

## Inhibition of *Staphylococcus aureus* biofilm by *Lactobacillus* supernatant and plant extracts

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### Summary

*Staphylococcus aureus* is a common food-borne pathogen associated with biofilm formation. Matrix adhesion genes related to the biofilm formation *isdB* and *sdrE* were detected in two strains or in three strains. Genes *hla* and *isdA* were found to be present in all staphylococcal strains. The objective of this study is to compare the stainless steel and microtiter plate surfaces for testing the anti-biofilm activity of cell-free supernatant from lactobacilli and plant extracts in four *Staph. aureus* strains of milk origin. The results showed high biofilm formation of *Staph. aureus* on stainless steel surfaces as well as the anti-biofilm effect of *Lactobacillus* supernatant and plant extracts.

### Keywords

biofilm; *Staphylococcus*; *Lactobacillus*; milk; plant extract; stainless steel

*Staphylococcus aureus* is a common food-borne pathogen associated with biofilm formation and also one of the abundant microbial contaminants in milk processing, cheese production and meat processing factories [1]. Consequently, the need for the development of new strategies and anti-biofilm agents allowing the prevention and control of biofilm formation by various microorganisms is of major importance in order to reduce the risk of contamination in food sector [2].

Main factors associated with biofilm formation are adhesive matrix proteins and the iron uptake system. The initial stage of bacterial adhesion was reported to be reversible because of the weakness of the interactions between bacterial cells and surfaces. More than twenty different microbial surface component recognizing adhesive matrix molecules (MSCRAMM), which can be expressed in *Staph. aureus*, have been identified [3]. Major proteins adhesins in this group include clumping factors A and B (ClfA, ClfB), fibronectin binding proteins A and B (FnBpA, FnBpB), collagen binding protein (Cna), bone sialoprotein binding protein (Bbp), iron regulated surface determinants A and B (IsdA, IsdB) and serine aspartate repeat gene proteins D and E (SdrD, SdrE) [4–7]. *Staph. aureus* also produces cytotoxins and hemo-

lysins ( $\alpha$ ,  $\beta$  and  $\gamma$ ), which possess the ability to form pores in host cells enabling lysis [8, 9]. Lactobacilli and plant extracts as food supplements are promising agents, which are able to control biofilm formation and the growth of pathogens. Moreover, lactobacilli could be used as a natural barrier or competitive exclusion organism in the food processing sector.

In this work, we evaluated the anti-biofilm activity of *Lactobacillus* cell-free supernatant and plant extracts against four *Staph. aureus* strains of milk origin, on stainless steel surface and polystyrene microplates.

## MATERIALS AND METHODS

### Bacterial strains

The following our own cultures were used: *Staphylococcus aureus* No. 11, *Staph. aureus* No. 12, *Staph. aureus* No. 14, all three isolated from ewes' milk; *Staph. aureus* No. 56 isolated from cows' milk; *Lactobacillus plantarum* was isolated from cows' milk. The used *Staph. aureus* strains were selected as strongest biofilm formers. All cultures were identified by matrix-assisted laser desorption/ionization (MALDI) biotyper (Bruker Dal-

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tonik, Leipzig, Germany). *Lactobacillus plantarum* was grown in de Man, Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, United Kingdom), by anaerobic incubation at 37 °C for 24 h. Staphylococci were cultured at 37 °C on Brain Heart Infusion (BHI) agar (Oxoid). Clonal relatedness of staphylococci was measured by principal component analysis (PCA) by MALDI Biotyper software, version 3.0 (Bruker Daltonik) [10].

### Polymerase chain reaction

Determination of the genetic background for virulence of staphylococci was carried out by polymerase chain reaction (PCR) amplification of  $\alpha$ -hemolysin gene *hla* (primers CTG ATT ACT ATC CAA GAA ATT CGA TTG and CTT TCC AGC CTA CTT TTT TAT CAG T), annealing at 58 °C, product of 209 bp [11], serine-aspartate repeat proteins E gene *sdrE* (primers AGA AAG TAT ACT GTA GGA ACT G and GAT GGT TTT GTA GTT ACA TCG T), annealing at 50 °C, product of 433 bp [12], iron-regulated surface determinants A gene *isdA* (primers CTG CGT CAG CTA ATG TAG GA and TGG CTC TTC AGA GAA GTC AC), annealing at 52 °C, product of 332 bp [13] and *isdB* gene (primers ACG AGA GTT TGG TGC GCT AT and GTT GAG GCC CCT ACT TCT GA), annealing at 55 °C, product of 192 bp [14].

