Journal of Dental Sciences 19 (2024) 1748–1757

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jds.com

Original Article

Effect of mouthwash containing poly L-Lysine and glycerol monolaurate on oral *Helicobacter pylori* relating to biofilm eradication, anti-adhesion, and pro-inflammatory cytokine suppression

Mutita Wongsuwanlert [a,b], Rawee Teanpaisan [a], Praphansri Ruangsri [a,b], Apichat Kaewdech [c], Somkiat Sunpaweravong [d], Nuntiya Pahumunto [a,e*]

[a] *Research Center of Excellence for Oral Health, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand*
[b] *Department of Conservative Dentistry, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand*
[c] *Gastroenterology and Hepatology Unit, Division of Internal Medicine, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand*
[d] *Department of Surgery, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand*
[e] *Department of Oral Diagnostic Sciences, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand*

Received 23 August 2023; Final revision received 10 October 2023
Available online 21 October 2023

KEYWORDS
Helicobacter pylori;
Mouthwash;
Poly L-Lysine;
Glycerol monolaurate;
Virulence factors;
Oral cavity

Abstract *Background/purpose: Helicobacter pylori* has been found to be related to periodontitis, and the oral cavity has been considered a reservoir for *H. pylori* gastritis infection. Thus, this study evaluated the effect of mouthwash containing poly L-Lysine and glycerol monolaurate on inhibiting *H. pylori* growth, biofilm formation, cell cytotoxicity, adhesion ability, *cag*A mRNA expression, and pro-inflammatory cytokines stimulated by *H. pylori*.
Materials and methods: Nineteen *H. pylori* strains were isolated from the oral cavity. The effectiveness of mouthwash containing poly L-Lysine and glycerol monolaurate was examined for its ability to inhibit *H. pylori* growth and biofilm formation and was tested for cell viability in oral epithelial cells (H357), gastric adenocarcinoma cells (AGS), and periodontal ligament cells (PDL). Additionally, the mouthwash was tested for reducing *cag*A mRNA expression,

* Corresponding author. Department of Oral Diagnostic Sciences, Faculty of Dentistry, Prince of Songkla University, 15 Karnjanavanich Road, Hat Yai, Songkhla, 90110. Thailand.
E-mail address: nuntiya.p@psu.ac.th (N. Pahumunto).

https://doi.org/10.1016/j.jds.2023.10.010
1991-7902/© 2023 Association for Dental Sciences of the Republic of China. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

adhesion ability to H357 and AGS cells, and pro-inflammatory cytokines stimulated with *H. pylori* in AGS and PDL cells.
Results: The mouthwash containing poly L-Lysine and glycerol monolaurate could eradicate the biofilm by 14.9–19.9% after incubation at 5 min, and cell viability revealed 77.2, 79.8, and 100.0% for AGS, H357, and PDL cells, respectively. Moreover, the mouthwash containing poly L-Lysine and glycerol monolaurate could down-regulate *cag*A mRNA expression, reduce adhesion of *H. pylori* by approximately 9.5–47.8% for H357 cells and 24.5–62.9% for AGS cells, and decrease pro-inflammatory cytokines, especially interleukin-8, stimulated with *H. pylori*.
Conclusion: Mouthwash containing poly L-Lysine and glycerol monolaurate could inhibit *H. pylori* growth and reduce their virulence expression. The mouthwash also revealed low cytotoxicity to oral and gastric cells.
© 2023 Association for Dental Sciences of the Republic of China. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

Helicobacter pylori is a pathogenic bacterium that causes gastritis and gastric cancer in humans. Fifty percent of the world's population is infected by *H. pylori*, 90% is found in populations of developing countries and 40% in developed nations.[1] The standard treatment for *H. pylori* infection is triple therapy using a proton pump inhibitor or ranitidine bismuth citrate combined with clarithromycin or amoxicillin or metronidazole. However, the success of this treatment has been seen to be lower than 70% due to *H. pylori* resistance to clarithromycin, metronidazole, and levofloxacin.[2,3] Recently, recurrent *H. pylori* infections in patients were found at least one year after eradication in the gastric tissue, and the global annual recurrence and reinfection of *H. pylori* were 4.3 and 3.1%, respectively.[4,5] Recurrence is related to being transmitted through an oral–oral or oral-gastro or oral-fecal route. The prevalence of *H. pylori* in oral samples (saliva and plaque) was found in 60% of patients with gastric *H. pylori* infection and 15% of patients without gastric *H. pylori* infection.[6]

In Thailand, the prevalence of *H. pylori* infection in oral samples in patients with gastric cancer presented as 84.8% by real-time PCR,[7] and there were 10 in 19 strains (52.6%) that were *cag*A gene positive. This gene is associated with adhesion to host cells and cell apoptosis.[8] The strains that were *cag*A positive displayed higher adhesion ability, *cag*A expression, and pro-inflammatory cytokine expression than *cag*A negative strains.[7] The oral cavity may serve as a potential reservoir, henceforward oral *H. pylori* eradication should be studied. A few studies examined the effect of mouthwash or oral rinse on oral *H. pylori* strains.[9,10] For example, oral rinses containing chlorhexidine (0.2% w/v) showed high potency to remove *H. pylori* and suppress *cag*A expression;[9] however, 0.2% chlorhexidine showed high toxicity to primary human fibroblast cells (96.6%), primary human myoblast cells (96.5%), and primary human osteoblast cells (98.3%) after 1 min incubation.[11] The side effects of 0.2% chlorhexidine included a change in taste and tooth staining. Thus, the recommendation of chlorhexidine is that it is not proposed to be used longer than two weeks. Wang et al.[10] reported that mouthwash containing poly L-Lysine and glycerol monolaurate could inhibit the growth of *H. pylori* in saliva samples. Both compounds are used as preservatives in the food industry, yet their properties can interrupt the distribution of cytoplasm, causing damage to the bacterial cells including *H. pylori* cells. However, there is a lack of data on biofilm eradication, anti-adhesion ability, cell cytotoxicity, and cytokine stimulation. Thus, the aims of this study were to investigate the effect of a mouthwash containing poly L-Lysine and glycerol monolaurate on inhibiting *H. pylori* growth, biofilm formation, cell cytotoxicity to oral and gastric cells, adhesion ability to oral and gastric cells, *cag*A mRNA expression, and pro-inflammatory cytokines stimulated with *H. pylori* in oral and gastric cells.

Materials and methods

H. pylori isolation and preparation

The study attained ethical approval (EC6410-066) from the Faculty of Dentistry, Prince of Songkla University. *H. pylori* strains were isolated from oral samples (saliva and plaque) from a previous study.[7] Of 19 clinical isolates, 10 isolates showed *cag*A gene-positive using PCR. All *H. pylori* strains were used in all experiments and were cultured in brain heart infusion (BHI) broth (Difco™, Sparks, MD, USA), and incubated for 48 h at 37 °C in anaerobic conditions. *H. pylori* cells were kept using centrifugation at 3000 rpm for 10 min and washed twice with a phosphate buffer (PBS) pH 7.0. The cells were used for tests in all experiments.

