Odontology (2020) 108:376–385
https://doi.org/10.1007/s10266-019-00478-8

ORIGINAL ARTICLE

A universal adhesive incorporating antimicrobial peptide nisin: effects on *Streptococcus mutans* and saliva-derived multispecies biofilms

Manduo Zhao[1] · Yang Qu[1] · Jia Liu[1] · Sui Mai[1] · Lisha Gu[1]

Received: 15 May 2019 / Accepted: 17 November 2019 / Published online: 7 January 2020

Abstract

For purpose of enhancing the antibacterial activity of a universal adhesive, the antimicrobial peptide nisin was incorporated into Single Bond Universal and its antibacterial effect on *Streptococcus mutans* monospecific biofilms and saliva-derived multispecies biofilms was studied. Nisin was incorporated into Single Bond Universal and the antibacterial activity was examined by confocal laser scanning microscopy (CLSM), reverse transcription-quantitative polymerase chain reaction (qRT-PCR), phenol–sulfuric acid method and lactate dehydrogenase enzymatic method. The bonding properties were tested by microtensile bond strength (μTBS) and degree of conversion (DC). Data were analyzed by one-way analysis of variance (ANOVA) and least significant difference multiple comparison tests ($P < 0.05$). The Single Bond Universal incorporated with 3% (w/v) nisin could significantly inhibit the growth of the *S. mutans* monospecific biofilms ($P < 0.01$) and decrease the expression of genes related to extracellular polysaccharide (EPS) synthesis (*gtfB, gtfC, gtfD* and *spaP*) and acidogenicity (*ldh*) ($P < 0.05$). 3% (w/v) nisin-incorporated Single Bond Universal could also inhibit the growth of saliva-derived multispecies biofilms and decrease the excretion of EPS and lactic acid ($P < 0.05$). μTBS and DC of 3% (w/v) nisin-incorporated Single Bond Universal did not deteriorate obviously ($P > 0.05$). In conclusion, 3% (w/v) nisin-incorporated Single Bond Universal substantially inhibited the growth of both *S. mutans* monospecific and saliva-derived multispecies biofilms without compromising the bonding properties.

Keywords Universal adhesive · Antimicrobial peptide · Single Bond Universal · Nisin · Saliva-derived multispecies biofilms

Introduction

Dental adhesives and resin composites have been long and extensively used to cure dental caries for their esthetics and strength [1]. However, failure of restorations in the long term is a major concern worldwide [2–5]. Secondary caries is the most common reason for restorations failure [6]. The shrinkage of dental adhesives during polymerization may lead to microcracks between the adhesives and the tooth tissue [7]. Cariogenic bacteria in the saliva, such as *Streptococcus mutans* (*S. mutans*), can reproduce in these microcracks, initiating biofilms formation and producing acid, which leads to secondary caries [8–10]. Therefore, inhibition of biofilm formation and acid production is an effective way to reduce secondary caries, expanding the life span of restorations and reducing the economic burden.

Introducing antibacterial components into dental adhesives is a common strategy to depress cariogenic biofilm accumulation [7, 11–14]. Nisin, an antimicrobial peptide produced by *Lactococcus lactis*, has aroused interest as a potential additional component in the dental adhesives, because it has antimicrobial effects on cariogenic bacteria with negligible cytotoxicity to human cells in the oral cavity [15, 16]. In addition, the acidic environment of dental adhesives can enhance the stability and antibacterial activity of nisin [17, 18]. In our pilot study [19], nisin additive shows an effective and durable inhibitive effect on *Streptococcus*

Manduo Zhao and Yang Qu authors contributed equally to this work.

✉ Sui Mai
maisui@mail.sysu.edu.cn

✉ Lisha Gu
gulisha@mail.sysu.edu.cn

[1] Guangdong Provincial Key Laboratory of Stomatology, Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, 56 Lingyuanxi Road, Yuexiu District, Guangzhou, Guangdong Province, China

mutans of a 5th generation etch-and-rinse dental adhesive. Although the aforementioned protocol yields promising results, monospecific biofilms are rare under natural conditions [20]. Dental plaque is composed of multiple microorganisms, thus multispecies biofilms should be examined from a clinical perspective. In addition, with the development of dental adhesives, the 8th generation universal adhesives were introduced to simplify bonding procedures and techniques [21, 22]. The antimicrobial property of the nisin-incorporated 8th generation dental adhesives will provide it with more clinical significance. As a representative of the 8th generation universal adhesives, Single Bond Universal (3 M, St. Paul, MN, USA) is one of the most commonly used adhesives in the clinical practice. To the best of our knowledge, so far there is no literature regarding the antibacterial effect on the saliva-derived multispecies biofilms of dental adhesives with the antimicrobial pepetide nisin-incorporated. In this paper, influences of nisin additive on the antibacterial activities of *S. mutans* monospecific biofilms and saliva-derived multispecies biofilms of Single Bond Universal were reported. The null hypotheses tested were as follows: (1) nisin-incorporated Single Bond Universal could inhibit *S. mutans* monospecies biofilms and saliva-derived multispecies biofilms; (2) nisin-incorporated Single Bond Universal could decrease the expression of virulence genes of *S. mutans*; and (3) incorporation of nisin into Single Bond Universal would not comprise its bonding properties.

Materials and methods

Flowchart of experimental process

For purpose of studying the antibacterial activity and bonding properties of nisin-incorporated universal adhesives, a flowchart of experimental process was designed, as shown in Fig. 1. Detailed experimental materials and methods are described below.

Preparation of nisin-incorporated adhesive specimens

Nisin-incorporated dental adhesives were prepared using nisin (Sigma-Aldrich, St. Louis, MO, USA) and Single Bond Universal adhesive (3 M, St. Paul, MN, USA). Nisin additives were mixed into the Single Bond Universal adhesives, and the mixtures were vibrated by means of a vertical oscillator to get a homogeneous dispersion system. The mixing and vibrating process were carried out under the room temperature and a light proof condition. Three experimental concentrations of nisin [1, 2 and 3% (w/v)] and a blank-control group [0% (w/v)] were selected for further tested.

Dental composite Z250 (3 M, St. Paul, MN, USA) was light-cured to fabricate wafer-like samples with the size of 15 mm in diameter and 1 mm in thickness. 10-μL of each group adhesive was spread onto the top of the Z250 samples, with soft air-blowing and light-curing for 10 s. All specimens were immersed in distilled phosphate buffer saline (PBS) and agitated for 2 h to remove un-polymerized monomers. Then, the adhesive samples were disinfected under ultraviolet light for 20 min in preparation for the following tests.

