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In vitro Evaluation of the Intestinal Cell Adhesion, Immunomodulatory Effect, and Cholesterol Assimilation of the Potential Probiotic and Postbiotic Isolated from Healthy Thai Children

Namfon Suebwongsa[1,3], Chamraj Kaewraemruaen[2,4], Panjamaporn Yotpanya[3], Viraphong Lulitanond[3] and Marutpong Panya[4]*

[1]National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani 12120, Thailand
[2]Department of Science and Bioinnovation, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, 73140, Thailand
[3]Research and Diagnostic Center for Emerging Infectious Diseases (RCEID), Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[4]Research Group for Biomedical Research and Innovative Development (RG-BRID), College of Medicine and Public Health, Ubon Ratchathani University, Warinchamrap, Ubon Ratchathani 34190, Thailand

*Corresponding author; E-mail: marutpong.p@ubu.ac.th

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Abstract

Several bacterial genera, including *Lactobacillus*, have been evaluated for their beneficial effects in humans. This study aimed to evaluate the "probiotic" and "postbiotic" potential of three bacterial strains: *Lactobacillus oris* RCEID28-3, *Limosilactobacillus fermentum* RCEID23-2, and *Limosilactobacillus fermentum* RCEID47-7. Probiotics are defined as live bacterial cells, whereas postbiotics are inactivated or killed probiotics. Probiotic potential was evaluated by assessing the adhesion ability of the Caco-2 and HT-29 cell lines, immunomodulatory effects, and cholesterol assimilation. Meanwhile, postbiotic potential was assessed by evaluating their immunomodulatory effects through measuring cytokine production in human peripheral blood mononuclear cells (PBMCs) using an enzyme-linked immunosorbent assay (ELISA). The human intestinal cell adhesion assay showed that all bacterial strains exhibited adhesion of more than 90% to both the Caco-2 and HT-29 cell lines, except for *L. oris* RCEID28-3. The highest cell adhesion level was observed in *L. fermentum* RCEID47-7. The cytokine production assay revealed that probiotic and postbiotic strains stimulated the production of three cytokines: IL-10, IFN- γ, and TNF-α, in PBMCs with the different cytokines, indicating the strain-dependent property. Moreover, cholesterol assimilation by live probiotics showed that all the strains, especially *L. fermentum* RCEID47-7, could reduce cholesterol levels. Therefore, this study provides scientific evidence to support the possibility of applying probiotics and their inactivated forms (postbiotics) in humans in the near future.

Keywords**:** *cytokine; cholesterol assimilation; human intestinal cell line; lactobacilli; Lactoplantibacillus postbiotic; probiotic*

1. Introduction

According to the International Scientific Association for Probiotics and Prebiotics (ISAPP), probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Probiotics used in humans have mostly been yeast and bacteria, although most are bacteria, mainly the

Lactobacillus and *Bifidobacterium* species, being predominant. The genus *Lactobacillus* has been reclassified into 23 genera and two emended genera: the *Lactobacillus delbrueckii* group and *Paralactobacillus* (Zheng et al., 2020). This classification is based on the core genome phylogeny, clade-specific signature genes, physiology, and ecology of organisms.

Lactobacillus has been isolated from various environments, such as fermented food products, cheese, yogurt, and the human gastrointestinal tract (Bazireh et al., 2020; Sanders, 2008). Some species, such as *Lacticaseibacillus rhamnosus* (Papizadeh et al., 2017), *Lactiplantibacillus plantarum* (Garcia-Gonzalez et al., 2021), *Lactiplantibacillus pentosus* (Benítez-Cabello et al., 2020), and *Lactobacillus helveticus* (Hassan et al., 2020), have been evaluated as probiotics. In general, the initial procedure for characterizing potential probiotics is based on survival ability in simulated human gastrointestinal acidic and enzymatic conditions, followed by the capacity to adhere to the intestine. The adhesion of a potential probiotic to the host cells mediates adhesion competition against a pathogen, production of antimicrobial substances, induction of mucus layer production, and stimulation of immune cells (Kaur et al., 2002; Leser, & Mølbak, 2009; Vélez et al., 2007; Wallace et al., 2003). The mucosal immune system, especially in the lower intestine, plays an essential role in both the innate and adaptive immune systems (Shi et al., 2017). Different cytokines produced by the mucosal immune cells play different roles in the mucosal immune system (Azad et al., 2018; Hardy et al., 2013). After oral administration of *L. rhamnosus* strain GG, LGG in mice stimulates the production of IL-10, which promotes mucosal immune homeostasis (Mirpuri et al., 2012). In addition, oral administration of LGG in a rat model demonstrated that LGG mediated the reduction of proinflammatory cytokines induced by an *Escherichia coli* lipopolysaccharide (Li et al., 2009). However, it has been demonstrated that differentially stimulated immune responses are strain-specific (Wells, 2011). Other beneficial effects of probiotics for specific purposes in humans, such as the reduction of serum cholesterol levels, have been evaluated (Nami et al., 2019; Palaniyandi et al., 2020).

