Abstract: Bacterial biofilms, formed on biotic or abiotic surfaces, can lead to serious environmental or medical problems. Therefore, it is necessary to find novel antimicrobial agents to combat biofilms, or more effective combinations of existing biocides. In this study, initial biofilms of *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 6538 in the presence of xylitol or xylitol and isothiazolones were determined using crystal violet staining in 96-well microplates and confocal laser scanning microscopy. Xylitol and isothiazolones exhibited enhanced synergistic inhibition of initial biofilm formation, and also the structure and production of extracellular polymeric substances by *P. aeruginosa* ATCC 9027 and *S. aureus* ATCC 6538 in a dose-dependent manner. In addition, xylitol and isothiazolones inhibited and restored the swimming motility of *P. aeruginosa* ATCC 9027, respectively. These findings show that a combination of xylitol and isothiazolones exerts pronounced antimicrobial activity against *P. aeruginosa* and *S. aureus* biofilms and may be applicable for preventing or reducing bacterial biofilms in vitro.

Keywords: *Pseudomonas aeruginosa*; *Staphylococcus aureus*; biofilm formation; isothiazolones; xylitol.

---

**Introduction**

*Pseudomonas aeruginosa* and *Staphylococcus aureus* are two opportunistic pathogens that have been implicated in a number of infectious diseases. *P. aeruginosa*, a versatile Gram-negative, rod-shaped bacterium, is one of the leading causes of opportunistic human infections (1). It frequently infects burn victims, and patients who have cystic fibrosis, hospital-acquired pneumonia, or those who are catheterized (2). Infections caused by *P. aeruginosa* are difficult to treat and are often severe and life-threatening due to its limited susceptibility to antimicrobial agents. Furthermore, the high frequency of emerging antibiotic resistance during therapy is largely attributed to the formation of biofilms (3,4).

*S. aureus*, a Gram-positive, coccos-shaped bacterium in the phylum Firmicutes, is frequently present in the human nose or respiratory tract, and on the skin. *S. aureus* has become the most common cause of nosocomial infec-
tions, and can infect any organ system due to its extensive virulence factors (5). It has been demonstrated that many human diseases, including endocarditis, osteomyelitis, and foreign body-related infections, can be caused by biofilm-associated S. aureus (6).

Microbial biofilms are cell-to-cell or surface-adherent assemblages of microorganisms that are encased in a three-dimensional matrix of self-produced polymers of hydrated extracellular polymeric substances (EPS), consisting mainly of polysaccharides, proteins, nucleic acids and lipids (7). Bacteria growing as biofilms are more resistant to antibacterial agents than corresponding planktonic cells (8); therefore, it is generally difficult to remove or inactivate developing and mature biofilms (9).

Biofilm formation can lead to various environmental and medical problems, including microbial food-safety risks, energy loss and blocked circuits or tubes in industrial water systems, and the emergence of antibiotic-resistant strains in hospitals (10). Biofilms have a great impact on public health because of their role in certain infectious diseases and a variety of device-related infections. A greater understanding of biofilm processes may lead to effective control strategies resulting in improved patient management (11). In previous studies, various antimicrobial agents such as dicloxacillin (12), azithromycin (13), chlorhexidine (14), lactoferricin (15), acidic and basic electrolyzed water (16), silver diamine fluoride (17) and isothiazolones (18) have been shown to inhibit or eliminate biofilms. Meanwhile, new solutions for biofilm control, such as enzymes, phages and antimicrobial molecules of microbial origin, are also constantly emerging (19-21). However, the bacterial biofilm phenotype provides intrinsic resistance to conventional disinfection regimens and new control strategies, thereby leading to eventual dissemination of resistance. Therefore, it is necessary to identify new agents or methods for preventing and eradicating bacterial biofilms.

The primary objective of this study was to evaluate the synergistic effects of xylitol and isothiazolones for inhibition of P. aeruginosa and S. aureus initial biofilm formation to provide further information that would lead to an effective strategy for countering bacterial biofilms.

Materials and Methods

Bacterial strains and chemicals

The bacterial strains P. aeruginosa ATCC 9027 and S. aureus ATCC 6538 used in this study were obtained from the American Type Culture Collection (ATCC) and stored at −80°C until use. Both of the above strains were cultured in Luria Bertani (LB) medium consisting of 10 g/L sodium chloride (Sigma, St. Louis, MO, USA), 10 g/L peptone powder (Oxoid, Hampshire, England) and 5 g/L yeast powder (Oxoid) or tryptic soy broth (TSB; Huankai, Guangzhou, P. R. China) at 30°C or 37°C in a shaking incubator at 160 rpm. Two representative isothiazolones, 14% (m/v) Kathon (a mixture of 5-chloro-N-methylisothiazolone (CMIT) and N-methylisothiazolone (MIT)), and 11% (m/v) 1, 2-benzisothiazolin-3-one (BIT), were generously provided by Guangdong Dimei Biology Technology Co., Ltd. (Guangzhou, China). Xylitol was purchased from Adams-Beta Reagent Co., Ltd. (Shanghai, P. R. China). All other chemicals used in this study were reagent grade and purchased from Sigma unless otherwise indicated.