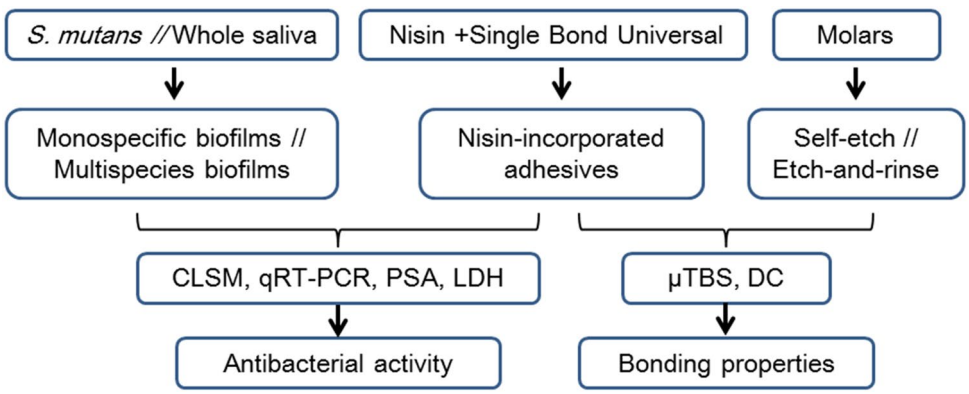

Fig. 1 Flowchart of the experimental process. Nisin-incorporated dental adhesives were prepared. For one thing, *S. mutans* was cultured and whole saliva was collected to form biofilms on the specimens, respectively. Then CLSM, qRT-PCR, PSA and LDH were applied to examine the antibacterial activity of nisin-incorporated adhesives. For another, the collected molars were prepared according to the etch-and-rinse mode or the self-etch mode. Then, μTBS and DC were applied to examine the bonding properties of nisin-incorporated adhesives

Effect on the *S. mutans* monospecific biofilms

Streptococcus mutans UA 159 was cultured overnight in Brain Heart Infusion (BHI) broth (Sigma-Aldrich, St. Louis, MO, USA) anaerobically (10% H_2, 5% CO_2, and 85% N_2; Forma Scientific, Inc., Marietta, OH) at 37 °C. The bacterial suspensions were diluted with 1% BHIS (BHI solution supplemented with 1% sucrose) to 10^6–10^7 CFU/ml.

The effect of nisin-incorporated adhesives on *S. mutans* monospecific biofilm formation and extracellular polysaccharide (EPS) synthesis was explored by confocal laser scanning microscopy (CLSM; CLSM 710, Zeiss, Jena, Germany). *S. mutans* suspensions were put into a 24-well cell culture plate in which three adhesive specimens from each group had been put inside in advance. Alexa Fluor 647-labeled dextran conjugate (Invitrogen Corp., Carlsbad, CA, USA) was added to the culture medium at a concentration of 1 μM for labeling EPS. After incubation at 37 °C anaerobically for 24 h, the specimens were gently rinsed with sterilized PBS for labeling of live and dead bacteria. Under a light proof condition, the specimens were inoculated with 100 μL of Live/Dead Baclight Bacterial Viability Stain (Kit L7012, Invitrogen, Carlsbad, CA, USA) and incubated for 15 min. After the reaction, each biofilm was observed using CLSM, with three random area with size of 500 × 500 μm being scanned. All three-dimensional reconstructions of biofilms were performed with Imaris 7.0.0 software (Bitplane, Zürich, Switzerland). The biovolumes of live and dead cells were quantified from the entire stack using COMSTAT image-processing software (http://www.imageanalysis.dk) [23]. The biovolume represented the volume of the biomass (μm^3) in the observation field (μm^2).

Expression of genes related to EPS synthesis (*gtfB*, *gtfC*, *gtfD* and *spaP*), acidogenicity (*ldh*) and aciduricity (*atpD* and *ffh*) of *S. mutans* biofilms cultured on the surface of nisin-incorporated adhesive specimens were tested with quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Three specimens from each group were used in the test. The specimens were incubated with *S. mutans* suspensions and BHIS in a 12-well plate as described above. After 24 h, the biofilms were harvested by centrifugation at 12,000 rpm for 5 min, and the total ribonucleic acid (RNA) was isolated by ultrasonic crushing with a RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). The total RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA, USA). Reverse transcription of the total RNA and quantitative PCR were performed to quantify the mRNA expression of *gtfB, gtfC, gtfD*, *spaP*, *ldh*, *atpD* and *ffh*, with the 16S rRNA gene as an internal control. The primer sequences were based on the literature and are listed in Table 1 [24–27]. The $2^{-\Delta\Delta Ct}$ method was used to calculate the fold changes in *S. mutans* gene expression.

Effect on the saliva-derived multispecies biofilms

Whole saliva was collected under the ethics-approved protocol of the Hospital of Stomatology, Sun Yat-sen University. Informed consent was obtained from all individual participants included in the study. For each repeated assay, five different donors with no evidence of present caries [decayed and missing (DMT) = 0] were included. All the donors had natural dentition and had not used antibiotics in the past 3 months. The donors did not brush their teeth or take any food or drink for at least 4 h prior to saliva donation [20]. Two milliliters of whole saliva was collected from each donor and pooled [28] to form saliva-derived multispecies dental biofilms.

The effect of the nisin-incorporated adhesives on the multispecies biofilm formation was explored by CLSM. The specimens were incubated at 37 °C for 24 h in a 24-well plate with 900 μL BHIS and 100 μL mixed saliva, as prepared previously. Live/dead assays were performed as described in the "Effect on the *S. mutans* monospecific biofilms" Section.

To further investigate the effect of the nisin-incorporated adhesives on the metabolism of the saliva-derived multispecies biofilms, phenol–sulfuric acid method (PSA) [23, 29] and lactate dehydrogenase enzymatic method (LDH) [13] were used to measure EPS synthesis and lactic acid production, respectively. For both assays, six adhesive specimens

Table 1 Nucleotide sequence of primers used for qPCR

Gene	Primer sequence (5′–3′)		Product length (bp)
	Forward	Reverse	
gtfB	ACACTTTCGGGTGGCTTG	GCTTAGATGTCACTTCGGTTG	127
gtfC	CCAAAATGGTATTATGGCTGTCG	GAGTCTCTATCAAAGTAACGCAGT	135
gtfD	TTGACGGTGTTCGTGTTGAT	AAAGCGATAGGCGCAGTTTA	219
spaP	TCCGCTTATACAGGTCAAGTTG	GAGAAGCTACTGATAGAAGGGC	121
ldh	AAAAACCAGGCGAAACTCGC	CTGAACGCGCATCAACATCA	255
atpD	TGTTGATGGTCTGGGTGAAA	TTTGACGGTCTCCGATAACC	176
ffh	TGGGAATGGGAGACTTGCTTA	GCTCGGAGTTAGGAGGTCAG	305

were tested for each group. The specimens were incubated at 37 °C for 72 h in 24-well plates. To assess EPS synthesis, the specimens were rinsed twice with PBS, and the sessile cells were collected using a sterile centrifuge tube and resuspended in 2 mL PBS followed by centrifugation for 2 min at 12,000 *g*. The precipitate was resuspended in 200 µL of deionized water, and 200 µL of 5% phenol and 1 mL of 95% sulfuric acid were then added. The solution was mixed by pipetting and incubated for 30 min. The $OD_{490\ nm}$ of the solution was determined by a spectrophotometer. Standard curves were prepared using standard glucose solutions. The concentration of EPS was determined by comparing the $OD_{490\ nm}$ with the standard curves. To assess lactic acid production, the specimens were washed twice with PBS to remove any planktonic bacteria and placed in 1.5 mL buffered peptone water (BPW; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.2% sucrose. After incubation at 37 °C for 3 h, the lactate concentrations in BPW were determined by measuring OD_{340nm} and compared to that of the lactic acid standard curves. Standard curves were prepared with a standard lactic acid.