Antibiotic susceptibility testing

H. pylori cells were adjusted to 0.5 MacFarland and spread on BHI agar. Antibiotic discs of erythromycin, amikacin, tetracycline, metronidazole, amoxicillin, ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole (Himedia, Mumbai, India) were placed and incubated overnight at 37 °C in anaerobic conditions. The inhibition zone was measured and reported as resistance (R), intermediate (I), and sensitive (S) by comparison to zone diameter standard breakpoints.

Antimicrobial activity of poly-L-Lysine, glycerol monolaurate, and $BiCl_3$

Agar well diffusion assay

H. pylori strains were adjusted to $OD_{600} = 0.2$ (10^8 CFU/mL) in PBS pH 7.0 using a spectrophotometer. One mL of cell suspension was added to 20 mL of melted BHI agar and poured on the sterile plate with metal cups. After solidification, metal cups were removed and 90 μL of 1 mg/mL of poly L-Lysine (Sigma, St. Louis, MO, USA) or glycerol monolaurate (Sigma, St. Louis, MO, USA) or $BiCl_3$ (Himedia, Mumbai, India) was added. The plate was incubated overnight at 37 °C in anaerobic conditions, and the clear zone was measured.

Broth microdilution assay

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were carried out as recommended by the instructions of the Clinical and Laboratory Standards Institute (CLSI). One hundred μL of 1 mg/mL of poly L-Lysine (Sigma) or glycerol monolaurate (Sigma) or $BiCl_3$ (Himedia) was diluted by 2-fold dilution with 100 μL of BHI broth in a 96-well plate, and 100 μL of *H. pylori* (10^8 CFU/mL) was added. The plate was incubated for 18–24 h at 37 °C in anaerobic conditions. The MIC was determined as the lowest concentration of poly L-Lysine or glycerol monolaurate or $BiCl_3$ required to fully inhibit growth as compared with the untreated control. The MBC was determined as the lowest concentration of wells that did not allow visible growth when 10 μL of the well contents were dotted on BHI agar and grown at 37 °C in anaerobic conditions.

Checkerboard assay

The checkerboard test was used to study the synergistic effect between poly L-Lysine (Sigma) and glycerol monolaurate (Sigma). Briefly, a two-fold serial dilution was used in the distribution of poly L-Lysine (solvent A) and glycerol monolaurate (solvent B) in a 96-well microtiter plate with a final concentration of individual solvent at 4x, 2x, 1/2x, 1/4x, and 1/8x MIC. Then 100 μL of inoculum equal to 10^8 CFU/mL from *H. pylori* was distributed into each well and incubated at 37 °C for 24 h in anaerobic conditions. The fractional inhibitory concentration index (FIC index) was computed by the accompanying equation: $\text{FIC index} = \frac{\text{MIC of antimicrobial solvent A in combination}}{\text{MIC of antimicrobial solventA alone}} + \frac{\text{MIC of antimicrobial solvent B in combination}}{\text{MIC of antimicrobial solventB alone}}$.

The FIC index showed a synergistic effect when it was ≤ 0.5, an indifferent effect when it was ≥ 1.0, and an antagonistic effect when it was > 4.0.

Mouthwash containing poly-L-Lysine and glycerol monolaurate, and mouthwash containing $BiCl_3$ preparation

The components for mouthwash base preparation comprised of 5.0% glycerol, 2.5% kolliphor®RH40, 0.001% peppermint oil, 0.001% saccharin sodium salt hydrate, 0.001% methyl 4-hydroxybenzoate, and water. The mixture was boiled at 60 °C for 1 h. Then 0.02 mg/mL glycerol monolaurate (Sigma) and 0.03 mg/mL poly-L-Lysine (Sigma) for formula 1 and 0.02 mg/mL $BiCl_3$ (Himedia) for formula 2 were added to the mouthwash base. The mouthwash containing poly-L-Lysine and glycerol monolaurate or mouthwash containing $BiCl_3$ were used to test anti-biofilm and cell viability to host cells. Additional testing for a mouthwash containing poly-L-Lysine and glycerol monolaurate was conducted for anti-adhesion ability, suppression of *cag*A mRNA expression, and pro-inflammatory cytokines. All experiments used a mouthwash base as a control.

Anti-biofilm formation by mouthwash containing poly-L-Lysine and glycerol monolaurate, and mouthwash containing $BiCl_3$

An amount of 200 μL of *H. pylori* (10^8 CFU/mL) was inoculated into each well of a 96-well plate and incubated for 24 h at 37 °C in anaerobic conditions for the development of a monolayer biofilm. The monolayer of biofilm was confirmed using staining with a Live/Dead *Bac*Light bacterial viability kit (Thermo Fisher Scientific, Eugene, OR, USA) and checked under a fluorescence microscope before testing.

Regarding biofilm eradication, the culture was removed and the well was carefully washed twice with PBS pH 7.0 to remove non-adherent cells. The biofilms were then exposed to 200 μL of mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate, and mouthwash containing 0.02 mg/mL $BiCl_3$. The plate was incubated for 24 h at 37 °C in anaerobic conditions and biofilm eradication was determined using a MTT assay. The MTT assay was according to Pahumunto et al.[12] and confirmed using a fluorescence microscope (mentioned above). The percentage of biofilm eradication was calculated by $[1 - (A_{570}$ of the test/A_{570} of non-treated control)] $\times$ 100.

Cell survival assay by mouthwash containing poly-L-Lysine and glycerol monolaurate, and mouthwash containing $BiCl_3$

Human oral squamous cell carcinoma (H357, kindly derived from Professor Paul Speight of the University of Sheffield, UK), human periodontal ligament cells (PDL), and human gastric adenocarcinoma cells (AGS, CRL-1739) were used in this study. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics, and incubated at 37 °C in 5% CO_2 for 3 days. Cells were sub-cultured, and 10^4 cells/mL of individual cells were seeded into a 96-well plate. The plate was incubated at 37 °C in 5% CO_2 for 3 days or until it showed 95% cell confluence for the cytotoxicity assay.

Mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate and mouthwash containing $BiCl_3$ were added to monolayer cells and incubated for 24 h in suitable conditions. Cell survival ability was measured by a MTT assay.[12] The percentage of cell viability was calculated by this formula: $\text{Cell viability} = \frac{\text{A570 of the treated cells} \times 100}{\text{A570 of untreated cells}}$.

