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Effect of *Lactobacillus acidophilus* and *Porphyromonas gingivalis* on proliferation and apoptosis of gingival epithelial cells

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ABSTRACT

Purpose: This study aimed to evaluate the possible antagonistic effects of *Lactobacillus acidophilus* on *Porphyromonas gingivalis*, and detect inhibition of *Lactobacillus acidophilus* on *Porphyromonas gingivalis* when they are co-cultured with human gingival epithelial cells.

Materials and methods: Human gingival epithelial cells were co-cultured with *Lactobacillus acidophilus* and *Porphyromonas gingivalis* alone or together. The amount of *Porphyromonas gingivalis* adhering to or invading the epithelial cells were determined by bacterial counts. The cellular proliferation was assayed by the MTT method. Apoptosis was detected by flow cytometry with apoptosis detection kit.

Results: On one hand, *Lactobacillus acidophilus* reduced the inhibitory effect of *Porphyromonas gingivalis* on the human gingival epithelial cells proliferation in a dose dependent manner. On the other hand, *Porphyromonas gingivalis* induced significant apoptosis on human gingival epithelial cells, and *Lactobacillus acidophilus* inhibited this apoptosis-inducing effect of *Porphyromonas gingivalis* in a dose dependent manner.

Conclusions: Porphyromonas gingivalis inhibits the proliferation and induces the apoptosis of human gingival epithelial cells. *Lactobacillus acidophilus* could attenuate this effect in a dose-dependent manner, and it thus reduces the destruction from pathogens. *Lactobacillus acidophilus* could be an effective candidate for probiotic therapy in periodontal diseases.

1. Introduction

Chronic periodontitis is a chronic inflammatory disease initiated mainly by periodontal microorganisms. Among pathogenic periodontal microorganisms, *Porphyromonas gingivalis* (*P. gingivalis*) could strongly invade the periodontal tissue and cause cellular apoptosis and periodontal tissue damage. In vitro studies indicated that the *P. gingivalis* can induce apoptosis of various types of cells, including fibroblasts [1–3], endothelial cells [4–7] and lymphocytes [8–10]. Cell apoptosis leads to widespread destruction of periodontal tissues in clinical cases [11]. To date, the effect of *P. gingivalis* on the apoptosis of epithelial cells is not yet fully understood. Several studies [12,13] agreed that *P. gingivalis* induces the apoptosis of epithelial cells, while other studies [14–16] indicated that *P. gingivalis* had an inhibitory effect on cell apoptosis.

In gastrointestinal tract infection situations, probiotics can inhibit the growth of pathogenic bacteria and prevent the cell apoptosis, ultimately reducing the damage that the pathogenic bacteria cause to the host. The invasive characteristics of *P. gingivalis* are similar to many intestinal pathogens. Given that *Lactobacillus acidophilus* (*L. acidophilus)* offsets the *P. gingivalis*-induced secretion of interleukins in a dose-dependent manner [17], as well as its benefits to epithelial cells under infection or inflammatory states [18–20], we are here interested in whether *L. acidophilus* can inhibit the damage effects of *P. gingivalis* on the epithelial cells, which remains unknown.

In this study, we co-cultured *L. acidophilus* and *P. gingivalis* with human gingival epithelial cells (GECs), and observed whether the *L. acidophilus* could attenuate the pathogenic effects of *P. gingivalis*.

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2. Materials and methods

2.1. Experimental bacterial strains

L. acidophilus (La, ATCC 4356) and *P. gingivalis* (Pg, ATCC 33277) were provided by the Shanghai Key Laboratory of Oral Medicine as previous described [17]. After 48 h inoculation in the brain-heart infusion (BHI) blood agar plate, the frozen stains were identified by Gram staining and biochemical identification. The recovered strains were seeded in the anaerobic BHI liquid medium, at 37 °C for 48 h. Strains were then centrifuged (4000 r/min for 5 min) and washed three times with phosphate-buffered saline (PBS) before experiments.

2.2. Human GEC isolation

GECs were obtained from clinical gingival tissues, using the enzyme-digestion method as previously described [17]. Patients' informed consents were obtained and the study was approved by the Ethics Committee of the Ninth People's Hospital (Approval number: 2014-90). Briefly, the harvested tissue samples were immediately washed with PBS and digested with 0.25% Dispase II (Sigma, Santa, Germany) at 4 °C overnight to obtain the gingival epithelial layers. Then the separated epithelial layers were cut into 0.5 mm × 0.5 mm pieces and further digested with 0.25% trypsin. Afterwards, the culture medium (containing 10% FBS) was added to terminate the digestion. And the samples were filtered and then centrifuged at 120 G for 5 min. The supernatant was discarded, and cells obtained from the pellet were cultured in DMEM supplemented with 10% FBS and 1% (w/v) penicillin/streptomycin in 5% CO2 humidified atmosphere at 37 °C. The medium was changed every 3 days. When the cells reached 80% confluence, the cells were collected through trypsin digestion.