Bonding properties

Bond strength of nisin-incorporated adhesives was tested using microtensile methods (µTBS) [30]. Adhesives-bonded teeth were prepared under the above ethics-approved protocol. Third molars with no caries were collected from informed donors. The collected molars were randomly separated into two groups, with 90 molars in each group, according to the bonding systems, namely the etch-and-rinse mode and the self-etch mode [31].

For the etch-and-rinse group, a 35% phosphoric acid etchant (Select Hv Etch, Bisco, Inc. Schaumburg, IL, USA) was applied for etching each dentin surface for 15 s. A separate etchant was not used for the self-etch bonding group. The experimental adhesives were then applied to the dentin surface and light-cured for 10 s. Then Dental composite Z250 (3 M, St. Paul, MN, USA) was added to form a 4-mm thick block and light-cured for 40 s. The composite–dentin specimens were stored in PBS at 37 °C for 24 h. Rectangular bar-shaped microtensile samples (0.9 × 0.9 × 8 mm) were cut from the above composite–dentin specimens with the resin–dentin interface in the middle. Microtensile tests were carried out with a tensile tester (Bisco, Schaumburg, IL, USA). The tensile strain rate is 0.5 mm/min till the specimen was broken. The µTBS values were calculated by dividing the breaking load by the cross-sectional area [21].

Degree of conversion (DC) of the nisin-incorporated adhesives was evaluated using Fourier transform infrared spectroscopy (FTIR, VECTOR-22, Bruker, Ettlingen, Germany) [32]. Specimens for each group were analyzed by applying the adhesives (20 µL) onto a potassium bromide disc, and the spectra before and after light-curing for 10 s were recorded. The DC% was calculated using the equation below [13]:

$$\mathrm{DC}\ (\%)=\left[1-\left\{\frac{\mathrm{Ac_{aliphatic}}/\mathrm{Ac_{aromatic}}}{\mathrm{Au_{aliphatic}}/\mathrm{Au_{aromatic}}}\right\}\right]\times 100\%,$$

where $Ac_{aliphatic}$ and $Au_{aliphatic}$ refer to the absorbance intensities of the aliphatic C=C bond peak at 1638 cm^{-1} of the cured and uncured specimens, respectively, and $Ac_{aromatic}$ and $Au_{aromatic}$ refer to the absorbance intensities of aromatic C=C bond peak at 1608 cm^{-1} of the cured and uncured specimens, respectively.

Statistical analyses

All experiments were performed in at least triplicate. All data were analyzed with GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, California) using one-way analysis of variance (ANOVA) and least significant difference (LSD) multiple comparison tests. Significance was set at a P value of 0.05.

Results

S. mutans monospecific biofilms characteristics

S. mutans biofilm was observed using CLSM, and three-dimensional images were reconstructed (Fig. 2a). The EPS is labeled blue, while the live bacteria are green, and the dead bacteria are red. The biofilm accumulation on the surfaces of the nisin-incorporated adhesive specimens was further evaluated by the biomass of both total bacteria and EPS. The total bacteria were calculated as the biomass sum of live and dead bacteria. According to Fig. 2b, the total bacteria in all experimental groups were reduced ($P < 0.01$), and it decreased in a dose-dependent manner. The biovolume of EPS, calculated as the biomass in the observation field, was quantified and illustrated in Fig. 2c. When the concentration of nisin was 3%, the total bacteria and the EPS production were decreased by 70.76% and 72.38%, respectively, compared with the control group ($P < 0.01$).

Figure 3 shows the effect of nisin-incorporated adhesives on the mRNA levels of *S. mutans* biofilm virulence factors. Compared with the levels observed in *S. mutans* biofilms grown on the blank-control specimens, the expression levels of EPS synthesis genes (*gtfB, gtfC, gtfD* and *spaP*) and an acidogenicity-related gene (*ldh*) were significantly decreased ($P < 0.01$) after exposure to 3% (w/v) nisin-incorporated adhesives for 24 h. In contrast, the expression levels of aciduricity-related genes (*atpD* and *ffh*) were not decreased significantly in the 3% nisin group.