Semi-quantitative determination of biofilms

P. aeruginosa ATCC 9027 and S. aureus ATCC 6538 biofilms were semi-quantified using crystal violet staining in pre-sterilized, polystyrene, flat-bottomed microtiter plates according to the previously described protocols with slight modifications (22). Briefly, eight wells of a 96-well microtiter plate (Corning Incorporated, Corning, NY, USA) were inoculated with 150 μL of an overnight culture suspension with an optical density (OD 600) of approximately 0.05. Negative control wells contained LB or TSB medium only. The inoculated plates were then transferred to a static incubator set at a constant temperature (30°C for P. aeruginosa ATCC 9027 and 37°C for S. aureus ATCC 6538) and cultured for 24 h. Subsequently, the optical density of planktonic cells in each well was measured using a MultiSkans G0 plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 600 nm. The planktonic cells were then discarded, and each well was gently washed with 250 μL of sterile distilled water at least three times and stained with 250 μL of 0.1% crystal violet (Shanghai Chemical Reagents Co. Ltd., Shanghai, P. R. China) for 30 min at room temperature. The excess crystal violet was removed by rinsing with sterile distilled water at least three times. The plates were dried at room temperature for another 30 min, and then 260 μL of 95% ethanol (vol : vol, Shanghai Chemical Reagents Co. Ltd.) was added to each well to re-solubilize the dye that bound to the biofilms. The OD 595 of each well was measured with the same MultiSkans GO reader. Each of the above experiments was performed with eight replicate wells and repeated at least three times.

Synergistic effects of isothiazolones and xylitol on biofilm inhibition

Both P. aeruginosa ATCC 9027 and S. aureus ATCC 6538 were cultured overnight in LB or TSB medium at 30°C or 37°C, respectively. A series of microtiter plate
wells were filled with 150-μL mixtures containing the diluted bacterial suspensions (final OD<sub>600</sub> = 0.05), solutions of the antimicrobial agents (Kathon or BIT), and xylitol so that the final concentrations of each reagent were as follows: Kathon (0, 0.5, 1.0, 2.0, 4.0, 8.0, and/or 16.0 mg/L), BIT (0, 2.0, 4.0, 8.0, 16.0, 32.0, and/or 64.0 mg/L), and xylitol (0, 50, 100, and 200 mM). Subsequently, all plates were transferred to a static incubator and cultured at 30°C (P. aeruginosa ATCC 9027) or 37°C (S. aureus ATCC 6538) for 24 h. The plates were then stained, rinsed and measured according to the protocols described above. In addition, wells without any antimicrobial agents or xylitol were used as negative controls.

Confocal laser scanning microscopy (CLSM) for analysis of biofilms
Biofilms formed on pre-sterilized glass microscope slides were observed by CLSM according to a previously described protocol with minor modifications (23). Briefly, coverslips were placed in the wells of a 24-well microtiter plate and each well was inoculated with 1 mg/L Kathon or 16 mg/L BIT (8 mg/L for S. aureus ATCC 6538), and 50 mM xylitol, and 2-mL aliquots of either P. aeruginosa ATCC 9027 or S. aureus ATCC 6538 suspensions (OD<sub>600</sub> = 0.05). The microtiter plates were then placed in an incubator and cultured statically at 30°C or 37°C for 24 h. Following 24 h of growth, biofilms of P. aeruginosa ATCC 9027 or S. aureus ATCC 6538 had formed on the coverslips. To assess the biomass and structures of the attached biofilms, the slides were stained with 5 μM SYTO9 fluorescent dye (Invitrogen, Carlsbad, CA, USA) and 30 μM propidium iodide (PI; Sigma Chemical Company) for at least 15-20 min in a dark room, according to the methods described previously (24). Subsequently, the stained biofilms were visualized using CLSM (LSM 710 Zeiss, Jena, Germany) and the live bacteria present in the biofilms were stained a fluorescent green color.

