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Original Article

Oleic acid impedes adhesion of *Porphyromonas gingivalis* during the early stages of biofilm formation

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Abstract: Oleic acid is a fatty acid thought to be cytotoxic to bacteria. This study aimed to investigate the antibacterial and antibiofilm activities of oleic acid against two strains of *Porphyromonas gingivalis*, a bacterial pathogen involved in chronic periodontitis. *P. gingivalis* W83 and ATCC 33277 were cultured in brain heart infusion culture media with or without oleic acid for 24 hours. Crystal violet staining was used to detect and quantify biofilms. Changes in morphology caused by oleic acid were assessed by scanning electron microscopy, and mRNA expression levels of virulence factor genes were detected by real-time PCR. When bacteria were exposed to oleic acid after biofilm formation, the adherent bacterial population of the preformed biofilm significantly increased in proportion to the amount of oleic acid ($P<0.05$); in contrast, the planktonic cell population significantly decreased in proportion to the amount of oleic acid ($P<0.05$). However, when bacteria were exposed to oleic acid prior to biofilm formation, the fatty acid significantly inhibited the formation of biofilm during the initial stage when the concentration was higher than 8× IC50 ($P<0.05$). Scanning electron microscopy revealed shrunken cells and deformed cell membranes. Genes encoding virulence factors in this form of biofilm (*hagA*, *rgpA*, *kgp*) were expressed at significantly lower levels when oleic acid was present ($P<0.05$). Oleic acid has strong antibacterial and antibiofilm activities against *P. gingivalis* and inhibits the early stage of biofilm formation by this organism.

Keywords: *P. gingivalis*, oleic acid, biofilm, antibacterial

Introduction

Periodontitis is caused by microorganisms embedded in the subgingival biofilm and is related to the complex interactions between bacteria and host [1, 2]. *Porphyromonas gingivalis*, the major etiological agent of chronic periodontitis, is a bacterial pathogen that not only invades and destroys host periodontal tissue, but also escapes the defense mechanisms of the host [3]. Bacteria can exist in two states, planktonic and adherent, with adherent bacteria resisting host immune defenses and drugs more effectively than bacteria in the planktonic state [4]. Bacteria adhering to the surface of an object can rapidly proliferate, leading to the formation of a complex microbial structure termed 'biofilm'. Bacterial adhesion is necessary for the formation of biofilms. For most bacteria, adhesion is promoted by auto-aggregation factors. Biofilms formed by *P. gingivalis* confer resistance to environmental stress, extreme drying, several drugs and exposure to ultraviolet light [5]. The biofilm of *P. gingivalis* contains virulence factors such as hemagglutinins, fimbriae and proteases [6, 7]. The acquisition of nutrients is facilitated by hemagglutinins, and in a previous study, the hemagglutinin adhesin, HagA, was shown to mediate adhesion to epithelial cells [8]. Gingipains, secreted by *P. gingivalis* and involved in the formation of biofilms and evasion of host defenses, consist of Arg-gingipain (Rgp) and Lys-gingipain (Kgp) [9, 10]. An increasing body of research has highlighted the role of these proteases and shown that they work in synergy to provide complete virulence, damaging the immune system of the host to elevate the risk of disease, as well as breaking down proteins in the host tissue and plasma [11].

In recent years, the destructive effects of oleic acid, an omega-9 (n-9) monounsaturated fatty acid, on the growth and morphology of bacteria has been explored [12, 13]. Oleic acid has also been reported to improve the efficacy of antibiotic formulations based on oleic acid liposomes in 32 multidrug-resistant strains of *Pseudomonas aeruginosa* (MDRPa) [14]. In addition, this fatty acid can enhance the effect of fluoride on the formation of biofilm extracellular polysaccharide (EPS) of *Streptococcus mutans* UA159, and act as an antibiofilm agent in *Escherichia coli* and *Bacillus* [15, 16]. Moreover, oleic acid is the principal unsaturated fatty acid innately present on the skin, as well as in abscesses caused by staphylococci [17]. This study aimed to investigate the antimicrobial activity of oleic acid on two strains of *P. gingivalis* (ATCC 33277 and W83), while evaluating the effects on biofilm formation, morphology, adhesion, and virulence-related gene expression.