Adhesion ability of *H. pylori* and anti-adhesion by mouthwash containing poly-L-Lysine and glycerol monolaurate

This experiment examined the effect of mouthwash containing poly-L-Lysine and glycerol monolaurate on inhibiting the adhesion ability of *H. pylori* because mouthwash containing $BiCl_3$ showed high cytotoxicity to host cells.

Human oral squamous cell carcinoma (H357) and human gastric adenocarcinoma cells (AGS) were used to study the adhesion ability of *H. pylori* strains. Both cells were cultured in suitable media (mentioned above) and incubated for three days in appropriate conditions. Cells were sub-cultured and 10^4 cells/mL were seeded into 24-well plates. The cells were incubated for three days or until 95% cell confluence. *H. pylori* cells (10^8 CFU/mL) or *H. pylori* cells (10^8 CFU/mL) combined with a sub-lethal dose of mouthwash containing poly L-Lysine and glycerol monolaurate were added to monolayer cells and incubated for 1 h. The treated cells were washed twice to remove unbound bacteria, and 0.25% trypsin was added to trypsinize cells. The adherence bacteria were counted using the plate count method and it was shown a percentage of adhesion ability calculated by this formula: $\frac{adhered\ bacteria x100}{bacterial\ cells\ at\ beginging}$.

*cag*A mRNA expression of *H. pylori* and its suppression by mouthwash containing poly-L-Lysine and glycerol monolaurate

H. pylori cells (10^8 CFU/mL) or *H. pylori* cells (10^8 CFU/mL) combined with a sub-lethal dose of mouthwash containing poly L-Lysine and glycerol monolaurate was incubated in BHI broth for 24 h at 37 °C in anaerobic conditions. The cells were kept using centrifugation at 3000 rpm for 10 min and were extracted for RNA using the RNA extraction kit (Thermo Fisher Scientific). A 100 ng/mL of total RNA was used to study *cag*A expression using real-time PCR with a specific primer for *cag*A (5'-ATAATGCTAAATTAGACAACTTGAGCGA-3' and 5'-AGAAACAAAAGCAATACGATCATTC-3')[13] and *ure*A genes (5-GCCAATGGTAAATTAGTT-3' and 5'-CTCCTTAATTGTTTTTAC-3').[14] The *ure*A gene is highly conserved for *H. pylori*, and this gene was used as a housekeeping gene in this study. The real-time PCR condition was 40 cycles for both genes with the denaturing temperature of 95 °C for 20s, annealing temperatures at 44 °C for 20s for the *cag*A and *ure*A genes, and the polymerizing temperature of 72 °C at 25s. *H. pylori* ATCC43504 was used as a control and the expression was set to 1.0.

Pro-inflammatory cytokines stimulation of *H. pylori* and its suppression by mouthwash containing poly-L-Lysine and glycerol monolaurate

Human periodontal ligament cells (PDL) and human gastric adenocarcinoma cells (AGS, CRL-1739) were used to study pro-inflammatory cytokines stimulation. Both cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics, and then incubated at 37 °C with 5% CO_2 for 3–5 days. Individual cells were sub-cultured using the trypsinization method, and 10^6 cells/mL were seeded into 6-well plates. Cells were incubated for 3 days or until 95% cell confluence. Then 100 μg/mL of *H. pylori* cell wall[12] or 100 μg/mL of *H. pylori* cell wall combined with 100 μL of mouthwash containing poly L-Lysine and glycerol monolaurate was added to the monolayer wells. The leftover untreated cells were used as a negative control. Treated cells were incubated for 24 h at 37 °C with 5% CO_2, and the total RNA was extracted from the cells to study cytokine expression.

A total RNA was extracted using the RNA extraction kit (Thermo Fisher Scientific, Carlsbad, CA, USA) following the manufacturer's instructions. The cDNA was synthesized by the Superscript™ first-strand cDNA system kit (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA levels of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α were measured with the CFX96 Touch™ Real-Time PCR detection system (BioRad, Foster, CA, USA). The real-time PCR reaction consisted of 5% cDNA (v/v), Sensifast™SYBR No-ROX reagent (Meridian Bioscience, Memphis, TN, USA), and 5 pM of each primer.[12] The real-time PCR condition was 40 cycles for all cytokines with the denaturing temperature at 95 °C for 20s, annealing temperatures at 60 °C for 20s, and the polymerizing temperature at 72 °C for 25s. For comparing gene expression, the GAPDH gene served as a housekeeping gene and the average induction of each cytokine was expressed from these separate experiments.

Statistical analysis

Antibiotic resistance was reported as description data. Anti-bacterial activity, cell viability, biofilm eradication, and pro-inflammatory cytokine suppression were shown as a mean ± SD. The results of *cag*A mRNA expression and adhesion ability showed box plots (25, 50, and 75 percentiles). Differences in anti-bacterial activity, cell viability, biofilm eradication, adhesion ability, *cag*A mRNA expression, and pro-inflammatory cytokine suppression between the groups were analyzed by the Mann–Whitney *U* test. *P*-value ≤0.05 revealed a significant difference.

Results

Nineteen *H. pylori* clinical strains were isolated from saliva (10 strains) and plaque (9 strains) from subjects having symptoms of gastritis or gastric cancer. All *H. pylori* strains, both *cag*A positive and *cag*A negative strains, demonstrated resistance to metronidazole and trimethoprim/sulfamethoxazole while being sensitive to tetracycline, erythromycin, amoxicillin, ampicillin, amikacin, and chloramphenicol.

All *H. pylori* strains were sensitive to poly-L-Lysine and glycerol monolaurate. The inhibition zone of both compounds was 28.5 ± 1.4 and 25.5 ± 0.6 mm for poly-L-Lysine and glycerol monolaurate, respectively. It was found that the *cag*A negative strains showed a clear zone greater than the *cag*A positive strains (Table 1). $BiCl_3$ showed the best anti-*H. pylori* zone compared to both compounds because the inhibition zone was 44.2 ± 0.2 mm.

The MIC and MBC values showed the same values of 0.3 mg/mL for poly-L-Lysine and 0.2 mg/mL for glycerol monolaurate. $BiCl_3$ revealed 0.02 mg/mL for MIC and MBC

Table 1 Sensitivity of poly-L-Lysine and glycerol monolaurate against oral *Helicobacter pylori* strains.

