Food Control 112 (2020) 107154

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

# Inhibition of multidrug-resistant foodborne *Staphylococcus aureus* biofilms by a natural terpenoid (+)-nootkatone and related molecular mechanism

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ARTICLE INFO

*Keywords:*
(+)-Nootkatone
Phytochemical
Multidrug resistance
*Staphylococcus aureus*
Biofilm
Mechanisms

ABSTRACT

*Staphylococcus aureus*, a foodborne pathogen, poses serious problems to the food industries owing to biofilm formation, and over 25% of the foodborne illnesses in China have been attributed to *S. aureus* only. Phytochemicals are widely used as anti-biofilm agents with promising efficacy, and most of them are widely available and safe. This study reported the anti-biofilm efficacy of (+)-nootkatone, a sesquiterpene ketone found in a common fruit grapefruit, against multidrug-resistant *S. aureus* and its potential molecular mechanism. (+)-Nootkatone exhibited bacteriostatic and bactericidal effects at 200 and 400 μg/mL, respectively, against *S. aureus* SJTUF 20758 and *S. aureus* ATCC 25923. Crystal violet staining indicated that (+)-nootkatone inhibited *S. aureus* biofilm formation ($p < 0.05$) at a sub-MIC of 50 μg/mL and reduced exopolysaccharide production. The thickness of biofilms was significantly reduced by (+)-nootkatone, which was supported by the light microscopy and confocal laser scanning microscopy. Growth curve of bacteria showed that the antibacterial activity of **(+)**-nootkatone was dose-dependent, and the sub-MIC concentrations did not affect the bacterial growth of planktonic cells. Besides, (+)-nootkatone affected the sliding motility of *S. aureus*. At 200 μg/mL, (+)-nootkatone led to the reduction of preformed biofilm mass by 50% and bacterial cell death of 79%, accompanied with a reduction of exopolysaccharide. The expression of biofilm-related genes, including *sarA, icaA*, *agrA*, *RNAIII,* and *spa*, was suppressed by (+)-nootkatone, as revealed by the transcriptional analysis. Additionally, MTT assay revealed that there was no toxicity of (+)-nootkatone to the human foreskin fibroblasts (HFF) cells. Therefore, (+)-nootkatone is a promising phytochemical against *S. aureus* biofilms, and has the potential to be used in food industry to fight against *S. aureus*-induced safety issues.

## 1. Introduction

Microbial biofilms characterized by the presence of extracellular polymeric substances (EPS), in which planktonic bacteria are embedded, are the major sources of food contamination and represent challenges to the food industry (Winkelströter, dos Reis Teixeira, Silva, Alves, & De Martinis, 2014; Flemming et al., 2016). EPS confers protection to the bacterial cells within the biofilm from the harsh environments, antimicrobial agents, and disinfectants.

*Staphylococcus aureus*, a well-known foodborne pathogen is capable of forming biofilms on food and food contact surfaces (Miao et al., 2019). Tang et al. (2015) reported that *S. aureus* was found at high levels in raw meat (95.5%), cooked meat products (93.5%), soybean products (86.7%), and pickled vegetables (78.1%). Recently, multidrug-resistant *S. aureus* has been reported in various kinds of foods. The prevalence of antibiotic-resistant *S. aureus* is high in raw and processed meat (Li et al., 2019), milk and dairy products (Dai et al., 2019), and ready-to-eat food products (Yang et al., 2016).

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https://doi.org/10.1016/j.foodcont.2020.107154
Received 18 October 2019; Received in revised form 31 January 2020; Accepted 1 February 2020
Available online 06 February 2020
0956-7135/ 

Many foodborne outbreaks have been reported due to staphylococcal contamination globally. *S. aureus* is the leading cause of approximately 25% of foodborne outbreaks in China (Wang et al., 2017). Biofilm formation and multidrug-resistance are the major causes of staphylococcal foodborne outbreaks. The currently available antibiotics against multidrug-resistant bacteria have limited efficacy in biofilm inhibition (Wu, Moser, Wang, Høiby, & Song, 2015). Therefore, there is a growing need to identify novel compounds as biofilm inhibitors, a requirement fueled by recent observations where phytochemicals such as shikimic acid (Bai, Zhong, Wu, Elena, & Gao, 2019), eugenol (Kim et al., 2016), tannic acid (Lee et al., 2013), and some flavonoids were shown to exhibit anti-biofilm properties against *S. aureus*.

The sesquiterpene ketone **(+)-**nootkatone present in essential oils from Alaska yellow cedar trees, some herbs, and grapefruit has been approved by the Food and Drug Administration (FDA) as a flavouring agent in citrus-flavored foods and beverages. Apart from its insecticidal activity, (+)-nootkatone can sensitize non-small-cell lung cancer A549 cells to Adriamycin (Hung, Moon, Ryu, & Cho, 2019). As an AMP-activated protein kinase (AMPK) activator, (+) nootkatone can stimulate the energy metabolism in liver and muscle cells, leading to the prevention of diet-induced obesity (Murase, Misawa, Haramizu, Minegishi, & Hase, 2010). Here, the current study examined the inhibitory activity of **(+)-**nootkatone against multidrug-resistant foodborne *S. aureus* biofilm and further elucidated the possible molecular mechanisms underlying its anti-biofilm activity.

