affected the *S. epidermidis* biofilm. On the other hand, hydrogen peroxide more effectively removed and devitalized *S. epidermidis* biofilm than *P. aeruginosa* biofilm. Highly resistant to hydrogen peroxide were biofilms containing *Burkholderia cenocepacia* complex [15]. When the duration of the exposure was prolonged to 30 min, effectiveness of some sanitizers was slightly enhanced with some strains, but did not change or even decreased with others. The enhancement was more pronounced at biofilms formed by Gram-positive bacteria.

In an attempt to improve the effectiveness of the removal and/or devitalization of the biofilm, selected sanitizers, which had been found effective in previous experiments, were applied at a concentration three times higher than that recommended by the manufacturers. Although enhanced efficiency was observed at these concentrations at devitalization of Gram-positive bacteria, no improvement but often a decrease in the efficiency of the removal of the biofilm biomass was observed (Tab. 3). This can be observed also according to the statistical significance and respective grouping of the tested strains. These results indicate that despite the decrease in viable cells numbers within the biofilm after the treatment with the sanitizer, there was a significant amount of intact cells and/or cellular debris that were still capable of retaining the crystal violet dye.

An increase in the biofilm mass, measured by crystal violet assay, was observed for some strains after the treatment with some sanitizers at higher concentrations (Tab. 3). This was probably only apparent and was caused by the fixation of organic matter to the well surface. Such phenomenon has been reported previously [5, 12, 18, 31] in particular when higher concentrations of benzalkonium chloride led to increased incorporation of crystal violet into biofilm [5, 31].

Further attempts to improve the effectiveness of the removal of the bacterial biofilm were done by applying the sanitizers at an increased temperature (45 °C), with mechanical treatment by scrub-

Tab. 3. Percentage of survival of live bacterial biofilm cells and percentage of remainder of the bacterial biofilm biomass after 10 min of exposure to selected sanitizers at three times increased concentration at 20 °C.

Strain	Percentage of survival based on fluorescence / percentage of remainder based on A_{570} [%]							
	Ban-Kaz 1.5%		5P plus 6%		Desana-Cid 3%		Desana-Max 3%	
	survival	remainder	survival	remainder	survival	remainder	survival	remainder
Staph. aureus CCM 2022	2 ± 0^a	108 ± 29^a	1 ± 0^a	124 ± 18^a	4 ± 1^a	147 ± 10^a	9 ± 3^a	105 ± 26^a
Staph. saprophyticus 10/1	0 ± 0^a	82 ± 8^a	2 ± 0^a	101 ± 15^a	0 ± 0^b	91 ± 13^b	37 ± 9^b	102 ± 29^a
Staph. saprophyticus 17/5	2 ± 0^a	58 ± 9^b	3 ± 2^a	140 ± 23^b	3 ± 1^a	122 ± 11^c	14 ± 2^a	110 ± 18^a
Staph. warneri 18/2	1 ± 0^a	73 ± 27^b	1 ± 0^a	144 ± 15^b	0 ± 0^b	96 ± 15^b	25 ± 8^c	115 ± 13^a
Sten. maltophilia 2/1	30 ± 7^b	90 ± 14^a	10 ± 4^a	88 ± 11^c	4 ± 1^a	48 ± 3^d	15 ± 4^a	53 ± 6^b
Prot. mirabilis 3/1	40 ± 5^c	85 ± 11^a	39 ± 15^b	90 ± 10^c	3 ± 0^a	39 ± 3^d	8 ± 2^a	41 ± 7^b
Ps. aeruginosa CCM 1961	49 ± 9^c	75 ± 13^b	48 ± 13^b	80 ± 9^c	3 ± 1^a	104 ± 9^b	25 ± 10^c	50 ± 10^b
Ps. aeruginosa 2/13	45 ± 8^c	72 ± 12^b	32 ± 14^c	111 ± 10^a	0 ± 0^b	95 ± 6^b	24 ± 5^c	71 ± 7^b

A_{570} – absorbance at 570 nm, output of the crystal violet staining assay. Values represent mean ± standard deviation.
abcd – for each column the same letters represent no significant difference ($p > 0.05$).

Tab. 4. Percentage of remainder of the bacterial biofilm biomass after 10 min of exposure to 0.5% Ban-Kaz at 20 °C and 45 °C, and treatment at 20 °C in ultrasonic bath, with an additional scrubbing, and with additional treatment with 1% Desana-Cid, respectively.