Sensitivity test	Glycerol monolaurate	Poly L-Lysine	$BiCl_3$
Inhibition zone (mm)			
All strains (n = 19)	28.5 ± 1.4^{B}	25.5 ± 0.6^{C}	44.2 ± 0.2^{A}
*cag*A positive strains (n = 10)	24.4 ± 0.6^{b}	25.1 ± 0.3^{a}	43.6 ± 0.1^{a}
*cag*A negative strains (n = 9)	30.2 ± 0.6^{a}	25.3 ± 0.4^{a}	44.8 ± 0.2^{a}
Minimum inhibitory concentration (MIC; mg/mL)			
All strains (n = 19)	0.2 ± 0.1^{B}	0.3 ± 0.0^{B}	0.02 ± 0.0^{A}
*cag*A positive strains (n = 10)	0.2 ± 0.0^{a}	0.3 ± 0.0^{a}	0.02 ± 0.0^{a}
*cag*A negative strains (n = 9)	0.2 ± 0.0^{a}	0.3 ± 0.0^{a}	0.02 ± 0.0^{a}
Minimum bactericidal concentration (MBC; mg/mL)			
All strains (n = 19)	0.2 ± 0.1^{B}	0.3 ± 0.0^{B}	0.02 ± 0.0^{A}
*cag*A positive strains (n = 10)	0.2 ± 0.0^{a}	0.3 ± 0.0^{a}	0.02 ± 0.0^{a}
*cag*A negative strains (n = 9)	0.2 ± 0.0^{a}	0.3 ± 0.0^{a}	0.02 ± 0.0^{a}
Fractional inhibitory concentration index (FIC index)			
Glycerol monolaurate	-	0.2 (0.02/0.03)	ND
Poly L-Lysine	0.2 (0.02/0.03)	—	ND

ND = not done; Capital letters presented a statistical difference between inhibition *H. pylori* strains of different derivative compounds; lowercase letters presented a significant difference between *cag*A positive strains and *cag*A negative strains ($P < 0.05$); FIC index showed a synergistic effect when it was $\leq$0.5, indifferent effect when it was $\geq$1.0, and antagonistic effect when it was >4.0.

values. The combination assay found synergism of poly-L-Lysine and glycerol monolaurate; it showed the FIC index as 0.20 (Table 1). Thus, the combination of poly-L-Lysine (0.03 mg/mL) and glycerol monolaurate (0.02 mg/mL) was formulated as a mouthwash for the next study. Before mouthwash formulation, the mouthwash base was used to test anti-bacterial activity to confirm the efficacy of poly-L-Lysine and glycerol monolaurate. The inhibition zone of the mouthwash base showed 6.8 $\pm$ 0.3 mm and biofilm eradication showed 6.5 $\pm$ 0.5% after incubation at 5 min and 20.7 $\pm$ 0.1% after incubation at 24 h.

Eradication of the biofilm formation from mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate (mentioned above) against *H. pylori* at various times is demonstrated in Table 2 and was confirmed under fluorescence microscope (Fig. 1). The results revealed that the biofilm eradication related to the incubation time; at 24 h it showed higher eradication compared to other times. Mouthwash containing $BiCl_3$ could remove biofilm better than the mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate at all times, and complete killing was revealed at 24 h incubation. The mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate could eliminate the biofilm by more than 50% at 2 h, and at 24 h it could remove approximately 78.0—83.0%. The *cag*A gene-positive strains showed resistance to mouthwash and $BiCl_3$ more than the *cag*A gene-negative strains.

Regarding the cell viability test, it was found that PDL cells showed a higher survival rate after being treated with the mouthwash base, mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate, and mouthwash containing $BiCl_3$ at all times compared to H357 and AGS cells. Mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate showed a higher survival rate of PDL, H357, and AGS cells compared to mouthwash containing $BiCl_3$ and mouthwash base. Mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate showed a survival rate of more than 50% at 15 min incubation for H357 and AGS cells

Table 2 *Helicobacter pylori* biofilm eradication treated with mouthwash containing poly L-Lysine and glycerol monolaurate or mouthwash containing $BiCl_3$.

Times	Biofilm eradication (%)			
	Mouthwash[a]		$BiCl_3$	
	*cag*A positive n = 10	*cag*A negative n = 9	*cag*A positive n = 10	*cag*A negative n = 9
5 min	$14.9 \pm 1.1^{a,A}$	$19.9 \pm 1.4^{b,A}$	$52.6 \pm 1.3^{a,A}$	$57.6 \pm 1.4^{b,A}$
15 min	$25.6 \pm 1.9^{a,B}$	$30.6 \pm 1.0^{b,B}$	$67.2 \pm 0.9^{a,B}$	$72.2 \pm 1.0^{b,B}$
60 min	$37.8 \pm 0.4^{a,C}$	$42.8 \pm 1.2^{b,C}$	$82.3 \pm 0.4^{a,C}$	$87.3 \pm 1.2^{b,C}$
120 min	$48.2 \pm 1.3^{a,D}$	$53.2 \pm 0.4^{b,D}$	$86.8 \pm 0.3^{a,D}$	$91.8 \pm 0.4^{b,D}$
360 min	$58.5 \pm 1.6^{a,E}$	$63.5 \pm 0.3^{b,E}$	$92.6 \pm 1.0^{a,E}$	$100.0 \pm 0.0^{b,E}$
24 h	$78.0 \pm 1.0^{a,F}$	$83.0 \pm 1.4^{b,F}$	$97.3 \pm 0.9^{a,F}$	$100.0 \pm 0.0^{b,E}$

h = hour; Capital letters presented a statistical difference in the same column ($P < 0.05$); Lowercase letters presented a significant difference between *cag*A positive and *cag*A negative strains ($P < 0.05$).

[a] Mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate.