## 2. Materials and methods

### 2.1. Chemicals, bacterial strains, and growth conditions

Multidrug-resistant *S. aureus* SJTUF 20758 (Zhang et al., 2019) and the standard strain *S. aureus* ATCC 25923 were used in the study. A total of 40 terpenoids were screened to find out the active compounds with anti-biofilm activities (Table 1). Based on the anti-bacterial, anti-biofilm and cytotoxic effects, the most active compound, (+)-nootkatone was chosen for further biofilm studies. (+)-Nootkatone (≥98%) (Fig. 1A) was purchased from Shanghai Yuanye Bio-Technology Co. Ltd., China. The stock solution was prepared in dimethyl sulfoxide (DMSO).

The bacteria were grown in tryptone soy broth (TSB) and maintained in a shaker at 37 °C and 200 rpm. For anti-bacterial studies, Mueller Hinton Broth (MHB) was used, while TSB supplemented with 0.25% glucose was used for anti-biofilm studies.

### 2.2. Anti-staphylococcal effects of (+)-nootkatone

The broth microdilution method was used for evaluating MICs of **(+)-**nootkatone according to guidelines of the Clinical Laboratory Standards Institute (Wikler et al., 2009). **(+)-**Nootkatone was serially diluted in the concentration range of 3.125–200 μg/mL in 96-well microtiter plate. The bacterial suspension ($1 \times 10^6$ CFU/mL) was inoculated into the wells and incubated at 37 °C for 24 h. MIC was considered as the lowest concentration of **(+)-**nootkatone that completely inhibits the visible growth of bacteria after 24 h of incubation. The MBC was evaluated by subculturing the content from each well showing no turbidity on MH agar plates and incubated for 24 h. The lowest concentration of **(+)-**nootkatone with no bacterial growth on the agar plate was considered as MBC.

### 2.3. Growth curve analysis

*S. aureus* ($1 \times 10^6$ CFU/mL) in the exponential phase was grown in 50 mL of TSB broth supplemented with various concentrations of (+)-nootkatone (0, 25, 50, 100, and 200 μg/mL) at 37 °C with 180 rpm for 24 h in a rotatory shaker. The bacterial cell density (OD at 600 nm) was measured at selected time intervals (0, 2, 4, 6, 8, 10, and 24 h) by spectrophotometer.

### 2.4. Crystal violet staining assay

The biofilm formation assay was performed in 96-well microtiter plates, as previously described (Xu et al., 2016). Briefly, *S. aureus* ($1 \times 10^6$ CFU/mL) was inoculated into wells containing sub-MICs of (+)-nootkatone (0, 25, 50, and 100 μg/mL) and incubated for 24 h. After incubation, the planktonic cells were removed and the wells were rinsed twice with sterile water. Biofilms were stained with 0.1% crystal violet (Aladdin, China). After 10 min, the excess stain was removed, the wells were rinsed twice with sterile water and crystal violet was dissolved in 200 μL of 33% glacial acetic acid. The absorbance was measured at 595 nm ($OD_{595}$).

To evaluate the effects of **(+)-**nootkatone on preformed biofilm, biofilms were established in 96-well microtiter plate as mentioned above. After 24 h of incubation, different concentrations of **(+)-**nootkatone (50, 100, and 200 μg/mL) were used to evaluate the biofilm eradication studies.

### 2.5. Microscopic analysis of biofilm

Biofilm was developed on sterile glass coverslip placed in 6-well polystyrene culture plates with or without **(+)-**nootkatone. For biofilm removal studies, biofilms were established on coverslips using the protocol described above followed by 24 h treatment with (+)-nootkatone. For light microscopy, the coverslips were stained with 0.1% crystal violet and visualized under Nikon microscope (Japan) (20 × magnification). For Confocal laser scanning microscopy (CLSM), the biofilms were stained with 100 μM of 5,6-Carboxyfluorescein diacetate (CFDA) (Beyotime Biotechnology, China) and visualized under CLSM system (Leica TCS SP2, Wetzlar, Hesse-Darmstadt, Germany). Images were acquired with 1024 × 1024 resolutions and COMSTAT software (Heydorn et al., 2000) was used to quantify the mean biofilm thicknesses (μm).

### 2.6. Exopolysaccharide production

The effect of **(+)-**nootkatone on exopolysaccharide production was studied as described previously with minor modification (Musthafa, Balamurugan, Pandian, & Ravi, 2012). Briefly, 1 mL of *S. aureus* culture ($1 \times 10^6$ CFU/mL) was incubated in 6-well microtiter plates with or without (+)-nootkatone at 37 °C for 24 h. At the end of incubation, the planktonic cells were removed and wells were washed with 0.9% saline solution. Then, the biofilms were harvested by adding 0.9% saline solution (0.5 mL) into the wells followed by scrapping the adhered biofilm with a sterile scraper. To the extracted mixture, 5% phenol (0.5 mL) and concentrated $H_2SO_4$ (2.5 mL) were added and incubated for 1 h in dark. The absorbance was measured at 490 nm. The percentage reduction in exopolysaccharide content of biofilm was calculated by using the following formula:

$$\% \text{ Reduction in exopolysaccharide} = (\text{Control}_{OD490\ nm} - \text{Test}_{OD490\ nm}) / \text{Control}_{OD490\ nm} \times 100$$

### 2.7. Metabolic activity detection by MTT assay

The metabolic activity of biofilms was determined by MTT assay as previously reported (Goswami, Thiyagarajan, Das, & Ramesh, 2014). Briefly, preformed biofilms were incubated with (+)-nootkatone at 37 °C for 24 h. Biofilms were stained with MTT solution (5 mg/mL) for 4 h, dissolved in 100 μL of DMSO, and absorbance at 570 nm was measured.

