Anti-*Helicobacter pylori* activity of a complex mixture of *Lactobacillus paracasei* HP7 including the extract of *Perilla frutescens* var. *acuta* and *Glycyrrhiza glabra*

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**Abstract**

The effect of standard therapeutic strategies on *Helicobacter pylori* infection is diminished over time owing to the emergence of drug resistant strains. In this study, we would like to confirm the enhanced effect of *L. paracasei* HP7, which has been reported to exert antibacterial and gastric mucosal protective effects, in combination with *Perilla frutescens* var. acuta (*P. frutescens*) and *Glycyrrhiza glabra* (*G. glabra*) extracts. *P. frutescens* extract and *G. glabra* extract were found to inhibit the growth of *H. pylori* in a concentration-dependent manner, and the combination of *L. paracasei* HP7 and *P. frutescens* extract and *G. glabra* extract effectively inhibited *H. pylori* from attaching to AGS a gastric epithelial cells. Moreover, *L. paracasei* HP7 complex mixture containing *P. frutescens* and *G. glabra* extracts has been shown to inhibit *H. pylori* virulence genes such as AlpA, CagA, FlaA and UreA. When *H. pylori*-infected mice were administered a complex mixture of *L. paracasei* HP7 containing *P. frutescens* and *G. glabra* extract, the infection rate of *H. pylori* was significantly reduced. In addition, the *L. paracasei* HP7 complex mixture significantly reduced serum IL-8 levels and stomach inflammation in *H. pylori* infected mice. These results suggest that a complex mixture of *L. paracasei* HP7 containing *P. frutescens* and *G. glabra* extracts may be an alternative to treating diseases caused by *H. pylori* infection.

**Keywords:** *Lactobacillus paracasei*, HP7, *Helicobacter pylori*, *Perilla frutescens* var. *acuta*, *Glycyrrhiza glabra*

**Introduction**

*Helicobacter pylori*, a major causative pathogen of chronic gastritis [1] and gastric ulcers [2], is a spiral of gram-negative bacteria associated with an increased risk of gastric cancer [3, 4]. Vaccination with antibiotics to remove gastric *H. pylori* can reduce *H. pylori*-associated gastrointestinal diseases [5, 6] and reduce the risk of gastric cancer [7]. The standard recommended therapy for *H. pylori* uses two antibiotics, usually a triple combination therapy, including clarithromycin and a proton pump inhibitor with amoxicillin or metronidazole [8, 9]. However, the efficacy of the triple therapy has currently reduced over time. Recent cure rates of less than 80% are mainly due to the increased prevalence of resistant *H. pylori* strains in metronidazole and clarithromycin [10–12]. In addition, some patients showed allergic side effects to antibiotics and can sometimes cause side effects if *H. pylori* is not treated [13]. Long-term vaccination with antibiotics is not recommended for the prevention of *H. pylori* infection. Therefore, it is important to develop new non-antibacterial agents for the treatment of *H. pylori* [14].
Lactobacillus spp. is recommended as an additive to the standard recommended treatment for H. pylori treatment, and it is possible to improve the patient’s adaptability by reducing the side effects of antibacterial agents [15, 16]. In our previous study, we reported that the lactic acid bacterium Lactobacillus paracasei HP7 (L. paracasei HP7) isolated from kimchi, a fermented vegetable widely consumed in Korea, had inhibitory effects against H. pylori in-vitro and in-vivo [17].

Recently, there has been a clear increase in demand for natural compounds from plant extracts that are effective antibacterial agents against a wide range of bacteria to control human infection and for the preservation of food [18]. Recently, the inhibitory effect of Glycyrrhiza glabra (G. glabra) on H. pylori and the therapeutic effect on infected patients have been reported [19–21]. In addition, antibacterial activities [22–24] and anti-inflammatory [25–27] effects of Perilla frutescens var. acuta (P. frutescens) have been reported.

In this study, we aimed to determine whether the combination of L. paracasei HP7 and P. frutescens and G. glabra extracts had a synergistic effect on the inhibition of H. pylori infection.

