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# Subtilosin Prevents Biofilm Formation by Inhibiting Bacterial Quorum Sensing

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**Abstract** Subtilosin, the cyclic lantibiotic protein produced by *Bacillus subtilis* KATMIRA1933, targets the surface receptor and electrostatically binds to the bacterial cell membrane. In this study, subtilosin was purified using ammonium sulfate ($(NH_4)_2SO_4$) precipitation and purified via column chromatography. Subtilosin's antibacterial minimum and sub-minimum inhibitory concentrations (MIC and sub-MIC) and anti-biofilm activity (biofilm prevention) were established. Subtilosin was evaluated as a quorum sensing (QS) inhibitor in Gram-positive bacteria using Fe(III) reduction assay. In Gram-negative bacteria, subtilosin was evaluated as a QS inhibitor utilizing *Chromobacterium voilaceum* as a microbial reporter. The results showed that *Gardnerella vaginalis* was more sensitive to subtilosin with MIC of 6.25 μg/mL when compared to *Listeria monocytogenes* (125 μg/mL). The lowest concentration of subtilosin, at which more than 90% of *G. vaginalis* biofilm was inhibited without effecting the growth of planktonic cells, was 0.78 μg/mL. About 80% of *L. monocytogenes* and more than 60% of *Escherichia coli* biofilm was inhibited when 15.1 μg/mL of subtilosin was applied. Subtilosin with 7.8–125 μg/mL showed a significant reduction in violacein production without any inhibitory effect on the growth of *C. violaceum*. Subtilosin at 3 and 4 μg/mL reduced the level of Autoinducer-2 (AI-2) production in *G. vaginalis*. However, subtilosin did not influence AI-2 production by *L. monocytogenes* at sub-MICs of 0.95–15.1 μg/mL. To our knowledge, this is the first report exploring the relationship between biofilm prevention and quorum sensing inhibition in *G. vaginalis* using subtilosin as a quorum sensing inhibitor.

**Keywords** Quorum sensing · Biofilm inhibition · Subtilosin · *Gardnerella vaginalis*

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## Introduction

Subtilosin A, a cyclic lantibiotic protein produced by *Bacillus subtilis*, was firstly isolated by Babasaki et al. [1]. Subtilosin is distinctively different from other bacteriocins that have a net cationic charge. The overall anionic properties guided researchers to a possible suggestion that subtilosin mainly targets surface receptors rather than electrostatic binding to bacterial cell membrane. The sulfide bridges may hold this binding in a conformation to target these receptors [2]. In addition to antimicrobial potential of subtilosin against *Listeria monocytogenes* and *Gardnerella vaginalis*, its biofilm bactericidal activity alone and in combination with natural antimicrobials against *G.*

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*vaginalis* was recently reported [3–5]. Since many studies noticed that some cyclic peptides [6, 7] mediated biofilm formation by downregulation of quorum sensing (QS)-associated gene expression, we hypothesized that subtilosin similarly inhibits QS signal production in bacterial cells.

In this study, subtilosin was evaluated as a QS inhibitor in Gram-positive, Gram-negative, and Gram-variable bacteria. *Chromobacterium violaceum* was used as a microbial reporter for QS inhibition in Gram-negative bacteria [8], while Fe(III) reduction method was utilized for Gram-positive microorganisms [9]. By conducting quantitative analyses to measure the quorum sensing effects of subtilosin on bacteria could lead to improved efficacy of the antimicrobial and implementation as an alternative to antibiotics.

## Materials and Methods

### Bacterial Strains and Growth Conditions

*L. monocytogenes* ScottA, *Escherichia coli* O157:H7, and *G. vaginalis* ATCC 14018 strains were utilized in this study as representatives of Gram-positive, Gram-negative, and Gram-variable bacteria. These strains were grown in brain-heart infusion (BHI) medium (Difco, Sparks, MD) supplemented with 3% (*v*/*v*) of horse serum (sBHI) (JRH Biosciences, KS) and incubated according to their requirements, aerobically for *L. monocytogenes* and *E. coli* and anaerobically (10% $H_2$, 5% $CO_2$, and 85% $N_2$) for *G. vaginalis*, using the anaerobic glove box (Coy Laboratory Products, Inc., Grass Lake, MI). BHI medium supplemented with 1% glucose (*w*/*v*) (BHIG) was used for biofilm inhibition assay. *C. violaceum* ATCC 12472 was grown in Luria-Bertani (LB) broth (ACROS, Miller, NJ) at 26 °C for 48 h aerobically. *Pseudomonas aeruginosa* ATCC 14213 was aerobically grown in LB broth at 37 °C for 24 h and used as positive control for QS inhibition in Gram-negative bacteria.

### Chemical and Antimicrobial Compounds

Subtilosin was isolated and purified according to Sutyak Noll et al. [10]. For auto-inducing peptide, the AI-2 detection in Gram-positive/variable bacteria, a working solution of Fe(III) was prepared following instructions reported by Wattanavanitchakorn et al. [9]. To prepare a 10 mM 1,10-phenanthroline/3.32 mM Fe(III) solution, 0.198 g of 1,10-phenanthroline was dissolved in 50 mL of distilled water (DW) and adjusted to pH 2 using 1 M HCl, followed by the addition of 0.16-g ferric ammonium sulfate. The final volume of the solution was completed to 100 mL with the addition of DW.