Materials and methods

Microorganisms and culture conditions

Two strains of *P. gingivalis* (ATCC 33177 and W83) were obtained from Professor Pan Yaping (China Medical University, Liaoning, China) and maintained in brain heart infusion (BHI) broth supplemented with 5 mg/mL hemin, 1 μg/mL vitamin K_1 and 50 mg/mL sterile defibrinated sheep blood. Anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) were used for culture at 37°C for 5 to 7 days. Following the inoculation of a single colony in BHI liquid medium, anaerobic culture was performed for 24 h, after which the optical density at 600 nm (OD_{600}) was measured. The inoculum was adjusted to 10^9 colony-forming units per milliliter (CFU/mL) followed by a 1:10 dilution in growth medium; the resulting diluted inoculum was used for all subsequent experiments [18].

Quantitative detection of biofilm

The concentrations of metronidazole and oleic acid that could respectively cause 50% inhibition (IC50) of *P. gingivalis* were determined. Diluted suspensions of *P. gingivalis* were exposed to metronidazole (11.7 mM) or oleic acid and cultured in 96-well polystyrene plates at 37°C for 24 h. The negative control was bacteria not exposed to treatment, while metronidazole served as a positive control. Following three washes in phosphate-buffered saline (PBS), bacteria in the plates were fixed with 4% paraformaldehyde for 15 min and stained with 1% crystal violet for 15 min at room temperature. Plates were then washed with water and photographed. The crystal violet was dissolved in 33% glacial acetic acid for 30 min prior to OD_{600} measurements (Thermo Fisher Scientific, Shanghai, China) [19]. Determination of the IC50 of biofilm inhibition involved curve fitting in GraphPad primer7 [20].

Scanning electron microscopy (SEM)

Cultures of *P. gingivalis* were adjusted from the logarithmic growth phase to a final concentration of approximately 1×10^8 CFU/mL in BHI, followed by treatment for 24 h with the IC50 of oleic acid. The cultures were then fixed with 4% (v/v) glutaraldehyde overnight at 4°C, followed by three washes in PBS and fixation with 2% (v/v) glutaraldehyde for 1 h at 4°C. Fixed cultures were sequentially dehydrated using an ethanol series (20, 50, 80 and 100%), sputter-coated using gold under vacuum, and observed with a Quanta™ 250 FEG microscope (Shanghai, China).

Quantification of virulence-related genes

RT-PCR was used to investigate the expression levels of *hagA*, *rgpA* and *kgp*. The primers used for RT-PCR (*hagA*: 5'-ACAGCATCAGCCGATATTCC-3' and 5'-CGAATTCATTGCCACCTTCT-3', *kgp*: 5'-AGCTGACAAAGGTGGAGACCAAAGG-3' and 5'-TGTGGCATGAGTTTTTCGGAACCGT-3', *rgpA*: 5'-GCCGAGATTGTTCTTGAAGC-3' and 5'-AGGAGCAGCAATTGCAAAG-3', 16S RNA: 5'-TGTAGATGACTGATGGTGAAA-3' and 5'-ACTGTTAGCAACTACCGATGT-3') were the same as those used previously by Fournier-Larente et al. [21]. After culture for 24 h with oleic acid, total RNA was extracted from *P. gingivalis* using TRIzol reagent (Life Technologies Thermo Fisher Scientific, Shanghai, China) in accordance with the manufacturer's protocol. RT-PCR was subsequently performed using HiScript® II Q RT SuperMix (Vazyme Biotech, Nanjing, China). The reverse transcription conditions were 25°C for 10 min, 50°C for 30 min and 85°C for 5 min. The resulting cDNA was diluted and amplified with ChamQ SYBR Color qPCR Master Mix (2×) (Vazyme Biotech, Nanjing, China) in a final volume of 20 μl, using 5 μM of each gene-specific primer and 2μl cDNA template. The cycling conditions were