**Table 1**
Anti-microbial, anti-biofilm, and cytotoxic effects of terpenoids.

| Compounds | | MIC(μg/mL) | MBC(μg/mL) | % Biofilm Inhibition at ½ MIC | % HFF cell viability at 20 μg/mL |
|---|---|---|---|---|---|
| Monoterpenoids | L-Menthol | – | – | – | – |
| | Paeoniflorin | – | – | – | – |
| | Cantharidin | – | – | – | – |
| | Norcantharidin | – | – | – | – |
| Sesquiterpenoids | Artemisinin | – | – | – | – |
| | Costunolide | – | – | – | – |
| | (+)-Nootkatone | 200 | 400 | 99.8 ± 0.60 | 99.8 ± 0.60 |
| | Dihydroartemisinin | – | – | – | – |
| | Parthenolide | 64 | 128 | 19.51 ± 2.07 | 19.51 ± 2.07 |
| | Patchouli alcohol | – | – | – | – |
| | Isoalantolactone | – | – | – | – |
| Diterpenoids | Andrographolide | – | – | – | – |
| | Tanshinone IIA | – | – | – | – |
| | Paclitaxel | – | – | – | – |
| | Tanshinone I | – | – | – | – |
| | Dehydroandrographolide | – | – | – | – |
| | Cryptotanshinone | – | – | – | – |
| | Oridonin | 32 | 64 | 21.57 ± 2.28 | 21.57 ± 2.28 |
| | Sclareolide | – | – | – | – |
| | Isosteviol | – | – | – | – |
| Triterpenoids | Betulinic acid | – | – | – | – |
| | Betulin | – | – | – | – |
| | Limonin | – | – | – | – |
| | Oleanolic acid | 32 | 32 | – | – |
| | Madecassoside | – | – | – | – |
| | Glycyrrhizic acid | – | – | – | – |
| | 18β-Glycyrrhetinic acid | 200 | > 200 | 81.2 ± 2.11 | 81.2 ± 2.11 |
| | Gipsoside | – | – | – | – |
| | Asiatic acid | 16 | 16 | – | – |
| | Ursolic acid | 16 | 16 | – | – |
| | Cycloastragenol | – | – | – | – |
| | Celastrol | 1 | 2 | 20.16 ± 1.79 | 20.16 ± 1.79 |
| | Ginsenoside Rb1 | – | – | – | – |
| | Ginsenoside Rg1 | – | – | – | – |
| | Ginsenoside Re | – | – | – | – |
| | Tubeimoside I | – | – | – | – |
| | Sodium Aescinate | – | – | – | – |
| | Astragaloside IV | – | – | – | – |
| | Panaxadiol | – | – | – | – |
| | Panaxatriol | – | – | – | – |

### 2.8. Motility assay

The sliding motility assay was performed according to Bai et al. (2019). Different concentrations of (+)-nootkatone were incorporated into the TSB plates containing 0.5% agar. Then, 5 μL of overnight culture of *S. aureus* ($1 \times 10^8$ CFU/mL) was placed in the center of the agar plates. The plates were incubated at 37 °C for 24 h and images were taken.

### 2.9. Quantitative real-time PCR

Total RNA was isolated from the control and **(+)**-nootkatone (50 μg/mL) treated *S. aureus* using RNAprep pure Kit (for cell/bacteria), (TIANGEN Biotech (Beijing) Co. Ltd., China). The quality and concentration of extracted RNA samples were assessed using Nano-300 Micro spectrophotometer. The $A_{260}/_{280}$ ratios of extracted RNA were about 2.0, indicating the good quality of them. cDNA was synthesized using PrimeScript™ Reverse Transcriptase reagent Kit (Takara, Japan). Real-time PCR analysis was performed using SYBR® Premix Ex Taq™ master mix (Takara, Dalian, Liaoning, China) for the candidate genes *agrA, icaA, spa* (Ma et al., 2012),16s rRNA (Kang, Liu, Liu, Wu, & Li, 2018), *sarA,* and *RNAIII* (Chen et al., 2016) as previously described (Aguilar et al., 2019). The relative gene expression levels of target genes were calculated by the comparative $2^{-\Delta\Delta Ct}$ method.

### 2.10. Cell cytotoxicity test

Cytotoxicity of **(+)**-nootkatone was evaluated using human foreskin fibroblasts (HFF) cells by MTT assay. HFF cells ($1 \times 10^4$) were inoculated in 96-well plate containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and incubated overnight in a humidified chamber at 37 °C and 5% $CO_2$. Different concentrations of **(+)**-nootkatone ranging from 12.5 to 200 μg/mL were added and incubated further for 24 h at 37 °C. MTT (5 mg/mL) dye was added and incubated for 4 h. DMSO (100 μL) was added to dissolve the formazan crystals and the absorbance at 570 nm was measured.

### 2.11. Statistical analysis

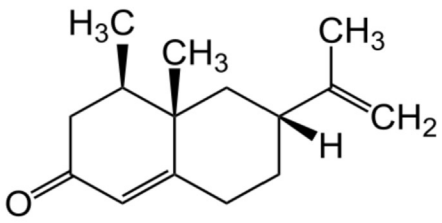
Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, Illinois, USA). Student's *t*-test was used to calculate the significant difference between control and treated samples. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. The anti-staphylococcal activity of (+)-nootkatone

**(+)**-Nootkatone at 200 μg/mL inhibited the visible growth of both *S. aureus* strains. No growth of *S.aureus* was observed on agar plates

A.