### Subtilosin Isolation

Previously described procedure [10] was followed with minor modifications. Briefly, *B. subtilis* KATMIRA1933 was inoculated in de Man, Rogosa and Sharpe (MRS) [11] broth (Difco BD, Franklin Lakes, NJ) and incubated aerobically for 24 h at 37 °C. After incubation, the cell-free supernatant was saturated with ($(NH_4)_2SO_4$) at 4 °C for 24 h, followed by subtilosin precipitation with 95% (*v*/*v*) methanol. The protein of interest was separated based on its assumed hydrophobic nature by column chromatography using Sep-Pak® Light C18 Cartridges (Waters, Milford, MA). The methanol from the fractions was removed using a Savant SC110 Speed Vac with UVS400 Universal Vacuum System (Savant Instruments, Farmingdale, NY). The protein concentration in the sample was quantified using a protein kit (Micro BCA Protein Assay Kit; Thermo Scientific, Rockford, IL) and was evaluated to be 6 mg/mL.

### Determination of Minimum Inhibitory Concentration

To determine the MIC of subtilosin against tested pathogens, broth micro-dilution assay was performed following Algburi et al. [5]. Briefly, the overnight cultures were diluted 1:1000 (*v*/*v*) into fresh BHI broth supplemented with 3% (*v*/*v*) horse serum (sBHI) to achieve approximately $10^6$ CFU/mL (the number of bacterial cells was confirmed by spot-plate method [4]). Subtilosin from the stock solution was serially diluted with sBHI in triplicates using a 96-well non-tissue culture microplate (Falcon, Corning Inc., Corning, NY) with 100 μL as the final volume. A 100-μL aliquot of the prepared bacterial suspension ($10^6$ CFU/mL) was added to each well in the 96-well microplate-treated with different concentrations of subtilosin. After incubation in the microplate reader for 24 h, a statistical analysis of the kinetic readings of bacterial growth in the microplate was performed in order to determine the MICs and sub-MICs of subtilosin.

### Biofilm Inhibition Assay

Biofilm inhibition assay was performed following Toole [12]. An overnight-grown culture of microorganisms were diluted into sBHI to achieve approximately $10^6$ CFU/mL. Subtilosin was twofold diluted with sBHI (for *E. coli* and *L. monocytogenes*) or with sBHIG (for *G. vaginalis*) in the 96-well tissue culture microplates (Falcon, Corning Inc., Corning, NY). A 100-μL aliquot of the bacterial suspension ($10^6$ CFU/mL) was added and mixed with the pre-determined dilutions of subtilosin. The microplate was incubated for 24 h at 37 °C. After incubation, the unattached cells were counted using the spot-plate

method. To prepare the biofilm mass for quantification, each well was washed with fresh broth to remove the remaining unattached cells. After washing, the biofilm's biomass was quantified according to Borucki et al. [13] with minor modifications. Once the intact biofilm was fixed using an incubator (New Brunswick Scientific Co., Inc., NJ) at 60 °C for 60 min in an inverted position, each well in the microplate was stained with 125 μL of 0.1% (*w*/*v*) crystal violet (CV). After the allotted incubation time of 15–20 min at room temperature, each well was rinsed three to four times with 200 μL of DW. Two hundred microliters of 95% (*v*/*v*) ethanol was added to solubilize the CV, and the micro-titer plate was incubated at 4 °C for 30 min. After incubation, 100 μL of solubilized CV were transferred to a flat 96-well microplate to quantify the absorbance of each sample using plate reader at 595 nm (Model 550, Bio-Rad Laboratories, Hercules, CA).

### Quorum Sensing Inhibition Assay in Gram-Negative Bacteria

This assay was performed according to Zhu et al. [14] with minor modifications. Briefly, the overnight-grown cells of *C. violaceum* were diluted in fresh LB broth to achieve $10^6$ CFU/mL. Subtilosin was serially twofold diluted with LB into a 48-well microplate (BD, Franklin Lakes, NJ). A bacterial suspension (500 μL) ($10^6$ CFU/mL) was mixed with 500 μL of pre-determined dilutions of subtilosin. Once the samples were prepared, the plate was aerobically incubated at 26 °C without shaking for 36–48 h. The cell-free supernatant (CFS) of *P. aeruginosa* grown in LB was used as a control, preventing violacein production by *C. violaceum*. After incubation, 750 μL from each well (test and control wells) was transferred to a 1-mL centrifuge tube and centrifuged at 8000*g* for 5 min in order to collect violacein and the producer cells. The supernatants were discarded and the pellets were vigorously vortexed with 750 μL of 100% DMSO to dissolve the insoluble violacein. The samples were centrifuged again at 8000*g* for 5 min in order to precipitate *C. violaceum* cells. To evaluate violacein production, 200 μL of violacein-containing supernatants was added into a 96-well microplate (Falcon, Corning Inc., Corning, NY) in triplicate and the $OD_{585}$ was measured using the plate reader (Model 550, Bio-Rad Laboratories, Hercules, CA). To ensure that the QS inhibition occurred without killing the targeted microorganisms by the subtilosin's sub-MICs, the precipitated bacterial cells were re-suspended in 750 μL of DW (pH 7.0) and the absorbance was measured at $OD_{595}$. The ODs of cells treated with sub-MICs of subtilosin were compared against the non-treated cells (positive control).