2.3. Experimental groups

The experimental groups were as follows:

- the *P. gingivalis* group (*Pg* group): a co-culture of *P. gingivalis* and human GECs; the multiplicity of infection (MOI) of *P. gingivalis* was 100;
- the *L. acidophilus* group (*La* group): a co-culture of *L. acidophilus* and human GECs at MOI = 100;
- the mixed bacterial groups (subgroups: 0.1 *L. acidophilus* + *P. gingivalis*, 1 *L. acidophilus* + *P. gingivalis*, and 10 *L. acidophilus* + *P. gingivalis*): *L. acidophilus* and *P. gingivalis* (MOI = 100) were co-cultured with human GECs, and the ratios of *L. acidophilus/P. gingivalis* were 0.1:1, 1:1, and 10:1, respectively;
- the control group consisted of human GECs alone.

2.4. Cell proliferation assay

The cells at passage 3 were suspended at the density of 3×10^4/ml, and seeded into the 48-well plates. The corresponding culture medium was added into each group and co-culture was performed for 2 h. The plates were washed three times with PBS and added the fresh culture medium for another 0 h, 4 h, 10 h, 22 h and 46 h, respectively (the total infection time was 2 h, 6 h, 12 h, 24 h and 48 h, respectively). At each time point, the supernatant was removed from the 48-well plate, and 200 ul MTT solution was added in each well and incubated for 4–6 h. The supernatant was removed and 150 ul DMSO was added in each well and incubated for 10 min at room temperature. The OD value at 490 nm was measured by a microplate reader.

2.5. Cell apoptosis assay

The cells at passage 3 were seeded in the 6-well plate at the density of 1×10^5 cells/ml. When the cells reached 80% confluence, the supernatant was discarded. The corresponding culture medium of six groups were added and cells were cultured for 2 h, 6 h and 24 h, respectively. The cells were harvested and washed three times with PBS. Then, they were suspended in the pre-cooling 100 ul Binding Buffer and bathed in the ice. Each reaction tube was added 10 ul Annexin V-FITC and 10 ul PI and mixed gently and incubated for 15 min. The 400 ul Binding Buffer was added and the cells were re-suspended and detected with flow cytometry.

The cells in four quadrants were defined as follows: the upper left quadrant (UL, Annexin V-/PI+) represented the dead cells; the lower left quadrant (LL, Annexin V-/PI-) represented the normal cells; the upper right quadrant (UR, Annexin V+/PI+) represented the late apoptosis cells, and the lower right quadrant (LR, Annexin V+/PI-) represent the early apoptotic cells.

2.6. Statistical analysis

All the experiments were repeated three times. The data were presented as mean ± SEM. Two-way ANOVA were performed to compare two groups, and a $P < 0.05$ was considered statistically significant.

3. Results

3.1. L. acidophilus elevates GEC proliferation when co-cultured with P. gingivalis

The proliferation of human GECs in different groups were detected with MTT assay (Fig. 1A). There was no significant difference between the groups at 6 or 12 h. After 12-h culture, *Pg* showed a significant suppressive effect on the GEC proliferation compared to GEC alone (*Pg* group vs the control group, two-way ANOVA $P < 0.01$ at 12, 24 and 48 h). While the cellular viability in the 10 *La* + *Pg* group was significantly higher compared to the *P. gingivalis* treated group at 24 h (*La* + *Pg* vs *Pg*, two-way ANOVA $P < 0.01$). At 48 h, a higher concentration of *L. acidophilus* (10 *La* + *Pg*) significantly elevated the cellular viability of GECs (two-way ANOVA $P < 0.01$ vs *Pg*). These results implied that *L. acidophilus* protects the GEC proliferation under *P. gingivalis*-induced toxic effects in a dose dependent manner.

3.2. L. acidophilus attenuates P. gingivalis-induced apoptosis

On the apoptosis aspect, we observed whether *L. acidophilus* affects the apoptosis of GEC under *P. gingivalis* stresses. The cell apoptosis was detected using Annexin V-FITC apoptosis assay kit and afterwards flow cytometry (Fig. 1 B & C). The percentage of apoptotic cells was derived from the sum of the percentages of late and early apoptotic cells. The apoptosis proportion in each group did not change with time (each group among 2, 6 and 24 h $P > 0.05$). Among groups, the apoptosis was significantly triggered by *P. gingivalis* treatment (at 2, 6 and 24 h compared to controls), and *L. acidophilus* co-culture significantly attenuated the cellular apoptosis in a dose-dependent manner, especially at 2 h (two-way ANOVA, at 2 h, 1 *La* and 10 *La* + *Pg* vs *Pg* $P < 0.01$; at 6 h, 10 *La* + *Pg* vs *Pg* $P < 0.01$; at 24 h 10 *La* + *Pg* vs *Pg* $P < 0.05$). Based on these, *L. acidophilus* fights the damage from *P. gingivalis* through not only enhancing the cell proliferation but also resisting the cell apoptosis of GECs. Also, our data strongly suggested that the *P. gingivalis*-induced toxic effects towards both proliferation and apoptosis.