Quantification of EPS inhibition
Inhibition of EPS production by P. aeruginosa ATCC 9027 and S. aureus ATCC 6538 was determined using the carbohydrate estimation method, in accordance with previously published protocols (25). Briefly, the biofilms were cultured with or without Kathon, BIT, xylitol or combinations thereof based on the CLSM observations in this study. After incubation for 24 h, each glass slide on which a biofilm had formed was transferred to a glass tube and suspended in 500 μL of 0.9% NaCl. Next, 5% phenol (500 μL) and 0.2% hydrazine sulfate (w/v; 2.5 mL in concentrated H<sub>2</sub>SO<sub>4</sub>) were added and incubated at room temperature in the dark to extract the EPS. One hour later, optical absorbance was read at OD<sub>490</sub> using the Multiskan GO reader, and EPS inhibitions were determined using the following formula: EPS inhibition percentage = (([Control OD<sub>490</sub> - Treated OD<sub>490</sub>] / Control OD<sub>490</sub>)) × 100%.

Swimming motility
The swimming motility of P. aeruginosa ATCC 9027 was determined according to methods reported previously, with slight modifications (26). Briefly, P. aeruginosa ATCC 9027 was cultured overnight on LB agar plates at 30°C. Subsequently, a single colony of P. aeruginosa ATCC 9027 was transferred to a swimming motility plate (10 g/L tryptone (Difco), 5 g/L NaCl, and 0.3% (wt/vol) agar) with a sterile toothpick. The plate was then wrapped with parafilm to prevent dehydration and incubated at 30°C for 24 h. All plates were photographed using a digital camera (Nikon D7200, AF-S DX 18-140 mm; Nikon, Tokyo, Japan).

Statistical analysis
All values obtained in this study were calculated as the mean of individual experiments and compared with those of the control groups using one-way analysis of variance. Differences at P < 0.05 were considered statistically significant. All statistical analyses were performed using Data Processing System (DPS) software.

Results
Synergistic effects of xylitol and isothiazolones for inhibition of initial biofilm formation by P. aeruginosa ATCC 9027
With increasing concentrations of xylitol (from 0 to 200 mM) in LB medium, both planktonic growth and initial biofilm formation by P. aeruginosa ATCC 9027 were inhibited in a concentration-dependent manner (Fig. 1A). In the presence of 200 mM xylitol, planktonic growth and initial biofilm formation by P. aeruginosa ATCC 9027 decreased by approximately 15.13% and 43.08%, respectively, relative to the control lacking xylitol (P < 0.05; Fig. 1A). When 2 mg/L Kathon or 32 mg/L BIT was added to the LB medium, both planktonic growth and biofilm formation, respectively, were repressed to different extents (Fig. 1A). Furthermore, isothiazolones and xylitol synergistically inhibited initial biofilm formation by P. aeruginosa ATCC 9027 in comparison with Kathon, BIT, or xylitol alone (Fig. 1A). For Kathon (2 mg/L) or BIT (32 mg/L) and xylitol, the synergistic biofilm-inhibitory effect was increased by 29.5-51.4% and 43.6-49.0%, respectively, in a dose-
dependent manner. The synergistic inhibitory effect of isothiazolones and xylitol on initial biofilm formation by *P. aeruginosa* ATCC 9027 was also observed in TSB medium, the results being similar, but not identical, to those obtained with LB medium (Fig. 1B). In particular, the growth of planktonic *P. aeruginosa* ATCC 9027 cells in TSB medium was slower than that in LB medium. For Kathon (2 mg/L) or BIT (32 mg/L) and xylitol, the synergistic inhibitory effect on biofilm formation was increased by 37.7-77.3% and 28.8-47.8%, respectively, in a dose-dependent manner.

**Synergistic effects of isothiazolones and xylitol for inhibition of initial biofilm formation by *S. aureus* ATCC 6538**

The Gram-positive organism *S. aureus* ATCC 6538 was also selected for use as a model bacterium to determine the synergistic effects of isothiazolones and xylitol for inhibition of initial biofilm formation. As shown in Fig. 2, both planktonic growth and initial biofilm formation by *S. aureus* ATCC 6538 were inhibited by xylitol in a concentration-dependent manner, irrespective of whether the bacteria were grown in LB or TSB medium. For Kathon (2 mg/L) or BIT (16 mg/L) and xylitol, the synergistic inhibitory effect on biofilm formation by *S. aureus* ATCC 6538 in LB medium was increased by 69.3-89.4% and 42.1-45.7%, respectively, in a dose-dependent manner (Fig. 2A). Similarly, the synergistic effects in TSB medium were increased by 14.7-49.2% for Kathon and by 27.2-40.2% for BIT (Fig. 2B).

