Abstract

Dental caries is a chronic progressive disease occurring in the tooth hard tissue due to multiple factors, in which bacteria are the initial cause. Both Streptococcus mutans and Streptococcus sanguinis are main members of oral biofilm. Helicobacter pylori may also be detected in dental plaque, playing an important role in the development of dental caries. Objective: The aim of this study was to investigate the effect of H. pylori culture supernatant on S. mutans and S. sanguinis dual-species biofilm and to evaluate its potential ability on affecting dental health. Material and methods: The effect of H. pylori supernatant on single-species and dual-species biofilm was measured by colony forming units counting and fluorescence in situ hybridization (FISH) assay, respectively. The effect of H. pylori supernatant on S. mutans and S. sanguinis extracellular polysaccharides (EPS) production was measured by both confocal laser scanning microscopy observation and anthrone-sulfuric acid method. The effect of H. pylori supernatant on S. mutans gene expression was measured by quantitative real-time PCR (qRT-PCR) assays. Results: H. pylori supernatant could inhibit both S. mutans and S. sanguinis biofilm formation and EPS production. S. sanguinis inhibition rate was significantly higher than that of S. mutans. Finally, S. mutans bacteriocin and acidogenicity related genes expression were affected by H. pylori culture supernatant. Conclusion: Our results showed that H. pylori could destroy the balance between S. mutans and S. sanguinis in oral biofilm, creating an advantageous environment for S. mutans, which became the dominant bacteria, promoting the formation and development of dental caries.

Keywords: Helicobacter pylori. Streptococcus mutans. Streptococcus sanguinis. Oral biofilm. Ecological balance.
Introduction

*Helicobacter pylori* is implicated in several diseases such as gastritis, gastric ulcers and gastric carcinoma. Approximately 10% of individuals suffer from gastritis or gastric ulcer due to *H. pylori* infection. *H. pylori* can also be detected in saliva, on the dorsum of the tongue, on the surface of oral ulceration and in dental plaque, the latter representing a crucial location, playing an important role in the development of dental caries. The prevalence of *H. pylori* infection in the oral cavity of gastric *H. pylori*-positive people is significantly higher than that of gastric *H. pylori*-negative people. Researchers increasingly consider *H. pylori* as a conditional pathogen that exists in the oral cavity of both healthy people and patients with gastritis. *H. pylori* infection in the oral cavity is associated with dental caries and poor oral hygiene. The caries rate in *H. pylori*-positive people is higher than that in *H. pylori*-negative people.

According to the World Health Organization (WHO), dental caries has been one of the most important global oral health issues, accounting for 60-90% school-aged children of most of the industrialized countries. *Streptococcus mutans* is considered a crucial agent in caries pathogenesis because of its cariogenic traits. Glucans are essential to the adhesion of *S. mutans* to the tooth surface and to other oral bacteria, as well as to the formation of dental biofilms matrix. Furthermore, *S. mutans* possesses acridic properties, allowing it to perform glycolysis at low pH values within the matrix of the biofilm, which result in dental enamel demineralization. *Streptococcus sanguinis* is usually colonizing oral biofilm, having been considered a “good” member in the oral biofilm, since its presence is associated with the absence of caries. *S. mutans* and *S. sanguinis* inversely affect each other in the formation of dental plaque. Previous studies have shown that the interspecies interaction between *S. mutans* and *S. sanguinis* is mediated by *S. mutans* acidogenicity (production of lactic acid by L-lactate dehydrogenase, encoded by *ldh*) and production of bacteriocin (two major mutacins, mutacin IV and mutacin V, encoded by *nlmAB* and *nlmC*, respectively). These two streptococci compete for teeth colonization, since elevated levels of *S. sanguinis* in the early colonization results in a delayed colonization by *S. mutans*. Conversely, *S. mutans* teeth colonization is associated with low levels of *S. sanguinis*. Indeed, caries-free children have high levels of *S. sanguinis* in their saliva and dental plaque compared to children with carious lesions who, instead, showed an elevated concentration of *S. mutans*. Therefore, the imbalanced microecology of dental plaque was considered a key factor leading to caries. Currently, several studies are available to demonstrate the relationship between oral *H. pylori* and dental caries, although the mechanism is still unclear. In this study we analyzed the effects of *H. pylori* culture supernatant on *S. mutans* and *S. sanguinis* dual-species biofilm formation.

