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In vitro synergistic antibacterial and anti-inflammatory effects of nisin and lactic acid in yogurt against *Helicobacter pylori* and human gastric cells

Abstract

Helicobacter pylori (*H. pylori*) is a bacterium that naturally thrives in acidic environments and has the potential to induce various gastrointestinal disorders in humans. The antibiotic therapy utilized for treating *H. pylori* can lead to undesired side effects, such as dysbiosis in the gut microbiota. The objective of our study was to explore the potential antibacterial effects of nisin and lactic acid (LA) in yogurt against *H. pylori*. Additionally, we investigated the anti-inflammatory effects of nisin and LA in human gastric (AGS) cells infected with *H. pylori*. Nisin and LA combination showed the strongest inhibitory activity, with confirmed synergy at 0.375 fractional inhibitory concentration index. Also, post-fermented yogurt with incorporation of nisin exhibited antibacterial effect against *H. pylori*. The combination of nisin and LA resulted in a significant reduction of mRNA levels of bacterial toxins of *H. pylori* and pro-inflammatory cytokines in AGS cells infected with *H. pylori*. Furthermore, this also increased bacterial membrane damage, which led to DNA and protein leakage in *H. pylori*. Overall, the combination of nisin and LA shows promise as an alternative therapy for *H. pylori* infection. Additionally, the incorporation of nisin into foods containing LA presents a potential application. Further studies, including animal research, are needed to validate these findings and explore clinical applications.

KEYWORDS:

yogurt, nisin, lactic acid, Helicobacter pylori, antibacterial effects

Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium that approximately has 50% global human infection rate (Suerbaum and Michetti, 2002). *H. pylori* easily survive in the gut's acidic environment. Thus, it can easily cause various gastrointestinal diseases such as gastritis, peptic ulcers, and stomach cancer (Danesh, 1999). The development of gastric cancer is known to be caused by inflammatory responses produced in gastric epithelial cells against chronic *H. pylori* infection (Wen and Moss, 2009). The primary causes of inflammatory responses in *H. pylori* infections are the cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA), which are associated with the progression to gastric cancer (Suriani et al., 2008). CagA is delivered into gastric epithelial cells through a type IV secretion system, and once inside the cell, it can activate the NF-kB pathway, leading to the production of pro-inflammatory cytokines (Jones et al., 2010). VacA induces the production of reactive oxygen species in gastric epithelial cells, which can also result in the production of pro-inflammatory cytokines (Jones et al., 2010). Due to the carcinogenic potential of *H. pylori*, the World Health Organization (WHO) and International Agency for Research on Cancer (IARC) classified *H. pylori* as a Group I carcinogen in 1994 (Lochhead and El-Omar, 2007).

Currently, commonly used eradication treatment for *H. pylori* infection is combination therapy with antibiotics such as ampicillin, amoxicillin, and metronidazole, along with acid-reducing medications (Mahony et al., 1992). However, antibiotic treatments have many health risks. Indeed, 22,292 patients infected with *H. pylori* who received these treatments, 22% experienced side effects such as a metallic taste, diarrhea, nausea, and vomiting (Nyssen et al., 2021). Therefore, the development and evaluation of safer alternatives to antibiotics are

necessary to help patients who are sensitive or allergic to prescription antibiotics (Hafeez et al., 2021).

A possible solution to the aforementioned problem is the utilization of bacteriocin, antibacterial peptides, produced by bacteria (Todorov et al., 2019). Nisin, the most well-known bacteriocin, is an antibacterial peptide produced by generally recognized as safe (GRAS) registered Lactococcus lactic strains and it has been approved as a food preservative through Food and Agriculture Organization (FAO) and WHO (Chalón et al., 2012). Thus, nisin is widely used as a natural food bio-preservative due to the antibacterial effect against a wide range of clinical and food-borne pathogens (Amiri et al., 2021). Recent studies have also suggested that nisin has anti-inflammatory effects by inhibiting the production of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF- α), interluekin6 (IL-6), and interleukin-1 beta (IL-1 β) in rat uterine (Jia et al., 2019). Also, nisin helped wound healing process by down regulating pro-inflammatory cytokines in human keratinocytes (Mouritzen et al., 2019).

However, fermentative production of nisin is complicated and requires additional purification step which increases overall cost of production. This makes nisin relatively more expensive to produce as compared to other antibacterial substances (Elshaghabee et al., 2016). Recently, ongoing research is exploring new ways to use nisin at a lower cost. Previous studies have sought to find substances that can enhance the antibacterial effect of nisin, which is possible to reduce the amount of nisin to help save costs (Kirazli and Tunca, 2022). For example, combination of nisin with essential oils, such as oregano, thyme, or rosemary, have been found to have a synergistic effect, resulting in a reduction in the required dosage of nisin (Bajpai et al., 2012).

77 Lactic acid (LA), produced by lactic acid bacteria (LAB), is known to effective against a
78 broad range of bacteria to increase the permeability of bacterial membrane (Alakomi et al.,
79 2000). In previous study, nisin and LA can work synergistically to increase the overall
80 antibacterial activity of Escherichia coli in red meat (De Martinez et al., 2002). Also, the
81 combination of nisin and other substances that can enhance the antibacterial effect of nisin
82 can allow for the use of lower concentrations of both agents, which can help to reduce costs
83 (Field et al., 2017).

84 Yogurt, a type of fermented milk, is a widely consumed fermented dairy product that is
85 popular around the world. During the fermentation process, LAB strains produce LA, which
86 transforms milk into yogurt by coagulation (Jeong et al., 2018). Trials adding nisin to yogurt
87 have not been attempted due to concerns that it may inactivate LAB in yogurt. However,
88 several evidence showed the positive health effects of inactivated LAB, called parabiotics
89 (Kazemi et al., 2021). Parabiotics have shown to have beneficial health effects such as
90 supporting the immune system, improving gut health by supporting the growth of beneficial
91 bacteria, reducing inflammation, and improving digestion (Kazemi et al., 2021). Therefore,
92 exploring the potential health benefits of incorporating nisin into post-fermented yogurt
93 would be a valuable research endeavor.

94 Consequently, our study aimed to investigate the antibacterial effects of yogurt, which
95 naturally contains LA, and explore the feasibility of supplementing it with nisin. This
96 investigation will assess the potential for incorporating nisin into post-fermented yogurt. We
97 also assessed the antibacterial effect of nisin and LA, as well as their impact on *H. pylori*
98 bacterial toxins, bacterial membrane damage and inflammatory cytokines in human gastric
99 (AGS) cells infected with *H. pylori*.

Materials and Methods

Preparation of nisin

A commercial nisin standard (Sigma Chemical Co., St. Louis, Mo.) was used in the present work. A stock nisin solution was prepared by commercial nisin standard into 10 mL of sterile 0.02 N HCl, centrifuging at 5,000 x g for 15 min and sterilizing by filtration through 0.22 μm filters. A stock nisin solution was stored at 4 °C until use.

