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ORIGINAL ARTICLE

Streptococcus strain C17^T as a potential probiotic candidate to modulate oral health

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Significance and Impact of the Study: Host tissue adhesion and pathogen inhibition are important selection criteria for bacterial strains with probiotic potential. In this study, we used human bronchial epithelial (HBE) cells as an *in vitro* oropharyngeal mucosal model to screen for potential oropharyngeal probiotics. *Streptococcus* strain C17^T effectively adapted to the oropharyngeal environment and inhibited the pathogen *Staphylococcus aureus* from adhering to the HBE cells. The foregoing results provide a theoretical basis for the development of innovative probiotics that regulate oropharyngeal health.

Keywords
adhesion to 16-HBE, anti-adhesion assay, antimicrobial activity, probiotic, *Staphylococcus aureus*.

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Abstract

In the microbiome, probiotics modulate oral diseases. In this study, *Streptococcus* strain C17^T was isolated from the oropharynx of a 5-year-old healthy child, and its potential probiotic properties were analysed using human bronchial epithelial cells (16-HBE) used as an *in vitro* oropharyngeal mucosal model. The results demonstrated that the C17^T strain showed tolerance to moderate pH ranges of 4–5 and 0·5–1% bile. However, it was more tolerant to 0·5% bile than 1% bile. It also demonstrated an ability to accommodate maladaptive oropharyngeal conditions (i.e. tolerating lysozyme at 200 μg ml^{-1}). It was also resistant to hydrogen peroxide at 0·8 mM. In addition, we found out that the strain possesses inhibitory activities against various common pathogenic bacteria. Furthermore, C17^T was not cytotoxic to 16-HBE cells at different multiplicities of infection. Scanning electron microscopy disclosed that C17^T adhesion to 16-HBE cells. Competition, exclusion and displacement assays showed that it had good anti-adhesive effect against *S. aureus*. The present study revealed that *Streptococcus* strain C17^T is a potentially efficacious oropharyngeal probiotic.

Introduction

Microbial communities exist on all surfaces of the human body including the respiratory mucosa. Specialized bacterial communities inhabit particular sites on the respiratory tract and play important roles in maintaining human health (Man *et al.* 2017). Beneficial bacteria strongly influence host metabolism, nutrition, physiology and immune function (Bustamante *et al.* 2020). Hundreds of microbial species inhabit the human oral cavity (Keijser *et al.* 2018). Most are commensals whilst others are mutual symbionts with oral mucosal barrier functions. They confer resistance to pathogenic bacterial colonization in the host (López-López *et al.* 2017). Oropharyngeal microbiota are dynamic and diverse (Lamont *et al.* 2018). Various risk factors such as bad food habits and poor oral hygiene can alter oral microbiota and disturb the balance between commensal and pathogenic microorganisms (Fan *et al.* 2018). These aberrations may lead to a predominance of opportunistic pathogenic bacteria in the oral cavity that cause pharyngitis, caries, gingivitis and other oral diseases and infections (Lu *et al.* 2018). It is reasonably safe to select bacterial species derived from the oropharynx of healthy humans for use as probiotics (Bidossi *et al.* 2018). Probiotics are living microorganisms that can confer health benefits to the host in the appropriate doses (Jäger *et al.* 2019). Probiotic