B.

| Compound | Bacteria | MIC (μg/mL ) | MBC (μg/mL ) |
|---|---|---|---|
| (+)-Nootkatone | Multidrug-resistant *S. aureus* SJTUF 20758 | 200 | 400 |
| | *S. aureus* ATCC 25923 | 200 | 400 |

C.

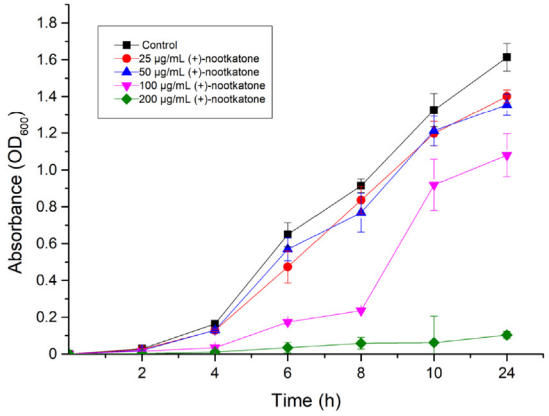



**Fig. 1. Antibacterial effects of (+)-nootkatone against *S. aureus*. (A)** Structure of (+)-nootkatone. **(B)** Anti-microbial effects of (+)-nootkatone against *S.aureus* strains. **(C)** Growth curve analysis of *S. aureus* with or without the presence of various concentrations of (+)-nootkatone.

after incubating with 400 μg/mL of **(+)**-nootkatone, thus 400 μg/mL was considered as the MBC values of both strains (Fig. 2B).

### 3.2. Non-toxic effects of (+)-nootkatone at sub-MICs

**(+)**-Nootkatone at 100 μg/mL slightly inhibited the viability of *S. aureus*. There were no significant differences in bacterial biomass between the untreated control, 25, and 50 μg/mL **(+)**-nootkatone treated *S. aureus*. These findings demonstrated that **(+)**-nootkatone at sub-MICs did not inhibit the viability of bacterial cells (Fig. 1C).

### 3.3. Effects of (+)-nootkatone on biofilm formation

(+)-Nootkatone at 50 and 100 μg/mL inhibited biofilm formation of *S. aureus* by > 90%, whereas, 47% inhibition was observed at 25 μg/mL with no activity seen when 12.5 μg/mL was used (Fig. 2A). Since 100 μg/mL of (+)-nootkatone impeded the bacterial growth, 50 μg/mL of (+)-nootkatone was used for the further biofilm-related studies. Light microscopy and CLSM images showed poor development of biofilm on the glass coverslips after (+)-nootkatone treatment in comparison to untreated control (Fig. 2B and 2C). COMSTAT analysis showed that the mean thickness of biofilm (μm) was decreased after treatment with (+)-nootkatone (0.08 ± 0.02) compared to the control (1.80 ± 0.14). Specifically, 95% reduction in biofilm thickness was observed ($p < 0.05$). A significant reduction (90%) in the exopolysaccharide production was observed in (+)-nootkatone treated *S. aureus* (Fig. 2D).

### 3.4. Effects of (+)-nootkatone on biofilm removal

(+)-Nootkatone was effective in disrupting the preformed biofilm at 200 μg/mL, which was revealed by a reduction in biomass percentage ($p < 0.05$) (Fig. 3A). Light microscopy and CLSM studies showed a reduction in the thickness of biofilm by (+)-nootkatone at 200 μg/mL in comparison to the control (Fig. 3B and 3C). Treatment with 200 μg/mL of (+)-nootkatone resulted in a decrease in the mean thickness (μm) of preformed biofilm (0.55 ± 0.105) compared with the control (2.05 ± 0.004) ($p < 0.05$). A reduction in exopolysaccharide content was observed in preformed *S. aureus* biofilm after the exposure to 200 μg/mL of (+)-nootkatone (Fig. 3D). Furthermore, MTT staining assay indicated a significant reduction in the viability of cells within the *S.aureus* matured biofilm upon treatment with (+)-nootkatone (Fig. 3E), which suggested that (+)-nootkatone could penetrate through the biofilm and cause killing of bacterial cells. The disintegration of biofilm structure after treatment with a higher concentration of (+)-nootkatone might be correlated with a reduction in exopolysaccharide content and a reduced number of viable cells in the biofilm. Collectively, these results showed the potential of (+)-nootkatone in disturbing the architecture of *S. aureus* matured biofilm.

### 3.5. Effects of (+)-nootkatone on bacterial motility

Motility assay indicated that (+)-nootkatone inhibited the sliding motility of *S. aureus* in a dose-dependent manner as observed by a decrease in the distance moved by the bacteria in the presence of varying concentrations of (+)-nootkatone (Fig. 4A).

### 3.6. Effects of (+)-nootkatone on biofilm-related gene expression

As shown in Fig. 4B, (+)-nootkatone significantly suppressed the expression levels of *sarA*, *icaA*, *agrA*, *RNAIII,* and *spa* genes, major genes involved in biofilm formation ($p < 0.05$). These data suggested that