Methods/experimental

Bacterial strains

L. paracasei HP7 was incubated at Man-Rogosa-Sharpe broth (Difco Laboratories, Detroit, Mich.) at 35 °C for 24 h. H. pylori strain SS1 (B0890; Korean Jeongeup Korean Collection) was cultured overnight at 37 °C. under microaerobic conditions in brain-heart infusion medium containing 10% fetal bovine serum (FBS) and grown to density ~ 2.0 × 10^8 CFU/mL. The cultured bacteria were then transferred to phosphate buffered saline (PBS) before the test.

Herbal extract

Each of the herbal extracts of G. glabra and P. frutescens were obtained from Korea Yakult Co., Ltd.

Cell culture

Human gastric cell line AGS cells (human gastric adenocarcinoma) were obtained from the Korean Cell Line Bank (cellbank.snu.ac.kr) and used. For maintenance and proliferation of cells, passage was performed every 2 days at 37 °C and 5% CO2 using Ham’s F-12 medium containing 10% FBS and 1% antibiotic. For analysis of H. pylori infection to gastric cells, antibiotics were not added to the culture medium.

H. pylori growth inhibition

To confirm the anti-H. pylori activity of P. frutescens and G. glabra extracts, Alamar blue assay was performed by referring to the study of Tsukasa M et al. [28]. H. pylori was suspended in DMEM / F-12 containing 5 mM L-lactic acid with a turbidity of 0.005 (1 × 10^5 CFU/mL). One hundred microliter suspension was added to 96 well culture plate and then incubated for 4 h at 37 °C with the test material (P. frutescens and G. glabra extracts) under micro-aerophilic conditions. After incubation, inhibition of H. pylori growth was measured by Alamar blue according to manufacturer’s criteria (Alamar Bio-Sciences, Sacramento, CA, U.S.A.). H. pylori inhibitory activity of the tested material was calculated by the following formula:

\[
\text{inhibition (％) = } \left(\frac{(A - B)}{(A - C)}\right) \times 100
\]

A: cultured without test sample. B: cultured with test sample. C: medium alone.

Inhibition of H. pylori adhesion to AGS cells

AGS cells were cultured in 6-well plates for 16 h. When the cells reached 90% confluence, the medium was replaced with serum and antibiotics-free F-12 medium. An overnight cultured H. pylori SS1 was suspended in Ham’s F-12 medium. For co-culture of bacteria and gastric epithelial cells, H. pylori SS1 (10^7 CFU) were added to wells containing 10^6 AGS cells and incubated for 4 h in the absence or presence of herbal extracts and L. paracasei HP7. The adhesion of H. pylori was measured using a Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) as in our previous paper [17]. Forward and reverse sequences of primers for amplifying the H. pylori 16S RNA gene were as follows: 5′-TCG GAA TCA CTG GGC GTA A-3′ and 5′-TTC TAT GGT TAA GCC ATA GGA TTC CAC-3′.

Detect of H. pylori virulence gene expression

H. pylori SS1 cells were cultured in brain-heart infusion broth at ~ 1.0 × 10^7 CFU/mL. Cultured H. pylori were treated with G. glabra extract (3µg / mL), P. frutescens extract (25µg/mL), and L paracasei HP7 (1.0 × 10^7 CFU/mL) and incubate at 37 °C for 2 h. cDNA was synthesized using murine leukemia virus reverse transcriptase with random hexamer. Primer sequence for H. pylori virulence genes were listed in Table 1. AlpA is genes that H. pylori attaches to the gastric mucosa, and CagA plays the role of H. pylori invading gastric cells. FlaA is related to the mobility of H. pylori, and UreA is genes that H. pylori uses to neutralize gastric acid [29].

Animals

Specific pathogen free (SPF) male C57BL/6 mice weighing 20–24 g were purchased from Samtako Co. (Osan, Korea) and were maintained at the inspection facility of Wonkwang University (Iksan, Korea) for 1 week before experiments. Thereafter, the mice were maintained in an...
SPF barrier room with regulated temperature (23 °C ± 1 °C) and humidity (50% ± 5%) and a 12:12-h light/dark cycle. The animals were fed a sterilized pellet diet (Purina, Seoul, Korea) and sterilized water ad libitum. All studies were performed in accordance with the Guide for Animal Experimentation of Wonkwang University and were approved by the Institutional Animal Care and Use Committee of Wonkwang University (approval no. WKU 2019-08-22).