Microorganism culture condition

H. pylori ATCC 43504 strains (obtained from American Type Culture Collection, Manassas, VA, USA) was used as the test organism. *H. pylori* was grown in Brucella agar (MB cell. Ltd., Seoul. Korea) supplemented with 5 % fetal bovine serum (FBS; Welgene Inc., Gyeongsan-si, Korea). A single colony from brucella agar was inoculated in 10 mL of brucella broth supplemented with 5 % FBS and incubated at 37°C with shaking at 150 rpm under microaerobic conditions (5–8% carbon dioxide) until the cell density reached 1 x 10^9 CFU/mL.

Broth microdilution method

Minimum inhibition concentration (MIC) values of nisin, LA, and ampicillin against *H. pylori* was determined using broth microdilution method on 96-well plates. Briefly, each well was inoculated with 100 μL of *H. pylori* adjusted to 1 x 10^6 CFU/mL. Serial 2-fold dilutions

of nisin, LA, and ampicillin were treated in each well at final concentrations of 0.01 to 40 μg/mL, 0.04 to 5 %, and 0.01 to 50 μg/mL, respectively. The final total volume of each well was 200 μL, and the final bacterial number was adjusted to 5 x 10^5 CFU/mL. The MIC value was defined as the lowest concentration of nisin and LA alone or in combination with nisin and LA that showed visible growth inhibition after 24 h incubating at 37°C under micro-aerobe condition.

Checkerboard assay

A checkerboard assay was carried out to evaluate the synergistic effects of nisin and LA (Zhao et al., 2023). Briefly, the rows on the x-axis of the 96-well plate contained 2-fold dilution of nisin, and the columns on the y-axis contained 2-fold dilution of LA The final concentration of *H. pylori* was adjusted to 5 x 10^5 CFU/mL for each well and then the plates were incubated at 37°C under micro-aerobe condition. The synergistic effects of each combination were determined by the fractional inhibitory concentration index (FICI) (Shi et al., 2017). The fraction inhibitory concentration index (FICI) was calculated as following formula: FICI = ΣFIC = FIC (nisin) + FIC (LA), where FIC (nisin) = MIC of nisin in combination/MIC of nisin alone, and FIC (LA) = MIC of LA in combination/MIC of LA alone. The interpretations of ΣFIC values were as follows: Synergy, if ΣFIC ≤ 0.5; no interaction, if ΣFIC > 0.5 to <2, and antagonism, if ΣFIC ≥ 2.

Yogurt and supernatant preparation

Yogurt was produced using 12% (w/v) of non-fat milk (Seoul Dairy cooperative, Seoul,

Korea). The milk was pasteurized at 85°C for 30 min and then cooled to 24°C. Next, a starter culture (2%, v/v) was added to the milk. The starter culture was prepared by incubating a mixture of 100 mL of non-fat milk (12%, w/v) and 0.34g of starter culture powder (Samik Dairy and Food Co. Ltd., Seoul, Korea) for 5 h. The starter culture contained mixed strains of Lactobacillus acidophilus (35%), Bifidobacterium longum (30%), and Streptococcus thermophilus (35%). The inoculated milk samples were then placed in an incubator at 42°C until they reached a pH of 4.5–4.6. The supernatants from the yogurt were separated by centrifuging samples (10 g) twice at 4,000 x g for 10 min at 4°C. Subsequently, the supernatants were further centrifuged at 10000 x g for 10 min at 4°C. They were then filtered through 0.45-μm syringe filter (Advantec, Tokyo, Japan) and stored at -80°C until use.

LA measurement

To determine the amount of LA in the yogurt, the titratable acidity was measured. At the end of the fermentation process, 10 g of yogurt was mixed with 10 mL of distilled water and titrated to a pH of 8.3 using 0.1 N NaOH. The TA was calculated using the following formula:

Titratable acidity (%) = (mL of NaOH used x 0.009) / (weight of yogurt in grams) x 100

Here, 0.009 is the conversion factor for LA, which is used to convert the volume of NaOH using into an equivalent amount of LA.

Comparative evaluation of antibacterial effects of yogurt supplemented with nisin and combination of nisin and LA

To evaluate the antibacterial effect, the agar well diffusion assay was used for both the yogurt supplemented with nisin and the combination of nisin and LA. A 100 µL aliquot of a 1x 10^8 CFU/mL *H. pylori* suspension was spread over brucella agar with 5% FBS, and then nisin (0, 1.25, 2.5, or 5 µg/mL) or nisin-supplemented yogurt supernatant (0, 1.25, 2.5, or 5 µg/mL) were added to each well on the agar. To further evaluate the effects of nisin, LA and their combination, and ampicillin (positive control), each was added to individual wells in the agar with different concentrations. The clear inhibition zone diameter (mm) was measured after 24 h of incubation at 37°C under micro-aerobe conditions.

Lactate dehydrogenase (LDH) activity assay

LDH assay was carried out by investigating the cytotoxicity of *H. pylori* (Sohn et al., 2020). In brief, AGS cells grown in 96-well plate were treated with different concentration (10^6, 10^7, 10^8, and 10^9 CFU/mL) of *H. pylori* and incubated at 37 °C for 24 h. 45 min before the end of incubation, cells were treated with lysis buffer for a positive control. After that, the medium was transferred to a 1.7 mL tube. Centrifugation was done to collect the supernatants. Supernatants 50 µL were transferred to a new 96-well plate and 50 µL of the Cytotox 96® reagent were added. The 96-well plate was incubated at 25 °C for 30 min in the dark. The optical value was measured at 490 nm with spectrophotometer, and the percentage of LDH was calculated as following formula:

LDH release (%) = [(ODsample LDH release/ODmaximum LDH release) × 100]

Cell culture and treatment

AGS cells were maintained in RPMI 1640 medium (Welgene Inc., Gyeongsan-si, Korea) containing 10% FBS, 1% penicillin /streptomycin (v/v) at 37 °C in humidified atmosphere containing 5% CO2. For the experiment, *H. pylori* was centrifuged and washed twice with PBS. Before cell treatment, *H. pylori* was resuspended in antibiotic-free RPMI 1640 medium and diluted to the desired concentration.

Cell viability assay

The cell viability was evaluated by the trypan blue dye exclusion assay (Jeong et al., 2021). AGS cells were grown in 6-well plates and treated with different concentration of nisin, LA, their combination, or ampicillin, and incubated at 37°C for 24 h. Viable cell number were counted using a hemocytometer (Hausser Scientific, Horsham, PA, USA) under an optical microscope.