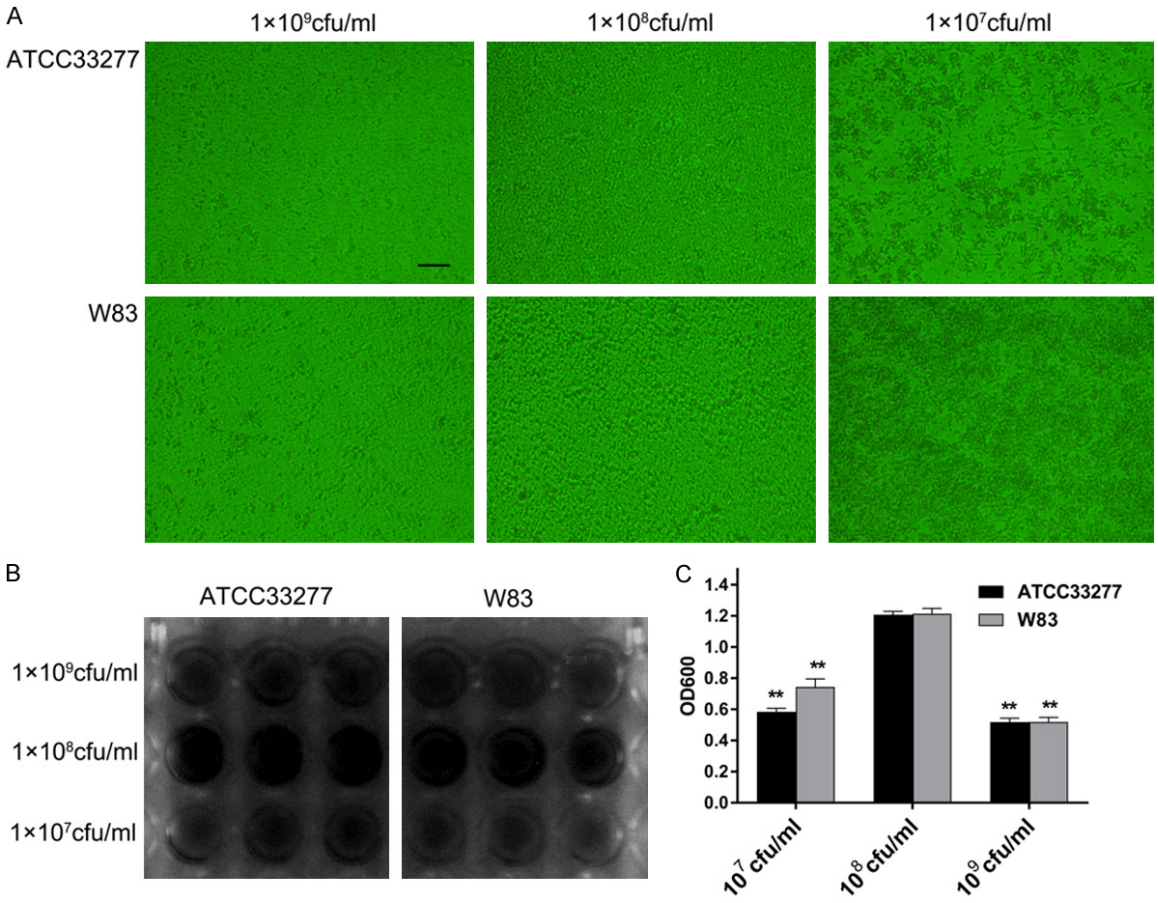

Figure 1. Optimal concentration of *P. gingivalis* for the formation of biofilms. A. 96-well plates were inoculated with 10^9, 10^8 or 10^7 CFU/mL of *P. gingivalis* ATCC 33277 or *P. gingivalis* W83 for 24 h, followed by observation using fluorescent microscopy (×40 magnification), scale bar=150 μm. B. Biofilms were stained with crystal violet and imaged. C. The OD_{600} of crystal violet for stained bacteria was measured. Data represent the mean of three independent determinations ± standard deviation. **P<0.01 *versus* 10^8 CFU/mL.

95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min. RT-PCR was performed using a Line-Gene 9600 Plus Detection System (Bioer Technology, Hangzhou, China). Normalization was carried out using 16S rRNA, and the $2^{-\Delta\Delta CT}$ approach was used to quantify changes in expression [22].

Statistical analysis

All experiments were performed in triplicate and data are represented as mean ± standard deviation (SD). Differences between groups were compared using the independent samples T-Test and Analysis of Variance (ANOVA). The Shapiro-Wilk test was used to check the normal distribution of data. SPSS version 18.0 for Windows (Chicago, IL, USA) was utilized for all statistical analyses and significance was set at P<0.05.

Results

Optimum concentration of oleic acid for inhibition of P. gingivalis ATCC 33277 and W83

It has been reported that oleic acid at IC50 can suppress biofilm formation in *Staphylococcus aureus*. To explore the effect that oleic acid had on the biofilm of *P. gingivalis*, the optimum concentration of strains ATCC 33277 and W83 required for biofilm formation were first determined. As shown in **Figure 1A**, the biofilm structure formed by 10^7 or 10^9 CFU/mL was almost connectionless and aggregated into clusters, while that formed with 10^8 CFU/mL showed a homogeneous and tightly connected structure. Moreover, following the addition of crystal violet, the 10^8 CFU/mL culture showed more intense staining (**Figure 1B**) and the absorbance at 600 nm was much higher than that of the other two bacterial concentrations (**Figure 1C**).