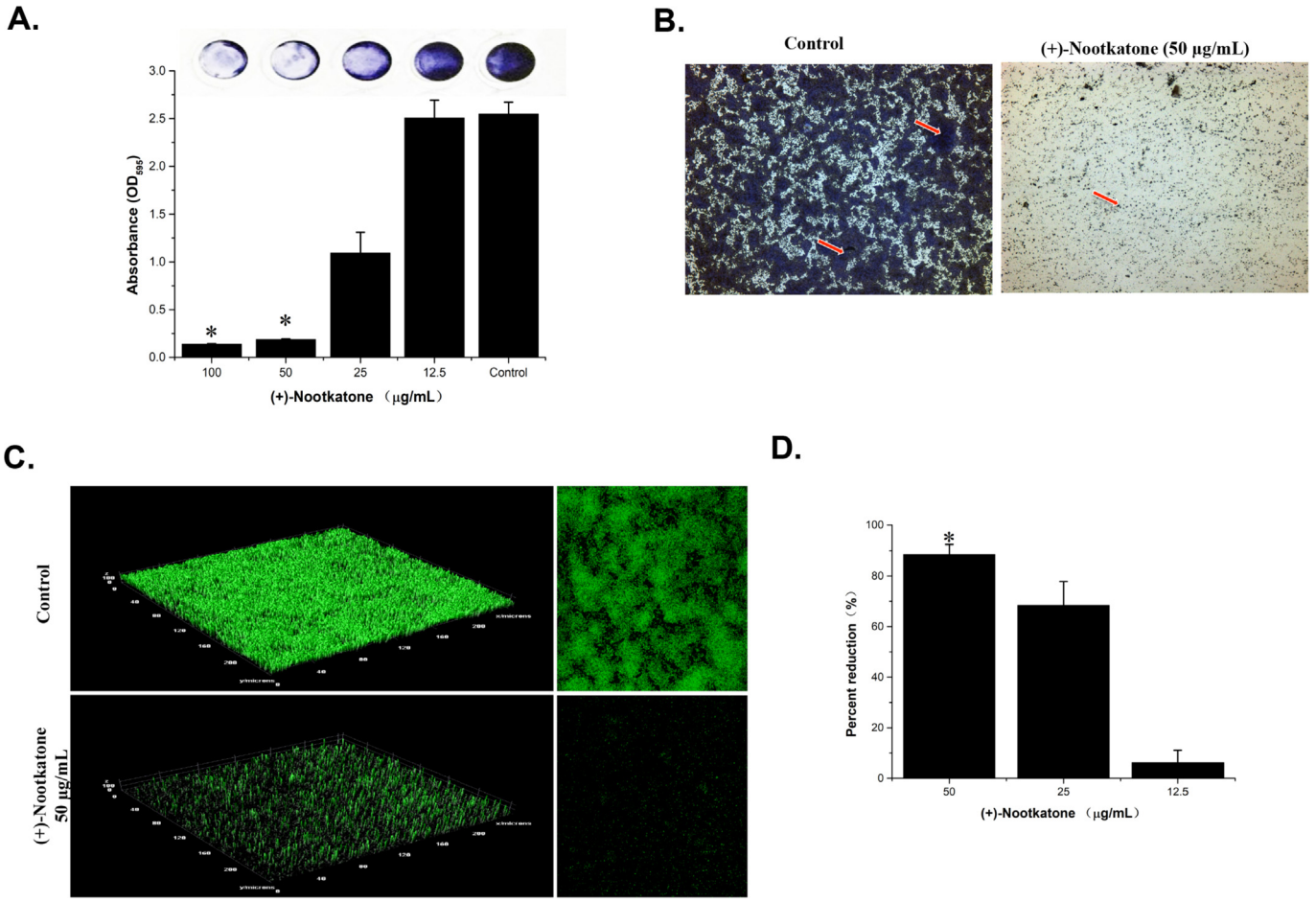



**Fig. 2. Anti-biofilm activity of (+)-nootkatone**. **(A)** Crystal violet staining assay of *S. aureus* treated with various concentrations of (+)-nootkatone. The biofilm was quantified as OD at 595 nm. **(B)** Light microscopic images of biofilm formation inhibitory effect of (+)-nootkatone. **(C)** Confocal laser scanning microscopy images of biofilm formation inhibitory effect of (+)-nootkatone. **(D)** Effect of (+)-nootkatone on exopolysaccharide production. Data represent mean ± S.D. (standard deviation). *p < 0.05, significant difference compared to untreated control.

(+)-nootkatone inhibited biofilm development by modulating the expression of genes that control bacterial quorum sensing, virulence, and biofilm development.

### 3.7. Cytotoxic evaluation of (+)-nootkatone

Up to 50 μg/mL, (+)-nootkatone was non-toxic to normal cells (Fig. 4C). Furthermore, at this concentration (50 μg/mL), (+)-nootkatone effectively inhibited biofilm formation of *S. aureus*.

## 4. Discussion

*S. aureus* is undoubtedly one of the leading foodborne pathogens that cause foodborne outbreaks worldwide (Brahma, Kothari, Sharma, & Bhandari, 2018). *S. aureus* can form biofilms on food and food contact surfaces by adherence, colonization, and development of extracellular matrix composed of polysaccharides, proteins, and eDNA, thereby affecting the quality of food. Importantly, relative to planktonic cells, biofilms are highly resistant to antibiotic treatments. Furthermore, classic antibiotics have failed to target planktonic bacteria within the biofilm because of the difference in metabolic rate among bacteria, nutrient deprivation, and impermeable nature of EPS. In light of this current scenario, inhibition of biofilm formation and eradication is an important strategy for controlling *Staphylococcus* food poisoning.