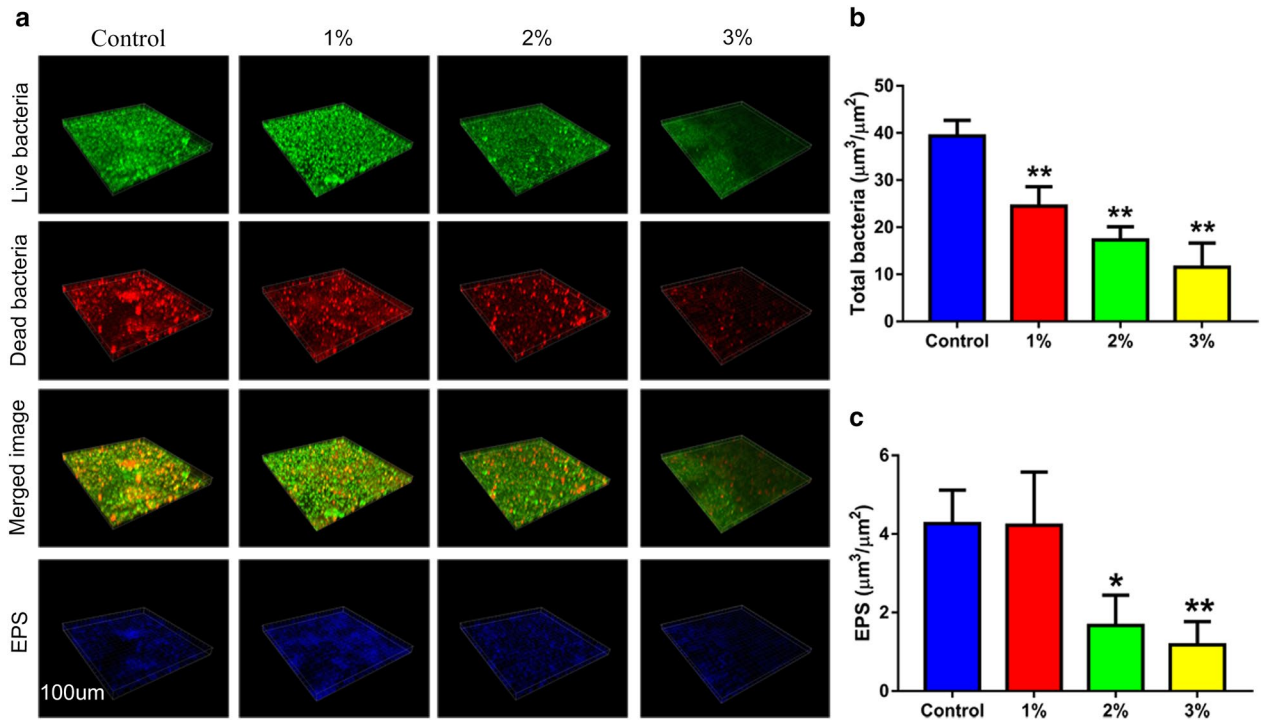

Fig. 2 CLSM images of *S. mutans* biofilms ($n=3$ samples per group). Each micrograph represents four optical sections **a** green (representing live bacteria), red (representing dead bacteria), yellow (representing the combined green and red from two channel images), and purple (representing EPS). The total bacterial and the EPS are quantified in panel **b** and **c**. Statistical differences compared with the control group are indicated with asterisks (*$P<0.05$; **$P<0.01$) (color figure online)

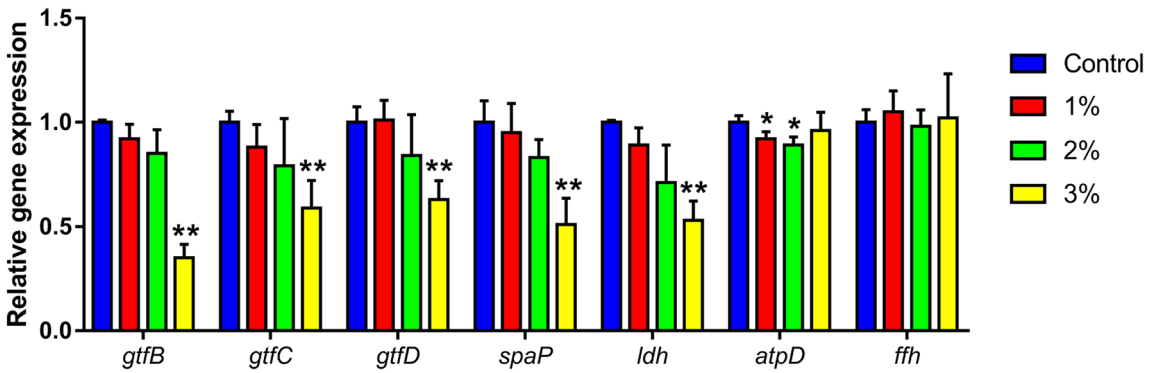

Fig. 3 qPCR of virulence genes of *S. mutans* bioflims. All targets were amplified using primers. Different gene expression levels were normalized to the level of 16S RNA gene transcripts. Statistical differences compared with the blank-control groÂup are indicated with asterisks (*$P<0.05$; **$P<0.01$)

Saliva-derived monospecific biofilms characteristics

Three-dimensional images of the 24 h saliva-derived multispecies biofilms were reconstructed in Fig. 4a. Quantization revealed that the total bacteria in the 3% nisin-incorporated adhesives were reduced by 65.70% compared with the control group ($P<0.05$, Fig. 4b), while the total bacteria did not decrease significantly at the concentration of 1% and 2%.

Figure 5 manifests the metabolism changes in the multispecies biofilms. With the increase in the nisin concentration in the adhesives, the excretion of EPS and lactic acid in the biofilms decreased. The EPS in the 3% group was decreased by 72.37% ($P<0.05$), and the production of lactic acid in the 2% and 3% groups was decreased by 25.67% and 29.84%, respectively ($P<0.05$).

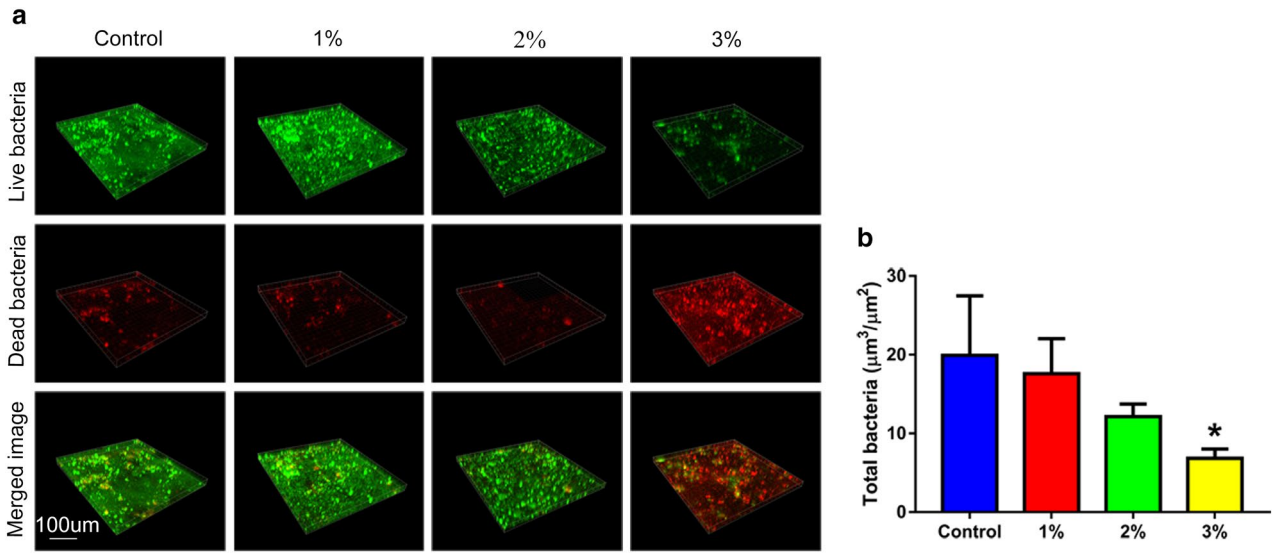

Fig. 4 CLSM images of saliva-derived multispecies biofilms ($n=3$ samples per group). Three optical sections are shown in each micrograph **a** green (representing live bacteria), red (representing dead bacteria), and yellow (representing the combined green and red from two channel images). The total bacterial is quantified in panel **b**. Statistical differences compared with the control group are indicated with asterisks ($P<0.05$) (color figure online)