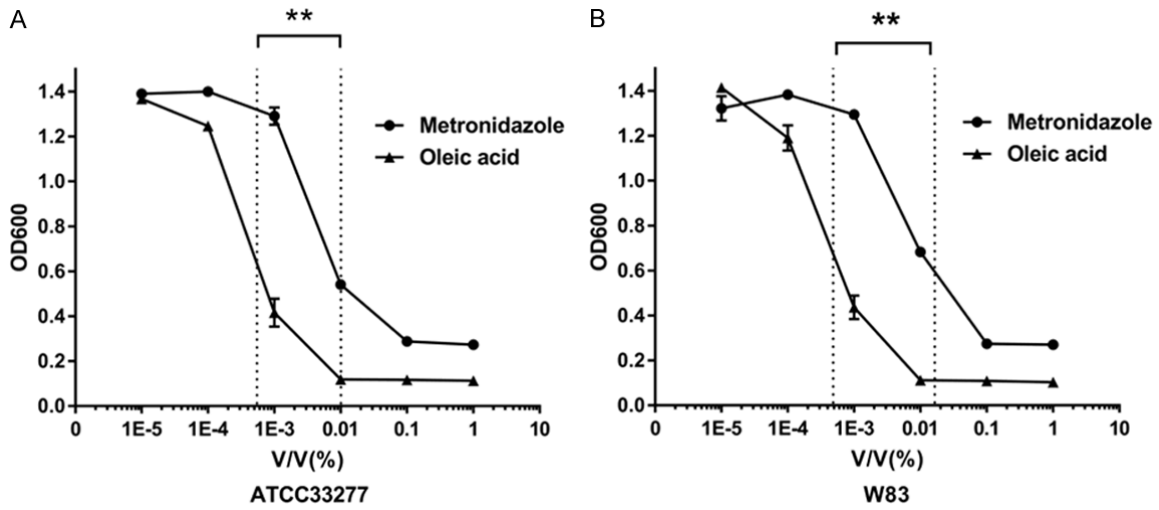

Figure 2. The IC50 of oleic acid and metronidazole for two strains of *P. gingivalis*. The IC50 of oleic acid was determined by the spectrophotometric measurement of crystal violet-treated biofilms of *P. gingivalis* ATCC 33277 and W83 exposed to varying concentrations of oleic acid. Data are the mean of three independent determinations ± standard deviation. **P<0.01 *versus* IC50 of metronidazole.

Therefore, bacterial strains were adjusted to 10^8 CFU/mL for subsequent experiments. **Figure 2A** and **2B** show that the IC50 of oleic acid for *P. gingivalis* ATCC 33277 and W83 was 0.00055% (1.76 nM) and 0.00048% (1.55 nM), respectively. These IC50s were applied to inhibit biofilm formation of *P. gingivalis* in subsequent experiments.

Biofilm formed by P. gingivalis is resistant to oleic acid

To investigate the effects of oleic acid on biofilm of *P. gingivalis*, the two strains of *P. gingivalis* were initially cultured for 24 h to allow biofilm formation before subsequent treatment with oleic acid for 24 h. Crystal violet staining indicated that biofilm formation did not decrease when the amount of oleic acid was increased (**Figure 3A**); in contrast, oleic acid enhanced the enlargement of the biofilms for both strains of *P. gingivalis*. Furthermore, cell numbers of the two strains decreased significantly (**Figure 3B**). This clearly suggested that oleic acid exerts differential effects on planktonic cells and adherent cells. Therefore, planktonic and adherent cells were examined after oleic acid treatment. While an increase in oleic acid concentration resulted in an increase in biofilm for the population of cells that were adherent, planktonic populations showed the opposite effect, although the overall number of bacteria was reduced slightly when both populations were taken into account (**Figure 3C**, **3D**). This suggested that oleic acid has better effects on planktonic cells than adherent cells, and that cells in the biofilm resisted the effect of oleic acid.

Oleic acid inhibits P. gingivalis during the early stages of biofilm formation

To explore whether oleic acid affected the adhesion of planktonic cells, the bacterial strains and oleic acid were incubated in culture media at the same time. Quantitative determination of biofilm formation revealed a significant inhibition of biofilm formation by oleic acid when the concentration was higher than 8x IC50 (**Figure 4A**). Cell growth analysis indicated that this inhibition on planktonic bacteria showed a gradient decline, and that growth of the bacteria was affected from the initial stages (**Figure 4B**). Moreover, the inhibition of *P. gingivalis* ATCC 33277 was greater than that for *P. gingivalis* W83. Together, these observations suggested that oleic acid limits the adhesion of planktonic cells of *P. gingivalis*.