Consumers always prioritize the use of natural compounds especially phytochemicals as antibacterial agents in real food systems (Gutiérrez-del-Río, Fernández, & Lombó, 2018). Easy availability, safety, and efficacy augment the importance of phytochemicals in the food industry and hence attract special attention. Previous studies showed that phytochemicals have potent antibacterial and anti-biofilm activities (Slobodníková, Fialová, Rendeková, Kováč, & Mučaji, 2016). (+)-Nootkatone is a well-known flavouring agent in grapefruit with bioactive potentials including antioxidant, anti-microbial (Yamaguchi, 2019), antiviral, insecticidal, and neuroprotective (Wang et al., 2018) activities. (+)-Nootkatone has been reported to exhibit antibiofilm effects (Yamaguchi, 2019), but its effects on bacterial motility, biofilm development and eradication, and related molecular mechanisms, particularly against foodborne multidrug-resistant *S. aureus*, remain largely unknown. The current study demonstrated that (+)-nootkatone exhibited antibacterial, anti-motility, and anti-biofilm activities against foodborne multidrug-resistant *S. aureus*.

(+)-Nootkatone effectively inhibited the growth of both multidrug-resistant and standard *S. aureus* strains. At sub-MIC concentrations, (+)-nootkatone caused the inhibition of biofilm formation by reducing the biofilm biomass without affecting the viability of planktonic cells. Notably, biofilm inhibitory concentration of (+)-nootkatone (50 μg/mL) against *S. aureus* was one-fourth of its MIC value (200 μg/mL). It indicated that the reduction in biofilm biomass was mainly due to the anti-biofilm effects rather than the antibacterial effect of (+)-nootkatone. Similar to our findings, some terpenoids such as celastrol (Woo et al., 2017) and geraniol (Kannappan et al., 2017) effectively inhibit the staphylococcal biofilm development. Inhibition of initial attachment of bacteria may restrict the formation of biofilm on the surface. Upon addition of (+)-nootkatone at sub-MICs, the number of *S. aureus*

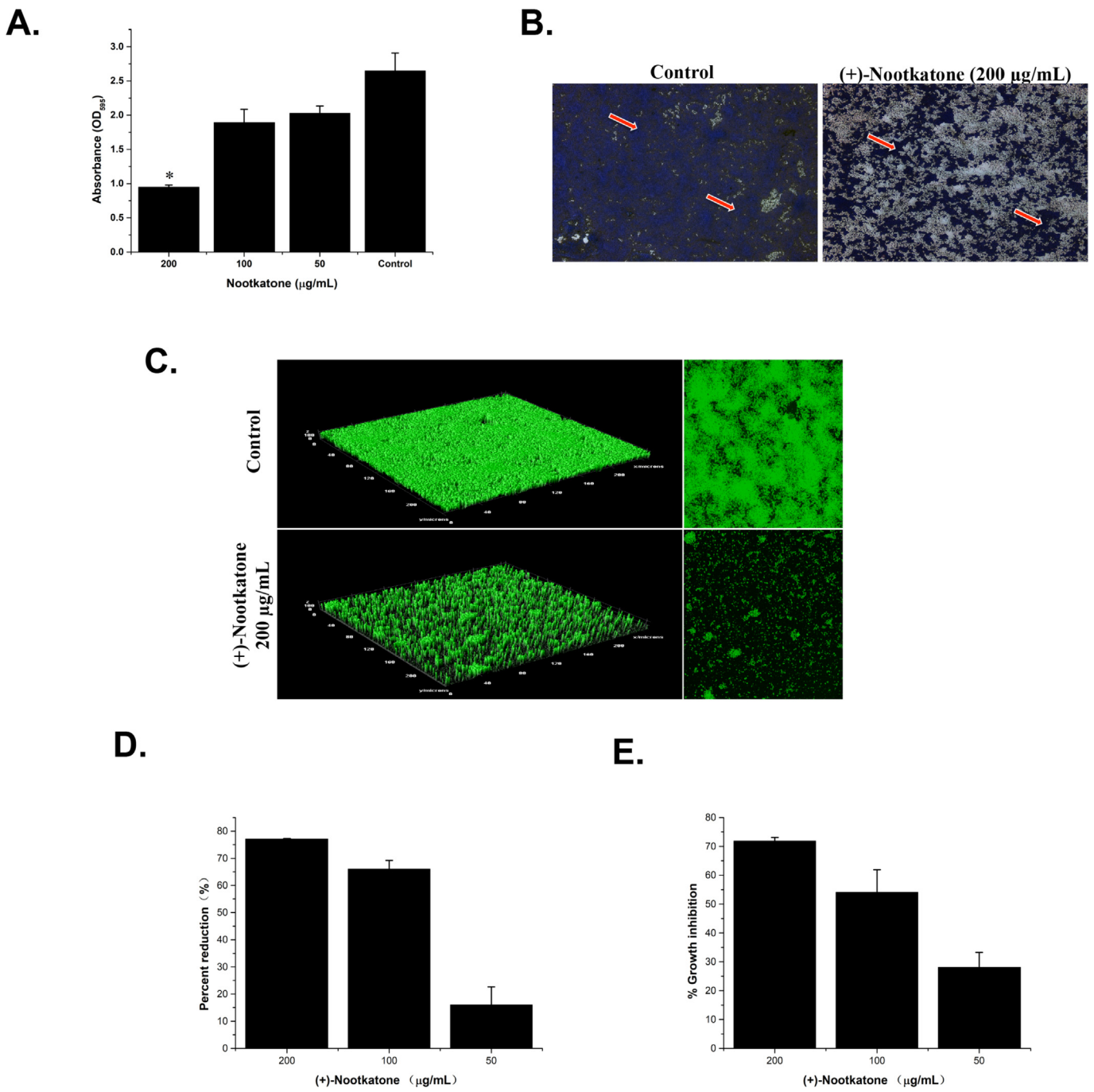



**Fig. 3. Eradication of preformed biofilms by (+)-nootkatone. (A)** Crystal violet staining of preformed staphylococcal biofilms treated with various concentrations of (+)-nootkatone. The biofilm was quantified as OD at 595 nm. **(B)** Light microscopic images of biofilm dispersal effect of (+)-nootkatone. **(C)** Confocal laser scanning microscopy images of biofilm dispersal effect of (+)-nootkatone. **(D)** Effect of (+)-nootkatone on exopolysaccharide content. **(E)** Effect of (+)-nootkatone on the metabolic activity of bacterial cells within the staphylococcal biofilm by MTT assay. Data represent mean ± S.D. (standard deviation). *$p < 0.05$, significant difference compared to untreated control.