Material and methods

Bacterial strains and growth conditions

*H. pylori* ATCC 43504 was incubated in brain heart infusion (BHI) fluid medium with 5% Fetal Bovine Serum (FBS) that represented the *H. pylori* medium, in a microaerophilic chamber (6% O$_2$, 10% CO$_2$, and 84% N$_2$; Thermo Fisher Scientific, Inc., Waltham, MA, USA). *S. mutans* UA159 (ATCC 700610) and *S. sanguinis* (ATCC 10556) were maintained in BHI fluid medium in an anaerobic chamber (10% H$_2$, 5% CO$_2$, and 85% N$_2$; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for planktonic growth. Both *S. mutans* and *S. sanguinis* were grown in BHI with 1% (w/v) sucrose as a supplemental carbohydrate source, to allow biofilm formation. Biofilms were incubated at 37°C without agitation.

*H. pylori* supernatant collection

*H. pylori* stored at -80°C was incubated in BHI fluid medium with 5% FBS in the mentioned microaerophilic environment to allow their recovery, and subsequently subcultured for four days. The bacterial solution was centrifuged at 4000 g ∙ min$^{-1}$ for 10 min; the supernatant was collected and subsequently filtered by a 0.22 μm filter under aseptic conditions. The supernatant, named *H. pylori* supernatant, was stored at -20°C until use.

Planktonic growth assay

*S. mutans* and *S. sanguinis* overnight bacterial cultures were diluted to an OD$_{600nm}$=0.2 (according to McFarland turbidity standards) in BHI and placed in a sterile 96-well microtiter plate to perform planktonic growth curve assay. Each well containing 190 μL S.
mutans or S. sanguinis culture (OD$_{600nm}$ = 0.2) plus 10 μL H. pylori supernatant represented the experimental group, while the control group was represented by the same 190 μL bacteria culture plus 10 μL H. pylori medium. Plates were incubated at 37°C and sampled at hourly intervals for 24 h. The optical density at 600 nm (OD600, transmittance) was recorded hourly using a microplate reader (BioTek, Winooski, VT, USA) as previously described. Four replicates of each bacterium for each group were used.

**Fluorescence in situ hybridization (FISH)**

Overnight bacterial cultures of S. mutans and S. sanguinis were adjusted to OD$_{600nm}$ = 0.2 in fresh BHI supplemented with 1% sucrose. Cultures of each bacterial species were inoculated either sequentially at a 3 h interval or simultaneously on saliva-coated glass coverslips in a 24-well cell culture plate. The experimental groups contained 1.5 mL BHI with 1% (w/v) sucrose, 200 μL S. mutans culture, 200 μL S. sanguinis culture and 100 μL H. pylori supernatant, while the control groups had 100 μL H. pylori medium (BHI with 5% FBS) instead of H. pylori supernatant. Finally, standard 24-well cell culture plates were incubated at 37°C under anaerobic condition for 24 h.

After the 24-h incubation period, biofilms were fixed in 4% paraformaldehyde, labeled with oligonucleotide probes (Probe 5′-ACTCCAGACTTTCCTGAC-3′ specific for S. mutans was labeled with FITC and probe 5′-GCATACTATGGTTAAGCCACAGCC-3′ specific for S. sanguinis was labeled with ROX) and analyzed by species-specific FISH as previously described. Micrographs from at least five randomly selected fields of each sample were captured. S. mutans to S. sanguinis ratio was calculated based on the coverage area of each species as determined by IMAGE PRO PLUS 6.0 analysis (Media Cybernetics, Silver Spring, MD, USA).