Morphology of P. gingivalis after oleic acid treatment

Scanning electron microscopy (SEM) revealed that oleic acid caused morphological changes in *P. gingivalis*. Untreated cells possessed a smooth and regular spherical morphology, with

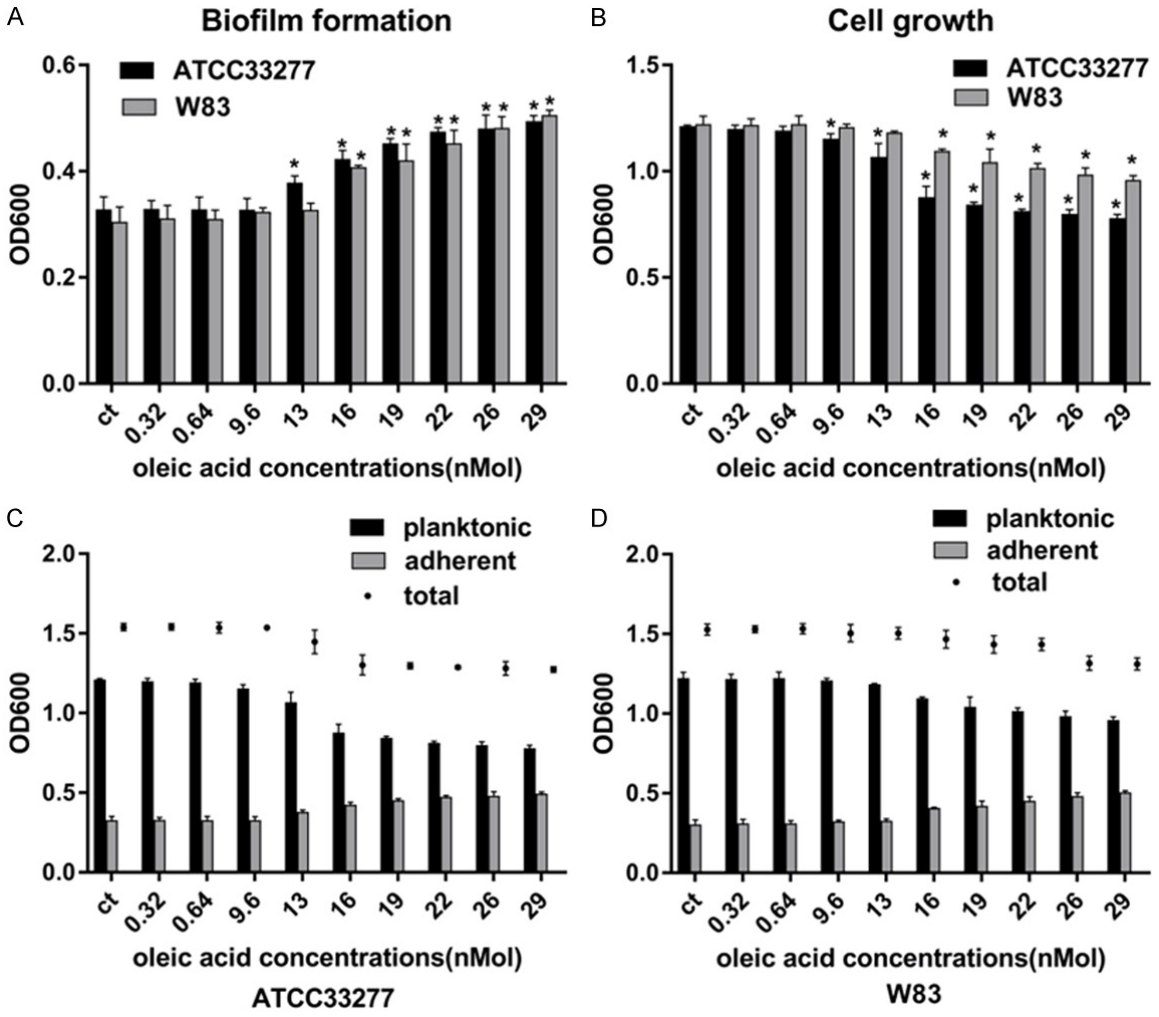

Figure 3. Exposure of *P. gingivalis* to oleic acid after biofilm formation for 24 h. *P. gingivalis* ATCC 33277 and W83 were cultured for 24 h then treated with increasing concentrations of oleic acid for 24 h, and biofilm formation and cell numbers were assessed. A. Biofilm formation was evaluated by crystal violet staining. B. Cell numbers were determined by measurement of OD_{600}. C, D. Plots of the square of counts of total bacteria, adherent and planktonic cells are shown on each graph based on OD_{600} measurements. Data represent means ± standard deviation from triplicate determinations. *P<0.05 *versus* untreated control group.