Real-time polymerase chain reaction analysis

The levels of mRNA expression for bacterial toxins (i.e., VacA and CagA) and pro-inflammatory cytokines (IL-6, IL-8, IL-1β, and TNF-α) in AGS cells were evaluated by RT-PCR. For quantification of mRNA expression of *H. pylori* toxin factors, 100 μL of *H. pylori* culture was inoculated in 10 mL of brucella broth containing 5 % FBS with different concentration of nisin, LA, its combination and ampicillin for 3 h. Then, the bacterial pellet was obtained through centrifugation at 8,000 x g for 5 min at 4°C. TRIZOL reagent (Life Technologies, Eugene, OR) 500 μL was used to extract RNA and TOPscript RT DryMIX kit (Enzynomics, Daejeon, Korea) was used for cDNA synthesis. The reference gene 16S rRNA

expression levels were used to quantify the relative mRNA expression levels. To quantify mRNA expression of pro-inflammatory cytokines, AGS cells were grown in a 6-well plate and treated with 10^8 CFU/mL of *H. pylori* in antibiotic-free RPMI medium for 6 h. Afterward, the bacteria-containing medium was withdrawn and rinsed thrice with PBS. Next, the cells were treated with nisin, LA, its combination or ampicillin for 3 h. After incubation, the medium was withdrawn and rinsed thrice with PBS. TRIZOL 500 µL was used to extract RNA, and TOPscript RT DryMIX kit was used for cDNA synthesis. The reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels were used to quantify the relative mRNA expression levels. The PCR conditions were as follows: 95°C for 15 min (denaturation) and 80 cycles of amplification at 95°C for 20 s, followed by respective annealing temperature and finally held at 4°C. The primer sequences used in current study are shown in Table 1.

Measurement of DNA and protein leakage

DNA and protein leakage was determined by measuring the absorbance of culture supernatants using spectrophotometer (Kirazli and Tunca, 2022). In brief, nisin (5 µg/mL), LA (0.6 %), their combination, or ampicillin (6.25 µg/mL) were added to the 10^8 CFU/mL of *H. pylori* suspensions in 1 x PBS. The suspensions were incubated at 37°C with shaking under microaerobic conditions for 3 h. Then, 0.22 µm microporous membrane was used to remove the bacteria. The leakage of DNA and protein was measured using Epoch microplate spectrophotometry at the absorbances of 260 and 280 nm, respectively.

Synergistic effect of nisin and LA on morphological changes in H. pylori

The morphology changes of *H. pylori* were observed by field emission scanning electron microscope (FE-SEM; SU-8010, Hitachi, Tokyo, Japan). *H. pylori* suspensions were treated with the antibacterial substances at 37 °C for 3 h under micro-aerobe condition (5–8% carbon dioxide). After incubation, the cells were collected and washed thrice with PBS and were fixed with 2.5% glutaraldehyde overnight at 4°C. After washing with PBS, the cells were dehydrated with 30, 50, 70, 90% ethanol solutions subsequently. After freeze drying, the cells were fixed on the FE-SEM support and sputtered with platinum under vacuum. Then, the morphologies were observed by FE-SEM.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed with SPSS-PASW Ver. 18.0 (SPSS Inc., IL, USA). Analysis of variance (ANOVA) was analyzed using one-way ANOVA with Dunnett's test post hoc test. Differences were considered statistically significant when p-values were < 0.05.

Results

MIC of nisin and LA, and their synergistic antibacterial effects in combination

The MIC of nisin, LA, and ampicillin alone or in combination against *H. pylori* was shown in Table 2. The MIC of nisin against *H. pylori* was determined to be 10 μg/mL and the MIC of LA and ampicillin was determined to be 0.6% and 0.195 μg/mL, respectively. The combined

effect of nisin and LA was calculated using checkerboard test (Table 2). The synergistic effect of nisin and LA was observed against *H. pylori*. After combining nisin and LA, MIC of nisin was 1.25 μg/mL (8 times lower than the MIC when used alone) and MIC of LA was 0.15% (4 times lower than the MIC when used alone). As shown in Table 2, the combined FICI value of nisin and LA was determined to 0.375, indicating synergism (FICI<0.5). The results suggest that combination of nisin and LA could effectively inhibit *H. pylori* growth and lower the concentration of nisin and LA.

Antibacterial effects of nisin-added yogurt supernatant, and nisin, LA or its combination against H. pylori

The yogurt contained 0.61 ± 0.01% LA based on titratable acidity. Agar well diffusion method was performed to determine the combined antibacterial effect of nisin (1.25, 2.5, and 5 μg/mL) and yogurt supernatant (Fig. 1A). The combination of yogurt supernatant with 5 μg/mL of nisin exhibited the highest inhibitory activity against *H. pylori*, with an inhibition zone diameter of 14.33 ± 0.33 mm. This inhibition zone was significantly greater than that of nisin alone as well as than that of yogurt supernatant alone (Fig. 1A). In fact, nisin alone did not exhibit any inhibition against *H. pylori* at all concentration. To further investigate the antibacterial effects of nisin, LA, nisin-LA combination, an agar well diffusion assay was conducted (Fig. 1B). Different concentrations of nisin (1.25, 2.5, and 5 μg/mL) were combined with 0.6% LA, and their antibacterial activities were compared with those of nisin and LA alone, as well as with ampicillin. The combination of nisin (5 μg/mL) with LA (0.6%) showed the highest antibacterial activity against *H. pylori*, with an inhibition zone diameter of 22.33 ± 0.33 mm, which was significantly greater than that of the same concentrations of

nisin and LA treated alone, and comparable to that of ampicillin (22.67 ± 0.67 mm).

Cytotoxicity of nisin, LA, combination of nisin and LA, and H. pylori in AGS cells

Trypan blue dye exclusion assay was used to evaluate the effect of nisin, LA, their combination, or ampicillin of AGS cell viability (Fig. 2A). Treatment of cells with nisin (1.25, 2.5, 5 μg/mL) and ampicillin (3.125, 6.25 μg/mL) for 24 h did not show any significant changes in cell viability compared to the control ($p > 0.05$). Similarly, treatment with LA (0.3, 0.6%) and combination of nisin and LA group did not significantly affect cell viability ($p > 0.05$). However, treatment with 1.2% of LA for 24 h resulted in a significant decrease in cell viability ($p < 0.001$) compared to the control. Based on these results, further in vitro studies were performed using 5 μg/mL nisin, 0.6% LA, and 6.25 μg/mL ampicillin. AGS cells treated with a concentration of 10^9 CFU/mL of *H. pylori* for 24 h showed significantly higher LDH release than control (Fig. 2B). Therefore, a concentration of 10^8 CFU/mL of *H. pylori* was used for further in vitro studies.

Reduction of mRNA levels of bacterial toxins in H. pylori

The results of the mRNA levels of bacterial toxins (i.e., CagA and VacA) in *H. pylori* are presented in Fig. 3. Treatment of *H. pylori* with 5 μg/mL of nisin resulted in a decrease in mRNA expression levels of CagA (Fig. 3A) and VacA (Fig. 3B), while LA or ampicillin alone did not affect mRNA levels ($p > 0.05$). However, when nisin and LA were used in combination, the mRNA level of CagA decreased significantly, compared to the control ($p < 0.001$). Similarly, the mRNA level of VacA decreased significantly compared to the control (p

297 < 0.05). These results suggest that the combination of nisin and LA effectively reduce
298 bacterial toxins in *H. pylori*.