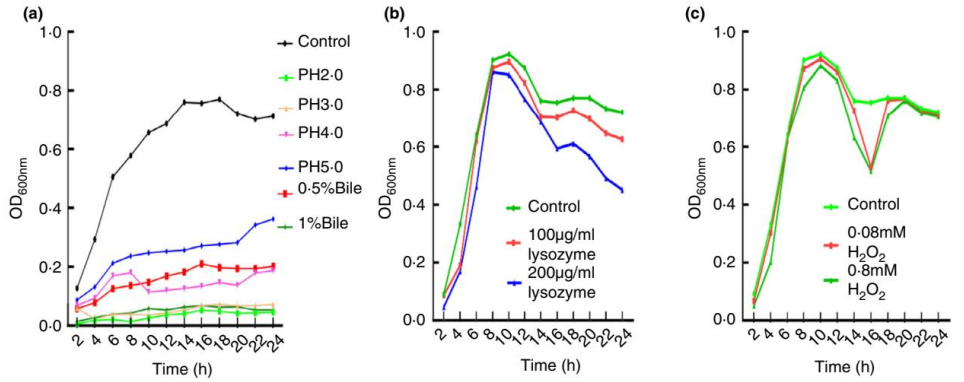

Figure 1 Growth curves plotting *Streptococcus* strain C17^T *vs* incubation time under different conditions. (a) Acidic pH and 0·5% or 1·0% bile. (b) 100 μg ml^{-1} or 200 μg ml^{-1} lysozyme. (c) 0·08 mM or 0·8 mM hydrogen peroxide (H_2O_2). OD = optical density.

consumption maintain bacterial balance and inhibits pathogen growth (Nazir *et al.* 2018).

Alpha-haemolytic streptococci such as *Streptococcus salivarius* and *S. oralis* are isolated from the human pharynx and are early colonizers of the upper respiratory mucosae. Their presence is indicative of healthy oral microflora (Wescombe *et al.* 2009; Sidjabat *et al.* 2016). They have a high affinity for the respiratory mucosae and protect epithelial cells from pathogen adhesion and potential cytotoxicity (Bidossi *et al.* 2018). The ability of probiotics to adhere to host cells is a classic selection criterion. Such probiotics can compete against pathogens for host cell binding sites and inhibit pathogenic bacterial adhesion (Monteagudo-Mera *et al.* 2019). Moreover, the superior adhesion capacity of efficacious probiotics enables them to interact with the host and confer beneficial effects upon it. *Staphylococcus aureus* is a pathogenic bacterium in the oral cavity. It usually causes microbiological dysbiosis and oropharyngeal tract dysfunction (Schenck *et al.* 2016). Hence, the oropharyngeal tract is a potential target for the development of novel probiotic products. This study aimed to investigate the oropharyngeal *Streptococcus isolate* C17^T as a novel candidate probiotic and analyse its potential probiotic properties *in vitro* with the objective of further regulating oral health.

Result and discussion

Resistance to acidic pH, bile, lysozyme and H_2O_2

We studied the potential probiotic properties of *Streptococcus* strain C17^T. A probiotic must resistance oropharyngeal stress conditions to maintain its activity and viability there. Strain survivability at various pH and bile salt concentrations is shown in Fig. 1a. C17^T tolerated moderately acidic pH 4–5 but its growth sharply declined at pH 2–3. C17^T had greater resistance to 0·5% bile than 1% bile. Moreover, it tolerated 0·8 mM H_2O_2 and 200 μg ml^{-1} lysozyme. Hence, it could overcome the hostile conditions of the oropharyngeal tract (Fig. 1b,c). The ideal probiotic strains are derived from humans, devoid of potential virulence genes, catalase-negative and sensitive to ordinary antibiotics; can tolerate oral and digestive tract conditions and are able to adhere to epithelial membranes and compete with other microbes (Nagpal *et al.* 2018). The results of this study indicated that C17^T survived moderately low pH, was resistant to bile salts and catalase and adapted to adverse oropharyngeal conditions.

Antibiotic susceptibility

Table 1 shows the sensitivity of C17^T to nine different antibiotics. C17^T was sensitive to chloramphenicol, vancomycin, clindamycin, linezolid and cefepime; strongly

Table 1 Antibiotic susceptibility of *Streptococcus* strain C17^T

Antibiotic	Dose (μg ml^{-1})	Sensitivity
Chloramphenicol	30	S
Cefepime	30	S
Vancomycin	30	S
Ampicillin	10	R
Cefatriaxone	30	R
Cindamycin	2	S
Linezolid	30	S
Cefotaxime	30	R
Erythromycin	15	I

S = susceptible; I = intermediate (moderately resistant); R = resistant.