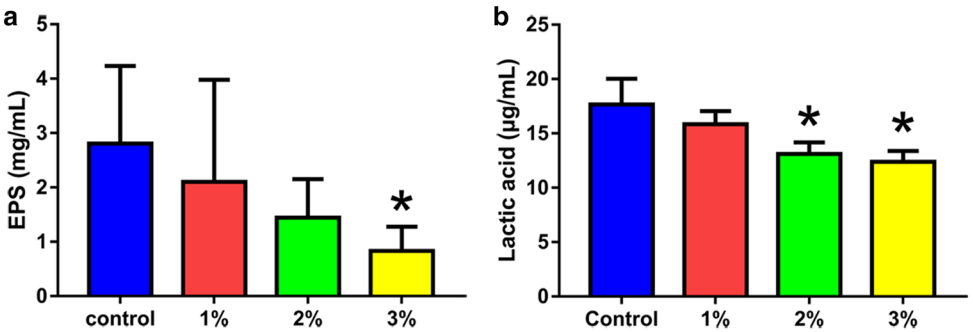

Fig. 5 Metabolism changes in saliva-derived multispecies biofilms. **a** EPS synthesis. **b** lactic acid production. Statistical differences compared with the control group are indicated with asterisks ($P<0.05$)

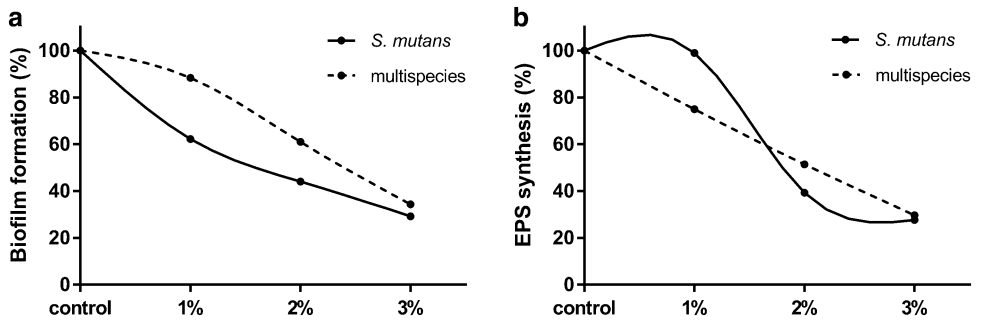

Fig. 6 Effects of nisin-incorporated adhesive specimens on the biofilm formation and the EPS synthesis of *S. mutans* and saliva-derived multispecies biofilms. Data were obtained by means of CLSM observation, COMSTAT image-processing and PSA. The percentage was calculated by the data of experimental groups divided by the data of blank control

Comparison between *S. mutans* and saliva-derived multispecies biofilms

To compare the effect of nisin-incorporated adhesives on *S. mutans* and saliva-derived multispecies biofilms, the biofilm formation and EPS synthesis are illustrated in Fig. 6. Compared with the blank control, 1% and 2% (w/v) nisin-incorporated adhesives exerted greater inhibitory effect on the formation of *S. mutans* biofilms than multispecies biofilms. However, when the concentration reached to 3%, the reduction rate in biofilm formation of *S. mutans* and multispecies biofilms were very close (70.76 ± 0.11% and 65.70 ± 0.05%). When comes to EPS synthesis, the reduction rate of *S. mutans* biofilms was approximately a constant, and at the concentration of 3%, the reduction rate of *S. mutans* and multispecies biofilms were very close (72.38 ± 0.11% and 72.37 ± 0.21%), which is consistent with the trend in the biofilm formation.

Bonding properties

The results of μTBS test are shown in Fig. 7. Twenty-eight specimens were tested in each subgroup. For both groups, the μTBS of 1–3% nisin subgroup did not decrease significantly compared to the control group ($P > 0.05$). However, the μTBS of both bonding mode groups drop rapidly when the concentration exceeds 3%, suggesting that the concentration of less than 3% (w/v) will not endanger the bond strength of Single Bond Universal.

Figure 7c presents FTIR results for the nisin-incorporated adhesives before (left) and after (right) light-curing. In the control group without nisin additive, the intensities of the peaks related to the stretching mode of the aliphatic C=C bond and aromatic C=C bond at 1638 cm^{-1} (number 1) and 1608 cm^{-1} (no. 2) increased after light-curing. Besides, no new peeks were observed before and after light-curing. Similar spectra were obtained in the groups with nisin additive at various concentrations. Based on the FTIR spectra calculation, the DCs for the blank control, 1%, 2% and 3% experimental groups were 47.52 ± 0.82%, 58.99 ± 0.93%, 41.54 ± 0.95% and 36.84 ± 0.52%, respectively.

Discussion

In the present study, nisin was incorporated into the 8th generation adhesive Single Bond Universal without compromising the bonding properties. Based on the literature [33, 34], nisin is a cationic antimicrobial peptide that can interact with the anionic lipids on the cytoplasmic membrane of bacterial cells, which conducive to its antibacterial activity. It can either bind to the nonspecific lipids on the cytoplasmic membranes or to the specific Lipid II, a crucial component of the cell membranes of Gram-positive bacteria [35–38]. The interaction causes pore formation on the cytoplasmic membranes and the efflux of vital components of bacteria, which leads to the cell death. Hence, nisin has more efficient antibacterial effect on Gram-positive bacteria.

As shown in our results, nisin-incorporated adhesives inhibited the biofilm formation and EPS synthesis of *S. mutans* monospecific biofilms. And the inhibitory effect increased as the nisin concentration increased. This result is in accordance with the literature [35, 36] that the nisin can inhibit *S. mutans* in a dose-dependent manner. *S. mutans* plays a vital role from the initial stage to the advanced stage of the biofilm formation, and is the main etiological microorganism involved in the dental caries [39, 40]. Therefore, the inhibition of *S. mutans* is a key objective in the prevention of secondary caries. EPS, produced by *S. mutans*, constitues a strong and sticky framework in the dental plaques and the decrease of EPS adds to the evidence of the loss of biofilm structure [41, 42].