299

300 ***Anti-inflammatory effect of nisin and LA in AGS cells infected with H. pylori***

301 The mRNA expression levels of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-8,
302 and IL-6, increased in AGS cells upon addition of 10^8 CFU/mL of *H. pylori* (Fig. 4A–D).
303 Treatment of AGS cells with nisin alone resulted in a decrease in the mRNA expression of
304 these cytokines, while LA alone didn't show a significant decrease in their mRNA
305 expressions. However, when nisin and LA were used in combination, a significant reduction
306 in the mRNA levels of the pro-inflammatory cytokines was observed, with levels similar to
307 the control without *H. pylori* infection ($p>0.05$) for all pro-inflammatory cytokines. In
308 contrast, the ampicillin-treated group showed no such effects. These results suggest that the
309 combination of nisin and LA may have a synergistic effect in downregulating the
310 inflammatory in *H. pylori* infected AGS cells.

311

312 ***DNA and protein leakage in H. pylori***

313 The effect of nisin and LA on the leakage of the DNA and protein from *H. pylori* is shown in
314 Fig 5. The results showed that the highest levels of DNA and protein leakage were observed
315 in samples treated with 5 μg/mL nisin and 0.6% of LA in combination (Fig. 5A and B).
316 Ampicillin showed similar degree of DNA and protein leakage like nisin-LA combination.
317 However, nisin or LA alone at the same concentration did not show any significant
318 differences in DNA and protein leakage, compared to the control ($p > 0.05$)

Disruption of H. pylori observed by FE-SEM

FE-SEM was used to visualize the morphology of *H. pylori* which was treated with nisin, LA, its combination or ampicillin (Fig. 6). The control group showed intact, smooth, and homogeneous bacterial surfaces (Fig. 6A). Treatment of *H. pylori* with LA alone caused slight deformation to the bacterial surface (Fig. 6B). Nisin alone resulted in slight membrane damage, with some areas of the bacterial surface appearing rough and uneven (Fig. 6C). However, the nisin and LA combination caused more extensive damage to the bacterial membrane, with significant deformation, and in some cases, visible fragments of the bacteria were observed (Fig. 6D). Ampicillin also resulted in membrane damage with strongly ruptured surfaces (Fig. 6E).

Discussion

The complete eradication of *H. pylori* is a challenging task due to its persistent nature in the gut. Antibiotic therapy is the primary approach for *H. pylori* eradication. However, it may cause dysbiosis in the gut microbiota and modulate the immune system, resulting in unwanted effects like metallic taste, diarrhea, nausea, or vomiting during the treatment process (Çalışkan et al., 2022). Consequently, researchers are seeking safe antibiotic alternatives that are devoid of such side effects (Seal et al., 2013). In this regard, nisin is not only safe but also has a broad antibacterial effect on bacteria (Norouzi et al., 2018). However, due to its complex purifying process and limited supply, nisin is relatively expensive compared to other antibacterial substances (Zhao et al., 2022). Thus, there are several

ongoing research regarding development and improvement of nisin production by LAB (Özel et al., 2018).

LA is a metabolite of LAB and is known to be a safe substance. It has been extensively studied for its ability as a permeabilizer to disrupt the outer membrane of bacteria by causing changes in the membrane lipid composition (Gyawali et al., 2011). Yogurt or fermented milk is a fermented dairy product, which possess plenty of LA due to the metabolites of LAB during fermentation. Although yogurt has a plenty of LA, there is no data regarding incorporation of nisin to yogurt due to concerns that nisin could inactivate LAB in fermented food. Although nisin can inactivate LAB in yogurt, recent findings suggest that the inactivated bacteria, known as parabiotics, can still confer beneficial effects in human (Kazemi et al., 2021). Some studies have found that parabiotics have a significant impact on serotonin secretion in the gut (Hara et al., 2018). Additionally, several strains of Lactobacillus parabiotics have demonstrated anti-inflammatory and anti-oxidative effects in in vitro and in vivo experimental models (Chung et al., 2019; Jang et al., 2018). Thus, both live probiotics and inactivated parabiotics can have beneficial effects on human health. Therefore, investigating the potential health benefits of incorporating nisin into post-fermented yogurt would be a valuable research endeavor.

While previous experiments have demonstrated the inhibitory effects of LAB and bacteriocins against *H. pylori* (El-Adawi et al., 2013; Kim et al., 2003), our study focused on the combination effect of nisin and LA for the growth inhibition of *H. pylori*. To investigate the possibility of incorporating nisin into yogurt, we evaluated the synergistic antibacterial effect of nisin and LA using checkerboard assay and agar well diffusion assay. MIC of nisin (μg/mL), LA (%) and ampicillin (μg/mL) against *H. pylori* was 10, 0.6, 0.195, respectively.

Previous studies indicated that the MIC of nisin was 0.39–25 μg/mL, and ampicillin was 0.015–0.25 μg/mL and it was similar to our data (Neshani et al., 2019; Weiss et al., 1998) The fractional inhibitory concentration index (FICI) of combined nisin and LA was 0.375, indicating that nisin and LA have synergistic effect.

The LA content in the yogurt was measured to be 0.6%. When the yogurt supernatant and nisin (5 μg/mL) were combined, the growth of *H. pylori* was inhibited as observed in the agar well diffusion assay. Among the concentrations of nisin tested (ranging from 1.25 to 5 μg/mL), 5 μg/mL of nisin exhibited better *H. pylori* growth inhibition effects than the MIC value of nisin (10 μg/mL). When nisin is combined with LA, 5 μg/mL of nisin demonstrated the most pronounced *H. pylori* growth inhibition effects. Based on these data, only 5 μg/mL of nisin was selected.

Nisin and LA are GRAS substances because they have been used in various foods for many years (Müller-Auffermann et al., 2015). However, to use these two substances together, the safety of their combined effect must be evaluated. As *H. pylori* is a bacterium that lives in the stomach, we conducted a safety evaluation using the trypan blue dye exclusion assay on AGS cells. Also, since it takes approximately 3 hours for food to be digested in the stomach, the material processing time was set to 3 hours. Live cells exclude trypan blue dye due to intact cell membrane, and blue-stained dye indicates cell death due to cell membrane damage. In previous studies, high concentrations of nisin were reported to possess anticancer properties by inducing apoptosis (Ahmadi et al., 2017). In our study, cell death was not observed in low concentrations of nisin (1.25 to 5 μg/mL), even when used in combination with 0.6% LA. Cell death was only observed in AGS cells treated with 1.2% LA. Based on these results, we selected the concentration of 5 μg/mL of nisin, 0.6% of LA, and 6.25 μg/mL of ampicillin for

387 further in vitro assays.