Table 2 Inhibition of common pathogens by *Streptococcus* strain C17^T

Pathogens Antagonistic strains	*Streptococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*K. pneumoniae*	*P. vulgaris*	*E. cloacae*	*A. Baumannii*	*S. pyogenes*
C17^T	+++	++	+++	++	+++	++	++	++

+++: bacteriostatic zone diameter ≥ 20 mm; ++: 15 mm ≤ inhibition zone diameter < 20 mm.

resistant to ampicillin, ceftriaxone and cefotaxime and moderately resistance to erythromycin. Antibiotic-resistant bacteria are generally regarded as unsafe for use as probiotics. Here, C17^T was susceptible to most antibiotics. A previous study reported similar findings (Kuebutornye *et al.* 2020).

Antimicrobial activity

Table 2 shows the antagonism of *Streptococcus* strain C17^T to eight common pathogenic bacteria. It was effective against *S. aureus*, *Pseudomonas aeruginosa, Escherichia coli, Streptococcus pneumoniae, P. vulgaris, S. pyogenes, A. baumannii* and *K. pneumoniae*.

Cytotoxicity assay

A key determinant of the probiotic efficacy of bacteria is their ability to adhere to host epithelial cells. Thus, we aimed to determine the toxic effects of probiotics on epithelial cells. Figure 2 shows no significant changes in cell viability when C17^T was co-incubated with human bronchial epithelial cells (16-HBE) at different MOI for 12 h. However, C17^T had >100% viability after incubation for at MOI 0·2 for 24 h. Furthermore, it was not cytotoxic to 16-HBE cells at MOI 2 or MOI 20. These findings resembled the results reported by Jia *et al.* (2019).

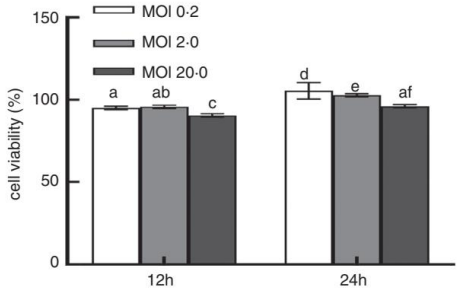

Figure 2 Effect of C17^T on 16-HBE cell proliferation at multiplicities of infection (MOI) = 0.2, 2, and 20. Means with different letters (a–f) differ significantly ($P < 0·05$).

Streptococcus strain C17^T adhesion to 16-HBE cells

Adhesion to host tissue is a crucial selection criterion for candidate probiotics with their own specific functions (Campana *et al.* 2017). Recent studies reported that the microbiome of the lower airway resembles that of the oropharynx (Cruz *et al.* 2014; Chang *et al.* 2015). It is difficult to evaluate adhesion *in vivo*. Hence, *in vitro* assays using human cell lines are widely used (Gopal *et al.* 2001; García-Ruiz *et al.* 2014; Vasiee *et al.* 2019). Here, we applied HBE cells to simulate respiratory epithelium *in vitro*. C17^T exhibited 43% adhesion to the 16-HBE cells. A previous investigation used fluorescein isothiocyanate (FITC) labelling to evaluate the adhesion of the oropharyngeal potential probiotics *Lactobacillus plantarum* AR113, *L. plantarum* AR195, and *L. salivarius* AR809 to a FaDu epithelial cell layer (Jia *et al.* 2019). Of these, *L. plantarum* AR809 and *L. salivarius* AR113 were the most (31·1%) and least (4·4%) adhesive strains, respectively. Bacterial adhesion to host epithelial cell surfaces was characterized by nonspecific physical binding followed by specific adhesion of the cell wall components (Haddaji *et al.* 2015). Scanning electron microscopy (SEM) revealed that C17^T adhered to the 16-HBE cells (Fig. 3). As a rule, adhesion involves interactions among bacteria-related molecular patterns such as surface layer proteins, peptidoglycans and so on (Johnson-Henry *et al.* 2007; Van Tassell