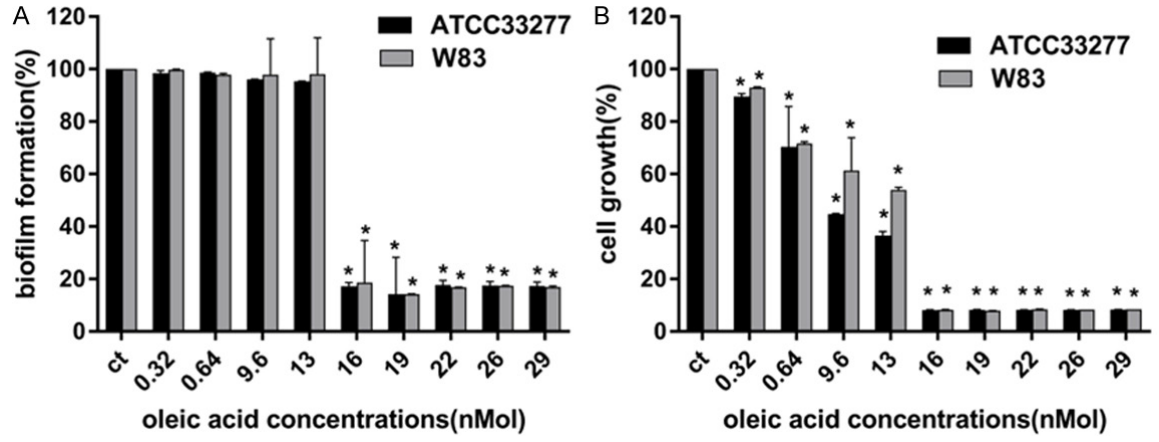

Figure 4. Exposure of *P. gingivalis* to oleic acid at the initial stage of biofilm formation. *P. gingivalis* ATCC 33277 and W83 were incubated with increasing concentrations of oleic acid for 24 h, then biofilm formation and cell numbers

were assessed. A. Formation of biofilm was evaluated by crystal violet staining. B. Cell numbers were determined by measurement of OD_{600}. Data are means ± standard deviation of triplicate determinations. *P<0.05 *versus* untreated control group.