388 To determine the optimal concentration of *H. pylori* to be treated in the cell, an LDH assay
389 was conducted. Cell membrane damage results in lactate dehydrogenase (LDH) release in the
390 culture medium, making it an indicator of damaged cells (Fotakis & Timbrell, 2006). It has
391 been suggested that VacA, which is secreted by *H. pylori*, regulates pro-apoptotic MMP-9
392 expression of cells, resulting in apoptosis (Gonciarz et al., 2019). Accordingly, the LDH
393 release increased when AGS cells were treated with 10^9 CFU/mL of *H. pylori*. Therefore, the
394 cell density of 10^8 CFU/mL of *H. pylori* was selected for further cell culture studies.

395 *H. pylori* is known to induce inflammation by modulating pro-inflammatory cytokine
396 production. In previous study, *H. pylori* treated AGS cells showed an increased mRNA level
397 of pro-inflammatory cytokines (Lamb and Chen, 2013). *H. pylori* bacterial toxin CagA and
398 VacA are major contributors to the increase in pro-inflammatory cytokines. CagA is
399 transported onto gastric epithelial cells via a type IV secretion system, where it activates the
400 NF-κB pathway, leading to the production of pro-inflammatory cytokines (Jones et al., 2010).
401 Meanwhile, VacA stimulated the production of reactive oxygen species in gastric epithelial
402 cells, which can lead to the production of pro-inflammatory cytokines (Jones et al., 2010). We
403 investigated the effect of nisin and LA on *H. pylori* infected AGS cells, with a focus on its
404 impact on the mRNA expression levels of pro inflammatory cytokines (IL-1β, IL-6, IL-8 and
405 TNF-α) and bacterial toxins (CagA and VacA). Our findings demonstrate that the
406 combination of nisin and LA was effective in downregulating the mRNA expression levels of
407 pro-inflammatory cytokines by reducing the mRNA expression levels of bacterial toxins. The
408 bacterial toxins have been shown to play a role in the development of pathogen-associated
409 diseases by leading to inflammation and tissue damage. Previous studies have shown that

410 nisin can downregulate the transcriptional levels of Staphylococcus aureus toxin genes,
411 leading to the reduction of bacterium adhesion and modulation of pro-inflammatory and anti-
412 inflammatory cytokines (Jia et al., 2019; Zhao et al., 2016). Additionally, exposure to nisin
413 could damage the cellular membrane and triggers DNA condensation of Staphylococcus
414 aureus (Jensen et al., 2020). Our data showed that the combination of nisin and LA is more
415 effective in downregulating the gene expressions of bacterial toxins compared to using them
416 alone. This may be attributed to nisin-LA combination ability to damage the bacterial
417 membrane of *H. pylori*, allowing nisin to enter the inside the cellular membrane and trigger
418 DNA condensation. The combination of nisin and LA led to the leakage of cytoplasmic
419 contents such as DNA and proteins, indicating membrane damage. Nisin and LA used
420 separately did show such antibacterial effects. These findings were supported by the results of
421 FE-SEM, which showed nisin alone did not cause significant damage to the bacterial
422 membrane, while the combination of nisin and LA resulted in a notable level of damage.

423 Incorporating nisin into post-fermented yogurt exhibited antibacterial effect against *H. pylori*.
424 Combination of nisin and LA was found to have a synergistic antibacterial effect against *H.*
425 *pylori*, compared to using them alone. This combination was also effective in downregulating
426 gene expressions of bacterial toxins and inflammatory cytokines in human gastric cells.
427 Based on our data, it is suggested that incorporating yogurt, which naturally contains LA,
428 with nisin could provide a safe approach to inhibit *H. pylori* infections. This combination
429 shows promise as a potential alternative therapy for *H. pylori* eradication. Further studies,
430 including animal studies, are necessary to validate our findings and assess the potential of
431 these combinations in clinical settings.

432

Author contributions

Conceptualization: Han SG (Han Seo Gu), Han SG (Han Sung Gu). Writing - original draft: Han SG (Han Seo Gu), Han SG (Han Sung Gu). Methodology: Han SG (Han Seo Gu). Investigation: Han SG (Han Seo Gu), Kwon HC, Kim DH, Hong SJ. Writing - review & editing: Han SG (Han Seo Gu), Kwon HC, Kim DH, Hong SJ. Han SG (Han Sung Gu).

Acknowledgments

None.

Conflict of interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

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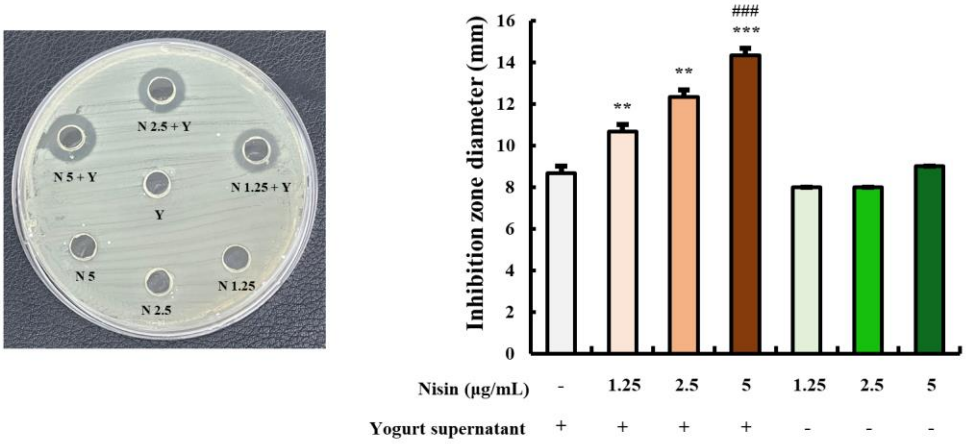
586 **Figure legends**