### Quroum Sensing Inhibition Assay in Gram-Positive Bacteria

This assay was performed using the methods described by Wattanavanitchakorn et al. [9] with minor modifications. The bacterial species, including *G. vaginalis* (tested microorganism), *S. aureus*, *L. monocytogenes* (controls, AI-$2^+$), and *E. coli* (negative controls, AI-$2^-$) were used in this study. The bacterial strains were inoculated into sBHI and incubated for 24 h at 37 °C according to their individual growth requirements. After incubation, bacterial suspensions were diluted in sBHI broth to achieve $10^6$ CFU/mL. The AI-2 production was measured after 0, 2, 4, 6, 8, 11, and 24 h of incubation in order to determine the time point at which the highest QS signals are produced. Subtilosin at its sub-MICs was prepared in sBHI and mixed with $10^6$ CFU/mL of each bacterial strain. The prepared mixtures were incubated for pre-determined time intervals at which the highest amount of AI-2 is produced. After incubation, tested strains were centrifuged (8000*g* for 5 min), 1 mL of their CFS were mixed with 1 mL of working solution (mentioned above) and left at room temperature for 15 min. After incubation, the volumes of mixtures were adjusted to 5 mL with the addition of DW and were centrifuged (8000*g* for 5 min). Aliquots of 200 μL were transferred to 96-well microplates (Falcon, Corning Inc., NY) and the absorbance was measured at $OD_{510}$ using a microplate reader. The absorbance of subtilosin-treated samples was compared against the non-treated control in order to evaluate the QS inhibition.

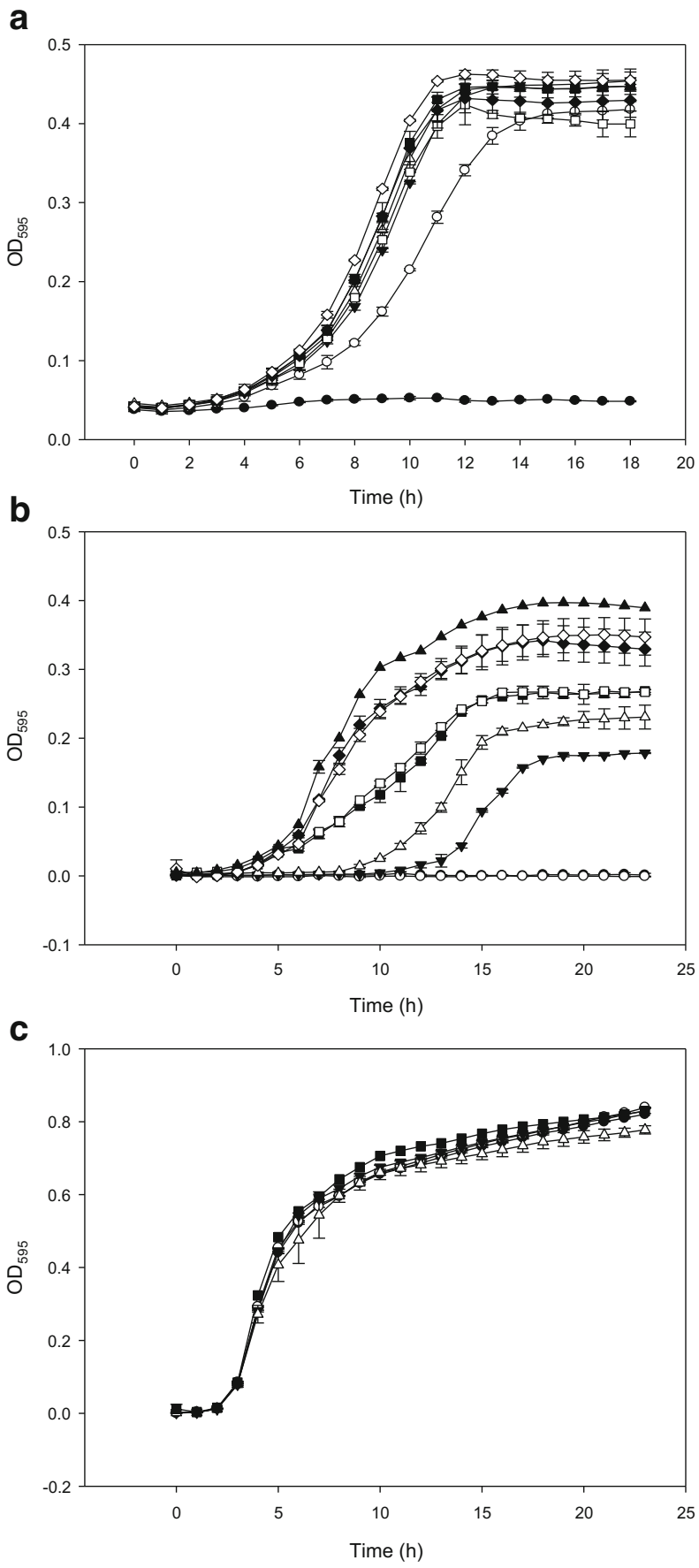
### Statistical Analysis

Each experiment was conducted at least three times in duplicate. The standard deviation in the figures is represented by error bars. All the calculations were performed in Microsoft Excel and then the statistical analysis was shaped with SigmaPlot 11.0 (Systat Software Inc., Chicago, IL).