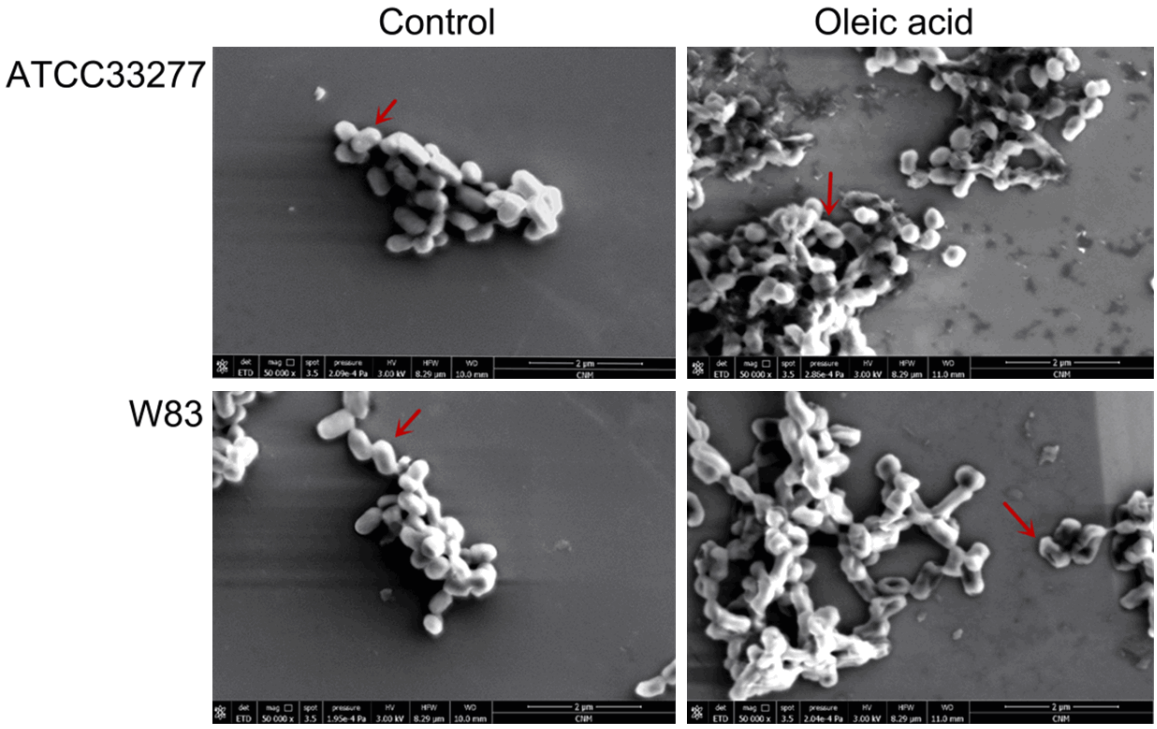

Figure 5. Morphology of *P. gingivalis* after exposure to oleic acid. *P. gingivalis* ATCC 33277 and W83 were incubated with 1.76 and 1.55 nM oleic acid, respectively, for 12 h. Images were obtained by scanning electron microscopy.

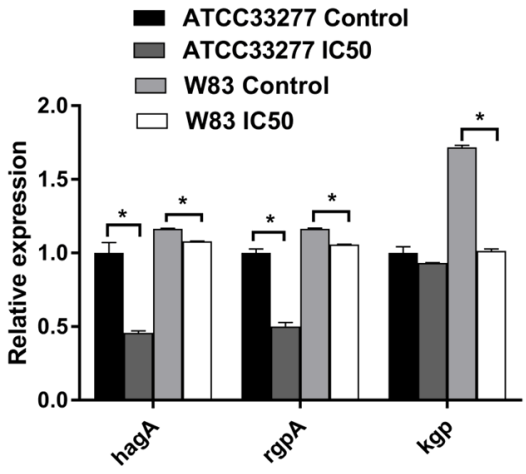

Figure 6. mRNA expression of virulence genes in *P. gingivalis* after oleic acid treatment. *P. gingivalis* ATCC 33277 and W83 (at 1×10^8 CFU/mL) were incubated with 1.76 and 1.55 nM oleic acid, respectively, for 12 h, then qRT-PCR was used to assess the mRNA expression levels of the virulence genes *hagA*, *rgpA* and *kgp*. 16S rRNA was used for normalization. Data represent means ± standard deviation of assays performed in triplicate. *P<0.05 *versus* untreated control group.

a cell wall and membrane that were intact. After exposure to oleic acid at IC50 for 12 h, cells of *P. gingivalis* were deformed and shrunken (**Figure 5**). This indicated that oleic acid deprived *P. gingivalis* of its normal cell structure and adhesion function.

Effect of oleic acid on adherence-related genes of P. gingivalis

Oleic acid reduced the expression of three key adherence-related genes in *P. gingivalis* (**Figure 6**). After treatment with oleic acid at the IC50, expression of *hagA* was downregulated to 45.6% in *P. gingivalis* ATCC 33277 and to 92.7% in *P. gingivalis* W83; expression of *rgpA* decreased to 50% and 90.8% in strains ATCC 33277 and W83, respectively. However, for the gene *kgp*, the opposite was observed in that expression was downregulated to 93.3% for strain ATCC 33277 and to 60% for strain W83. Thus, oleic acid inhibited the genes *hagA* and *rgpA* of *P. gingivalis* ATCC 33277 to a greater extent than in *P. gingivalis* W83. For the gene

kgp, oleic acid had a clear inhibitory effect in *P. gingivalis* W83 but there was no obvious change in expression of this gene in *P. gingivalis* ATCC 33277.

Discussion

This research showed that oleic acid exhibited antibacterial and antibiofilm effects in two strains of *P. gingivalis* - ATCC 33277 and W83. The antibiofilm effect of oleic acid was weak when the fatty acid was added after biofilm formation; however, the two strains were both significantly inhibited by oleic acid during the initial stages of biofilm formation. Moreover, oleic acid limited the adhesion of planktonic cells of *P. gingivalis*. Using SEM, shrinking and lysis of cells of *P. gingivalis* were observed after oleic acid treatment. In addition, expression of adherence-related genes decreased significantly after oleic acid treatment. These results indicated that oleic acid not only kills planktonic bacteria, but also reduces biofilm formation by inhibiting the adhesion of *P. gingivalis*.