A



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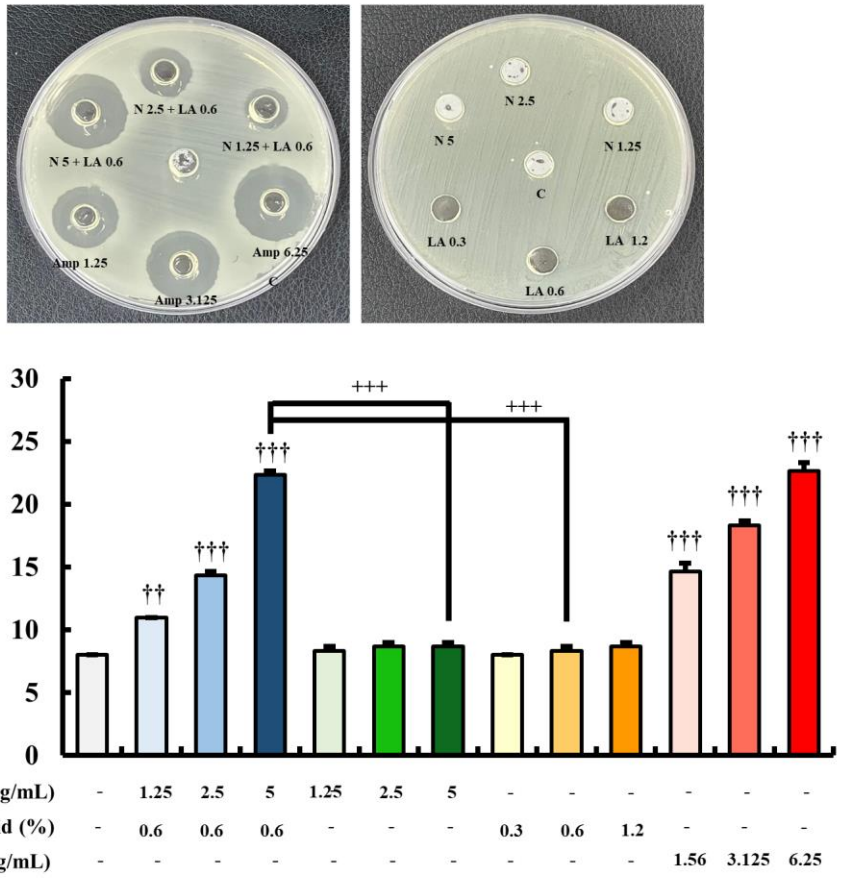
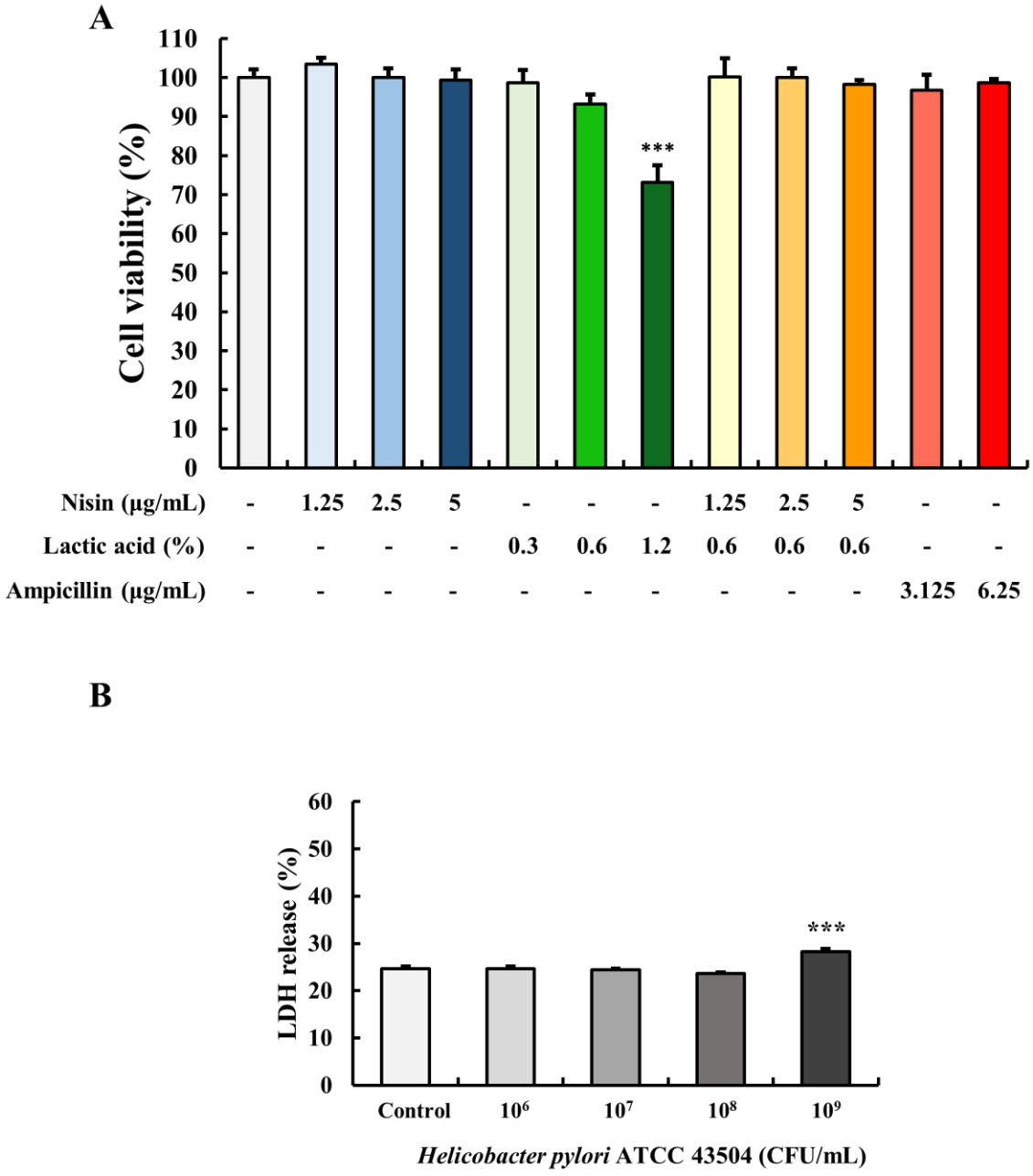


587

588 **Fig. 1. Antibacteral effects of nisin, LA, and nisin-supplemented yogurt against *H.***
589 ***pylori*.**

590 (A) Agar well diffusion assay was conducted to determine nisin, yogurt supernatant and their
591 combination effects against *H. pylori* (N: nisin μg/ml; Y: yogurt supernatant) and inhibition
592 zone diameters (mm). (B) Agar well diffusion assay conducted to determine nisin, LA and
593 their combination effects against *H. pylori* (N: nisin μg/ml; LA: lactic acid %; C: control;
594 Amp: ampicillin μg/ml) and inhibition zone diameters (mm). Values represent means ± SEM
595 (n=3). Statistical significance was calculated via one-way ANOVA with Dunnett's test post
596 hoc test. ** ($p < 0.01$) and *** ($p < 0.001$) indicate a significant difference compared to the
597 yogurt supernatant only. ### ($p < 0.001$) shows a significant difference compared to the nisin
598 5 μg/mL treated only. † † ($p < 0.01$) and ††† ($p < 0.001$) indicate a significant difference
599 compared to the control. +++ ($p < 0.001$) shows a significant difference compared to the nisin
600 at 5 μg/ml and LA at 0.6%, respectively.