**Biofilm single colony forming units (CFU) count**

Standard 24-well cell culture plates were filled with 200 μL S. mutans or S. sanguinis culture (OD$_{600nm}$ = 0.2) and H. pylori supernatant in BHI to a final amount of 2 mL containing 1% (w/v) sucrose in total. The control group had the same composition except the 5% H. pylori medium that replaced the 5% H. pylori supernatant. Plates were incubated at 37°C under anaerobic conditions for 24 h. The plate was incubated at 37°C under anaerobic conditions for 24 h. Next, we removed the planktonic bacteria, washed the coverslips with sterile PBS and dried them with a sterile filter paper, keeping them in the dark for the entire procedure. Bacteria were stained with SYTO 9 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) as previously reported and coverslips were washed using deionized sterile water to remove the residual dye, dried with a sterile filter paper, and sealed with immersion oil type-F for laser scanning confocal microscopy (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany) provided with a 63× oil immersion objective lens. Image collection gates were set at 655 to 690 nm for Alexa Fluor 647 and at 495 to 515 nm for SYTO 9. During imaging, amplifier gain (1.0), detector gain (500 V), and offset (0%) were kept constant. Five randomly selected fields were scanned for each sample. The quantification of EPS/bacteria biomass was performed with IMARIS 7.0.0 (Bitplane, Zurich, Switzerland), as previously described.

**Anthrone-sulfuric acid method to determine biofilm insoluble EPS production**

Standard 24-well cell culture plates contained 200 μL S. mutans or S. sanguinis culture (OD$_{600nm}$ = 0.2) and H. pylori supernatant in BHI to a final amount of 2 mL containing 1% (w/v) sucrose in total. The
control group had the same composition except that 5% \textit{H. pylori} supernatant was replaced by 5% \textit{H. pylori} medium. The plate was incubated at 37°C under anaerobic conditions for 24 h. Next, planktonic bacteria were removed and the adherent biofilm was resuspended in 2 mL PBS. The detailed procedure was performed as previously described\textsuperscript{19}. Water-insoluble extracellular polysaccharides were extracted from the sample using 1.0 M NaOH with agitation for 2 h at 37°C\textsuperscript{19}. The concentration of alkali-soluble carbohydrate was determined in the supernatant using the anthrone-sulfuric method. Briefly, the alkali-soluble carbohydrate solution was mixed with three volumes of anthrone-sulfuric acid reagent and heated in a water bath at 95°C for 5 min until the reaction was complete\textsuperscript{19}. Then, the solution was allowed to cool-down to room temperature, and its absorbance was measured in a 96-well cell culture plate at 625 nm using a microplate reader (BioTek).

Quantitative real-time PCR (qRT-PCR)

Gene-specific primers for \textit{nlmA}, \textit{nlmC}, and \textit{ldh} were designed, as shown in Figure 1. Total bacterial RNA was isolated, purified, cDNA was reverse transcribed, and PCR reactions were performed as previously described\textsuperscript{6}. Different gene expressions were normalized to 16S rRNA gene levels. Data were analyzed according to the 2^{-ΔΔCT} method\textsuperscript{3}.

Data analysis and statistics

In this study, all of the \textit{in vitro} experiments include biological and technical triplicates. Exploratory data analysis was performed to determine the most appropriate statistical tests. Assumptions of equal variances and normal distribution of errors were also checked. Data were further analyzed using SPSS 16.0 (SPSS, Inc, Chicago, IL, USA), and unpaired Student’s \textit{t}-test was used to compare data of two groups. Results are calculated as average values ±SD (standard deviation). Data were considered significantly different if the two-tailed \textit{P}-value was <0.05.

Results

Effect of \textit{H. pylori} culture supernatant on \textit{S. mutans} or \textit{S. sanguinis} single-species biofilm formation

In the single-species biofilm formation, \textit{S. mutans} and \textit{S. sanguinis} CFU levels showed a statistically significant reduction due to the presence of \textit{H. pylori} supernatant and not to \textit{H. pylori} medium. Indeed, \textit{S. mutans} CFU levels were 222×10^7 CFU/mL and 169×10^7 CFU/mL in the presence of \textit{H. pylori} medium and \textit{H. pylori} supernatant respectively, whereas \textit{S. sanguinis} CFU levels were 230×10^7 CFU/mL and 25×10^7 CFU/mL, respectively. The inhibition rate exerted on \textit{S. sanguinis} by \textit{H. pylori} supernatant was statistically significantly higher than that exerted on \textit{S. mutans} (Figure 2A) (\textit{F}(3,20)=1.576; \textit{R}^2=0.9935; \textit{P}<0.05). The inhibition of \textit{S. sanguinis} and \textit{S. mutans} by \textit{H. pylori} supernatant was also confirmed by crystal violet dye staining of single-species biofilm (Figure 2B) (\textit{F}(3,20)=0.9551; \textit{R}^2=0.9779; \textit{P}<0.05).