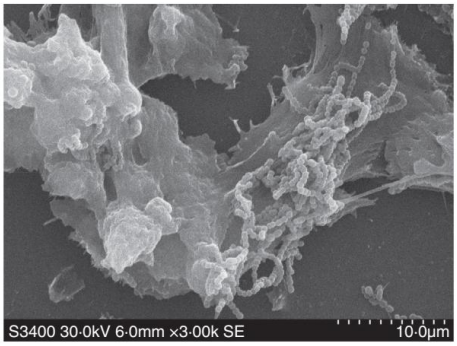

Figure 3 Scanning electron microscopy (SEM) of adherence of *Streptococcus* strain C17^T to 16-HBE cells. Magnification: ×3 000.

and Miller 2011). Certain surface proteins enhance lactic acid bacterial adhesion (Muñoz-Provencio *et al.* 2012; Wlodarska *et al.* 2015). Bacterial cell wall adhesins also participate in epithelial cell binding.

Anti-adhesion effects of *Streptococcus* strain C17^T against *Staphylococcus aureus*

Adhesion is a crucial stage in infection by pathogens requiring hosts to colonize, grow and produce toxins (Falah *et al.* 2019). A vital feature of probiotics is their capacity to prevent pathogen adhesion and colonization on host epithelial cells. Here, competition, exclusion and displacement assays were used to estimate the inhibition of *S. aureus* CGMCC10201 by C17^T. The anti-adhesion properties of C17^T are presented in Fig. 4. The competition assay demonstrated the ability of the probiotic to compete against pathogenic bacteria for epithelial cell adhesion receptors. A significant reduction in *S. aureus* adhesion to 16-HBE cells was observed in the presence of C17^T. The relative rate of adhesion of *S. aureus* to 16-HBE cells was 8·1 ± 2·2%. The exclusion experiment showed that C17^T strongly inhibited *S. aureus* adhesion to 16-HBE cells. The relative rate of adhesion of *S. aureus* to 16-HBE cells was reduced to 9·6 ± 0·5%. The displacement test disclosed that C17^T was significantly antagonistic to *S. aureus* and lowered its adhesion to 16-HBE cells to 31 ± 4·2%. An earlier report evaluated the ability of the probiotic candidate strain Lactobacilli CCMA0743 to inhibit host cell adhesion by the pathogens *Salmonella* and *E. coli* (Fonseca *et al.* 2020). Probiotics may inhibit pathogen adhesion to host cells via competition for nutrition and cell receptors, immunomodulation, co-aggregation, antimicrobial substances such as bacteriocins, hydrogen peroxide, organic acids, polysaccharides, anti-adhesion compounds, degradation of carbohydrate receptors through secreted proteins or biosurfactants (Jayashree *et al.* 2018). The competition assay showed greater inhibition of *S. aureus* by C17^T than the exclusion or displacement assays. Several studies showed that both probiotic and pathogenic bacteria have similar surface adhesins and may, therefore, compete for the same adhesion sites (Chen *et al.* 2007; Gueimonde *et al.* 2007). Probiotics can effectively displace pathogens adhering to epithelial cells provided that the former are present at high concentrations or have greater affinity for adhesion receptors than the latter (Lee *et al.* 2000). Several factors may influence the adhesion ability of both pathogens and probiotics. These include buffer compositions, incubation times, normal intestinal microbiota and food matrices (Ouwehand and Salminen 2009). Anti-adhesion properties also depend on the probiotic and pathogen strains and the evaluation methods used (Jankowska *et al.* 2008; Zhang *et al.* 2016; Falah *et al.* 2019).