4. Discussion

GECs are the natural barrier in the periodontal tissue. When they come in contact with bacteria and their products, they secrete antimicrobial peptide, cytokine and protease to resist the foreign risk factors from invading. *P. gingivalis* is one of the main pathogens of chronic periodontitis. Its virulence factors such as cilia, capsule, outer membrane vesicles and endotoxin can stimulate gingival tissue cells, which

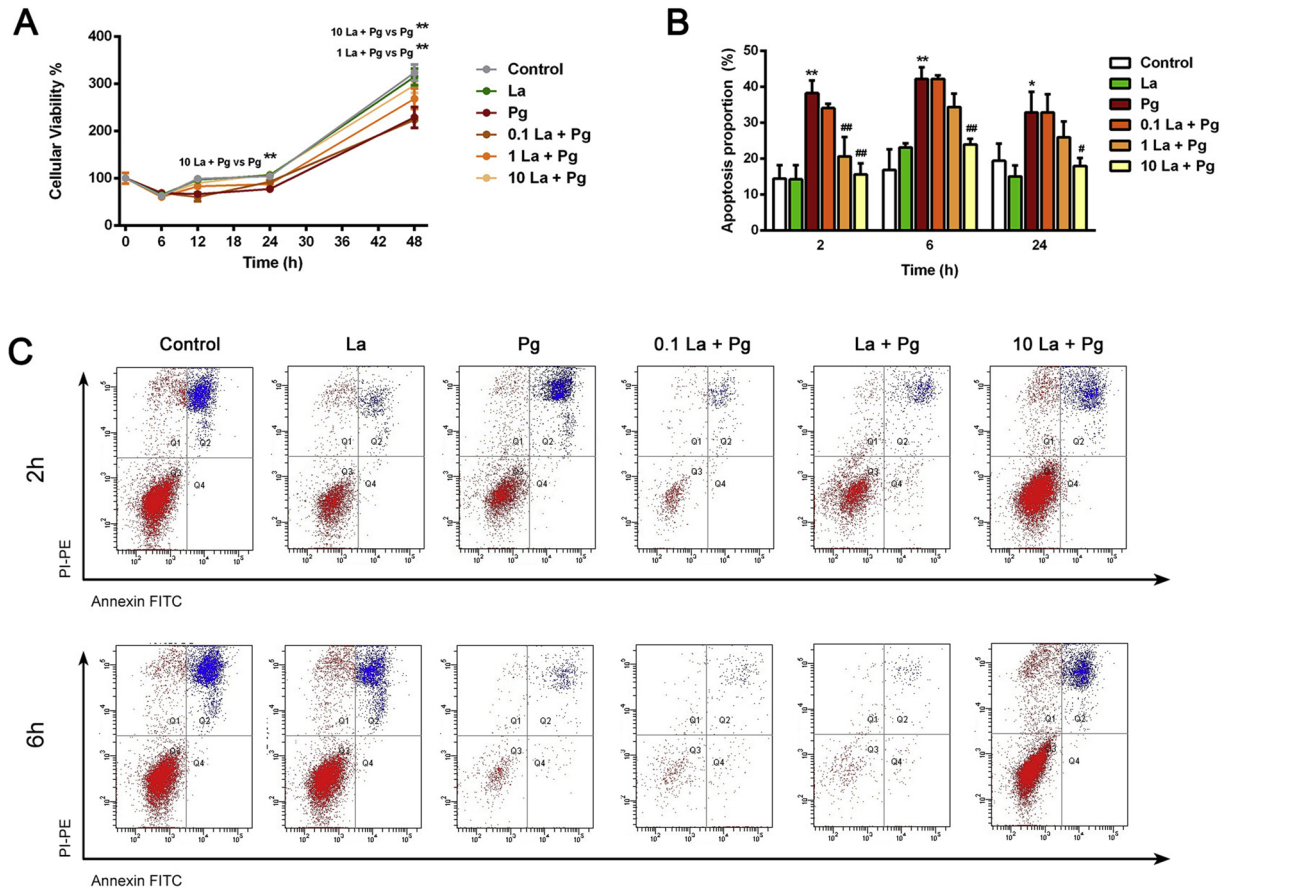

Fig. 1. *L. acidophilus* (La) protects the gingival epithelial cells (GECs) from the damage induced by *P. gingivalis* (Pg). (A) La elevates GEC proliferation when co-cultured with Pg. After 12-h culture, Pg showed a significant suppressive effect on the GEC proliferation compared to GEC alone; the cellular viability in the 10 La + Pg group was higher compared to the Pg treated group. Higher concentrations of La (1 La and 10 La + Pg) significantly elevated the cellular viability of GECs. (B) La attenuates Pg-induced the apoptosis at 2, 6 and 24 h. (C) Representative figures demonstrating the flow cytometry analysis of different g roups. * $P < 0.05$, * * $P < 0.01$.