Effect of \textit{H. pylori} culture supernatant on dual-species biofilm

Since \textit{H. pylori} could be detected in dental plaque and is related to the presence of dental caries, we examined the effect of \textit{H. pylori} culture supernatant on the ecological balance of a dual-species biofilm composed by \textit{S. mutans} and \textit{S. sanguinis}. The 24-h dual-species bacteria biofilm formation results showed that the \textit{S. mutans}/\textit{S. sanguinis} ratio in the experimental groups treated with \textit{H. pylori} supernatant was higher than that in the control group treated with \textit{H. pylori} medium (Figures 3A and 3B) (\textit{F}(5,30)=0.5442;
The $S. \text{mutans}/S. \text{sanguinis}$ ratio was highest in the group in which $S. \text{mutans}$ was incubated 3 h earlier than $S. \text{sanguinis}$, while the lowest ratio was in the group in which $S. \text{sanguinis}$ was incubated 3 h earlier than $S. \text{mutans}$ (F(5,30)=0.5442; $R^2=0.9959$; P<0.05). In other words, the microbial colonization was dominated by $S. \text{mutans}$ in the groups treated with $H. \text{pylori}$ supernatant.

**Effect of $H. \text{pylori}$ culture supernatant on $S. \text{mutans}$ or $S. \text{sanguinis}$ EPS production**

We found, as expected, a decrease in both the biofilm and the EPS after $H. \text{pylori}$ supernatant treatment if compared with the biofilm after $H. \text{pylori}$ medium treatment. In addition, $H. \text{pylori}$ supernatant inhibited EPS production in both bacteria, although the inhibiting effect on $S. \text{sanguinis}$ was statistically significantly stronger than that on $S. \text{mutans}$ (Figures 4A and 4B) (P<0.05). The Results of the anthrone-

![Figure 2](image1.png)

**Figure 2** - Effect of $H. \text{pylori}$ supernatant on $S. \text{mutans}$ and $S. \text{sanguinis}$ biofilm formation. Planktonic bacteria were removed from $S. \text{mutans}$ or $S. \text{sanguinis}$ culture and the adherent biofilm was resuspended and diluted. A quantity of 100 μL of the final bacterial solution was placed onto the BHI agar plate. Colony forming units (CFU) were quantified to evaluate $H. \text{pylori}$ supernatant inhibition ratio. Each group was performed in triplicate. Data were considered significantly different if the two-tailed P-value was <0.05.

(A) Effect of $H. \text{pylori}$ supernatant and $H. \text{pylori}$ medium on single-species biofilm measured by CFU counting.

(B) Effect of $H. \text{pylori}$ supernatant and $H. \text{pylori}$ medium on single-species biofilm measured by crystal violet dye staining

![Figure 3](image2.png)

**Figure 3** - Effect of $H. \text{pylori}$ supernatant on dual-species oral biofilm. Overnight bacterial cultures of $S. \text{mutans}$ and $S. \text{sanguinis}$ were inoculated either sequentially at a 3 h interval, or simultaneously on saliva-coated glass cover slips. After 24 h incubation, biofilms were fixed, labeled and analyzed by species-specific FISH assays as described in “Material and methods”. Data were considered significantly different if the two-tailed P-value was <0.05.

(A) FISH images of dual-species biofilm were taken by confocal laser scanning microscopy (60× magnification). Green: $S. \text{mutans}$; Red: $S. \text{sanguinis}$.