**H. pylori inoculation**
Animals were intragastrically inoculated three times, with a 3-day interval between inoculations, with *H. pylori* at ~ 1.0 × 10^9 CFU in 0.5 mL broth. The challenged animals were confirmed as *H. pylori*-positive by stool antigen analysis using the Bioline *H. pylori* Ag kit (Standard Diagnostics, Suwon City, Korea) as previously described [30].

**In vivo study protocol**
The inhibition of *H. pylori* growth by *L. paracasei* HP7 was investi in a mouse model. The mice were divided into six groups: negative control (NC, *n* = 10); *H. pylori*-infected without treatment (*C*, *n* = 10); *H. pylori*-infected with positive control Deglycyrrhizinated Licorice (DGL) [20] treatment (*D*, *n* = 10); *H. pylori*-infected with *P. frutescens* extract (PFE) 5 mg/kg + *G. glabra* extract (GGE) 1.2 mg/kg (COM 1, *n* = 10); *H. pylori*-infected with PFE 10 mg/kg + GGE 1.2 mg/kg (COM 2, *n* = 10); and *H. pylori*-infected with *L. paracasei* HP7 2.0 × 10^7 CFU + PFE 10 mg/kg + GGE 1.2 mg/kg (COM 3, *n* = 10). All substances were administered orally once daily for 4 weeks. At the end of the experiment, the animals were euthanized with ether, and then dissected. The stomach was further incised along the taiwanese valley, and washed with saline. The remaining portion was formalin fixed and inserted into paraffin for histological analysis. *H. pylori* colonies were confirmed by the aforementioned quick urease test (CLO-test) [30].

**Blood analysis**
Blood samples were collected from the hearts of sacrificed animals, centrifuged at 1000×g for 15 min at 4 °C, and the isolated plasma was stored at ~ 80 °C. Serum titers of anti-*H. pylori* antibodies were measured using a mouse anti-*H. pylori* antibody (IgG-1) ELISA kit (Cusbio Biotech, Wuhan, China) in accordance with the manufacturer’s instructions. IL-8 levels in mice were measured using the Mouse Interleukin 8 ELISA Kit (R&D System, Minneapolis, USA) in accordance with the manufacturer’s instructions.

**Statistical analysis**
Experimental results were compared between groups using Minitab (State College, PA, USA) and one-way ANOVA, a parametric multiple comparison procedure. The results were expressed as mean ± standard error and statistically significant when *P* < 0.05.

**Results**

**H. pylori growth inhibition**
We measured the *H. pylori* growth inhibitory activity of 140 plant extracts including *G. glabra* and *P. frutescens*. Excluding non-edible plants, *G. glabra* and *P. frutescens* extracts showed the best inhibitory effect on the growth of *H. pylori*. In particular, *G. glabra* 90% ethanol extract and *P. frutescens* 50% ethanol extract showed high activity (data not shown).

There have been several reports of antibacterial and Helicobacter pylori inhibitory activity of *P. frutescens* and *G. glabra* [19–24]. However, there have been few reports of synergistic effects of *H. pylori* inhibitory activity of *P. frutescens* and *G. glabra*. Therefore, the *H. pylori* growth inhibitory activity of each of the *P. frutescens* extract (PFE) and *G. glabra* extracts (GGE) was investigated, and whether the two extracts had a synergistic effect on *H. pylori* inhibition was examined. PFE and GGE inhibited the growth of *H. pylori* in a concentration-dependent manner. PFE and GGE almost completely inhibited the growth of *H. pylori* at concentrations of 12.5 μg/mL and 50 μg/mL, respectively, and the IC₅₀ of each extract was 23.84 μg/mL.

| Gene name | Sequence | Tm (°C) | Reference |
|-----------|----------|---------|-----------|
| alpA | F: AAACGGCTCTGTGGATATGG | 55.0 | NZ_CP009259.1 |
|  | R: GAACTGGAAGTGGTGTTATTG | 45.6 |         |
| cagA | F: TCACCTCTGGCGGATATGGAAT | 57.5 |         |
|  | R: ACACAGAAGACAGGCTTTAT | 57.7 |         |
| flaA | F: GCTAAGAGCATCAATGTTTC | 58.3 |         |
|  | R: CGTAAACATCCCGCAATT | 58.5 |         |
| ureA | F: AGTTGGTATTGAAGCGATTT | 57.6 |         |
|  | R: AAGAACAATCTACCAAGGAAC | 57.6 |         |
and 2.88 \( \mu g/mL \) (Fig. 1a). When the extract corresponding to IC\(_{50}\) was co-treated, the growth of H. pylori was inhibited by about 90% (Fig. 1b). This suggests that P. frutescens and G. glabra are synergistic in inhibiting the growth of H. pylori.