## Results

### Determination of MIC and Sub-MICs of Subtilosin

Broth micro-dilution method was used to determine the MIC and sub-MICs of subtilosin against three tested bacterial strains. The results illustrated that *G. vaginalis* was more sensitive to subtilosin with MIC of 6.25 μg/mL (Fig. 1a) as compared to *L. monocytogenes* (125 μg/mL) (Fig. 1b). The subtilosin concentration as high as 250 μg/mL was insufficient in inhibiting growth of *E. coli* cells (Fig. 1c). The sub-MICs of subtilosin against tested pathogenic strains were also identified in order to be utilized in the biofilm inhibition assay.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◀ **Fig. 1** **a** Antimicrobial activity of subtilosin against *G. vaginalis* growth. Subtilosin concentrations are as follows: 6.25 μg/mL (*closed circles*), 3.12 μg/mL (*open circles*), 1.56 μg/mL (*closed reverse triangles*), 0.78 μg/mL (*increments*), 0.39 μg/mL (*closed squares*), 0.19 μg/mL (*open squares*), 0.097 μg/mL (*closed diamonds*), 0 μg/mL (*lozenges*). **b** Antimicrobial activity of subtilosin against *L. monocytogenes* growth. Subtilosin concentrations are as follows: 250 μg/mL (*closed circles*), 125 μg/mL (*open circles*), 62.5 μg/mL (*closed reverse triangles*), 31.25 μg/mL (*increments*), 15.6 μg/mL (*closed squares*), 7.8 μg/mL (*open squares*), 3.9 μg/mL (*closed diamonds*), 1.9 μg/mL (*lozenges*), 0 μg/mL (*closed triangles*). **c** Antimicrobial activity of subtilosin against *E. coli* growth. Subtilosin concentrations are as follows: 250 μg/mL (*closed circles*), 125 μg/mL (*open circles*), 62.5 μg/mL (*closed reverse triangles*), 31.25 μg/mL (*increments*), 0 μg/mL (*black squares*)

### Biofilm Inhibition

In this assay, we determined biofilm integrity percent of each bacterial strains when the sub-MICs of subtilosin were applied. For *G. vaginalis*, the tested concentrations of subtilosin ranged from 3.31 to 0.19 μg/mL, and for both *L. monocytogenes* and *E. coli*, they ranged from 15.1 to 0.95 μg/mL (Fig. 2a–c). The lowest concentration of subtilosin, at which more than 90% of *G. vaginalis* biofilm was inhibited without impacting the growth of planktonic cells, was 0.78 μg/mL. In addition, about 80% and more than 60% of *L. monocytogenes* and *E. coli* biofilm, respectively, were inhibited when 15.1 μg/mL (sub-MIC) of subtilosin was applied without any effect on bacterial growth in planktonic population.

### Quorum Sensing Inhibition

#### *Inhibition of Violacein Production by Subtilosin*

Inhibition of violacein production of *C. violaceum*, the bacterial reporter, is used as an indicator of quorum sensing inhibition in Gram-negative bacteria. Subtilosin at sub-MICs ranged from 7.8 to 125 μg/mL showed significant reduction in violacein production by *C. violaceum* without any inhibition of bacterial growth (Fig. 3). For *E. coli*, 15.1 μg/mL of subtilosin was enough to prevent about 60% of biofilm formation and inhibited 85% of violacein production as compared to the control. These data suggest that quorum sensing was probably inhibited by subtilosin.

#### *Inhibition of AI-2 Production in Gram-Positive and Gram-Variable Bacteria*

The AI-2 production by *G. vaginalis* in the presence of subtilosin was evaluated. The sub-MICs of subtilosin (3 and 4 μg/mL) showed strong inhibition of AI-2 production in *G. vaginalis* compared to the positive control (untreated bacteria) (Fig. 4a). These concentrations did not affect the bacterial growth.

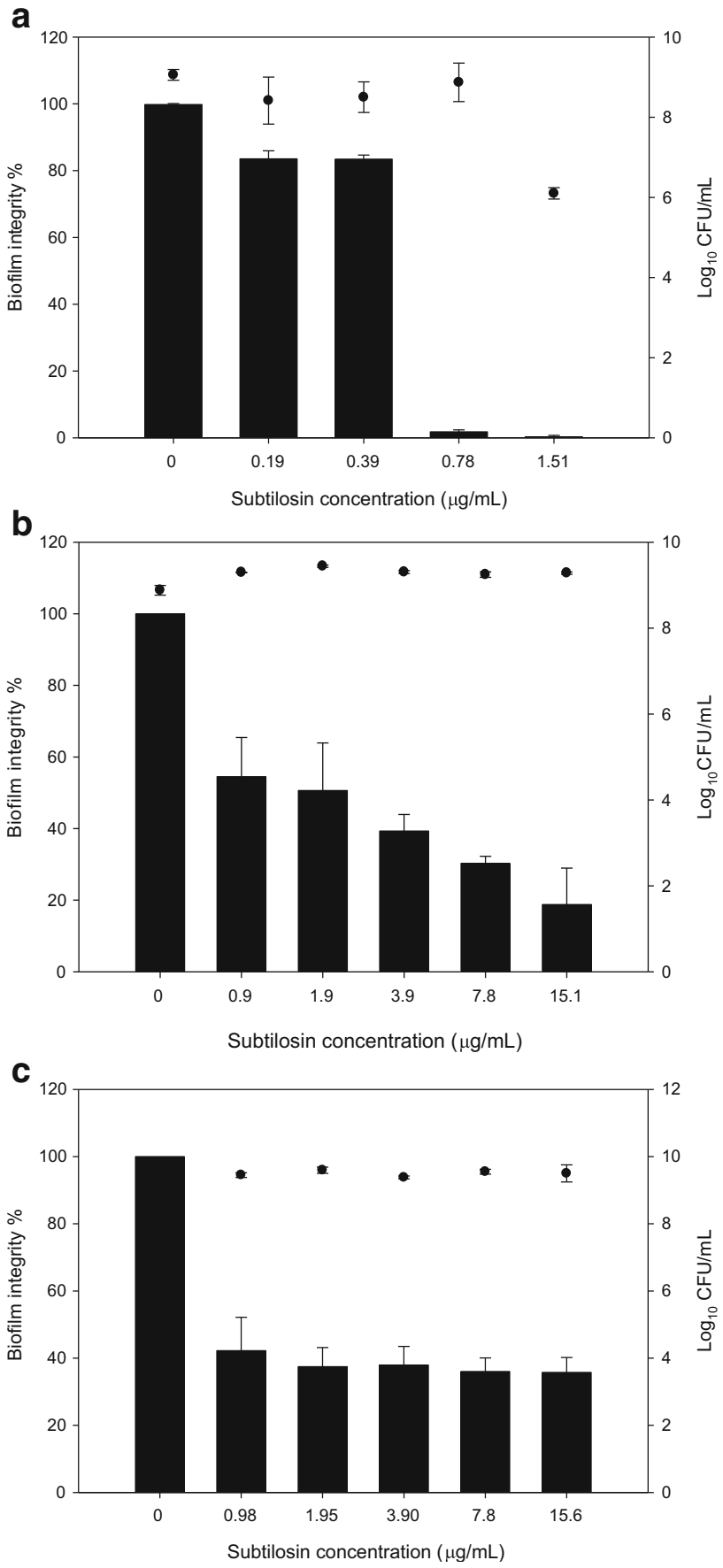
The effect of subtilosin on AI-2 production in *L. monocytogenes* was different from what we observed in *G. vaginalis*. Subtilosin at sub-MICs 0.95–15.1 μg/mL did not influence the AI-2 production by *L. monocytogenes* (Fig. 4b). In addition, the AI-2 production correlated with bacterial growth.