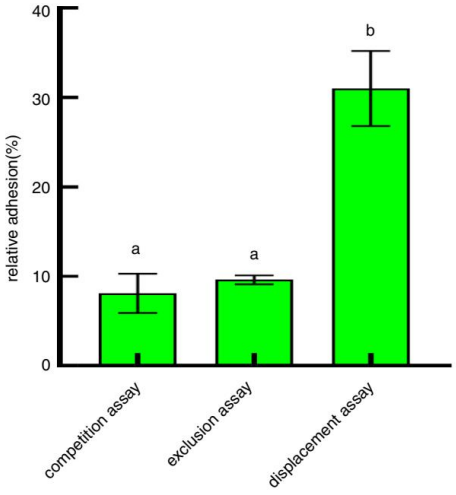

Figure 4 Effects of *Streptococcus* strain C17^T on the adhesion behaviour of *Staphylococcus aureus* to 16-HBE cells. Means with different letters (a–b) are significantly different at $P < 0·05$.

Conclusion

Streptococcus strain C17^T survived in moderately acidic conditions, was resistance to bile salts as well as catalase and adapted well to adverse oropharyngeal conditions. Moreover, it effectively inhibited the adhesion of the pathogen *S. aureus* to HBE cells. Although this strain is highly promising as a novel putative probiotic for the regulation of oral health, further *in vitro* and *in vivo* testing on it is required. Concurrently, we expect the strain to be used in the production of probiotic foods such as powder and chewing gum.

Materials and methods

Bacterial strains and culture conditions

Bacterial samples were collected with pharyngeal swabs. C17^T was obtained from the oropharynx mucosa of a healthy five-year-old child. *S. aureus* CGMCC10201, *P. aeruginosa* CGMCC10104, *E. coli* CGMCC10003, *K. pneumoniae* CGMCC31001, *P. vulgaris* CGMCC1.1651, *E. cloacae* 1.8726, *A. baumannii* CGMCC1.10395 and *S. pyogenes* CGMCC98001 were procured from the China Industrial Strain Preservation Center (Shang hai).

Streptococcus strain C17^T was grown in brain heart infusion (BHI; HyClone Laboratories, Logan, UT, USA) broth at 37°C for 24 h and stored at −40°C until further use. Pathogenic bacteria used in the adhesion inhibition assay were cultured in BHI broth for 6–8 h.

Isolation and identification of the strain

Using the culturomics approach, the novel *Streptococcus* strain C17^T was isolated from the oropharyngeal mucosa of a healthy 5-year-old child in Shenyang, China. The strain was an inactive, aerobic and catalase-negative Gram-positive coccus. A 16srRNA gene sequencing analysis indicated that this bacterium was a new member of the genus *Streptococcus* and showed 99·8% similarity with the newly identified *S. pseudopneumoniae* ATCC BAA-960T. The dDDH between C17^T and its closest strain was 52·9%. The average nucleotide homology (ANI) between C17^T and its related *Streptococcus* strains was in the range of 82·21–93·40%. Hence, C17^T was unique among all streptococci named *Streptococcus symcisp*. nov. (=GDMCC 1.1633 = JCM 33582). The 16S rRNA sequence of C17^T was deposited in GenBank under accession no. MN068913.1 (Qi *et al.* 2021).

Resistance to acid and bile salts

The pH tolerance assay was slightly modified from the method reported by Jia *et al.* (2019). Overnight C17^T cultures with density = 10^8 CFU ml^{-1} were either inoculated into BHI broth at 3% (v/v) and adjusted to 2·0, 3·0, 4·0 and 5·0 with 1 M HCl or inoculated into BHI broth containing 0·5% (w/v) and 1% (w/v) bile. Uninoculated BHI broth (pH 7·0) served as the control. The bacteria were incubated at 37°C for 24 h. Absorbance was spectrophotometrically (UV-6800,5%, Lichen Technology Co., LTD, China) measured at 600 nm every 2 h.

Resistance to lysozyme and H_2O_2

The effects of lysozyme and H_2O_2 were determined by spectrophotometry as previously described (Jia *et al.* 2019), with minor modifications. Overnight C17^T cultures corresponding to a density of 10^8 CFU ml^{-1} were inoculated into BHI broth at 3% (v/v) and incubated at 37°C. Then C17^T was subjected to lysozyme (100 μg ml^{-1} and 200 μg ml^{-1}) or hydrogen peroxide (H_2O_2; 0·08 mM and 0·8 mM) for 24 h and absorbance was spectrophotometrically measured at 600 nm every 2 h. BHI broth alone was the control.