601



602

603 **Fig. 2. Cell viability of nisin, LA, and their combination and cytotoxicity of AGS cells**

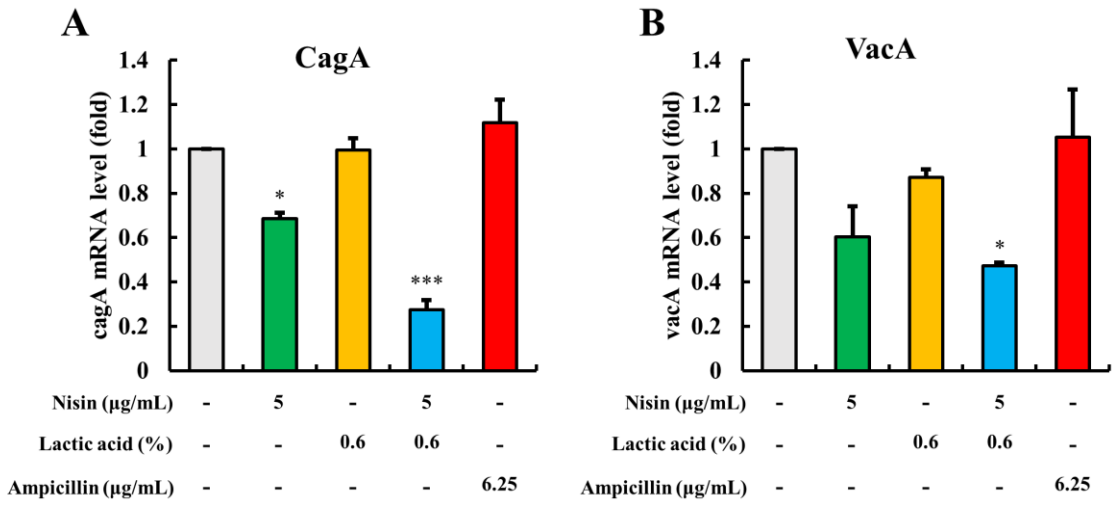
604 **treated with *H. pylori*.**

605 (A) The percentage of viable cells treated with nisin, LA, their combination, or ampicillin

606 was calculated relative to the untreated control. (B) The cytotoxicity of *H. pylori* was

607 determined by measuring LDH release after treatment with various concentrations of *H.*
608 *pylori* (10^6, 10^7, 10^8, and 10^9 CFU/mL). Values represents means ± SEM (n=3). Statistical
609 significance was calculated via one-way ANOVA with Dunnett's test post hoc test. *** ($p <$
610 0.001) show a significant difference, compared to the control.

611



612

613 **Fig. 3. Relative mRNA expression levels of bacterial toxins in *H. pylori* following**

614 **treatment with nisin, LA, and their combination.**

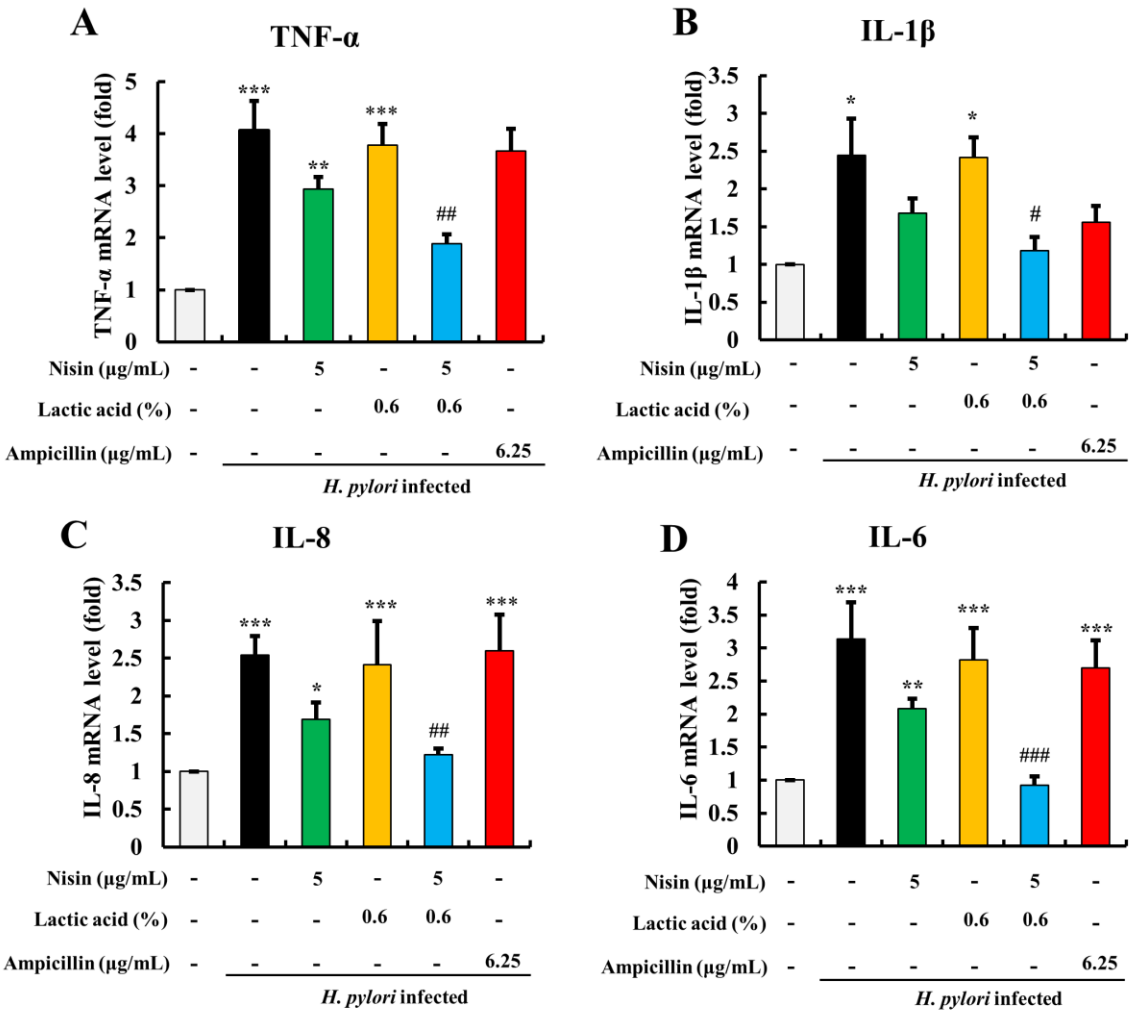
615 (A) CagA and (B) VacA mRNA expression levels in *H. pylori*. *H. pylori* were treated with

616 nisin, LA, their combination, or ampicillin for 3 h. Values represent means ± SEM (n=3).

617 Statistical significance was calculated via one-way ANOVA with Dunnett's test post hoc test.

618 * ($p < 0.05$), and *** ($p < 0.001$) indicate a significant difference compared to control.