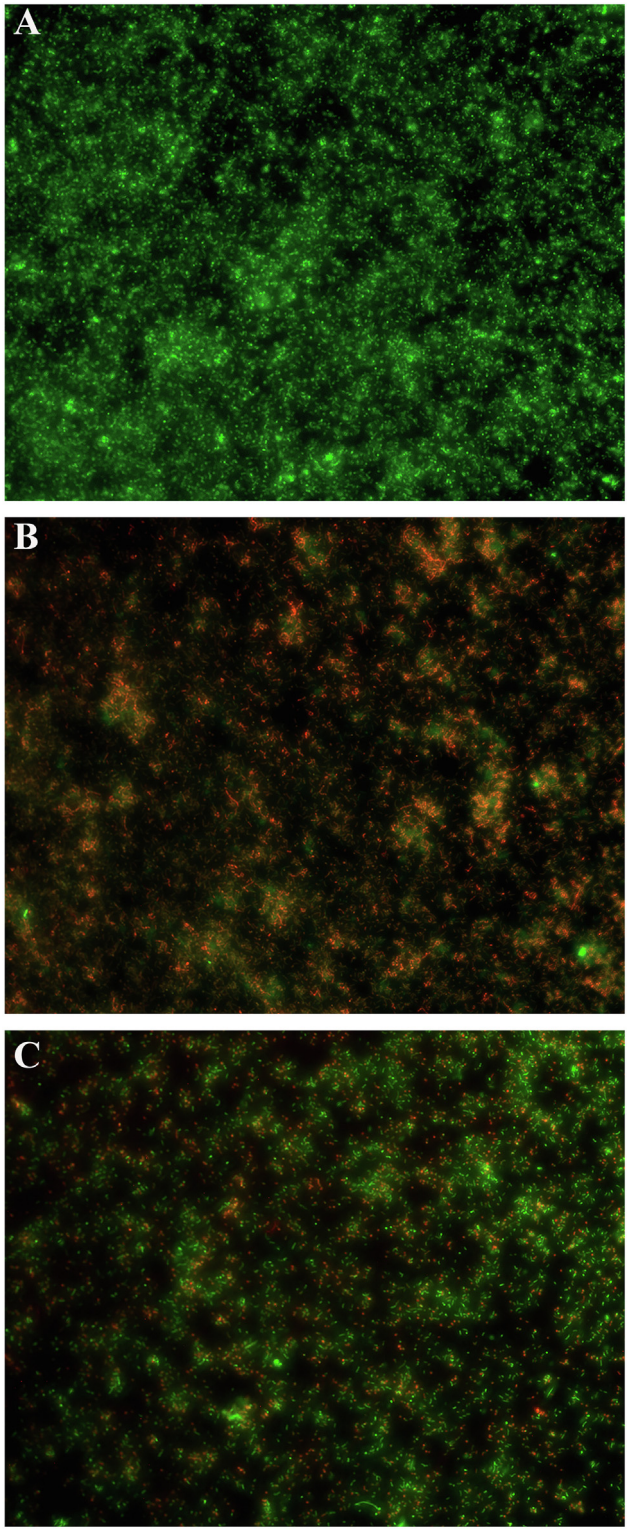

Figure 1 Biofilm formation of clinical oral *Helicobacter pylori* at 24 h (A), biofilm eradication by mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate after 24 h incubation (B), and biofilm eradication by mouthwash base (without 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate) after 24 h incubation (C) was investigated by fluorescence microscope (40X) staining with a Live/Dead *Bac*Light bacterial viability kit.

and 100% for PDL cells (Table 3). Mouthwash containing $BiCl_3$ showed complete killing of cells at 1 h incubation, and at 15 min incubation cell viability showed 4.2, 7.7, and 15.5% for AGS, H357, and PDL cells, respectively. While the mouthwash base showed cell viability of 63.0 ± 0.6, 77.0 ± 1.1, and 79.3 ± 0.7% for AGS, H357, and PDL cells after incubation for 15 min. The mouthwash base also showed lower amounts of cell viability than mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate.

The adhesion ability of *H. pylori* strains on H357 and AGS cells is shown in Fig. 2A. Overall, all *H. pylori* strains exhibited significantly higher adhesion on AGS cells compared to H357 cells, and *H. pylori* with *cag*A strains adhered to both cells higher than strains without *cag*A genes. After being combined with mouthwash, the adhesion of *H. pylori* with the *cag*A gene (from 61.1 ± 10.8 to 40.6 ± 7.7% for H357 cells and from 67.2 ± 13.7 to 36.0 ± 4.1% for AGS cells) or without the *cag*A gene (from 52.0 ± 9.0 to 44.7 ± 9.2% for H357 cells and from 56.4 ± 5.9 to 45.0 ± 3.1% for AGS cells) showed a significant decrease in both cells ($P < 0.05$). *H. pylori* with *cag*A gene-positive strains revealed high expression levels of *cag*A mRNA expression after incubation at anaerobic conditions, ranging from 117.0 to 9351.0 folds of induction (Fig. 2B). The *cag*A mRNA expression decreased significantly after being combined with mouthwash, ranging from 2.1 to 164.1 folds.

All pro-inflammatory cytokine expression in PDL cells showed lower expression than in AGS cells. It is interesting that the mouthwash base showed high pro-inflammatory cytokine stimulation, especially IL-8, which showed 22.8 ± 0.2 and 257.0 ± 0.3 folds for PDL and AGS cells, respectively. However, the mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate stimulated PDL and AGS cells to express all pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) lower than the mouthwash base, which ranged from 1.5 to 8.5 folds and 0.6 to 3.4 folds, respectively (Table 4). *H. pylori* strains that were *cag*A gene-positive expressed all pro-inflammatory cytokines higher than *H. pylori* strains that were *cag*A gene-negative. IL-8 mRNA (29.4–46.5 folds for AGS cells and 13.3–18.9 folds for PDL cells) showed greater expression than other cytokines, whilst IL-1β mRNA showed the lowest cytokine expression (2.7–3.2 folds for AGS cells and 1.1–1.2 folds for PDL cells). After combination with mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate, all pro-inflammatory cytokines were reduced compared to *H. pylori* alone (Table 4). The reduction in AGS and PDL cells were 1.5–1.9 folds and 1.1–1.5 folds for IL-1β, 1.4–1.6 folds and 1.2–1.6 folds for IL-6, 1.2–1.4 folds and 1.2–1.4 folds for IL-8, as well as 1.7–2.6 folds and 1.6–1.9 folds for TNF-α, respectively.

Discussion

H. pylori is a pathogen closely related to gastric cancer, and it has been confirmed that *H. pylori* eradication after treatment reduces the risk of gastric cancer. Our finding found that poly L-Lysine and glycerol monolaurate inhibited *H. pylori* growth, and a synergistic effect of a combination

Table 3 Cell viability treated with mouthwash containing poly L-Lysine and glycerol monolaurate or mouthwash containing $BiCl_3$.

Times	Cells survival rate (%)					
	Mouthwash[a]			$BiCl_3$		
	H357	PDL	AGS	H357	PDL	AGS
5 min	$79.8 \pm 2.9^{b,A}$	$100.0 \pm 0.0^{a,A}$	$77.2 \pm 1.4^{b,A}$	$15.5 \pm 1.7^{b,A}$	$29.3 \pm 1.1^{a,A}$	$10.3 \pm 1.8^{c,A}$
15 min	$53.9 \pm 0.6^{c,B}$	$100.0 \pm 0.0^{a,A}$	$61.2 \pm 1.5^{b,B}$	$7.7 \pm 1.1^{b,B}$	$15.2 \pm 2.3^{a,B}$	$4.2 \pm 0.1^{c,B}$
60 min	$32.0 \pm 2.0^{c,C}$	$95.4 \pm 0.0^{a,B}$	$42.3 \pm 1.1^{b,C}$	$0.0 \pm 0.0^{b,C}$	$0.0 \pm 0.0^{b,C}$	$2.7 \pm 0.0^{a,C}$
120 min	$20.8 \pm 1.0^{c,D}$	$82.8 \pm 0.0^{a,C}$	$34.5 \pm 1.6^{b,D}$	$0.0 \pm 0.0^{b,C}$	$0.0 \pm 0.0^{b,C}$	$2.2 \pm 0.0^{a,C}$
360 min	$16.9 \pm 0.8^{c,E}$	$55.6 \pm 0.0^{a,D}$	$28.5 \pm 1.6^{b,E}$	$0.0 \pm 0.0^{a,C}$	$0.0 \pm 0.0^{a,C}$	$0.0 \pm 0.0^{a,D}$
24 h	$12.5 \pm 0.8^{c,F}$	$35.6 \pm 0.0^{a,E}$	$17.4 \pm 1.2^{b,F}$	$0.0 \pm 0.0^{a,C}$	$0.0 \pm 0.0^{a,C}$	$0.0 \pm 0.0^{a,D}$

h = hour; Capital letters presented a statistical difference in the same column ($P < 0.05$); Lowercase letters presented a significant between cell survival rates of H357, PDL, and AGS cells ($P < 0.05$).