**Effects of xylitol, isothiazolones, and their combinations on biofilm structure**

The structure and surface topography of bacterial biofilms can be stained with fluorescent dyes and observed by CLSM. Therefore, *P. aeruginosa* ATCC 9027 and *S. aureus* ATCC 6538 biofilms that formed on the surfaces of glass slides in the presence of xylitol, isothiazolones, and their combinations were stained with SYTO9 and PI, and imaged. In addition, the percentages of EPS inhibition under the same conditions were also determined. It was found that typical biofilms of *P. aeruginosa* ATCC
259 (control) were formed on the surfaces of glass slides after 24 h of culture in LB medium (Fig. 3A). In the presence of 50 mM xylitol, 1 mg/L Kathon or, in particular, 16 mg/L BIT, less biofilms and smaller amounts of EPS were formed by *P. aeruginosa* ATCC 9027 relative to the control (Figs. 3B, 3C, 5A). After treatment for 24 h with a combination of 1 mg/L Kathon (or 16 mg/L BIT) and 50 mM xylitol, most *P. aeruginosa* ATCC 9027 biofilm formation (including EPS) on the glass slide was effectively inhibited (Figs. 3D, 3E, 3F, 5A). As expected, xylitol (50 mM), Kathon (1 mg/L) and BIT (8 mg/L) showed similar inhibitory effects on *S. aureus* ATCC 6538 biofilms when used alone or in combination (Figs. 4, 5B).

**Effects of xylitol, isothiazolones, and their combinations on swimming motility of *P. aeruginosa* ATCC 9027**

It has been reported that the swimming motility of some bacteria can partially contribute to biofilm formation (27). The swimming motility of *P. aeruginosa* ATCC 9027 in the presence of isothiazolones and xylitol, either alone or in combination, was therefore evaluated. It was found that 50 mM and 100 mM xylitol did not inhibit the swimming motility of *P. aeruginosa* ATCC 9027 (Fig. 6A, 6B, 6C, Table 1). However, when the concentration of xylitol was increased to 200 mM, the swimming motility was slightly affected (Fig. 6D, Table 1). On the other hand,
Swimming motility was significantly inhibited by 2 mg/L Kathon or 32 mg/L BIT (Fig. 6E, 6I, Table 1). When xylitol was added to the plates containing Kathon or BIT, the swimming motility of *P. aeruginosa* ATCC 9027 was partially restored (Fig. 6F-H, J-L, Table 1).

### Discussion

Isothiazolones oxidize accessible thiols of various metabolic proteins or enzymes and inhibit their activities, thus repressing microbial cell growth and metabolism, resulting in loss of viability (28). Two representative isothiazolones, Kathon and BIT, are heterocyclic chemical compounds that are used extensively to combat Gram-positive and Gram-negative bacteria, fungi, viruses, and algae in industrial, agricultural and medical environments (29). It has been reported that either Kathon or BIT might have potential for inhibiting bacterial biofilm formation and development. A Kathon concentration of 15 mg/L inhibited all microbial activity and resulted in the death of biofilms established on laboratory-scale rotating biological contactors (30). Kathon and BIT stimulated initial biofilm formation by *Enterobacter cloacae* at low concentrations and had inhibitory effects on both biofilm formation and planktonic growth at higher concentrations (18). Unfortunately, the above two isothiazolones were unable to remove pre-formed or fully mature *E. cloacae* biofilms (18). Kathon was shown to be effective against pure *Hormoconis resinae* biofilms at 50 and 100 mg/L, but was only effective against mixed biofilms (*H. resinae* and anaerobic sulphate-reducing bacteria [SRB]) at higher concentrations (31). In addition, isothiazolones and a direct current have been reported to exert enhanced synergistic inhibition of biofilm formation. Direct current alone has little or no effect on the survival of *P. aeruginosa* biofilms grown on stainless steel studs; however, the combination of direct current and biocides, including Kathon, glutaraldehyde, and quaternary ammonium compounds, exhibited significantly enhanced killing efficacy (32). In the present study, the results demonstrated that both planktonic cells and biofilms of *P. aeruginosa* ATCC 9027 and *S. aureus* ATCC 6538 cultured in LB or TSB medium were inhibited by Kathon and BIT (Figs. 1, 2), which is consistent with previous reports.

Xylitol, a type of sugar alcohol, is an important substitute for sucrose and is widely used in the food and drink industries. Recent studies have shown that xylitol alone

### Table 1 Diameter of *P. aeruginosa* ATCC 9027 colonies in swimming motility assays

| Xylitol      | 0 mM     | 50 mM     | 100 mM    | 200 mM    |
|--------------|----------|-----------|-----------|-----------|
| No biocides  | 1.95 ± 0.15 a | 1.95 ± 0.13 a | 1.82 ± 0.03 a | 0.8 ± 0.10 b |
| Kathon (2 mg/L) | 0.28 ± 0.03 d | 1.63 ± 0.15 a | 1.37 ± 0.06 b | 0.67 ± 0.21 c |
| BIT (32 mg/L) | 0.12 ± 0.03 b | 0.40 ± 0.10 a | 0.23 ± 0.06 b | 0.17 ± 0.06 b |

Results are listed as the means of datasets obtained from analysis of three swimming motility colonies. Standard deviations (SD) are shown after each of the means. Different superscript letters represent significant differences within a row (Tukey’s HSD: *P* < 0.05).