(B) $S. \text{mutans}$ to $S. \text{sanguinis}$ ratio in dual-species biofilm. Results were averaged from five randomly selected fields of each sample and are expressed as mean ±standard deviation
sulfuric acid method to determine the biofilm insoluble EPS showed that *H. pylori* supernatant could inhibit the production of biofilm insoluble EPS in both bacteria, and also in this experiment we found that the inhibition rate on *S. sanguinis* was statistically significantly higher than that on *S. mutans* (Figure 3C) $(F(3,20)=1.968; R^2=0.9985; P<0.05)$. The results of anthrone-sulfuric acid method and confocal laser scanning microscopy techniques were consistent. Both experiments showed that *H. pylori* supernatant could inhibit the production of EPS in *S. mutans* and *S. sanguinis* biofilm.

**Effect of *H. pylori* culture supernatant on *S. mutans* bacteriocin- and acidogenicity-related genes expression**

Previous studies have shown that interspecies interaction between *S. mutans* and *S. sanguinis* is mediated by *S. mutans* acidogenicity and production of bacteriocin$^{13}$. *H. pylori* supernatant was able to increase the percentage of *S. mutans* in a dual-species biofilm. Therefore, to validate whether *S. mutans* bacteriocin- and acidogenicity-related gene expression was induced by *H. pylori* supernatant, we further investigated the expression of some *S. mutans* genes such as *nlmA*, encoding mutacin IV, *nlmC*, encoding...
mutacin V, and ldh, encoding L-lactate dehydrogenase (Figure 5). Although the expression of nlmA showed a 0.7-fold change after the H. pylori supernatant treatment, ldh expression had a 3.8-fold increase, and nlmC expression had a 14.3-fold increase (P<0.05).

Discussion

H. pylori is the first bacterium identified as a potential human carcinogenic pathogen18,26. It occurs in childhood by oral ingestion and persists for a lifetime in the host unless treated10. Several studies have demonstrated that H. pylori can be detected in dental plaque and saliva, making the oral cavity as the primary extra-gastric reservoir, which may be the source of infection and transmission4,10,30. The oral cavity is the starting point of the digestive tract, thus, because of the tight connection between oral cavity and digestive tract, the relationship between oral H. pylori and oral cavity diseases has caught increasing attention5. The association between H. pylori infection, dental caries and recurrent aphthous mouth ulcerations has been investigated in other researches6,25. A previous study showed that in H. pylori positive participants, caries prevalence rate was two times higher than in the participants without H. pylori8. Previous studies already have epidemiologic surveys regarding the relationship between dental caries and oral H. pylori, but the interaction between oral H. pylori and dental caries-related bacteria such as S. mutans and S. sanguinis has not been explored.

Oral biofilm can be defined as a diverse community of microorganisms, working as a system allowing bacterial adhesion and antibiotic resistance16. Oral biofilm is the key factor that causes dental caries, not bacterioplankton2,17. Thus, in this study we focused on oral biofilm to understand its role in caries formation. However, our attempts to culture H. pylori with other oral bacteria have failed because of the rigorous growth conditions needed by this bacterium1. Thus, in this study H. pylori supernatant was used to explore the influence of H. pylori on S. mutans and S. sanguinis biofilm. Our results showed that H. pylori supernatant could inhibit both S. mutans and S. sanguinis biofilm formation and EPS production. However, in a dual-species biofilm model, S. mutans showed a superior competitive advantage over S. sanguinis under H. pylori supernatant treatment. The observations in gene expression assays suggested that H. pylori supernatant could induce the production of mutacin and enhance the acidogenicity of S. mutans, alluding the creation of an advantageous environment for S. mutans, which became the dominant bacteria. We also found that the production of EPS of S. mutans and S. sanguinis was inhibited by H. pylori supernatant. Thus, we hypothesized that H. pylori supernatant contained some specific substances that may be secreted effectors, small molecules or metabolites, and that these substances could inhibit streptococcal EPS synthesis, affecting, therefore, the biofilm formation. However, this hypothesis needs further studies to be confirmed.

We also found that H. pylori supernatant had no significant effect on planktonic growth although having clear effects on biofilm and EPS formation. The biofilm formation and EPS production of S. mutans were known to be regulated by several signal transduction systems, like two-component system and second messenger signaling7. It is possible that the function of these signal systems was affected by substances in H. pylori supernatant. Our further research will focus on the specific mechanisms of the anti-biofilm effects of H. pylori supernatant.