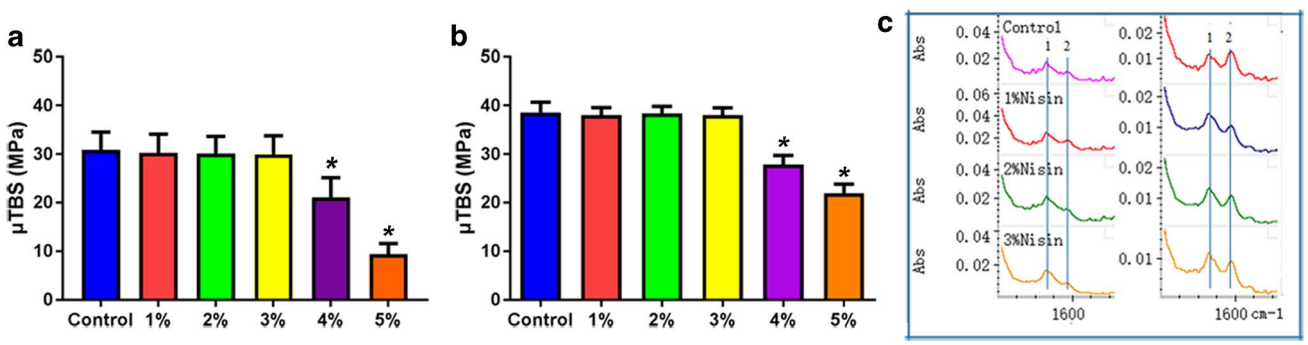

Fig. 7 Bonding properties. **a** μTBS in the self-etch mode. **b** μTBS in the etch-and-rinse mode. **c** FTIR graphs. Number 1 refers to the absorbance intensities of the aliphatic C=C bond peak at 1638 cm^{-1}, and number 2 refers to the absorbance intensities of aromatic C=C bond peak at 1608 cm^{-1}. Statistical differences compared with the control group are indicated with asterisks (*$P < 0.0001$)

Nevertheless, under natural conditions, monospecific biofilms are rare. Dental plaque is a dynamic complex composed of multispecies microorganisms with interspecies and intraspecies interactions [20, 42]. The bacterial species alter constantly throughout the formation of biofilms [40]. To mimic the situations in vivo, whole saliva was collected as an inoculum and incubated on the surface of the nisin-incorporated adhesive specimens. Similar trends were observed with the saliva-derived multispecies biofilms. The biofilm formation, EPS synthesis and lactic production were all inhibited by the nisin-incorporated adhesives at the concentration of 3%. Thus, the first null hypothesis cannot be rejected. EPS synthesis and acidogenicity promote biofilm formation and demineralization of the teeth, thus leading to the formation of caries in teeth [43, 44]. The results indicated that the cariogenic ability of the multispecies bacteria in the saliva was decreased and the components in the saliva will not disturb with the antibacterial activity of nisin [16]. Although there are more than 700 bacterial species in the oral cavity, most of the cariogenic bacteria are Gram-positive ones [45]. As the antimicrobial peptide nisin generally has a strong antibacterial effect on Gram-positive bacteria, the cariogenic bacteria may be specially targeted, which leads to the loss of cariogenic ability in the saliva.

To better understand the antibacterial mechanism of nisin-incorporated adhesives on the dental biofilms, the expression profiles of virulence genes of *S. mutans* were examined and the comparison of the antibiofilm effect on the monospecific and multispecies biofilms was also made. The cariogenic virulence of *S. mutans* is due to its acidogenic, aciduric, and EPS synthetic ability [44]. Thus, the down-regulation of the EPS synthesis genes *gtfB, gtfC, gtfD* and *spaP* and the acidogenicity-related gene *ldh* in the results implied that the cariogenic potential of *S. mutans* might be inhibited, which is consistent with observation in other tests mentioned above. Accordingly, the second null hypothesis cannot be rejected. Peschel et al. [46] stated that nisin might have other antibacterial activities based on the cytoplasmic membrane interactions, such as inhibition of cell wall biosynthesis and activation of autolytic enzymes. Nevertheless, there is little information about the anti-virulence activities of nisin against *S. mutans* [24], which should be further explored in the future studies. Furthermore, the comparison of the antibiofilm effect on *S. mutans* and saliva-derived multispecies biofilms was made. According to Fig. 6, the reduction rate of EPS in *S. mutans* biofilms of increasing concentration of nisin is approximately a constant, while the reduction rate of multispecies biofilms is not. This phenomenon might indicate that the mechanism beneath the inhibitory effect on the EPS synthesis is much more complicated in the multispecies biofilms than in the monospecific biofilms due to the intraspecies interactions. As a cationic peptide, nisin can inhibit the coaggregation of multispecies bacteria, thus disturbing the colonization and development of biofilms [47]. Nevertheless, further studies are needed to clarify the mechanisms of the interactions between nisin and oral biofilm species.

The bonding properties of nisin-incorporated Single Bond Universal were also examined for its future clinical applications. For both self-etch and etch-and-rinse modes, the μTBS of 1–3% nisin-incorporated adhesives showed no significant difference in comparison with the control group, indicating that 1–3% nisin-incorporated adhesives would not adversely affect the bond strength. For a given nisin concentration, the μTBS of the etch-and-rinse mode is always higher than that of the self-etch mode. This is because in the etch-and-rinse system, 37.5% phosphoric acid can eliminate the smear layer and demineralize enamel to induce a robust surface that enhances the mechanical and chemical bonding, while in the self-etch system, the smear layer is partly solved by acidic monomers in the universal adhesives [48, 49]. The etching ability of phosphoric acid is stronger than that of the acidic monomers in the adhesive, and consequently, the resin tags in the demineralized layer are thicker [50]. Hence, the bond strength of the etch-and-rinse system is usually better than that of the self-etch system [51, 52].

DC examination was supplemented by FTIR to get an insight on how nisin influence the bonding properties. Relative DC value reflects the polymerization degree of the monomers contained in experimental adhesives [53]. The higher the DC value is, the higher the bond strength of the adhesives might be. The maximum appears when the concentration of nisin is 1%. As there are C=C bonds in both nisin molecules and resin monomers [54], these controversial findings may be due to the ring being opened for both components and reacted during light-curing. Furthermore, in the FTIR spectra, no new peeks were found before and after light-curing. Therefore, the nisin molecules were possibly bound non-covalently to the resin monomers. But overall, the concentration of nisin within 3% has little effect on the relative DC value, which provides a plausible explanation for the observation of μTBS.

Within the limitation of the present study, 3% (w/v) nisin-incorporated Single Bond Universal achieved substantial antibacterial effects on both *S. mutans* monospecific and saliva-derived multispecies biofilms without compromising the bonding properties, indicating that the nisin-incorporated Single Bond Universal can be a potential antibacterial adhesive. Further investigations of secondary caries animal models are necessary to determine the potential of nisin-incorporated dental adhesives as therapeutic agents against secondary caries in the clinical practice.

Acknowledgements This study was supported by the National Natural Science Foundation of China (Grant No. 81873712) and the Science Foundation of Guangzhou (Grant No. 201904010057).

Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Hospital of Stomatology, Sun Yat-sen University (Grant No. ERC-[2016]-43) and with the 1964 Helsinki Declaration and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Tyas MJ, Anusavice KJ, Frencken JE, Mount GJ. Minimal intervention dentistry–a review. FDI Commission project 1–97. Int Dent J. 2000;50(1):1–12.
2. Demarco FF, Correa MB, Cenci MS, Moraes RR, Opdam NJ. Longevity of posterior composite restorations: not only a matter of materials. Dent Mater. 2012;28(1):87–101. https://doi.org/10.1016/j.dental.2011.09.003.
3. Elderton RJ. The prevalence of failure of restorations: a literature review. J Dent. 1976;4(5):207–10.
4. Van Dijken JW, Pallesen U. Posterior bulk-filled resin composite restorations: a 5-year randomized controlled clinical study. Br Dent J. 2016;221(7):409. https://doi.org/10.1038/sj.bdj.2016.731.
5. Moraschini V, Fai CK, Alto RM, Dos Santos GO. Amalgam and resin composite longevity of posterior restorations: a systematic review and meta-analysis. J Dent. 2015;43(9):1043–50. https://doi.org/10.1016/j.jdent.2015.06.005.
6. Kopperud SE, Tveit AB, Gaarden T, Sandvik L, Espelid I. Longevity of posterior dental restorations and reasons for failure. Eur J Oral Sci. 2012;120(6):539–48. https://doi.org/10.1111/eos.12004.
7. Dutra-Correa M, Leite A, de Cara S, Diniz IMA, Marques MM, Suffredini IB, Fernandes MS, Toma SH, Araki K, Medeiros IS. Antibacterial effects and cytotoxicity of an adhesive containing low concentration of silver nanoparticles. J Dent. 2018;77:66–71. https://doi.org/10.1016/j.jdent.2018.07.010.
8. Totiam P, Gonzalez-Cabezas C, Fontana MR, Zero DT. A new in vitro model to study the relationship of gap size and secondary caries. Caries Res. 2007;41(6):467–73. https://doi.org/10.1159/000107934.
9. Bourbia M, Ma D, Cvitkovitch DG, Santerre JP, Finer Y. Cariogenic bacteria degrade dental resin composites and adhesives. J Dent Res. 2013;92(11):989–94. https://doi.org/10.1177/0022034513504436.
10. Kramer N, Mohwald M, Lucker S, Domann E, Zorzin JI, Rosentritt M, Frankenberger R. Effect of microparticulate silver addition in dental adhesives on secondary caries in vitro. Clin Oral Investig. 2015;19(7):1673–81. https://doi.org/10.1007/s00784-014-1396-x.
11. Wu T, Li B, Zhou X, Hu Y, Zhang H, Huang Y, Xu HHK, Guo Q, Li M, Feng M, Peng X, Weir MD, Cheng L, Ren B. Evaluation of novel anticaries adhesive in a secondary caries animal model. Caries Res. 2018;52(1–2):14–21. https://doi.org/10.1159/000481832.
12. Zhang K, Wang S, Zhou X, Xu HH, Weir MD, Ge Y, Li M, Wang S, Li Y, Xu X, Zheng L, Cheng L. Effect of antibacterial dental adhesive on multispecies biofilms formation. J Dent Res. 2015;94(4):622–9. https://doi.org/10.1177/0022034515571416.
13. Brandao NL, Portela MB, Maia LC, Antonio A, Silva V, Silva EMD. Model resin composites incorporating ZnO-NP: activity against *S. mutans* and physicochemical properties characterization. J Appl Oral Sci. 2018;26:e20170270. https://doi.org/10.1590/1678-7757-2017-0270.
14. Garcia IM, Leitune VCB, Visioli F, Samuel SMW, Collares FM. Influence of zinc oxide quantum dots in the antibacterial activity and cytotoxicity of an experimental adhesive resin. J Dent. 2018;73:57–60. https://doi.org/10.1016/j.jdent.2018.04.003.
15. Tong Z, Ni L, Ling J. Antibacterial peptide nisin: a potential role in the inhibition of oral pathogenic bacteria. Peptides. 2014;60:32–40. https://doi.org/10.1016/j.peptides.2014.07.020.
16. Shin JM, Ateia I, Paulus JR, Liu H, Fenno JC, Rickard AH, Kapila YL. Antimicrobial nisin acts against saliva derived multi-species biofilms without cytotoxicity to human oral cells. Front Microbiol. 2015;6:617. https://doi.org/10.3389/fmicb.2015.00617.
17. Cerrutti P, Terebiznik MR, Huergo MSD, Jagus R, Pilosof AMR. Combined effect of water activity and pH on the inhibition of *Escherichia coli* by Nisin. J Food Prot. 2001;64(10):1510–4. https://doi.org/10.4315/0362-028x-64.10.1510.
18. Rollema HS, Kuipers OP, Both P, De Vos WM, Siezen RJ. Improvement of solubility and stability of the antimicrobial peptide nisin by protein engineering. Appl Environ Microbiol. 1995;61(8):2873–8.
19. Su M, Yao S, Gu L, Huang Z, Mai S. Antibacterial effect and bond strength of a modified dental adhesive containing the peptide nisin. Peptides. 2018;99:189–94. https://doi.org/10.1016/j.peptides.2017.10.003.
20. Huang X, Zhang K, Deng M, Exterkate RAM, Liu C, Zhou X, Cheng L, Ten Cate JM. Effect of arginine on the growth and biofilm formation of oral bacteria. Arch Oral Biol. 2017;82:256–62. https://doi.org/10.1016/j.archoralbio.2017.06.026.
21. Munoz MA, Luque I, Hass V, Reis A, Loguercio AD, Bombarda NH. Immediate bonding properties of universal adhesives to dentine. J Dent. 2013;41(5):404–11. https://doi.org/10.1016/j.jdent.2013.03.001.
22. Mena-Serrano A, Kose C, De Paula EA, Tay LY, Reis A, Loguercio AD, Perdigao J. A new universal simplified adhesive: 6-month clinical evaluation. J Esthet Restor Dent. 2013;25(1):55–69. https://doi.org/10.1111/jerd.12005.
23. Xie T, Liao Z, Lei H, Fang X, Wang J, Zhong Q. Antibacterial activity of food-grade chitosan against Vibrio parahaemolyticus biofilms. Microb Pathog. 2017;110:291–7. https://doi.org/10.1016/j.micpath.2017.07.011.
24. Wang Y, Wang X, Jiang W, Wang K, Luo J, Li W, Zhou X, Zhang L. Antimicrobial peptide GH12 suppresses cariogenic virulence factors of Streptococcus mutans. J Oral Microbiol. 2018;10(1):1442089. https://doi.org/10.1080/20002297.2018.1442089.
25. Zeng H, Liu J, Ling J. Efflux inhibitor suppresses *Streptococcus mutans* virulence properties. FEMS Microbiol Lett. 2017. https://doi.org/10.1093/femsle/fnx033.
26. Cao L, Zhang ZZ, Xu SB, Ma M, Wei X. Farnesol inhibits development of caries by augmenting oxygen sensitivity and suppressing virulence-associated gene expression inStreptococcus mutans. J Biomed Res. 2017;31(4):333–43. https://doi.org/10.7555/jbr.31.20150151.
27. Zhou Y, Millhouse E, Shaw T, Lappin DF, Rajendran R, Bagg J, Lin H, Ramage G. Evaluating *Streptococcus mutans* Strain Dependent Characteristics in a Polymicrobial Biofilm Community. Front Microbiol. 2018;9:1498. https://doi.org/10.3389/fmicb.2018.01498.
28. Kolderman E, Bettampadi D, Samarian D, Dowd SE, Foxman B, Jakubovics NS, Rickard AH. L-arginine destabilizes oral multi-species biofilm communities developed in human saliva.