**Suppression of H. pylori adhesion to gastric epithelial cells**

In a previous study, we confirmed that hp7 inhibits Helicobacter pylori adhesion to gastric epithelial cells [17]. The complex mixture of L. paracasei HP7 containing PFE and GGE significantly inhibited H. pylori adhesion to gastric cells than L. paracasei HP7 or PFE or GGE alone (Fig. 2a). These results demonstrate that L. paracasei HP7 and P. frutescens and G. glabra extracts are synergistic in inhibiting bacterial adhesion to gastric epithelial cells.

**Inhibition of H. pylori virulence factor**

H. pylori produces urease to decompose the urea in the stomach, reduce the acidity around it, move using flagella, and attach to epithelial cells through adhesion factors such as AlpA. In addition, the CagA protein secreted by H. pylori inflames gastric epithelial cells and

![Fig. 1](image1.png)

**Fig. 1** Inhibitory effects of P. frutescens and G. glabra extract against growth of H. pylori. **a** H. pylori growth inhibitory activity of P. frutescens and G. glabra extracts at various concentrations. **b** H. pylori inhibitory activity following co-treatment of P. frutescens and G. glabra extract. PFE, P. frutescens extract; GGE, G. glabra extract. **Significantly different from the non treated control C (P < 0.01). ***Significantly different from the non treated control C (P < 0.001). #Significantly different from the PFE and GGE treated group (P < 0.01).

![Fig. 2](image2.png)

**Fig. 2** Effect of complex mixture of L. paracasei HP7 containing P. frutescens and G. glabra extract on H. pylori adhesion and H. pylori virulence genes expression. **a** Degree of H. pylori attached to AGS cells (b) alpA, cagA, flaA and ureA mRNA expression in L. paracasei HP7, P. frutescens extract, G. glabra extract and complex mixture treated H. pylori SS1. PFE, P. frutescens extract 25 \( \mu g/mL \); GGE, G. glabra extract 3 \( \mu g/mL \); HP7, L. paracasei HP7 1.0 \times 10^7 CFU/ML. *Significantly different from non-treated control C (P < 0.05). **Significantly different from the non-treated control C (P < 0.01). ***Significantly different from the non-treated control C (P < 0.001). #Significantly different from the PFE, GGE and HP7 treated group (P < 0.05).
causes gastric cell changes known as the “hummingbird phenomenon” [1, 2, 29]. Therefore, we investigated the effect of a complex mixture of L. paracasei HP7 containing PFE and GGE on the mRNA expression of genes encoding AlpA, Cag, FlaA, and UreA of H. pylori.

PFE significantly reduced ureA and GGE decreased alpA and cagA. HP7 significantly reduced flaA associated with H. pylori motility. Meanwhile, the HP7 complex mixture significantly reduced H. pylori virulence genes compared to PPE or GGE or HP7 alone (Fig. 2b).

**Anti-H. pylori antibody titer in serum**

To confirm the colonization of H. pylori in mice, the absorbance of IgG serum against H. pylori was also related to H. pylori colonization, so anti-Helicobacter IgG-1 serum levels were measured [31]. The serum antibody titers were elevated 4 weeks after H. pylori inoculation, to values of 1.48 ± 0.06, 0.94 ± 0.07, and 0.95 ± 0.04 in the H. pylori infection (Group C), positive control DGL (Group D), and H. pylori infection/L. paracasei.

HP7 + PPE + GGE (Group COM3) treatment groups, respectively, as compared with 0.25 ± 0.01 in control animals (Group NC) (Fig. 3).

These results indicated that H. pylori infection was significantly reduced by treatment with a complex mixture of L. paracasei HP7 containing extracts of PPE and GGE.