## Discussion

Emergence of antibiotic resistance and infection recurrence are urging the need for finding alternative antimicrobials instead of the commonly used and prescribed antibiotics. Antimicrobial proteins (AMPs) have been proposed as an alternative targeting the antibiotic-resistant pathogens [15–17]. AMPs are naturally produced by eukaryotes and prokaryotes [18] attacking pathogens either by pore-formation mechanism or perturbation of bacterial cellular membrane [19]. Some studies referred to the anti-biofilm activity of AMPs which are preventing and/or eradicating biofilm by binding to extra-cellular DNA and facilitating biofilm detachment [20, 21] or by down-regulating the genetic expression of quorum sensing system, rhamnolipids synthesis, and type IV pili formation [22, 23]. Subtilosin, the cyclic peptide produced by *B. subtilus* KATMIRA1933, showed antibacterial and anti-biofilm activity against pathogens such as *L. monocytogenes* and *G. vaginalis* [3, 4]. The safety of subtilosin to the human tissues [24, 25] and the selectivity of its bactericidal potential [4] attracted our attention to study its anti-biofilm potential as a QS inhibitor.

In this study, we identified MIC of subtilosin against tested *L. monocytogenes* and *G. vaginalis* at 125 and 6.25 μg/mL, respectively. The MIC of subtilosin against *L. monocytogenes* was reported by van Kuijk et al. [3] to be 19.56 and 7.21–9.22 μg/mL against *G. vaginalis* by Turovskiy et al. [4] and Sutyak Noll et al. [26]. The difference of subtilosin's MIC values among other studies is possibly because of the variability of antimicrobial's preparations, experimental designs, and strategies used in each study.

In order to identify quorum sensing inhibitory effect of subtilosin, the sub-MICs were determined and used as concentrations that did not inhibit the growth activity of planktonic cells. Interestingly, we found that sub-MICs of subtilosin which were required to inhibit biofilm formation of tested pathogenic bacteria were much lower than its MICs. In addition to avoid bacterial mutations which lead to antibiotic resistance, using sub-MICs of antimicrobials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Biofilm integrity %
Log10 CFU/mL
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Biofilm integrity %
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Subtilosin concentration (μg/mL)

◀ **Fig. 2** **a** Inhibition of *G. vaginalis* biofilm by subtilosin. Biofilm integrity % (*bars*), $Log_{10}$ CFU/mL (*closed circles*). **b** Inhibition of *L. monocytogenes* biofilm by subtilosin. Biofilm integrity % (*bars*), $Log_{10}$ CFU/mL (*closed circles*). **c** Inhibition of *E. coli* biofilm by subtilosin. Biofilm integrity % (*bars*), $Log_{10}$ CFU/mL (*closed circles*)

may assist with increasing of the viability of the eukaryotic host's beneficial microbiota.

The antimicrobial action of bacteriocins, such as nisin, against pathogenic bacteria is associated with membrane perturbation after their interaction with bacterial receptors [27]. Subtilosin has been noticed to deplete the transmembrane pH gradient (pH), disrupting the portion of the proton motive forces (PMF) and then killing the targeted cells due to the leakage of intracellular ATP, essential ions, and metabolites in *G. vaginalis* [28]. However, subtilosin has been reported to have a species-specific mode of action against *L. monocytogenes* [3] which is different from what Noll et al. [28] had reported. Van Kuijk et al. [3] noticed that subtilosin has a slight effect on the transmembrane electrical potential ($\Delta\Psi$) and pH gradient ($\Delta$pH), but no significant effect on the efflux of intracellular ATP in *L. monocytogenes*. In the same study, subtilosin was found to embed itself in the phospholipid bilayer of the cellular membrane, causing intracellular damage and killing of bacterial cells.