PLoS One. 2015;10(5):e0121835. https://doi.org/10.1371/journal.pone.0121835.
29. Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S, Lee YC. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal Biochem. 2005;339(1):69–72. https://doi.org/10.1016/j.ab.2004.12.001.
30. Sauro S, Osorio R, Watson TF, Toledano M. Assessment of the quality of resin-dentin bonded interfaces: an AFM nano-indentation, muTBS and confocal ultramorphology study. Dent Mater. 2012;28(6):622–31. https://doi.org/10.1016/j.dental.2012.02.005.
31. Thanaratikul B, Santiwong B, Harnirattisai C. Self-etch or etch-and-rinse mode did not affect the microshear bond strength of a universal adhesive to primary dentin. Dent Mater J. 2016;35(2):174–9. https://doi.org/10.4012/dmj.2015-109.
32. Eshmawi YT, Al-Zain AO, Eckert GJ, Platt JA. Variation in composite degree of conversion and microflexural strength for different curing lights and surface locations. J Am Dent Assoc. 2018;149(10):893–902. https://doi.org/10.1016/j.adaj.2018.06.004.
33. Mai S, Mauger MT, Niu LN, Barnes JB, Kao S, Bergeron BE, Ling JQ, Tay FR. Potential applications of antimicrobial peptides and their mimics in combating caries and pulpal infections. Acta Biomater. 2017;49:16–35. https://doi.org/10.1016/j.actbio.2016.11.026.
34. Hagiwara A, Imai N, Nakashima H, Toda Y, Kawabe M, Furukawa F, Delves-Broughton J, Yasuhara K, Hayashi SM. A 90-day oral toxicity study of nisin A, an anti-microbial peptide derived from *Lactococcus lactis* subsp. lactis, in F344 rats. Food Chem Toxicol. 2010;48(8–9):2421–8. https://doi.org/10.1016/j.fct.2010.06.002.
35. Tong Z, Dong L, Zhou L, Tao R, Ni L. Nisin inhibits dental caries-associated microorganism in vitro. Peptides. 2010;31(11):2003–8. https://doi.org/10.1016/j.peptides.2010.07.016.
36. Tong Z, Zhou L, Jiang W, Kuang R, Li J, Tao R, Ni L. An in vitro synergetic evaluation of the use of nisin and sodium fluoride or chlorhexidine against Streptococcus mutans. Peptides. 2011;32(10):2021–6. https://doi.org/10.1016/j.peptides.2011.09.002.
37. Tong Z, Zhang L, Ling J, Jian Y, Huang L, Deng D. An in vitro study on the effect of free amino acids alone or in combination with nisin on biofilms as well as on planktonic bacteria of Streptococcus mutans. PLoS One. 2014;9(6):e99513. https://doi.org/10.1371/journal.pone.0099513.
38. Shin JM, Gwak JW, Kamarajan P, Fenno JC, Rickard AH, Kapila YL. Biomedical applications of nisin. J Appl Microbiol. 2016;120(6):1449–65. https://doi.org/10.1111/jam.13033.
39. Li M, Huang R, Zhou X, Qiu W, Xu X, Gregory RL. Effect of nicotine on cariogenic virulence of Streptococcus mutans. Folia Microbiol (Praha). 2016;61(6):505–12. https://doi.org/10.1007/s12223-016-0465-8.
40. Selwitz RH, Ismail AI, Pitts NB. Dental caries. The Lancet. 2007;369(9555):51–9. https://doi.org/10.1016/S0140-6736(07)60031-2.
41. Klein MI, Hwang G, Santos PH, Campanella OH, Koo H. Streptococcus mutans-derived extracellular matrix in cariogenic oral biofilms. Front Cell Infect Microbiol. 2015;5:10. https://doi.org/10.3389/fcimb.2015.00010.
42. Sutherland IW. Biofilm exopolysaccharides: a strong and sticky framework. Microbiology. 2001;147(1):3–9. https://doi.org/10.1099/00221287-147-1-3.
43. Xiao J, Klein MI, Falsetta ML, Lu B, Delahunty CM, Yates JR 3rd, Heydorn A, Koo H. The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. PLoS Pathog. 2012;8(4):e1002623. https://doi.org/10.1371/journal.ppat.1002623.
44. Banas JA. Virulence properties of Streptococcus mutans. Front Biosci. 2004;9:1267–77.
45. Kuramitsu HK, He X, Lux R, Anderson MH, Shi W. Interspecies interactions within oral microbial communities. Microbiol Mol Biol Rev. 2007;71(4):653–70. https://doi.org/10.1128/mmbr.00024-07.
46. Peschel A, Sahl HG. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat Rev Microbiol. 2006;4(7):529–36. https://doi.org/10.1038/nrmicro1441.
47. Smith RN, Andersen RN, Kolenbrander PE. Inhibition of intergeneric coaggregation among oral bacteria by cetylpyridinium chloride, chlorhexidine digluconate and octenidine dihydrochloride. J Periodontal Res. 1991;26(5):422–8.
48. Salz U, Mucke A, Zimmermann J, Tay FR, Pashley DH. pKa value and buffering capacity of acidic monomers commonly used in self-etching primers. J Adhes Dent. 2006;8(3):143–50.
49. Chan KM, Tay FR, King NM, Imazato S, Pashley DH. Bonding of mild self-etching primers/adhesives to dentin with thick smear layers. Am J Dent. 2003;16(5):340–6.
50. Arslan S, Ertas H, Zorba YO. Effect of a plant-based hemostatic agent on microleakage of self-etching adhesives. Med Oral Patol Oral Cir Bucal. 2013;18(1):e124–9.
51. Meerbeek BV, Yoshihara K, Yoshida Y, Mine A, De MJ, Van LKL. State of the art of self-etch adhesives. Dent Mater. 2011;27(1):17–28.
52. Pashley DH, Tay FR, Breschi L, Tjäderhane L, Carvalho RM, Carrilho M, Tezvergil-Mutluay A. State of the art etch-and-rinse adhesives. Dent Mater. 2011;27(1):1–16.
53. Wendl B, Droschl H, Kern W. A comparative study of polymerization lamps to determine the degree of cure of composites using infrared spectroscopy. Eur J Orthod. 2004;26(5):545–51.
54. Okuda K, Zendo T, Sugimoto S, Iwase T, Tajima A, Yamada S, Sonomoto K, Mizunoe Y. Effects of bacteriocins on methicillin-resistant *Staphylococcus aureus* biofilm. Antimicrob Agents Chemother. 2013;57(11):5572–9. https://doi.org/10.1128/aac.00888-13.