Strain	Percentage of remainder [%]				
	20 °C	45 °C	ultrasonic bath	scrubbing	Desana-Cid
Staph. aureus CCM 2022	39 ± 10	32 ± 10	16 ± 1 ^{A}	18 ± 4 ^{A}	85 ± 13 ^{A}
Staph. saprophyticus 10/1	31 ± 21	20 ± 9	10 ± 4 ^{A}	15 ± 4 ^{A}	48 ± 15
Staph. saprophyticus 17/5	53 ± 3	17 ± 3 ^{A}	3 ± 1 ^{A}	10 ± 4 ^{A}	58 ± 13
Staph. warneri 18/2	45 ± 9	25 ± 6 ^{A}	9 ± 3 ^{A}	10 ± 5 ^{A}	26 ± 9 ^{A}

Values represent mean ± standard deviation. A – for each row A represents significant difference ($p < 0.05$).

Tab. 5. Percentage of remainder of the bacterial biofilm biomass after 10 min of exposure to 1% Desana-Max at 20 °C and 45 °C, and treatment at 20 °C in ultrasonic bath and with an additional scrubbing.

Strain	Percentage of remainder [%]			
	20 °C	45 °C	ultrasonic bath	scrubbing
Sten. maltophilia 2/1	12 ± 2	15 ± 2	0 ± 1 ^{A}	7 ± 5 ^{A}
Prot. mirabilis 3/1	12 ± 2	15 ± 6	2 ± 3 ^{A}	11 ± 6
Ps. aeruginosa CCM 1961	43 ± 7	81 ± 19 ^{A}	1 ± 0 ^{A}	22 ± 9 ^{A}
Ps. aeruginosa 2/13	63 ± 6	65 ± 15	1 ± 1 ^{A}	18 ± 5 ^{A}

Values represent mean ± standard deviation. A – for each row A represents significant difference ($p < 0.05$).

bing or in an ultrasonic bath. The sequential use of two sanitizers was also studied. In these conditions, the benzalkonium chloride-based sanitizer Ban-Kaz was tested with biofilms formed by Gram-positive bacteria, and the peroxide-based sanitizer Desana-Max was tested with biofilms formed by Gram-negative bacteria. Results are summarized in Tab. 4 and Tab. 5. We considered 20 °C standard temperature of the application of sanitizers. With Ban-Kaz, an on average 42% increase in the removal efficiency was observed at an increased temperature, an increase in efficiency of 60–90% was observed when the treatment took place in an ultrasonic bath, and an increase in efficiency of 50–80% was observed when scrubbing was also applied. This observation is similar to results of a previous study when authors found routine cleaning poorly effective, while using mechanical scrubbing or high-pressure spray reduced the biofilm coverage of factory area to less than 1% [7]. The scrubbing step was recommended to be applied to critical sites also by other authors [27, 28]. The sequential treatment with Ban-Kaz and Desana-Cid resulted in a lower efficiency of individual sanitizers at three out of four tested strains of Gram-positive bacteria. This result corresponds with previously published data [4], when authors observed a reduction in anti-biofilm effectiveness of the industrial sanitizer Brillo Degragerm (Brillo, Johnson Wax Professional, Cergy Pointoise, France) containing quaternary ammonium compounds when mixed with a natural biocide thymol. With Desana-Max, no increase in the removal efficiency was observed at an increased temperature. An increase in efficiency of 85–100% was observed when the treatment took place in an ultrasonic bath, and an increase in efficiency of 42–72% was observed when scrubbing was also applied. When devitalization of the biofilm was followed at these conditions, effects proportional to those on the removal of the biofilm were observed (data not shown). However, sequential application of Ban-Kaz and Desana-Cid led to a devitalization of 100% in all tested Gram-positive strains (data not shown).

The observed fact that none of the tested sanitizers was sufficiently effective at the devitalization and removal of bacterial biofilm, when applied without mechanical treatment, underlines the need for performing mechanical cleaning in order to achieve good hygiene in food processing plants.

CONCLUSIONS

None of the tested sanitizers was sufficiently effective with all tested strains, either at the devitalization of the biofilm or at its removal, when used at a concentration recommended by the manufacturer and at duration of the exposure of 10 min. Neither a prolonged exposure of 30 min, nor a three-fold increase in the concentration led to a significant improvement in the efficiency of the sanitizers. The efficiency of a benzalkonium chloride-based sanitizer Ban-Kaz at the removal of the biofilm formed by Gram-positive bacteria was enhanced by on average 42% when applied at an increased temperature of 45 °C. Best results at the removal of bacterial biofilm were achieved applying Ban-Kaz and a peroxide-based sanitizer Desana-Max in an ultrasonic bath or with scrubbing. However, our study of the sanitizing effectivity on biofilms, which represent difficult conditions, does not necessarily negate the data on the effectivness of the sanitizers as obtained by testing according to valid standards [32, 33].

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