### Preparation of *Lactobacillus* supernatants

Preparation of cell-free supernatant was carried out as described previously [15]. The grown culture was centrifuged to remove all cells and then, the supernatant was filtered through a syringe filter of a pore size of 0.22  $\mu$ m (TPP, Trasadingen, Switzerland). To eliminate the effect of lactic acid, pH of the supernatant was neutralized. The final supernatant was stored at 4 °C. Protein concentration of the supernatant was quantified by measuring the absorbance at 280 nm using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

### Biofilm formation on stainless steel surface

Sterile stainless steel coupons (type 304) of 20 mm  $\times$  50 mm, 1 mm thick were used to study biofilm formation. Before carrying the biofilm assay, the stainless steel coupons were cleaned by 70% ethanol and sterilized. Each *Staph. aureus* strain was cultured with coupons individually in BHI broth (Oxoid) for 24 h at 37 °C. After incubation, stainless steel coupons were washed to eliminate unattached cells. To quantify the adhered bacteria, coupons were sonicated (105 W for 10 min) in an ultrasonic bath PSO3000A (Notus-

PowerSonic, Vráble, Slovakia) and counting of the colony-forming units (CFU) was performed using the spiral plater (Eddy Jet, Barcelona, Spain). Surface density of bacterial contamination was calculated in colony forming units per square centimetre.

### Microtiter plate assay

Quantification of the biofilm production was performed using MaxiSorp polystyrene U-bottomed 96-well microtiter plates with a high protein binding capacity and hydrophilic surface (Nunc, Roskilde, Denmark) by a previously published method [16] with slight modifications. In brief, staphylococci were grown on BHI agar (Oxoid), colonies were transferred to BHI broth (Oxoid) to reach the density equivalent to McFarland standard 0.5. Volumes of 200  $\mu$ l of these cell suspensions were transferred to wells of the microplate and incubated statically for 24 h at 37 °C. Following incubation, the content of each well was removed and the wells were washed three times with 250  $\mu$ l of phosphate buffered saline solution (PBS, Thermo Fisher Scientific). Adherent cells were stained with 0.1% crystal violet (Mikrochem, Pezinok, Slovakia) solution for 15 min. Afterwards, excess stain was rinsed off by filling the wells with sterile distilled water. The adhering dye was dissolved with 30% acetic acid. The optical density of wells was measured at 570 nm using Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, Vermont, USA).

### Anti-biofilm activity

The effect of commercially available water extracts of *Allium cepa*, *A. sativum* and *Silybum marianum*, as well as of cell-free *Lactobacillus* supernatant (CFLS) on staphylococcal biofilm formation was tested using sterile stainless steel coupons as the first method. Biofilm formation was assessed by counting the colony forming units as described above.

The second method used polystyrene microtiter plates. The effect of CFLS on the formation of *Staph. aureus* biofilm was tested by addition of different volumes (10  $\mu$ l, 100  $\mu$ l) of CFLS using 96-well MaxiSorp microtiter plates (Nunc). Control wells contained culture medium and the tested strain without adding CFLS. After 24 h of incubation at 37 °C, biofilm was quantified in the same manner as described above. To detect the effects of commercially available water extracts of *A. cepa*, *A. sativum* and *S. marianum* (200 IU·ml<sup>-1</sup>) on staphylococcal biofilm formation, 10  $\mu$ l of extracts were added and the plates were incubated for 24 h at 37 °C. Biofilm was quantified as de-

scribed above. Control wells contained culture medium and the tested strain without adding plant extracts.

### Statistical analysis

All assays were performed in eight replicates and mean as well as standard deviation were calculated. One-way ANOVA and Tukey's test were used to compare data utilizing Statistica 9.0 software (StatSoft, Tulsa, Oklahoma, USA).

## RESULTS AND DISCUSSION

Biofilm formation of staphylococci and anti-biofilm activity of lactobacilli and plant extracts in four *Staph. aureus* strains isolated from cows' and ewes' milk were examined.

In the present study, genes *hla* and *isdA* were found to occur in all staphylococci. The presence of *isdB* gene was detected in two strains and *sdrE* gene was present in three strains from four. The positivity rates of *sdrE* in the present study were higher than in the previous study that showed positivity rates of *sdrD* and *sdrE* in *Staph. aureus* isolates of 48 % and 56 %, respectively [17]. LIU et al. [18] reported positivity rates of *sdrC*, *sdrD* and *sdrE* among *Staph. aureus* isolates 87.8 % (253/288), 63.9 % (184/288) and 68.1 % (196/288), respectively. Results of our study are in agreement with KATEETE et al. [19] and SHUKLA et al. [20] who reported that 100 % isolates had *hla* gene. In contrast, the study performed by RUSENOVA et al. [21] showed that 56.2 % of isolates contained *hla* and *hly*.