[a] Mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate.

between poly L-Lysine and glycerol monolaurate was found. Thus, mouthwash containing poly L-Lysine and glycerol monolaurate was used to study the reduction of virulence factors of *H. pylori* strains. Mouthwash containing poly L-Lysine and glycerol monolaurate eradicated biofilm by more than 50% at 2 h depending on incubation time, and the cell viability showed more than 50% at 15 min incubation for H357 and AGS cells, and 100% for PDL cells. In addition, the mouthwash could restrain the adhesion of *H. pylori* on host cells, suppress the expression of *cag*A, and reduce pro-inflammatory cytokine expression after being treated with *H. pylori*.

Nowadays, *H. pylori* strains are found to increase antibiotic resistance such as metronidazole, levofloxacin, and clarithromycin, but the resistance to amoxicillin and tetracycline is usually low according to our study.[2,15] The treatment has to use triple therapy using a proton pump inhibitor or ranitidine bismuth citrate combined with clarithromycin or amoxicillin or metronidazole to provide a high-efficacy treatment. However, the recurrence of *H. pylori* still increased because it can be transmitted from the oral to gastrointestinal tract,[4,5] so *H. pylori* in the oral cavity needs to be removed. Mouthwash is an alternative way to remove or reduce the number of *H. pylori* in the oral cavity (saliva and plaque). In observation of anti-*H. pylori* activity in planktonic and biofilm forms, the mouthwash base could inhibit the growth of *H. pylori* in both forms because the mixture of mouthwash base included methyl 4-hydroxybenzoate, an anti-microbial preservative in cosmetics. However, after adding poly L-Lysine and glycerol monolaurate in the mouthwash base as in our study, there was high anti-*H. pylori* activity in planktonic form and *H. pylori* biofilm was eradicated. Poly L-Lysine is adsorbed electrostatically to the cell surface of the bacterial outer membrane causing damage to the *H. pylori* cells and glycerol monolaurate. This is a biological activity because of increased bacterial membrane permeability or disruption of electron transportation and oxidative phosphorylation or inhibition of membrane enzymatic activities.[10,16] In addition, this study revealed that a combination of poly L-Lysine and glycerol monolaurate exhibited a synergistic response compared to a single compound. In addition, a combination of both compounds could inhibit the growth of other oral pathogens such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Streptococcus mutans* (unpublished results).

Biofilm formation in this study used a monolayer of *H. pylori* on 96 well plates. Mouthwash containing poly L-Lysine and glycerol monolaurate demonstrated biofilm eradication of more than 50% at 2 h while mouthwash containing $BiCl_3$ (standard treatment for *H. pylori* infection) was used for 5 min for 50% biofilm eradication. Bismuth is used for bactericidal effects on *H. pylori* by forming complexes in the bacterial cell wall and periplasmic space, inhibiting different enzymes, ATP synthesis, and adherence of the bacteria to the gastric mucosa.[17] Although $BiCl_3$ showed a high biofilm eradication, it showed low cell viability of host cells, which may be due to bismuth-induced oxidative stress, mitochondrial dysfunction, and consequently, apoptosis-like mode of cell death in human cells.[18] According to our study, it was found that mouthwash containing $BiCl_3$ showed high toxicity to both oral and gastric cells. While the mouthwash containing poly L-Lysine and glycerol monolaurate has low cytotoxicity to human periodontal ligament cells (PDL cells), human oral keratinocyte cells (H357 cells), and human gastric adenocarcinoma cells (AGS cells), glycerol monolaurate showed no cytotoxicity to dendritic cells and human gingival fibroblast cells according to a previous study.[16,19] Although a report showed that glycerol monolaurate has shown to be toxic to cells by inducing cell apoptosis, that study used high concentrations of glycerol monolaurate.[20] The concentration of glycerol monolaurate showed toxicity to human gingival fibroblast cells of more than 640 μM,[19] and which began to aggregate on the bottom of a microtiter plate.

H. pylori infection can be found in the oral cavity, especially dental plaque. The first channel of *H. pylori* for humans is the oral cavity, but its function in *H. pylori* infection is generated in the gastric tissue. A previous study showed a high prevalence of *H. pylori* in the plaque (supra- and sub-gingival plaque) in patients with gastritis and gastric cancer.[7] It may be due to there being a correlation between *H. pylori* infection in periodontal disease and gastric reinfection. Thus, this study would like to examine the anti-adhesion and immune responses of two types of cells (oral and gastric cells). Adhesion ability is the first