In conclusion, our results showed the ability of H. pylori to destroy the balance between S. mutans and S. sanguinis in oral biofilm, creating an environment in which S. mutans is the dominant bacteria, promoting the formation and development of dental caries.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (31200985, 81430011), and State Key Laboratory of Oral Diseases (SKLOD201414, SKLOD201609). The authors declare no conflict of interests.

References

1- Agarwal S, Jithendra KD. Presence of Helicobacter pylori in subgingival plaque of periodontitis patients with and without dyspepsia, detected by polymerase chain reaction and culture. J Indian Soc Periodontol. 2012;16(3):398-403.
2- Barnabé M, Saraceni CH, Dutra-Correa M, Suffredini IB. The influence of Brazilian plant extracts on Streptococcus mutans biofilm. J Appl Oral Sci. 2014;22(5):366-72.
3- Cheng X, Zheng X, Zhou X, Zeng J, Ren Z, Xu X, et al. Regulation of oxidative response and extracellular polysaccharide synthesis by a diadenylate cyclase in Streptococcus mutans. Environ Microbiol. 2016;18(3):904-22.
4- Desai HG, Gill HH, Shankaran K, Metha PR, Prabhu SR. Dental plaque: a permanent reservoir of *Helicobacter pylori*? Scand J Gastroenterol. 1991;26(11):1205-8.
5- Dowsett SA, Kowolik MJ. Oral *Helicobacter pylori*, can we stomach it? Crit Rev Oral Biol Med. 2003;14(3):226-33.
6- Elsheikh MM, Mahfouz ME. Prevalence of *Helicobacter pylori* DNA in recurrent aphthous ulcersations in mucosa-associated lymphoid tissues of the pharynx. Arch Otolaryngol Head Neck Surg. 2005;131(9):804-8.
7- Ge Y, Caufield PW, Fisch GS, Li Y. *Streptococcus mutans* and *Streptococcus sanguinis* colonization correlated with caries experience in children. Caries Res. 2008;42(6):444-8.
8- Gebara EC, Faria CM, Pannuti C, Chelker L, Mayer MP, Lima LA. Persistence of *Helicobacter pylori* in the oral cavity after systemic eradication therapy. J Clin Periodontol. 2006;33(5):329-33.
9- Klein MI, Hwang G, Santos PH, Camanella OH, Koo H. *Streptococcus mutans*-derived extracellular matrix in cariogenic oral biofilms. Front Cell Infect Microbiol. 2015;5:10.
10- Kolho KL, Hölttä P, Alaluusua S, Lindahl H, Savilahti E, Rautelin H. Dental caries is common in Finnish children infected with *Helicobacter pylori*. Scand J Infect Dis. 2001;33(11):815-7.
11- Koo H, Falsetta ML, Klein MI. The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. J Dent Res. 2013;92(12):1065-73.
12- Kreth J, Gacaman RA, Raghavan R, Merritt J. The road less traveled - defining molecular commensalism with *Streptococcus sanguinis*. Mol Oral Microbiol. 2017;32(3):181-96.
13- Kreth J, Merritt J, Shi W, Qi F. Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. J Bacteriol. 2005;187(21):7193-203.
14- Liu Y, Lin H, Bai Y, Qin X, Zheng X, Sun Y, et al. Study on the influence of time, toothpaste and saliva in the retention of *Streptococcus mutans* and *Streptococcus sanguinis* on different toothbrushes. J Appl Oral Sci. 2014;22(3):152-8.
15- Liu P, Yue J, Han S, Deng T, Fu C, Zhu G, et al. Molecule targeting glucosyltransferase inhibits *Streptococcus mutans* biofilm formation and virulence. Antimicrob Agents Chemother. 2015;60(1):126-35.
16- Polk DB, Peek RM Jr. *Helicobacter pylori*: gastric cancer and beyond. Nat Rev Cancer. 2010;10(6):403-14.
17- Ren Z, Cui T, Zeng J, Chen L, Zhang W, Xu X, et al. Molecule targeting glucosyltransferase inhibits *Streptococcus mutans* biofilm formation and virulence. Antimicrob Agents Chemother. 2015;60(1):126-35.