cells attached on the surface was gradually decreased. This observation suggests that (+)-nootkatone might have the ability to inhibit the primary attachment of cells on the surface. Motility plays a crucial role in bacterial surface colonization and subsequent formation of biofilms (O'May & Tufenkji, 2011). As the non-flagellated bacteria, *S. aureus* shows sliding motility. Our study indicated that (+)-nootkatone could inhibit the motility of *S.aureus* and thereby limiting biofilm development. Exopolysaccharide, a major contributor to EPS formation, is responsible for the structural integrity and protection required for the survival of planktonic bacteria within the biofilm. Previous reports have shown the ability of phytochemicals to reduce the exopolysaccharide content in *S. aureus* (Al-Shabib et al., 2017). A similar phenomenon was observed in our studies demonstrating that (+)-nootkatone couldprevent the production of exopolysaccharide by *S. aureus*.

Dispersal of existing biofilms by phytochemicals has been described before (Jia, Xue, Duan, & Shao, 2011). An effective anti-biofilm agent should kill the bacteria within the biofilm, reducing the biofilm biomass and modifying the EPS matrix (Skogman, Vuorela, & Fallarero, 2012). EPS can restrict the diffusion of antibiotics, making the bacterial cells within the biofilm more resistant to antibiotics. Compared to planktonic

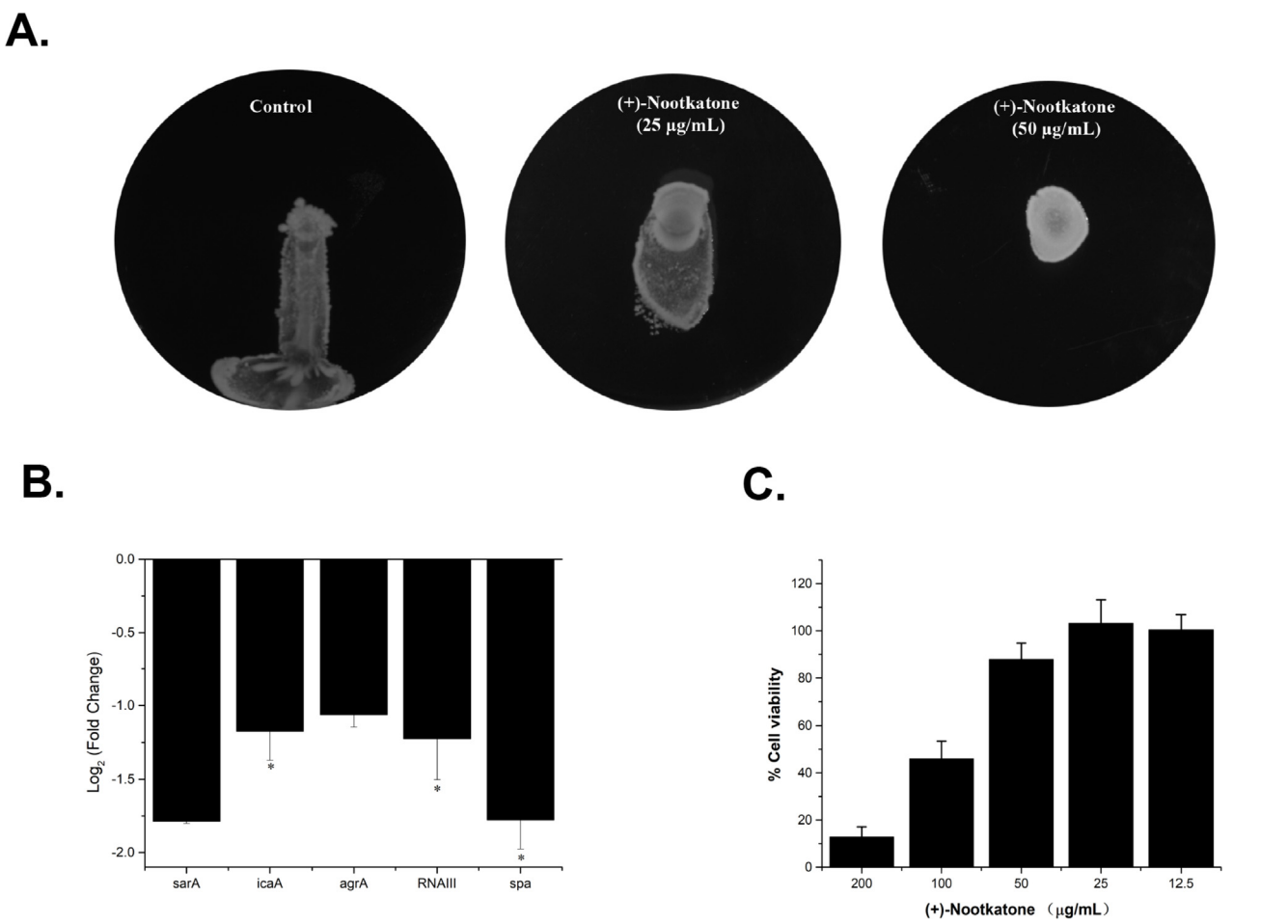



**Fig 4. (+)-Nootkatone inhibited bacterial sliding motility and reduced the expression of genes associated with quorum sensing, virulence, and biofilm development. (A)** Effect of (+)-nootkatone on sliding motility of *S. aureus* (B) Real-time PCR quantification of biofilm associated genes **(C)** Cytotoxicity study of (+)-nootkatone using HFF cell line by MTT assay. Data represent mean ± S.D. (standard deviation). *p < 0.05, significant difference compared to untreated control.