Biofilm formation ability of four *Staph. aureus* isolates on stainless steel surface was determined

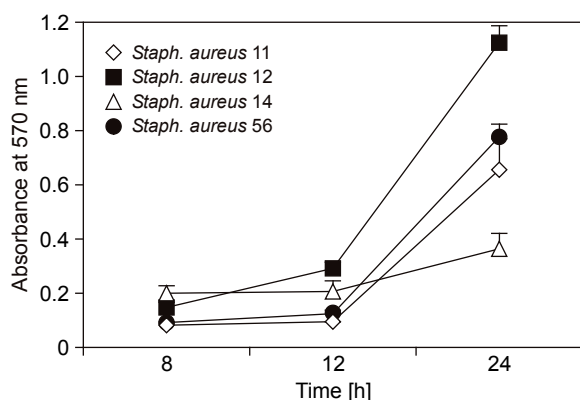
by enumeration of colony forming units per square centimetre carried out on biofilms formed during 24 h. The highest value of  $4.6 \times 10^5$  CFU·cm<sup>-2</sup> was observed for the biofilm of *Staph. aureus* 12, while lower values were recorded for the biofilms of *Staph. aureus* 56, *Staph. aureus* 14 and *Staph. aureus* 11 ( $3.4 \times 10^5$  CFU·cm<sup>-2</sup>,  $2.7 \times 10^5$  CFU·cm<sup>-2</sup> and  $9.5 \times 10^4$  CFU·cm<sup>-2</sup>, respectively).

Since the anti-biofilm activity of plant extracts and CFLS is already known, it was important to test the effect of these compounds on biofilm formation on stainless steel coupons. For that reason, we compared the decreased numbers of cultivable attached bacterial cells on the surfaces of stainless steel. *Staph. aureus* 12 exposed to *A. sativum*, *A. cepa*, *S. marianum* and CFLS reached maximum cell densities of  $7.9 \times 10^4$  CFU·cm<sup>-2</sup>,  $1.5 \times 10^5$  CFU·cm<sup>-2</sup>,  $2.4 \times 10^5$  CFU·cm<sup>-2</sup> and  $3.9 \times 10^5$  CFU·cm<sup>-2</sup>, respectively. The second tested, *Staph. aureus* 56 reached maximum cell densities of  $7.7 \times 10^4$  CFU·cm<sup>-2</sup>,  $1.5 \times 10^5$  CFU·cm<sup>-2</sup>,  $2.5 \times 10^5$  CFU·cm<sup>-2</sup> and  $2.7 \times 10^5$  CFU·cm<sup>-2</sup>, respectively.

The values determined in our study are somewhat higher than those published by KORENOVÁ et al. [1], who reported that staphylococci and enterococci dominated before sanitation on stainless steel at  $10^3$ – $10^4$  CFU·cm<sup>-2</sup> as well as on plastic surfaces at  $10^1$ – $10^3$  CFU·cm<sup>-2</sup> in ewes' milk processing small and medium-sized enterprises. This is in accordance with results of a study by BONSAGLIA et al. [22], where bacteria were found to adhere and form biofilm more effectively on hydrophilic surfaces including stainless steel and glass than on hydrophobic materials such as plastics. Also LEE et al. [23] suggested that *Staph. aureus* cells attach more readily to hydrophilic surfaces than hydrophobic surfaces. The results of microtiter plate assay used for assessment of biofilm-forming ability of four isolates of *Staph. aureus* (11, 12, 14 and 56) are presented in Fig. 1. All the tested strains showed a better growth of the biofilm in last 12 h.

To evaluate the effect of the cell-free *Lactobacillus* supernatant on biofilm formation, four strains of staphylococci were assayed at two different volumes. Our results demonstrate that CFLS in both volumes used in the present study caused significant reduction in biofilm formation of tested isolates ( $p < 0.001$ ). The biofilm was inhibited by 43.1 % and 78.8 % at 10  $\mu$ l and 100  $\mu$ l volumes, respectively. The observed anti-biofilm effect apparently referred to inhibiting adhesion of the bacterial strain to microtiter plate surface.