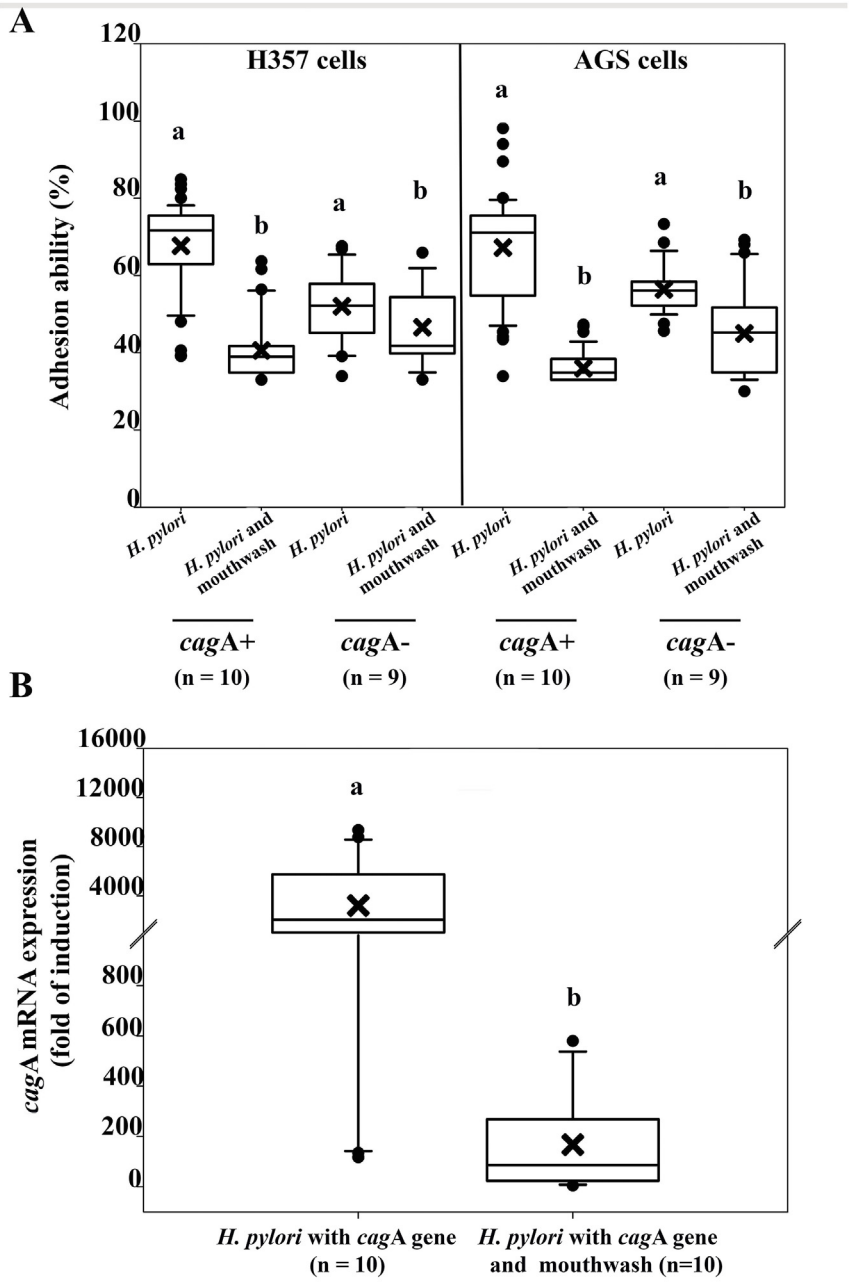

Figure 2 Reduction of adhesion ability (A) and *cag*A mRNA expression (B) of *Helicobacter pylori* single strain and a combination with mouthwash containing poly L-lysine and glycerol monolaurate (mouthwash). Lowercase letters represent significant differences between adhesion ability to host cells or *cag*A mRNA expression of *Helicobacter pylori* individual strain and a combination of *Helicobacter pylori* and the mouthwash ($P < 0.05$).

mechanism of *H. pylori* in order to colonize the oral cavity and gastric tissue, and inhibition in this process can protect the initial growth or progression of the disease. Mouthwash containing poly L-Lysine and glycerol monolaurate decreased the adhesion of *H. pylori* to H357 and AGS cells due to the inhibiting function of the *vac*A gene (unpublished results). This gene may be associated with the adhesion ability or block adhesion or flagellar filament of *H. pylori* to adhere to host cells. However, the mechanism of the mouthwash that inhibited *H. pylori* adhesion is still unclear and needs to be clarified.

This study found that the mouthwash base highly stimulated pro-inflammatory cytokines in both cells, particularly IL-8, because of methyl 4- hydroxybenzoate. The pro-inflammatory cytokines decreased after diluting the mouthwash base with water (1:100) showing low pro-inflammatory cytokines (unpublished results). It is interesting to find that mouthwash containing poly L-Lysine and glycerol monolaurate induced low pro-inflammatory cytokines and suppressed pro-inflammatory cytokine stimulation (IL-1β, IL-6, IL-8, and TNF-α) after being induced by *H. pylori* to PDL and AGS cells. Pro-inflammatory cytokine

Table 4 Pro-inflammatory cytokine mRNA expression (folds of induction) induced by oral *Helicobacter pylori* single strain and in combination with mouthwash containing poly L-Lysine and glycerol monolaurate on AGS and PDL cells.

Samples	IL-1β median (min–max), mean ± SD	IL-6 median (min–max), mean ± SD	IL-8 median (min–max), mean ± SD	TNF-α median (min–max), mean ± SD
AGS cells				
Mouthwash[a]	1.5 (0.9–2.1), 1.5 ± 0.4	2.3 (2.0–2.5), 2.3 ± 0.2	8.5 (8.1–8.8), 8.5 ± 0.3	1.5 (0.3–2.7), 1.5 ± 1.3
*cag*A negative	2.7 (2.5–2.8), 2.7 ± 0.1[a]	3.3 (2.9–5.0), 3.6 ± 0.8[a]	29.4 (25.4–34.1), 29.6 ± 4.0[a]	2.8 (2.3–6.8), 3.7 ± 1.7[a]
*cag*A negative and mouthwash[a]	1.7 (1.6–2.0), 1.8 ± 0.2[b]	2.3 (1.6–4.1), 2.5 ± 0.8[b]	23.7 (16.4–36.7), 24.9 ± 7.0[b]	1.2 (1.0–1.7), 1.4 ± 0.3[b]
*cag*A positive	3.2 (2.8–3.7), 3.2 ± 0.3[a]	4.7 (4.4–4.8), 4.6 ± 0.2[a]	44.8 (34.2–61.7), 46.5 ± 8.9[a]	5.7 (4.3–6.5), 5.5 ± 0.7[a]
*cag*A positive and mouthwash[a]	1.7 (0.9–3.3), 1.8 ± 0.6[b]	2.9 (2.8–2.9), 2.9 ± 0.2[b]	34.1 (13.1–46.0), 32.5 ± 9.5[b]	3.1 (1.8–5.3), 3.3 ± 1.2[b]
PDL cells				
Mouthwash[a]	0.1 (0.0–2.1), 0.9 ± 0.9	0.6 (0.6–0.7), 0.6 ± 0.1	3.1 (1.9–5.4), 3.4 ± 1.7	0.0 (0.0–1.2), 0.4 ± 0.5
*cag*A negative	1.1 (1.0–1.1), 1.1 ± 0.1[a]	1.3 (0.9–1.5), 1.1 ± 0.2[a]	13.3 (11.3–15.3), 13.3 ± 1.9[a]	1.5 (1.3–1.5), 1.4 ± 0.1[a]
*cag*A negative and mouthwash[a]	0.6 (0.1–1.6), 1.0 ± 0.6[b]	1.0 (0.2–1.7), 0.9 ± 0.5[b]	11.2 (1.1–15.0), 9.8 ± 5.0[b]	0.9 (0.8–1.0), 0.9 ± 0.1[b]
*cag*A positive	1.2 (1.2–1.3), 1.2 ± 0.1[a]	1.4 (1.1–1.4), 1.3 ± 0.1[a]	19.2 (16.5–20.9), 18.9 ± 2.3[a]	2.8 (2.6–3.5), 3.9 ± 0.5[a]
*cag*A positive and mouthwash[a]	0.8 (0.6–0.9), 0.8 ± 0.1[b]	0.8 (0.6–1.0), 0.8 ± 0.2[b]	14.4 (10.8–27.4), 15.9 ± 4.9[b]	2.0 (1.9–2.4), 2.1 ± 0.2[b]