**Fig. 6** Swimming motility of *P. aeruginosa* ATCC 9027 with different concentrations of xylitol, Kathon, BIT or their combinations. Cells were inoculated with a toothpick from an overnight LB agar plate onto a swimming motility plate (tryptone broths plus 0.3% agarose) and photographed over a 24-h incubation at 30°C. (A): Control; (B), (C), (D): 50 mM, 100 mM and 200 mM xylitol, respectively; (E): 2 mg/L Kathon; (F), (G), (H): 2 mg/L Kathon + 50 mM, 100 mM, or 200 mM xylitol, respectively; (I): 32 mg/L BIT; (J), (K), (L): 32 mg/L BIT + 50 mM, 100 mM, or 200 mM xylitol, respectively.
or in combination with other antimicrobial agents inhibits or eliminates bacterial biofilms. Xylitol (1% and 3%) demonstrated a clear inhibitory effect on biofilm formation by six bacterial species (Streptococcus mutans, Streptococcus sobrinus, Lactobacillus rhamnosus, Actinomyces viscosus, Porphyromonas gingivalis, and Fusobacterium nucleatum) that were prepared on hydroxyapatite (HA) discs according to the Zürich Biofilm Model (33). Biofilm formation by P. aeruginosa, Enterococcus faecalis and S. aureus was completely inhibited in the Lubbock Chronic Wound Biofilm (LCWB) model following treatment with 20% xylitol, and xylitol targeted P. aeruginosa preferentially (34). Combined treatment with lactoferrin and xylitol significantly decreased the viability of established P. aeruginosa biofilms in vitro, and the antimicrobial mechanism was considered to include both disruption of biofilm structure and permeabilization of the bacterial membranes (35). Recently, using a colony-drip-flow reactor biofilm model, the same group also found that for biofilms composed of both a single species and two species, lactoferrin/xylitol hydrogel in combination with the silver wound dressing Acticoat significantly reduced biofilm viability relative to the commercially available wound hydrogel (36). They also demonstrated that whilst lactoferrin treatment of P. aeruginosa biofilms destabilized the bacterial cell membrane though iron chelation, combined treatment with lactoferrin and xylitol inhibited the ability of P. aeruginosa biofilms to respond to environmental iron restriction (37). Farnesol combined with xylitol and lactoferrin was the most effective treatment against biofilms of the endodontic Enterococcus faecalis strain MB35 and reduced the biomass of Staphylococcus epidermidis biofilms (38). Xylitol and ursolic acid or ribose can be potentially useful for enhancement of antimicrobial and anti-biofilm efficacy against S. mutans and S. sobrinus in the oral environment (39,40).

The effects and efficiency of xylitol on bacterial biofilm formation are still controversial, and a number of studies have obtained contradictory results. One study demonstrated the inhibitory influence of xylitol on model-based multispecies biofilms, whereas a separate study did not support the inhibitory effect of xylitol on S. mutans cells, although this work also used a multispecies biofilm model with repeated xylitol exposure (41). Xylitol inhibits the growth of Streptococcus pneumoniae; however, when the test medium was supplemented with glucose or fructose, biofilm formation was enhanced and the inhibitory effect of xylitol on biofilm formation was not observed (42). A reduced effect on S. mutans biofilms was also found when xylitol was used with glucose (43). Xylitol was reported to exhibit weak activity against preformed biofilms of 20 clinical P. aeruginosa isolates, in terms of the minimum biofilm inhibitory concentration (44). No differences were detected in EPS production by xylitol-sensitive (Xs) S. mutans grown with or without xylitol, nor between xylitol-resistant (Xr) and Xs S. mutans strains. Thus, it seems that xylitol does not affect EPS synthesis in S. mutans strains (45). The above results are difficult to compare because of the substantial differences in the experimental conditions, bacterial isolates and procedures employed.