**Decrease of H. pylori colonization**

Repeated intragastric inoculation of C57BL/6 mice treated with H. pylori (1.0 × 10^9 CFU/mouse, three times) led to a positive reaction in the gastric mucosal campylobacter-like organism (CLO) test (Table 2). Positive percentages were increased 4 weeks after H. pylori inoculation, with values of 100% (CI 72.2–100), 30% (CI 10.8–60.3), 10% (CI 1.8–40.4) in the H. pylori infection (Group C), positive control DGL (Group D) and H. pylori infection/L. paracasei HP7 + PPE + GGE (Group COM3) treatment groups, respectively, compared with 0% (CI 0–27.6) in control animals (Group NC) (Table 2).

CLO scores were decreased by H. pylori infection/L. paracasei HP7 + PPE + GGE (Group COM3) relative to H. pylori-infected animals without treatment (Group C) (P < 0.01; Fig. 4). Therefore, L. paracasei HP7 + PPE + GGE may reduce the colonization rate of H. pylori.

**Alleviation of gastric mucosal lesions caused by H. pylori**

Pathological changes in the gastric mucosa were minimal in animals not infected with H. pylori (Group NC). In contrast, Group C (H. pylori inoculated) mice exhibited gastric atrophy and severely shortened villi. However, mice in Group COM3 (H. pylori infected/L. paracasei HP7 + PPE + GGE) showed a significant improvement in gastric mucosa. These results were confirmed by an increase in villus length in Group COM3 compared with Group C (Fig. 5).

**Suppression of H. pylori-induced IL-8 production**

Blood IL-8 levels were elevated 4 weeks after H. pylori inoculation, with values of 7.39 ± 0.70, 5.73 ± 0.63, 5.16 ± 0.49 in the H. pylori infection (Group C), positive control DGL (Group D) and H. pylori infection/L. paracasei HP7 + PPE + GGE (Group COM3) treatment groups, respectively, as compared to 5.36 ± 0.59 in control animals (Group NC) (Fig. 6).

**Discussion**

Lactic acid bacteria suppress the growth of human bacterial pathogens by secreting compounds, such as antibiotics, organic acids, and bacteriocins, to lower the pH of the environment and control gastrointestinal infections [31, 32]. The inhibitory activity of H. pylori has been reported in several Lactobacillus spp., including L. acidophilus [32], L. casei [33], L. johnsonii [34], L. reuteri [35], and L. salivarius [36].

A new Lactobacillus spp. isolated from kimchi by Korea Yakult Co. Ltd. was identified as L. paracasei and was named strain HP7. Kimchi is considered a healthy food as it is enriched in vitamins A, B, and C, and is high in fiber, but also contains a number of lactic acid bacteria [37]. The two herbs selected in this study were P. frutescens var. acuta and G. glabra, which showed the strong antibacterial activity of H. pylori by measuring the Helicobacter antibacterial activity (growth suppression) in the extraction of natural product candidates through the inhibitory clear zone test of H. pylori (data not shown).

G. glabra (licorice) was reported to exhibit antimicrobial activity against several gram-negative and gram-
positive bacterial strains including *H. pylori* [38]. In addition, licorice also exerted beneficial effects against *H. pylori* through its antiadhesive properties [39]. Activity against ulcer and cancer, and clinical outcomes of *H. pylori* infection were also exhibited by licorice. The curative effect of deglycyrrhizinated licorice (DGL) on ulcers has been reported in vivo and in clinical studies [40–42], and the anticancer effect of licorice extract was shown in an in vitro study [43]. *G. glabra* was shown to possess anti-ulcerogenic properties that may be conferred by the cytoprotective mechanism of its antioxidant properties. These results supported the ethnomedical uses of licorice in the treatment of gastric ulcer [44].

Traditionally, *P. frutescens var. acuta* has been prescribed to treat depression-related disease, anxiety, asthma, chest stuffiness, vomiting, cough, cold, flu, phlegm, tumors, allergies, intoxication, fever, headache, stuffy nose, constipation, abdominal pain, and indigestion, and acts as an analgesic, anti-abortive agent, and a sedative [23]. The antibacterial activity of *P. frutescens var. acuta* has also been reported [24].