Tab. 1 shows results of the inhibitory effect of plant extracts on biofilm formation by the studied



**Fig. 1.** Biofilm formation of staphylococci detected by the microtiter plate assay with crystal violet staining.

Mean values  $\pm$  standard deviation are presented.

strains. All extracts caused significant reduction in biofilm formation of the studied strains ( $p < 0.001$ ). In the present study, *A. cepa*, *A. sativum* and *S. marianum* extracts inhibited biofilm formation by 79.9%, 76.7% and 74.3%, respectively. Among the extracts, that from *A. cepa* showed the highest anti-biofilm activity against staphylococci ( $p < 0.001$ ). The strongest inhibition was found for *A. cepa* extract against *Staph. aureus* 12 (90.1%). The differences in the inhibitory effect of the set of plant extracts on staphylococci were not significant ( $p > 0.05$ ).

The efficiency of *A. sativum* extracts in preventing biofilm formation were reported previously [24]. According to the mean of the inhibitory effects of *A. sativum* extract at the selected concentrations, it had an ability to inhibit 50% of biofilm formation in the tested bacteria. The highest inhibition of biofilm formation was observed against *S. pneumoniae* (88.5%) and *B. cereus* (88.6%) and the lowest inhibition was observed for biofilm formation of *Staph. aureus* (52.8%). To investigate the effect of *A. sativum* extract on biofilms, Wu et al. [25] applied live/dead differential staining and could show that the decrease in biofilm formation was accompanied by a strong bactericidal effect toward biofilm-embedded bacteria, with a 100% loss of viability observed already at a concentration of  $3.13 \mu\text{g}\cdot\text{ml}^{-1}$  allicin. EVREN et al. [26] showed anti-biofilm effects of silymarin extract from *S. marianum* against *Staph. epidermidis*, *Staph. aureus* and methicillin-resistant *Staph. aureus*. Biofilm formation was decreased at  $15 \mu\text{g}\cdot\text{ml}^{-1}$  silymarin concentration, when compared with silymarin-untreated group. Silymarin reduced the biofilm viability to 13% and 46% at  $1 \text{ mmol}\cdot\text{l}^{-1}$  and  $0.5 \text{ mmol}\cdot\text{l}^{-1}$  concentrations, respectively. Flavonolignans from *S. marianum*, specifically silybin and isosilybin, showed weak inhibition of biofilm formation [27]. Many reports describe antimicrobial effect of *A. cepa*, but this is the first report describing its inhibition effect on biofilm formation of *Staph. aureus*.

A comparison of two methods for detection of anti-biofilm activity against *Staph. aureus* No. 12

**Tab. 1.** Anti-biofilm activity of plant extracts.

Strain	Biofilm inhibition [%]		
	<i>Allium cepa</i>	<i>Allium sativum</i>	<i>Silybum marianum</i>
<i>Staph. aureus</i> 11	70.3	61.1	63.2
<i>Staph. aureus</i> 12	90.1	78.6	78.3
<i>Staph. aureus</i> 14	88.6	83.7	66.7
<i>Staph. aureus</i> 56	70.5	83.2	89.1
Mean $\pm$ SD ( $n = 4$ )	$79.9 \pm 11.0$	$76.7 \pm 10.6$	$74.3 \pm 11.8$

Anti-biofilm activity was detected by the microtiter plate assay with crystal violet staining.  
SD – standard deviation.

and No. 56 is presented in Tab. 2. Both methods showed a significant reduction in biofilm formation of tested isolates. However, it is important to note that the stainless steel assay quantified only living cells, while the microtiter plate assay measured the absorbance of bound crystal violet, which depends on the total mass of the biofilm. Therefore, values determined using stainless steel surface could be lower than those observed on microtiter plates.

## CONCLUSIONS

The development of concepts and the discovery of novel agents against staphylococcal biofilm are promising issues in food safety. MSCRAMM are important virulence factors associated with biofilm production in *Staph. aureus* of milk origin. Cell-free *Lb. plantarum* supernatant and plant extracts reduced *Staph. aureus* biofilm formation under subinhibitory conditions. Anti-biofilm properties of *Lb. plantarum* supernatant against four biofilm producers suggest its potential applicability as an anti-biofilm agent in the food sector.

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**Tab. 2.** Anti-biofilm activity of the agents determined by two methods on different surfaces.

Strain	Biofilm inhibition [%]							
	Stainless steel				Polystyrene			
	<i>Allium cepa</i>	<i>Allium sativum</i>	<i>Silybum marianum</i>	CFLS	<i>Allium cepa</i>	<i>Allium sativum</i>	<i>Silybum marianum</i>	CFLS
<i>Staph. aureus</i> 12	67.4	82.8	47.8	15.2	90.1	78.6	78.3	81
<i>Staph. aureus</i> 56	55.9	77.4	26.5	20.6	70.5	83.2	89.1	39

CFLS – cell-free *Lactobacillus* supernatant with a protein concentration of  $13.01 \text{ mg}\cdot\text{ml}^{-1}$ .



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