In the present study, the growth of both planktonic cells and biofilms (structures and EPS) of P. aeruginosa ATCC 9027 and S. aureus ATCC 6538 was affected by xylitol alone or in combination with Kathon and BIT (Figs. 1-5), suggesting that isothiazolones and xylitol exert synergistic effects. Although a lot of effort has been devoted to clarifying the inhibitory effect of xylitol, the underlying plaque-reducing mechanisms have yet to be fully elucidated. The cellular uptake of xylitol by the flexible phosphotransferase system is an important step in the energy-consuming futile cycle believed to be responsible for its inhibitory effect (46). Depending on the energy available, xylitol may be phosphorylated intracellularly. Xylitol 5-phosphate may compete with phosphofructokinase, thus inhibiting glycolysis. Because of its cellular toxicity, xylitol 5-phosphate must be dephosphorylated and expelled as xylitol, requiring a major input of energy. Furthermore, xylitol seems to cause disruption of protein synthesis (47). As a metabolic consequence, several studies have shown that S. mutans cells reduce their growth, thereby decreasing the production of both acid and extracellular polysaccharides (48). The mechanisms responsible for xylitol inhibition of P. aeruginosa ATCC 9027 and S. aureus ATCC 6538, and the enhanced synergistic effect of xylitol and isothiazolones require further research.

P. aeruginosa is able to swim in liquid by using flagella and to move on surfaces by using type IV pili (49,50). Using the microtiter plate assay, O’Toole and Kolter (1998a) showed that flagella or flagellum-driven motility is required for biofilm formation by P. aeruginosa, and that type IV pili are required for biofilm and microcolony formation by this organism (51,52). In this study, the results showed that Kathon and BIT inhibited the swimming ability of P. aeruginosa ATCC 9027, and that addition of xylitol restored this ability (Fig. 6, Table 1), suggesting that the inhibitory effect of isothiazolones on P. aeruginosa ATCC 9027 biofilm formation is partly due to inhibition of swimming motility, and that xylitol and isothiazolones may antagonize each other during the swimming assay.
In this study, xylitol and isothiazolones (Kathon and BIT) exhibited enhanced synergistic inhibitory effects on initial biofilm formation (including biofilm structures and EPS production) and planktonic growth of *P. aeruginosa* ATCC 9027 and *S. aureus* ATCC 6538 in a dose-dependent manner. It was also found that isothiazolones and xylitol inhibited the swimming motility of *P. aeruginosa* ATCC 9027. Thus, a combination of xylitol and isothiazolones shows increased antimicrobial activity against *P. aeruginosa* and *S. aureus* biofilms *in vitro*. Barraud and colleagues have reported that co-addition of tobramycin and mannitol enhanced the killing of *P. aeruginosa* biofilms when grown *in vitro* on an abiotic surface (53). However, mannitol does not enhance tobramycin-mediated killing of *P. aeruginosa* in a cystic fibrosis model system of biofilm formation (54). This suggests that the bacterial biofilms formed *in vivo* and on biotic materials may exhibit different and more complex responses to sugar alcohol and antimicrobial agents than those formed *in vitro* and on abiotic surfaces. Therefore, further studies are required to determine the validity of these xylitol and isothiazolone mixtures for practical or *in vivo* applications.