[a] Mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate; Lowercase letters presented significant differences in pro-inflammatory cytokines between the *H. pylori* single strain and a combination with mouthwash containing 0.03 mg/mL poly-L-Lysine and 0.02 mg/mL glycerol monolaurate ($P < 0.05$).

suppression might be caused by the mouthwash block of the function of the *cag*A gene, which is associated with changes in cytokine signaling and cell cycle control of *H. pylori* with cytokine stimulation. Our study showed that *cag*A mRNA expression decreased after being treated with mouthwash containing poly L-Lysine and glycerol monolaurate, which had the effect of reducing pro-inflammatory cytokines. Moreover, a previous study reported that glycerol monolaurate suppressed human gingival fibroblast to express IL-1β, IL-6, and TNF-α after being stimulated with *A. actinomycetemcomitans*.[16] Thus, mouthwash containing poly L-Lysine and glycerol monolaurate in this study may indicate a positive modulation in the inflammatory response in the oral cavity and gastric tissue.

In conclusion, mouthwash containing poly L-Lysine and glycerol monolaurate could reduce the virulence of *H. pylori* strains including having anti-growth, anti-biofilm, anti-inflammation, and anti-adhesion properties. Moreover, mouthwash containing poly L-Lysine and glycerol monolaurate has low cytotoxicity to host cells, human oral epithelial cells, human periodontal ligament cells, and human gastric cells. This means that the mouthwash is safe to use in humans. However, as this study lacks data on mouthwash in volunteers, further research needs to study the effects of such.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was granted by the National Science, Research and Innovation Fund (MED6505193a) and the Research fund from the Faculty of Dentistry, Prince of Songkla University for Dr. Nuntiya Pahumunto. The authors would like to acknowledge the Faculty of Dentistry, Prince of Songkla University for facilitating the study.

References

1. Kleanthous H, Lee CK, Monath TP. Vaccine development against infection with *Helicobacter pylori*. *Br Med Bull* 1998; 54:229–41.
2. Agudo S, Perez-Perez G, Alarcon T, Lopez-Brea M. High prevalence of clarithromycin resistant *Helicobacter pylori* strains and risk factors associated with resistance in Madrid, Spain. *J Clin Microbiol* 2010;48:3703–7.
3. De Francesco V, Giorgio F, Hassan C, et al. Worldwide *H. pylori* antibiotic resistance: a systematic review. *J Gastrointestin Liver Dis* 2010;19:409–14.
4. Hu Y, Wan JH, Li XY, Zhu Y, Graham DY, Lu NH. Systematic review with meta-analysis: the global recurrence rate of *Helicobacter pylori*. *Aliment Pharmacol Ther* 2017;46:773–9.
5. Xue Y, Zhou LY, Lu HP, Liu JZ. Recurrence of *Helicobacter pylori* infection: incidence and influential factors. *Chin Med J* 2019;132:765–71.
6. 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;16:398—403.
7. Wongsuwanlert M, Teanpaisan R, Pahumunto N, Kaewdech A, Ruangsri P, Sunpaweravong S. Prevalence and virulence factors of *Helicobacter pylori* isolated from oral cavity of non-disease, gastritis, and gastric cancer patients. *J Dent Sci* 2023 (in press).
8. Shirin H, Moss SF. *Helicobacter pylori* induced apoptosis. *Gut* 1998;43:592—4.
9. Kashyap D, Baral B, Verma TP, et al. Oral rinses in growth inhibition and treatment of *Helicobacter pylori* infection. *BMC Microbiol* 2020;20:1—18.
10. Wang XM, Yee KC, Hazeki-Taylor N, et al. Oral *Helicobacter pylori*, its relationship to successful eradication of gastric *H. pylori* and saliva culture confirmation. *J Physiol Pharmacol* 2014;65:559—66.
11. Liu JX, Werner J, Kirsch T, Zuckerman JD, Virk MS. Cytotoxicity evaluation of chlorhexidine gluconate on human fibroblasts, myoblasts, and osteoblasts. *J Bone Jt Infect* 2018;3:165—72.
12. Pahumunto N, Chotjumlong P, Makeudom A, Krisanaprakornkit S, Dahlen G, Teanpaisan R. Pro-inflammatory cytokine responses in human gingival epithelial cells after stimulation with cell wall extract of *Aggregatibacter actinomycetemcomitans* subtypes. *Anaerobe* 2017;48:103—9.
13. Tomasini ML, Zanussi S, Sozzi M, Tedeschi R, Basaglia G, De Paoli P. Heterogeneity of *cag* genotypes in *Helicobacter pylori* isolates from human biopsy specimens. *J Clin Microbiol* 2003;41:976—80.
14. Clayton CL, Pallen MJ, Kleanthous H, Wren BW, Tabaqchali S. Nucleotide sequence of two genes from *Helicobacter pylori* encoding for urease subunits. *Nucleic Acids Res* 1990;18:362.
15. Francesco VD, Zullo A, Hassan C, Giorgio F, Rosania R, Ierardi E. Mechanisms of *Helicobacter pylori* antibiotic resistance: an updated appraisal. *World J Gastrointest Pathophysiol* 2011;2:35—41.
16. Silva VO, Pereira LJ, Pasetto S, da Silva MP, Meyers JC, Murata RM. Effects of monolaurin on oral microbe-host transcriptome and metabolome. *Front Microbiol* 2018;9:1—9.
17. Alkim H, Koksal AR, Boga S, Sen I, Alkim C. Role of bismuth in the eradication of *Helicobacter pylori*. *Am J Therapeut* 2017;24:751—7.
18. Akhtar MJ, Ahamed M, Alhadlaq H. Bismuth Oxide (Bi_2O_3) Nanoparticles cause selective toxicity in a human endothelial (HUVE) cell line compared to epithelial cells. *Toxics* 2023;11:1—12.
19. Poulsen C, Mehalick LA, Fischer CL, et al. Differential cytotoxicity of long-chain bases for human oral gingival epithelial keratinocytes, oral fibroblasts, and dendritic cells. *Toxicol Lett* 2015;237:21—9.
20. Johnson CR, Chun J, Bittman R, Jarvis WD. Intrinsic cytotoxicity and chemomodulatory actions of novel phenethylisothiocyanate sphingoid base derivatives in HL-60 human promyelocytic leukemia cells. *J Pharmacol Exp Therapeut* 2004;309:452—61.