In this study, we confirmed in vitro and in vivo experiments of *H. pylori* inhibitory activity of a *L. paracasei* HP7 complex mixture containing *P. frutescens var. acuta* and *G. glabra* extracts. *P. frutescens* extract and *G. glabra* extract inhibited the growth of *H. pylori* in a dose-dependent manner, and the *H. pylori* growth inhibitory effect was increased when the two extracts were mixed at IC50 concentration. In addition, the inhibitory effect of adhesion of gastric epithelial AGS cells of *H. pylori* by the *L. paracasei* HP7 or *P. frutescens* extract and *G. glabra* extract, when applied in a complex mixture, rather than each individually, was confirmed to be larger. Also, we confirmed the inhibitory activity of a complex mixture of *L. paracasei* HP7 including the extracts of *P. frutescens* and *G. glabra* against *H. pylori* in a mouse model; a rapid urease test of mouse stomachs showed decreased *H. pylori* colonization. Thus, the eradication of *H. pylori* reduced inflammation and epithelial damage in the stomach, although it is also possible that a complex mixture of *L. paracasei* HP7 including the extract of *P. frutescens* and *G. glabra* had direct anti-inflammatory effects on the gastric mucosa.
Although triple therapy consisting of two antibiotics and a proton pump inhibitor is effective over a short term and helps to maintain patient compliance, many patients experience undesirable side effects such as diarrhea, epigastric pain, nausea, and bloating [45].

In comparison, a complex mixture of L. paracasei HP7, including the extracts of P. frutescens and G. glabra, is safe and therefore appropriate for the prevention and treatment of H. pylori infection. In this study, the therapeutic effect of a complex mixture of L. paracasei HP7 including the extract of P. frutescens and G. glabra, was partial, at 90%. However, H. pylori adhesion and a reduced inflammatory response was shown. Other researchers reported also that probiotics alone could not completely eliminate H. pylori, but could reduce the load of H. pylori in the stomach, and alleviate gastric mucosal inflammation [46, 47]. Accumulating evidence suggests an important role of IL-8 in H. pylori infection-associated chronic atrophic gastritis, peptic ulcer and gastric cancer [48]. The suppression of IL-8 by a complex mixture of L. paracasei HP7, including the extract of P. frutescens and G. glabra, can potentially prevent H. pylori-induced gastritis and carcinogenesis in the stomach.

Previously, the results of our study reported that L. paracasei HP7 alone was able, to some extent, suppress H. pylori infection [17]. This study was performed to confirm the elevation effect of compounds mixed with P. frutescens and G. glabra extract, which are known to have antibacterial and gastric mucosal protective effects other than L. paracasei HP7.

**Conclusions**
The administration of a complex mixture of L. paracasei HP7 containing an extract of P. frutescens and G. glabra was more effective than that of L. paracasei HP7 alone or P. frutescens extract or G. glabra extract, and the administration of a higher antibacterial effect of H. pylori and inflammation induced by H. pylori or it was confirmed to reduce the damage to the mucous membrane. The mechanism of this action resulted from the inhibitory effect of L. paracasei HP7 on the adhesion of H. pylori to the gastric mucosa, the antibacterial effect and antioxidative effect of G. glabra and P. frutescens extract, and the increased secretion of gastric mucosal mucin. It can be assumed that the anti-H. pylori effect and the protective effect on the gastric mucosa were induced. Thus, a complex mixture of L. paracasei HP7, including the extract of Perilla frutescens and Glycyrrhiza glabra can be used to treat patients with gastric symptoms, including ulcers caused by H. pylori.

These results demonstrated that treatment with a complex mixture of L. paracasei HP7, including the extract of P. frutescens and G. glabra could inhibit the growth of H. pylori and is thus a promising treatment for patients with gastric symptoms, such as gastritis, that are caused by H. pylori infection.

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**Authors’ contributions**
We confirmed all authors’ contributions. The author(s) read and approved the final manuscript.

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**Availability of data and materials**
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**Competing interests**
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**Fig. 6** Serum IL-8 levels of mice infected with H. pylori followed by treatment with complex mixture of L. paracasei HP7 and herbal extracts. **Significantly different from the infection control Group C (P < 0.01). ***Significantly different from the infection control Group C (P < 0.001).
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