Acknowledgements

This work was funded by the Natural Science Foundation of Guangdong Province (No. 2015A030313713), and the National Natural Science Foundation of China (No. 31500036 and 31770091).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrenre P, Hickey MJ et al. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature 406, 959-964.
2. Sadikot RT, Blackwell TS, Christman JW, Prince AS (2005) Pathogen-host interactions in *Pseudomonas aeruginosa* Pneumonia. Am J Respir Crit Care Med 171, 1209-1223.
3. Drenkard E (2003) Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. Microbes Infect 5, 1213-1219.
4. Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O’Toole GA (2003) A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature 426, 306-310.
5. Archer GL (1998) *Staphylococcus aureus*: a well-armed pathogen. Clin Infect Dis 26, 1179-1181.
6. Götz F (2002) *Staphylococcus* and biofilms. Mol Microbiol 43, 1367-1378.
7. Fleming HC, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8, 623-633.
8. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995) Microbial biofilms. Annu Rev Microbiol 49, 711-745.
9. Hamanaka D, Onishi M, Genkawa T, Tanaka F, Uchino T (2011) Effects of temperature and nutrient concentration on the structural characteristics and removal of vegetable-associated *Pseudomonas* biofilm. Food Control 24, 165-170.
10. Mattila-Sandholm T, Wirataen G (1992) Biofilm formation in the industry: a review. Food Rev Int 8, 573-603.
11. Donlan RM (2002) Biofilms: microbial life on surfaces. Emerg Infect Dis 8, 881-890.
12. Cerca N, Martins S, Sillankorva S, Jefferson KK, Pier GB, Oliveira R et al. (2005) Effects of growth in the presence of subinhibitory concentrations of dicloxacillin on *Staphylococcus* epidermidis and *Staphylococcus* haemolyticus biofilms. Appl Environ Microbiol 71, 8677-8682.
13. Ichimiya T, Takeoka K, Hiramatsu K, Hirai K, Yamasaki T, Nasu M (1996) The influence of azithromycin on the biofilm formation of *Pseudomonas aeruginosa* in vitro. Chemotherapy 42, 186-191.
14. Modesto A, Drake DR (2006) Multiple exposures to chlorhexidine and xylitol: adhesion and biofilm formation by *Streptococcus* mutans. Curr Microbiol 52, 418-423.
15. Xu G1, Xiong W, Hu Q, Zuo P, Shao B, Lan F et al. (2010) Lactoferrin-derived peptides and lactoferricin chimera inhibit virulence factor production and biofilm formation in *Pseudomonas aeruginosa*. J Appl Microbiol 109, 1311-1318.
16. Sun JL, Zhang SK, Chen JY, Han BZ (2012) Efficacy of acidic and basic electrolyzed water in eradicating *Staphylococcus aureus* biofilm. Can J Microbiol 58, 448-454.
17. Savas S, Kucukyilmaz E, Celik EU, Ates M (2015) Effects of different antibacterial agents on enamel in a biofilm caries model. J Oral Sci 57, 367-372.
18. Zhou G, Li L, Shi Q, Ouyang Y, Chen Y, Hu W (2014) Efficacy of metal ions and isothiazolones in inhibiting Enterobacter cloacae BF-17 biofilm formation. Can J Microbiol 60, 5-14.
19. Simões M, Simões LC, Vieira MJ (2010) A review of current and emergent biofilm control strategies. LWT-Food Sci Technol 43, 573-583.
20. Kaplan JB, LoVetri K, Cardona ST, Madhyastha S, Sadovskaya I, Jabbouri S et al. (2012) Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococcal biofilms. J Antimicrob Chemother 66, 55-60.
21. Stepanović S, Vuković D, Dakić I, Savić B, Švabić-Vlahović M (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 40, 175-179.
22. Shukla SK, Rao TS (2013) Dispersal of Bap-mediated *Staphylococcus aureus* biofilm by proteinase K. J Antimicrob 66, 55-60.
23. Shukla SK, Rao TS (2013) Effect of calcium on *Staphylococcus* biofilm architecture: a confocal laser scanning microscopic study. Colloid Surf B Biointerfaces 103, 448-454.
24. GrayMerod R, Hendrickx L, Mueller L, Xavier J, Wuertz S (2005) Effect of nucleic acid stain Syto9 on nascent biofilm architecture of *Acinetobacter* sp. BD413. Water Sci Technol 52, 195-202.
25. Padmavathi AR, Abinaya B, Pandian SK (2014) Phenol, 2, 4-bis (1,1-dimethylethyl) of marine bacterial origin inhibits quorum sensing mediated biofilm formation in the uropathogen Serratia marcescens. Biofouling 30, 1111-1122.

26. Rashid MH, Kornberg A (2000) Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of Pseudomonas aeruginosa. Proc Natl Acad Sci USA 97, 4885-4890.

27. Hölscher T, Bartels B, Lin YC, Gallegos-Monterroso R, Price-Whelan A, Kolter R et al. (2015) Motility, chemotaxis and aerotaxis contribute to competitiveness during bacterial pellicle biofilm development. J Mol Biol 427, 3695-3708.

28. Williams TM (2007) The mechanism of action of isothiazolone biocide. Power Plant Chem 9, 14-22.

29. Pucci MJ, Podos SD, Thanassi JA, Leggio MJ, Bradbury BJ, Deshpande M (2011) In vitro and in vivo profiles of ABC-702, an isothiazoloquinolone, against bacterial pathogens. Antimicrob Agents Chemother 55, 2860-2871.

30. Laopaiboon L, Smith R, Hall S (2001) A study of the effect of isothiazolones on the performance and characteristics of a laboratory-scale rotating biological contactor. J Appl Microbiol 91, 93-103.

31. Guiamet PS, Gaylarde CC (1996) Activity of an isothiazolone biocide against Hormoconis resinae in pure and mixed biofilms. World J Microbiol Biotechnol 12, 395-397.

32. Blenkinsopp SA, Khoury A, Costerton J (1992) Electrical enhancement of biocide efficacy against Pseudomonas aeruginosa biofilms. Appl Environ Microbiol 58, 3770-3773.

33. Badet C, Furiga A, Thebaud N (2008) Effect of xylitol on an in vitro model of oral biofilm. Oral Health Prev Dent 6, 337-341.

34. Dowd SE, Sun Y, Smith E, Kennedy JP, Jones CE, Wolcott R (2009) Effects of biofilm treatments on the multi-species Lbubock chronic wound biofilm model. J Wound Care 18, 508-512.