The sub-MIC of subtilosin is not enough to kill or inhibit the growth of pathogens but it possibly downregulates some of their chemo-physiological activities such as quorum sensing systems. Disruption of PMF has been reported to inhibit quorum sensing in the pathogenic *E. coli* cells [29]. Since subtilosin has been reported as proton pump inhibitor in *G. vaginalis* [28], it is possible for subtilosin to interfere with PMF in *E. coli* which may lead to inhibition of quorum sensing system without killing the bacterial cells. However, we have noticed that subtilosin did not influence the level of AI-2 production by *L. monocytogenes*. Several studies referred to the inconsistency regarding *luxS* and AI-2 effect on biofilm formation [30–32]. Some investigators showed that mutation in *luxS* gene increases both the mass and thickness of biofilm more than the wild strains [33–35]. In agreement with these findings, the data of Sela et al. [36] indicated the role of *luxS* gene in repression of biofilm formation by *L. monocytogenes*; the more LuxS and AI-2 are produced, the more biofilm formation is prevented/repressed. In addition, it has been reported that bacterial cells have species-specific AI-2 [37]. Based on our data, we speculate that perhaps, subtilosin inhibits cell-to-cell communication signals, "the competitor's intracellular communication" rather than *L. monocytogenes* specific AI-2, and thus inhibits biofilm formation. In the same regard, Pearson et al. [38] found that inhibition of proton efflux pumps led to the accumulation of intracellular autoinducers which possibly interfere with the biofilm formation of bacterial cells by downregulation of quorum sensing genes. The perturbation of the lipid bilayer of bacterial membrane by sub-MICs of subtilosin [3] may also inhibit the attachment of bacterial cells, the first step of biofilm development. The antimicrobial properties of subtilosin including its anionic charge, selectivity, and synergistically acting feature alongside its safety should attract the attentions of researchers to study this bacteriocin as alternative antimicrobials in food and pharmaceutical applications.

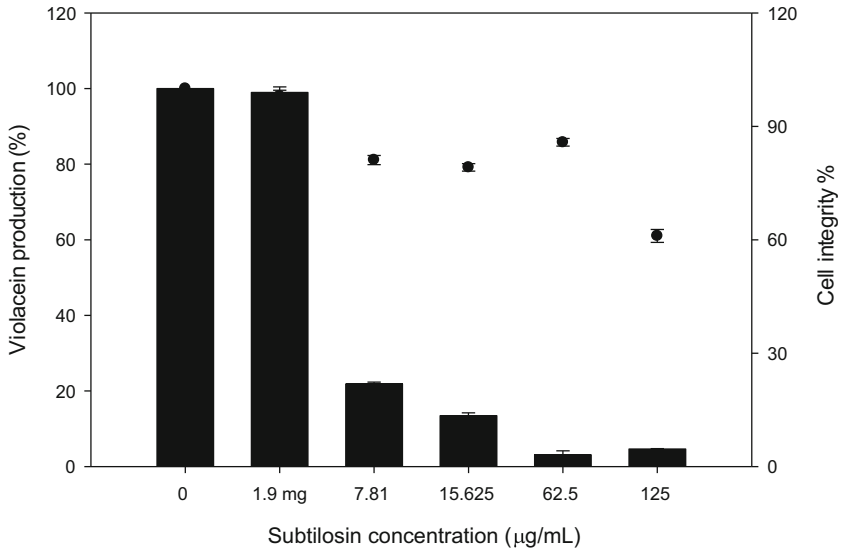


**Fig. 3** Inhibition of violacein 120 production by subtilosin. Violacein production % (*bars*) and cell integrity % (*closed circles*)