There are known health benefits offered by polyunsaturated and monounsaturated fatty acids (PUFAs and MUFAs, respectively), especially in reducing inflammation and as antioxidants [23]. Indeed, a recent study demonstrated that the anti-inflammatory effect of unsaturated fatty acids led to a clear improvement in oral health [24]. In this study, the IC50 of oleic acid on *P. gingivalis* biofilms was determined and used to set reference concentrations for subsequent experiments. The IC50 of oleic acid on *P. gingivalis* was far lower that of metronidazole, indicating that the antibiofilm effect of oleic acid was much stronger than that of conventional antibiotics. While the effect of n-9 fatty acids against bacteria has yet to be established, there is a resemblance between these molecules and the bipolar lipids of the bacterial cell membranes, both in terms of their heads and tails, which are hydrophilic and hydrophobic, respectively. This point of similarity is suggestive of the fact that fatty acids can penetrate the membrane and cause a breakdown in cell membrane function in both bacteria and fungi. The inhibition of biofilm formation by addition of oleic acid to bacteria before primary adhesion was proven by using different concentrations of oleic acid. The inhibition rate of the biofilm of *P. gingivalis* W83 was higher than that of *P. gingivalis* ATCC 33277. When oleic acid was added after primary adhesion, formation of biofilm was more reduced under aerobic conditions than an anaerobic environment. Statistically significant differences were observed when the concentration of oleic acid was greater than 8× IC50, and furthermore, the formation of biofilm was inversely proportional to the dose of oleic acid.

Mechanical removal of damaged tissue is the normal process utilized for removing oral biofilms [25]. However, the results of this study suggest a potential role of oleic acid in preventing biofilm formation, or as an antibiofilm compound which could be used to avoid infections such as those involving periodontitis. The mechanism associated with the observations of this study has yet to be fully elucidated. A previous study investigated potential regulatory mechanisms of *P. gingivalis* in biofilms and found that the transcriptional activity of virulence genes can be modified by macromolecules via a range of reactions, including sialylation and glycosylation, and by aggregating and entering into a biofilm growth phase [26]. Consequently, the resistance of bacteria that have established a biofilm is higher than that of free bacteria that have not formed a biofilm. Such discoveries highlight the potential role of oleic acid as a supplement with which to prevent periodontitis when biofilms are at an initial stage of development.

The morphology of *P. gingivalis* exposed to oleic acid treatment was examined by SEM. Oleic acid is known to act upon *P. gingivalis* by penetrating the cell wall and causing destruction of the cell membrane [27, 28]. The results from SEM were in lieu of earlier studies that used other antibacterial compounds. We believe that disruption to the cell envelope structure causes a reduction in the adhesion of *P. gingivalis*.

As *P. gingivalis* plays a key role in chronic periodontitis, the search for molecules that can prevent or inhibit the virulence of this microbe is of vital importance. In the present study, expression of three genes involved in virulence and the formation of biofilms, *hagA*, *rgpA* and *kgp*, was investigated to gain a better understanding of how oleic acid exerts action on *P. gingivalis*. Expression of *hagA*, which is involved in host colonization, was reduced by oleic acid treatment, suggesting the potential role of this fatty acid in preventing pathogens from

adhering to the mucosa and causing infection. Incubation of *P. gingivalis* W83 with the IC50 of oleic acid caused a reduction in expression of *rgpA* and *kgp*. These two genes encode proteases involved in the inactivation of the host response, the degradation of host tissue and the acquisition of nutrients. However, for *P. gingivalis* ATCC 3327, only the expression of *rgpA* decreased after exposure to the IC50 of oleic acid; no changes were evident for *kgp*. This interesting strain-specific difference requires further attention.

The initial step of periodontitis involves the formation of biofilms in the subgingival tissues by pathogens that specifically target this location. This disease involves chronic inflammation which can cause destruction of the gums and the potential loss of teeth. Although antagonists are available which can target anaerobiosis, such as ornidazole, tinidazole, and metronidazole, these molecules have lower efficacy at low doses and can have undesirable effects [29]. An increase in the prevalence of diseases, coupled with resistance to current antibiotic regimens, dictates the need to explore strategies that are more effective, affordable and can safely prevent and treat diseases [30]. Data from this study indicate that oleic acid has the potential to serve as an innovative agent that targets biofilm formation, and could therefore be used in the prophylaxis of periodontitis. The complexities of the environment of different periodontal pathogens necessitates further research in order to assess the performance of oleic acid on biofilms formed by several bacteria.

Acknowledgements

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Disclosure of conflict of interest

None.

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