Antibiotic susceptibility testing

The antibiotic susceptibility of C17^T was determined by disc diffusion assays according to the 2018 recommendations of the Clinical Laboratory Standards Institute (Qi *et al.* 2021). Colonies were selected and cultured for 24 h on agar plates containing 5% (w/v) defibrinated sheep blood and suspended in 5 ml sterile normal saline solution with turbidity = 0·5 McFarland. The bacterial fluid was applied to M-H medium with sterile cotton swabs. The antibiotics tested were chloramphenicol (30 μg), cefepime (30 μg), vancomycin (30 μg), ceftriaxone (30 μg), ampicillin (10 μg), clindamycin (2 μg), erythromycin (15 μg), cefotaxime (30 μg) and linezolid (30 μg). All assays were performed in triplicate.

Antimicrobial activity

C17^T was cultured on 5% (w/v) defibrinated sheep blood plate for 24 h. Monoclonal colonies were inoculated into BHI liquid medium and cultured at 37°C and 180 rpm for 24 h. The final concentration was adjusted to 1×10^9 CFU ml^{-1}. The indicator strains were incubated to the log phase and their concentration was adjusted to 1×10^6 CFU ml^{-1}. The Oxford cup method (Bhola and Bhadekar 2019) was used to observe the inhibition of C17^T on the indicator strains. One hundred microlitres indicator bacteria suspension was applied to each agar plate with sterile cotton swabs. Four Oxford cups were placed at equal distances on the agar plates with sterile tweezers. Then 200 μl fermentation liquid from each antagonistic strain was placed in two holes, and equal amounts of BHI medium were added to the other two holes as a control. The culture plate was then kept in a 37°C incubator for 18 h. There were three replicate plates per indicator strain.

Cell culture assays

The 16-HBE cell line was procured from the laboratory of Shenyang Medical College, Liaoning Province, China. The cells were cultured and maintained in Endothelial Cell Medium (ECM; HyClone) containing 5% (v/v) foetal bovine serum, 1% (w/v) penicillin/streptomycin (P/S) solution, and 1% (w/v) endothelial cell growth supplement (ECGs) at 37°C under a 5% CO2 atmosphere. The 16-HBE cells were then lifted from a 25-cm^2 culture bottle containing 0·25% (v/v) trypsin-EDTA solution (Sigma-Aldrich Corp., St. Louis, MO, USA) at 37°C for 13 min and collected by centrifugation (4 000 ***g***, 4°C, 1 min). The 16-HBE cells were subcultured in a six-well tissue culture plate at a density of 8×10^4/well and grown at 37°C with under 5% CO_2 for 3 days until confluence.

Cytotoxicity assay

The cytotoxicity of C17^T to 16-HBE was determined by the colourimetric assay with 3-(4,5-dimethylthiazol-2

-yl)-2,5-diphenyltetrazolium bromide (MTT) (Jia *et al.* 2019). The 16-HBE cells were prepared in a 96-well tissue culture plate at a density of 8×10^4/well until confluence. Then C17^T was added at MOI (bacteria: HBE cell) = 0·2:1, 2:1, and 20:1 before co-incubation at 37°C under 5% CO_2 for 24 h. Then 10 µl MTT (5 mg ml^{-1}) was added to each well, and the suspensions were incubated at 37°C under 5% CO_2 for 4 h. Then 150 µl dimethyl sulfoxide (DMSO) was added to each well and the formazan crystal product was completely dissolved by shaking for 10 min. The absorbance (A) of each well was measured in a microplate reader (TECAN Infinite200PRO, Beijing Long yue Biological Technology Development Co. LTD, USA) at 570 nm. Cell viability was calculated as follows:

$$\text{Cell viability } (\%) = (A_{sample}/A_{control}) \times 100 \quad (1)$$

where A_{sample} is the absorbance of the 16-HBE cells co-incubated with C17^T and $A_{control}$ is the absorbance of C17^T alone.