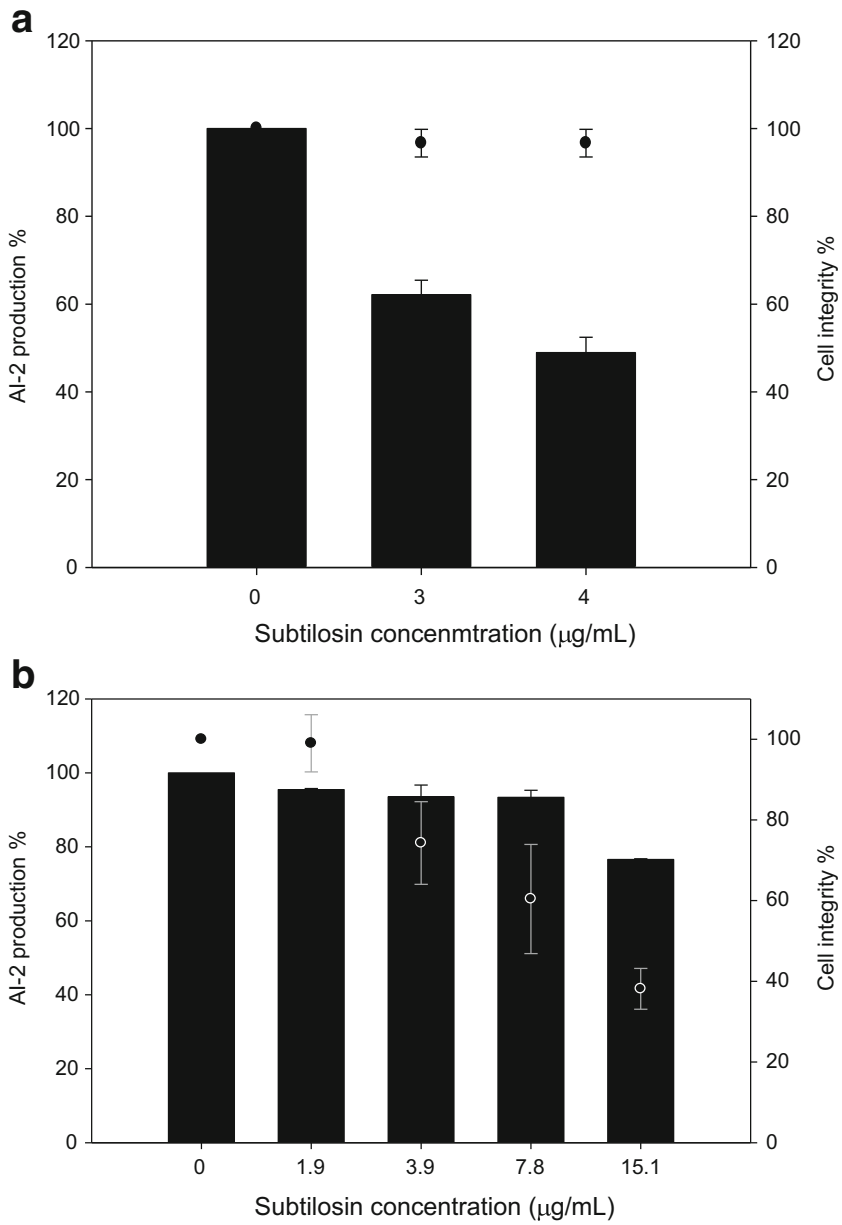


**Fig. 4** **a** Inhibition of AI-2 production in *G. vaginalis* by subtilosin. AI-2 production % (*bars*) and cell integrity % (*closed circles*). **b** Effect of subtilosin on AI-2 production by *L. monocytogenes*. AI-2 production % (*bars*) and cell integrity % (*closed circles*)

## Conclusion

Biofilm control is still a big challenge urging the need to find a reasonable method for avoiding infection recurrence and antibiotic resistance. Prevention of biofilm formation, which is a more advantageous strategy than biofilm killing, is achieved either by stopping cell-cell communication (quorum sensing inhibition) or inhibiting cell-cell/cell-surface attachment. Advantages of biofilm prevention include using lower doses of antimicrobials which is ultimately minimizing the cost, reducing the bacterial mutation frequency, and enhancing the viability of beneficial microbes. The role of quorum sensing inhibition in microbial biofilm prevention was reported in several studies. To our knowledge, this is the first study showing the relationship between inhibiting quorum sensing and biofilm prevention using subtilosin which is reported as a QS inhibitor in Gram-positive, variable, and Gram-negative bacteria inhibiting their biofilm formation. Our data suggests the use of subtilosin as an anti-biofilm agent in future food and medical applications to avoid persistent infections—associated with biofilm.

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**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no conflict of interest.