consequently produce various cytokines and arouse the inflammatory and immune response, ultimately leading to periodontal tissue destruction. When *P. gingivalis* invades the GECs, the cells undergo morphological changes, including becoming round and separating from the culture dish. However, the integral function of cell can still remain for a short period. In the current study, the effect of *P. gingivalis* on the proliferation of GECs was assessed by MTT assay. The result indicated that the cytotoxicity of *P. gingivalis* may lead to partial cell death and reduction of the cell number, while the survived cells still maintained the ability of cell division and proliferation. In our study, we also found that *L. acidophilus* itself does not promote the proliferation of GECs. When the concentration ratio of *L. acidophilus* and *P. gingivalis* was 1:1 or 10:1, the cell viability of GECs was significantly higher than the *P. gingivalis* group. These results highlighted that the *L. acidophilus* could attenuate the inhibitory effect of *P. gingivalis* on GEC proliferation in a dose-dependent manner.

P. gingivalis is known to induce cell apoptosis of fibroblasts, cells and lymphocytes [21–23]. The cell apoptosis might be a mechanism underlying the chronic periodontitis lesion. It is still controversial whether the *P. gingivalis* could cause apoptosis of human GECs. Several studies suggest that *P. gingivalis* inhibits the GEC apoptosis while others indicate that *P. gingivalis* promotes the apoptosis. In this study, *P. gingivalis* was used for infection, and dynamic results showed that the *P. gingivalis* induced the apoptosis of GECs from 2 to 24 h. However, the time-dependent effect described by Stathopoulou et al. [11] did not appear in our study. Our results support an apoptosis-promoting role of *P. gingivalis*, but further studies are needed to make a convincing conclusion.

P. gingivalis had similar invasion mechanisms to many intestinal pathogenic bacteria. In the infectious diseases of the gastrointestinal tract, probiotics could inhibit cell apoptosis through blocking the adhesion of pathogen, inhibiting the intestinal inflammation, and regulating the transduction signal pathway and the expression of apoptotic gene. We found no significant differences in the apoptosis rates between the *L. acidophilus* group and the control group, which indicates that the *L. acidophilus* itself has no effects on GEC apoptosis. However, when *L. acidophilus* and *P. gingivalis* were co-cultured with GECs, *L. acidophilus* attenuated the apoptosis effect induced by *P. gingivalis* in a dose-dependent manner, and this trend was much more significant at 2 and 6 h. Stathopoulou et al. [11] confirmed that only the living *P. gingivalis* could induce the apoptosis effect, while the heat-killed bacteria has no effect. *L. acidophilus* may reduce the number of living *P. gingivalis* by inhibiting the bacteria growth, thereby inhibiting the apoptotic effect from *P. gingivalis*. Further experiments are needed to elucidate whether the *L. acidophilus* can inhibit the apoptosis through regulating known apoptosis signal pathways, apoptosis proteases and apoptosis genes.

4.1. Study limitations

The major study limitation is that we have not probed the clear mechanism of the protective effects from *L. acidophilus.* We will further study the inhibiting role of *L.acidophilus* on *P.gingivalis,* to reveal whether it could degrade the secretory products of *P.gingivalis*, or whether it could suppressthe expression of apoptotic protease/ genes of GEC.

5. Conclusions

In summary, the current study identified that *P.gingivalis* inhibits the proliferation and triggers the apoptosis of the human GECs. *L.acidophilus* could attenuate the above adverse effects in a dose-dependent manner. Our study suggests that *L.acidophilus* may serve as an innovative approach towards therapeutics in periodontal disease.

Author contribution

Study Design: Jun-jun Zhao,Xi-ping Feng
Data Collection: Jun-jun Zhao, Ya-qin Zhu
Statistical Analysis: Long Jiang
Data Interpretation: Jun-jun Zhao, Ya-qin Zhu
Manuscript Preparation: Jun-jun Zhao, Ya-qin Zhu
Literature Search: Jun-jun Zhao, Long Jiang
Funds Collection: Jun-jun Zhao, Ya-qin Zhu

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Conflict of interest

The authors declare that they have no conflict of interests.

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