619



620

621 **Fig. 4. Anti-inflammatory effect of nisin, LA, and their combination on pro-**

622 **inflammatory cytokine expression in AGS cells infected with *H. pylori*.**

623 (A)TNF-α, (B) IL-1β, (C) IL-8, and (D) IL-6 mRNA expression in AGS cells. AGS cells

624 were pretreated with 10^8 CFU/mL of *H. pylori* for 6 h and then treated with nisin, LA, their

625 combination, or ampicillin for 3 h. Values represent means ± SEM (n=3). Statistical

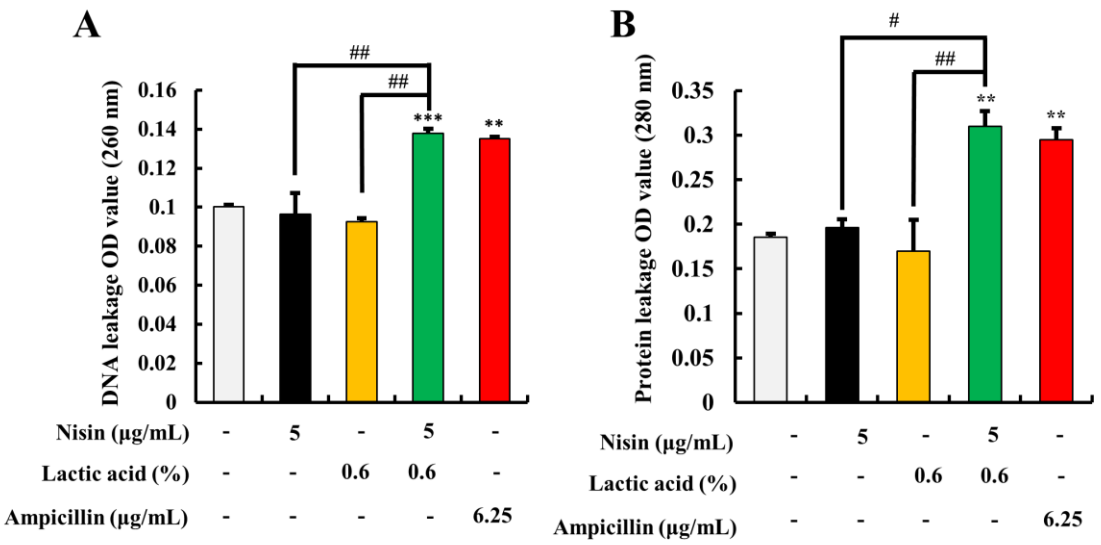
626 significance was calculated via one-way ANOVA with Dunnett's test post hoc test. * ($p <$

627 0.05), ** ($p < 0.01$) and *** ($p < 0.001$) indicate a significant difference compared to control.

628 # ($p < 0.05$), ## ($p < 0.01$) and ### ($p < 0.001$) show a significant difference compared to

629 cells only treated with *H. pylori*.

630



631

632 **Fig. 5. Effect of nisin, LA, and their combination on DNA and protein leakage from *H.***

633 ***pylori*.**

634 (A) DNA leakage and (B) protein leakage were measured by absorbance at 260 and 280 nm,

635 respectively. *H. pylori* was cultured for 3 h in the presence of nisin, LA, their combination, or

636 ampicillin. Values represents means ± SEM (n=3). Statistical significance was calculated via

637 one-way ANOVA with Dunnett's test post hoc test. ** ($p < 0.01$), *** ($p < 0.001$) show a

638 significant difference, compared to the control. # ($p < 0.05$) and ## ($p < 0.01$) show a

639 significant difference compared to the nisin at 5 μg/ml and LA at 0.6%, respectively.

640

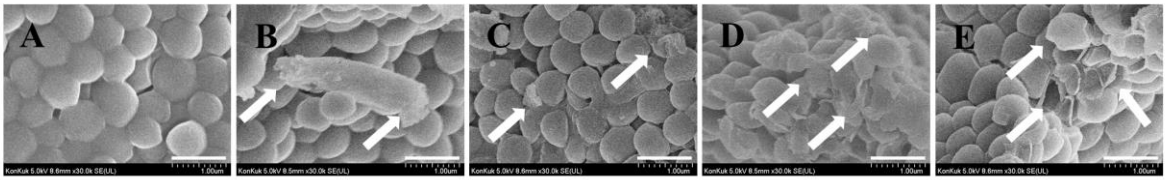


Fig. 6. Effect of nisin, LA, and their combination on the ultrastructure of *H. pylori*.

The membrane damage (white arrow) of *H. pylori* in FE-SEM image. (A): control; (B): nisin (5 μg/ml); (C): LA (0.6%); (D): nisin (5 μg/ml) + LA (0.6%); (E): ampicillin (6.25 μg/ml). The scale bars in the images are 1 μm.

Table 1. Primers used in the current study

Name of genes		Primer Sequence (5'-3')
IL-1β[a]	(F)	TGT ACC TGT CCT GCG TGT TGA AAG
	(R)	CTG GGC AGA CTC AAA TTC CAG CTT
IL-6[b]	(F)	ACA GCC ACT CAC CTC TTC AGA AC
	(R)	TTT TCT GCC AGT GCC TCT TTG C
IL-8[c]	(F)	ACT TCC AAG CTG GCC GTG GCT CT
	(R)	GTG TTG GCG CAG TGT GGT CCA CT
TNF-α[d]	(F)	AAG CCC TGG TAT GAG CCC ATC TAT
	(R)	AGG GCA ATG ATC CCA AAG TAG ACC
VacA[e]	(F)	GCA AAA TCA ATC GCC CTC TGG T
	(R)	CTG TAG CGA TCC CCC CAA CA
CagA[f]	(F)	CAA CCA CAA ACC GAA GCG GCT TTT
	(R)	AAA GCT TGC CTG TTA TCC CTA TCA
GAPDH[g]	(F)	GAC CCC TTC ATT GAC CTC AAC TAC
	(R)	ATG ACA AGC TTC CCG TTC TCA G
16S rRNA[h]	(F)	TGT GGG AGA GGT AGG TGG AA
	(R)	CAT CGT TTA GGG CGT GGA CT

Abbreviations: [a] IL-1β, interleukin-1 beta; [b]IL-6, interleukin 6; [c]IL-8, interleukin 8; [d]TNF- α, tumor necrosis factor-alpha; [e]VacA, vacuolating cytotoxin A; [f]CagA, cytotoxin-associated gene A; [g]GAPDH, glyceraldehyde 3-phosphate dehydrogenase; [h]16s rRNA, 16s ribosomal ribonucleic acid

Table 2. MIC of nisin, lactic acid, their combination, or ampicillin and the FICI values of combinations against *H. pylori*

Microorganism	MIC Individual			MIC Combination		FICI	Result
	Nisin (μg/mL)	Lactic acid (%)	Ampicillin (μg/mL)	Nisin (μg/mL)	Lactic acid (%)		
Helicobacter pylori **ATCC 43502**	10	0.6	0.195	1.25	0.15	0.375	Synergism

Abbreviations: MIC, minimum inhibitory concentration; FICI, fractional inhibitory concentration index