Adhesion assay

The 16-HBE cells were prepared in a six-well tissue culture plate at a density of 8×10^4/well. The cells were inoculated at 37°C and under 5% CO_2 until confluence. One millilitre bacterial suspension (10^8 CFU ml^{-1}) was added to a confluent 16-HBE monolayer and incubated at 37°C and under 5% CO_2 for 2 h (Chen *et al.* 2018). The unbound bacteria were gently removed with phosphate-buffered saline (PBS; pH 7·4). The remaining bacteria and 16-HBE cells detached with 0·25% (v/v) trypsin-EDTA solution at 37°C for 13 min, diluted with sterile PBS and coated on a 5% (w/v) defibrinated sheep blood plate for enumeration(Singh *et al.* 2017). C17^T adhesion was calculated as follows:

$$\text{Adhesion } (\%) = [(\text{Adhered bacteria/initial bacteria})] \times 100 \,(\text{Falah et\_al.2019}) \quad (2)$$

Scanning electron microscopy

After the adhesion of C17^T to the 16-HBE cell monolayer was assessed, the latter were cultured on sterile glass coverslips in a six-well tissue plate for 7 days. After 2 h incubation, the 16-HBE monolayers were washed thrice with PBS (pH 7·4) and fixed with 2·5% (w/v) glutaraldehyde at 25°C for 2 h. Post-fixation was performed with 2% (w/v) osmium tetraoxide at 4°C overnight followed by rinsing thrice with PBS, dehydration in a graded ethanol concentrations series (50, 70, 80, 90, 95 and 100% ethanol) and subjected to SEM(LEO, Germany, model VP 1450,20 ~ 20 000).

Anti-adhesion activity of C17^T against *Staphylococcus aureus*

Three different assays (competition, exclusion and displacement) were used to evaluate the ability of C17^T to inhibit *S. aureus* adhesion. For the competition assay, 1 ml each C17^T (1×10^9 CFU ml^{-1}) and *S. aureus* suspension (1×10^6 CFU ml^{-1}) were co-incubated with a 16-HBE monolayer at 37°C under 5% CO_2 for 2 h. For the exclusion assay, a 16-HBE monolayer was preincubated with 1 ml C17^T suspension (1×10^9 CFU ml^{-1}) for 1 h, and the unbound bacteria were washed thrice with PBS (pH 7·4). Then 1 ml *S. aureus* (1×10^6 CFU ml^{-1}) was added, and the suspension was incubated for 1 h. The displacement assay was performed in the same manner as the aforementioned adherence exclusion assay. *S. aureus* (1×10^6 CFU ml^{-1}) was added to a 16-HBE monolayer and incubated for 1 h. Unbound bacteria were removed and C17^T (1×10^9 CFU ml^{-1}) was added, and the suspension was incubated for 1 h. Separate *S. aureus* cultures served as the control.

For all the foregoing experiments, the unbound bacteria were removed with sterile PBS and subjected to 0·25% (v/v) trypsin-EDTA solution for 13 min to detach the 16-HBE cells. Serial dilutions were then plated onto blood agar to enumerate the *S. aureus* (Piwat *et al.* 2015; Singh *et al.* 2017).

Statistical analysis

All experiments were conducted independently at least in triplicate. Data were presented as mean ± standard deviation (SD). Data were analysed by one-way ANOVA, and Duncan's test was used to compare the overall differences ($P < 0·05$).

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Conflict of Interest

Not required.

Author contributions

CX: Conceptualization (lead); WZ: Resources (lead); Data Curation (lead); Formal Analysis (lead); Writing – original draft (lead); Writing – review and editing (lead); BY: Methodology (equal); Formal Analysis (equal); Writing – original draft (equal); XB: Methodology (equal); Formal

Analysis (equal); Writing – original draft (equal); NW: Methodology (equal); Resources (equal); Data Curation (equal); HT: Methodology (equal); Validation (equal); Writing – original draft (equal). All authors read and approved the final manuscript.

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