cells, bacterial cells within the biofilm are safer and require higher concentrations of drugs to eliminate them. In the present study, (+)-nootkatone at 200 μg/mL dispersed preformed *S. aureus* biofilm. Interestingly, (+)-nootkatone at 200 μg/mL showed the ability to detach the biofilm from the glass surface, reducing exopolysaccharide content, and killing the bacteria within the biofilm.

The major global regulators, the accessory gene regulator (*agr*) quorum-sensing system and staphylococcal accessory regulator (*sarA*) transcriptional regulator, play an important role in biofilm formation (Beenken et al., 2010). The *agrBDCA* operon consists of two units P2 and P3 which encode RNAII and RNAIII transcripts that produce *agrB, D, C, A* gene products and delta-hemolysin toxin (*hld*) respectively. As an internal effector molecule, *RNAIII* can regulate the expression of agr-controlled virulence factors (Le & Otto, 2015). As a transcriptional activator, *sarA* activates biofilm development by enhancing the transcription of *ica* operon and its mutation reduces biofilm formation (Tormo et al., 2005). The *icaADBC* operon, which encodes four proteins responsible for the production of polysaccharide intercellular adhesion (PIA) or polymeric N-acetyl-glucosamine (PNAG) is involved in biofilm synthesis (Hoang et al., 2019; Ma et al., 2012). N-acetylglucosaminyl-transferase, encoded by *icaA*, is a major factor involved in the synthesis of N-acetylglucosamine oligomers, a major component of PIA (O'Gara, 2007). *Spa* (staphylococcal protein A), one of the major surface proteins, is essential for bacterial aggregation and biofilm development (Merino et al., 2009). Our study demonstrates that (+)-nootkatone treatment down-regulates the expression of *agrA,* and subsequently reduces virulence-related regulator *RNAIII* expression in *S. aureus*. Additionally, (+)-nootkatone reduces *sarA* and *icaA* gene expression, thereby decreasing the PIA production and biofilm formation. As such, (+)-nootkatone suppressed the expression of *spa* gene. Previous studies showed that phytochemicals have the potential to inhibit the activity of global regulators. For instance, phytochemicals like gallic acid (Liu et al., 2017), cinnamaldehyde (Jia et al., 2011), tannic acid (Lee et al., 2013), and so forth have been shown to be excellent anti-biofilm agents, which show their biofilm inhibitory effect in a similar pattern.

Excellent antibacterial activity and safety make the phytochemicals as worthy candidates of antibacterial agents and reinforce their use in the food industry. However, phytochemicals are not acceptable as food additives if they show any toxicity to humans, particularly at higher concentrations. Due to its non-toxic nature, (+)-nootkatone is approved as Generally Recognized As Safe (GRAS) by FDA. In our cytotoxicity study, (+)-nootkatone did not show any measurable toxicity to the HFF cells at a concentration of 50 μg/mL in agreement with recent literature data (Hung et al., 2019). At this concentration, (+)-nootkatone could inhibit 90% biofilm formation and suppress the expression of biofilm-related genes. It's also more feasible to use (+)-nootkatone as an anti-biofilm agent since it cannot show any toxic effects at sub-MIC values.

## 5. Conclusion

The study demonstrated that (+)-nootkatone exhibited remarkable

antibacterial activity against multidrug-resistant foodborne *S. aureus*. Furthermore, (+)-nootkatone prevented the biofilm formation, inhibited the swarming motility, disrupted the preformed biofilms, and killed the bacteria within biofilms of *S. aureus*. Due to its low toxicity and improved antibacterial activity, (+)-nootkatone represents an alternative agent for biofilm inhibition and provides a promising new strategy for combating foodborne outbreaks of *S. aureus*. Future studies are needed to assess the efficacy of (+)-nootkatone as an anti-biofilm agent in the real food system to further strengthen its potential application in the food industry.

## Funding

This work was supported by the National Key R&D Program of China (Grant No. 2017YFC1600100), China Central Public-interest Scientific Institution Basal Research Fund (Grant No.Y2020XK05), the Shanghai Basic and Key Program (Grant No. 18JC1410800), and the Shanghai Pujiang Talent Plan (Grant No. 18PJ1404600).

## CRediT authorship contribution statement

**Arakkaveettil Kabeer Farha:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft. **Qiong-Qiong Yang:** Investigation. **Gowoon Kim:** Investigation. **Dan Zhang:** Investigation. **Vuyo Mavumengwana:** Writing - review & editing. **Olivier Habimana:** Writing - review & editing. **Hua-Bin Li:** Writing - review & editing. **Harold Corke:** Conceptualization, Funding acquisition, Writing - review & editing. **Ren-You Gan:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing.

## Declaration of competing interest

The authors declare no conflict of interest.

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