35. Ammons MCB, Ward LS, Fisher ST, Wolcott RD, James GA (2009) In vitro susceptibility of established biofilms composed of a clinical wound isolate of Pseudomonas aeruginosa treated with lactoferrin and xylitol. Int J Antimicrob Agents 33, 230-236.

36. Ammons MCB, Ward LS, Dowd S, James GA (2011) Combined treatment of Pseudomonas aeruginosa biofilm with lactoferrin and xylitol inhibits the ability of bacteria to respond to damage resulting from lactoferrin iron chelation. Int J Antimicrob Agents 37, 316-323.

37. Ammons MC, Ward LS, James GA (2011) Anti-biofilm efficacy of a lactoferrin/xylitol wound hydrogel used in combination with silver wound dressings. Int Wound J 8, 268-273.

38. Alves FR, Silva MG, Rôças IN, Siqueira Jr JF (2013) Biofilm biomass disruption by natural substances with potential for endodontic use. Braz Oral Res 27, 20-25.

39. Zou Y, Lee Y, Huh J, Park JW (2014) Synergistic effect of xylitol and ursolic acid combination on oral biofilms. Restor Dent Endod 39, 288-295.

40. Lee HJ, Kim SC, Kim J, Do A, Han SY, Lee BD et al. (2015) Synergistic inhibition of Streptococcal biofilm by ribose and xylitol. Arch Oral Biol 60, 304-312.

41. Giertsen E, Arthur R, Guggenheim B (2011) Effects of xylitol on survival of mutants streptococci in mixed-six-species in vitro biofilms modelling supragingival plaque. Caries Res 45, 31-39.

42. Kurola P, Tapiaiten T, Sevander J, Kairelailen T, Leinonen M, Uhari M et al. (2011) Effect of xylitol and other carbon sources on Streptococcus pneumoniae biofilm formation and gene expression in vitro. APMIS 119, 135-142.

43. Decker EM, Klein C, Schwindt D, von Ohle C (2014) Metabolic activity of Streptococcus mutans biofilms and gene expression during exposure to xylitol and sucrose. Int J Oral Sci 6, 195-204.

44. Abbas HA, Berry FM, EL-Masry EM (2012) Combating Pseudomonas aeruginosa biofilms by potential biofilm inhibitors. Asian J Res Pharm Sci 2, 66-72.

45. Martinen AM, Ruas-Madiedo P, Hidalgo-Cantabrana C, Saari MA, Ihalin RA, Söderling EM (2012) Effects of xylitol on xylitol-sensitive versus xylitol-resistant Streptococcus mutans strains in a three-species in vitro biofilm. Curr Microbiol 65, 237-243.

46. Assev S, RÖLla G (1986) Further studies on the growth inhibition of Streptococcus mutans OMZ 176 by xylitol. APMIS 94, 97-102.

47. Trahan L, Néron S, Bareil M (1991) Intracellular xylitol-phosphate hydrolysis and efflux of xylitol in Streptococcus sobrinus. Mol Oral Microbiol 6, 41-50.

48. Lee YE, Choi YH, Jeong SH, Kim HS, Lee SH, Song KB (2009) Morphological changes in Streptococcus mutans after chewing gum containing xylitol for twelve months. Curr Microbiol 58, 332-337.

49. O’Toole GA, Kolter R (1998) Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol 28, 449-461.

50. Klausen M1, Heydorn A, Ragas P, Lambertsen L, Aaes-Jørgensen A, Molin S et al. (2003) Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. Mol Microbiol 48, 1511-1524.

51. O’Toole GA, Kolter R (1998) Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol Microbiol 30, 295-304.

52. Barken KB, Pamp SJ, Yang L, Gjermansen M, Bertrand JJ, Jørgensen A, Molin S et al. (2003) Contribution of flagella and type IV pili in Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. Mol Microbiol 48, 1511-1524.

53. Barksen KB, Pamp SJ, Yang L, Gjermansen M, Bertrand JJ, Klausen M et al. (2008) Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in Pseudomonas aeruginosa biofilms. Environ Microbiol 10, 2331-2343.

54. Barraud N, Buson A, Jarolimek W, Rice SA (2013) Mannitol enhances antibiotic sensitivity of persister bacteria in Pseudomonas aeruginosa biofilms. PLoS One 8, e84220.

55. Price KE, Orazi G, Ruoff KL, Hebert WP, O’Toole GA, Mastoridis P (2015) Mannitol does not enhance tobramycin killing of Pseudomonas aeruginosa in a cystic fibrosis model system of biofilm formation. PLoS One 10, e0141192.