## References


1. Babasaki K, Takao T, Shimonishi Y et al (1985) Subtilosin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: isolation, structural analysis, and biogenesis. J Biochem 98: 585–603
2. Kawulka KE, Sprules T, Diaper CM et al (2004) Structure of subtilosin A, a cyclic antimicrobial peptide from *Bacillus subtilis* with unusual sulfur to α-carbon cross-links: formation and reduction of α-thio-α-amino acid derivatives. Biochemist 43:3385–3395. doi:10.1021/bi0359527
3. Van Kuijk S, Noll KS, Chikindas ML (2012) The species-specific mode of action of the antimicrobial peptide subtilosin against *Listeria monocytogenes* Scott A. Lett Appl Microbiol 54:52–58. doi:10.1111/j.1472-765X.2011.03170.x
4. Turovskiy Y, Cheryian T, Algburi A et al (2012) Susceptibility of *Gardnerella vaginalis* biofilms to natural antimicrobials subtilosin, ε-poly-L-lysine, and lauramide arginine ethyl ester. Infect Dis Obstet Gynecol 2012:284762. doi:10.1155/2012/284762
5. Algburi A, Volski A, Chikindas ML (2015) Natural antimicrobials subtilosin and lauramide arginine ethyl ester synergize with conventional antibiotics clindamycin and metronidazole against biofilms of *Gardnerella vaginalis* but not against biofilms of healthy vaginal lactobacilli. Pathog Dis 73(5) doi: 10.1093/femspd/ftv018
6. Ryan RP, Dow JM (2008) Diffusible signals and interspecies communication in bacteria. Microbiol 154:1845–1858. doi:10.1099/mic.0.2008/017871-0
7. Li J, Wang W, Xu SX et al (2011) *Lactobacillus reuteri*-produced cyclic dipeptides quenchagr-mediated expression of toxic shock syndrome toxin-1in staphylococci. Proc Nat Acad Sci 108:3360–3365. doi:10.1073/pnas.1017431108
8. McClean KH, Winson MK, Fish L (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiol 143:3703–3711
9. Wattanavanitchakorn S, Prakitchaiwattana C, Thamyongkit P (2014) Rapid and simple colorimetric method for the quantification of AI-2 produced from *Salmonella Typhimurium*. J Microbiol Methods 99:15–21. doi:10.1016/j.mimet.2014.01.014
10. Sutyak KE, Wirawan RE, Aroutcheva AA et al (2008) Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-derived *Bacillus amyloliquefaciens*. J Appl Microbiol 104: 1067–1074. doi:10.1111/j.1365-2672.2007.03626.x
11. de Man JD, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. J Appl Bact 23:130–135. doi:10.1111/j.1365-2672.1960.tb00188.x
12. O'Toole GA (2011) Microtiter dish biofilm formation assay. JoVE. 47. doi:10.3791/2437
13. Borucki MK, Peppin JD, White D et al (2003) Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl Enviro Microbiol 69:7336–7342. doi:10.1128/AEM.69.12.7336-7342.2003
14. Zhu H, He CC, Chu QH (2011) Inhibition of quorum sensing in *Chromobacterium violaceum* by pigments extracted from *Auricularia auricular*. Lett Appl Microbiol 52:269–274. doi:10.1111/j.1472-765X.2010.02993.x
15. Jenssen H, Hamill P, Hancock REW (2006) Peptide antimicrobial agents. Clin Microbiol Rev 19:491–511. doi:10.1128/CMR.00056-05
16. Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3:238–250. doi:10.1038/nrmicro1098
17. Brogden NK, Brogden KA (2011) Will new generations of modified antimicrobial peptides improve their potential as pharmaceuticals? Int J Antimicrob Agents 38:217–225. doi:10.1016/j.ijantimicag.2011.05.004
18. Rossi LM, Rangasamy P, Zhang J et al (2008) Research advances in the development of peptide antibiotics. J Pharm Sci 97:1060–1070. doi:10.1002/jps.21053
19. Wimley W, Hristova K (2011) Antimicrobial peptides: successes, challenges and unanswered questions. J Membrane Biol 239:27–34. doi:10.1007/s00232-011-9343-0
20. Das T, Sharma PK, Busscher HJ et al (2010) Role of extracellular DNA in initial bacterial adhesion and surface aggregation. Appl Environ Microbiol 76:3405–3408. doi:10.1128/AEM.03119-09
21. Al Atya AK, Belguesmia Y, Chataigne G, Ravallec R, Vachée A, Szunerits S, Boukherroub R, Drider D (2016) Anti-MRSA activities of enterocins DD28 and DD93 and evidences on their role in the inhibition of biofilm formation. Fron Microbiol 7:817. doi:10.3389/fmicb.2016.00817
22. Drider D, Bendali F, Naghmouchi K et al (2016) Bacteriocins: not only antibacterial agents. Probiotics Antimicrob Proteins 1:1–6. doi:10.1007/s12602-016-9223-0
23. Overhage J, Campisano A, Bains M et al (2008) Human host defense peptide LL-37 prevents bacterial biofilm formation. Infect Immun 76:4176–4182. doi:10.1128/IAI.00318-08
24. Sutyak KE, Anderson RA, Dover SE et al (2008) Spermicidal activity of the safe natural antimicrobial peptide subtilosin. Infect Dis Obstet Gynecol 2008:540758. doi:10.1155/2008/540758
25. Torres NI, Noll KS, Xu S et al (2013) Safety, formulation and in vitro antiviral activity of the antimicrobial peptide subtilosin against herpes simplex virus type 1. Probiotics Antimicrob Proteins 5:26–35. doi:10.1007/s12602-012-9123-x
26. Noll KS, Prichard MN, Khaykin A et al (2012) The natural antimicrobial peptide subtilosin acts synergistically with glycerol monolaurate, lauric arginate, and ε-poly-L-lysine against bacterial vaginosis-associated pathogens but not human lactobacilli. Antimicrob Agents Chemother 56:1756–1761. doi:10.1128/AAC.05861-11
27. Wiedemann I, Breukink E, van Kraaij C et al (2001) Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J Biol Chem 276:1772–1779. doi:10.1074/jbc.M006770200
28. Noll KS, Sinko PJ, Chikindas ML (2011) Elucidation of the molecular mechanisms of action of the natural antimicrobial peptide subtilosin against the bacterial vaginosis-associated pathogen *Gardnerella vaginalis*. Probiotics Antimicrob Proteins 3:41–47. doi:10.1007/s12602-010-9061-4
29. Varga ZG, Armada A, Cerca P et al (2012) Inhibition of quorum sensing and efflux pump system by trifluoromethyl ketone proton pump inhibitors. In Vivo 26:277–285
30. Daines DA, Bothwell M, Furrer J et al (2005) Haemophilus influenzae luxS mutants form a biofilm and have increased virulence. Microb Pathog 39:87–89. doi:10.1016/j.micpath.2005.06.003
31. McNab R, Ford SK, El-Sabaeny A et al (2003) LuxS-based signaling in *Streptococcus gordonii*: autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. J Bacteriol 185:274–284. doi:10.1128/JB.185.1.274-284.2003
32. Prouty AM, Schwesinger WH, Gunn JS (2002) Biofilm formation and interaction with the surfaces of gallstones by *Salmonella* spp. Infect Immun 70:2640–2649. doi:10.1128/IAI.70.5.2640-2649.2002
33. Cole SP, Harwood J, Lee R et al (2004) Characterization of monospecies biofilm formation by *Helicobacter pylori*. J Bacteriol 186:3124–3132. doi:10.1128/JB.186.10.3124-3132.2004
34. Tannock GW, Ghazally S, Walter J et al (2005) Ecological behavior of *Lactobacillus reuteri* 100-23 is affected by mutation of the *luxS* gene. Appl Environ Microbiol 71:8419–8425. doi:10.1128/AEM.71.12.8419-8425.2005

35. Xu L, Li H, Vuong C et al (2006) Role of the *luxS* quorum-sensing system in biofilm formation and virulence of *Staphylococcus epidermidis*. Infect Immun 74:488–496. doi:10.1128/IAI.74.1.488-496.2006
36. Sela S, Frank S, Belausov E et al (2006) A mutation in the *luxS* gene influences *Listeria monocytogenes* biofilm formation. Appl Environ Microbiol 72:5653–5658. doi:10.1128/AEM.00048-06
37. Hardie KR, Heurlier K (2008) Establishing bacterial communities by 'word of mouth': LuxS and autoinducer 2 in biofilm development. Nat Rev Microbiol 6:635–643. doi:10.1038/nrmicro1916
38. Pearson JP, Van Delden C, Iglewski BH (1999) Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J Bacteriol 181:1203–1210