In addition to using live bacteria as probiotics, postbiotics, which are defined as preparations of inanimate (inactivated) microorganisms and/or their components, including microbial bioactive substances that confer a health benefit to the host, have recently been explored for application in humans along with probiotics (Salminen et al., 2021). Killed microorganisms can effectively modulate the human immune response, leading to health-promoting effects. Clinical trials of postbiotics have evaluated their benefits; evidence includes a *Lactobacillus*-derived postbiotic that alleviates the severity of atopic dermatitis in humans after oral administration (Tanojo et al., 2023), treatment of sepsis (Lou et al., 2023), and modulating the human gut microbiota. Postbiotics should be derived from probiotic strains (Tsilingiri, & Rescigno, 2013).

Based on the above background and rationale, specific bacterial strain(s) need to be evaluated initially using *in vitro* experiments to support the possibility of using these strains as either probiotics or postbiotics in humans.

2. Objectives

This study aimed to evaluate the probiotic and postbiotic potential of three bacterial strains: *Lactobacillus oris* RCEID28-3, *Limosilactobacillus fermentum* RCEID23-2, and *Limosilactobacillus fermentum* RCEID47-7. Probiotic potential was evaluated by assessing the adhesion ability to the Caco-2 and HT-29 cell lines, immunomodulatory effects, and cholesterol assimilation. Postbiotic potential was evaluated only by evaluating the immunomodulatory effects.

3. Materials and methods

3.1 Ethics approval

Ethical permission for the isolation of peripheral blood mononuclear cells (PBMCs) from the heparinized blood samples of healthy volunteers was obtained from the Khon Kaen University Ethics Committee for Human Research (number HE591324).

3.2 Bacterial strains

The bacterial strains used in this study and their characteristics are listed in Table 1. All strains were first recovered from -80°C frozen stock by performing the cross-streak plate technique on De Man, Rogosa, and Sharpe (MRS) agar (Difco™, MD, USA). The purified colonies were used for further experiments.

Table 1 Bacterial strains and their relevant characteristics

Strains	Relevant characteristics	Reference
Lactobacillus oris [a]RCEID28-3	The strain was isolated from human feces. It exhibited growth inhibition activity against *Salmonella enterrica* subsp. *enterica* serovar Typhimurium [b]ATCC13311	Yotpanya et al. (2016)
Limosilactobacillus fermentum RCEID23-2	The strain was isolated from human feces	Yotpanya et al. (2016)
Limosilactobacillus fermentum RCEID47-7	The strain was isolated from a human that had been used as a bacterial host for heterologous protein expression. Its complete genome was determined and reported in the NCBI[c] database accession number	Konyanee et al. (2019)
Lacticaseibacillus rhamnosus ATCC53103 (GG)	Reference probiotic strain	Silva et al. (1987)

[a]Research and Diagnostic Center for Emerging Infectious Diseases, Khon Kaen University
[b]American Type Culture Collection
[c]National Center for Biotechnology Information

3.3 Human intestinal cell line preparation

Two human intestinal cell lines, Caco-2 and HT-29, were used as model cell lines for the adhesion assays. Caco-2 cells, which are an immortalized human colorectal adenocarcinoma cell line, were purchased from the American Type Culture Collection (ATCC). The Caco-2 cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibgo®, MA, USA) supplemented with 20% (v/v) fetal bovine serum (FBS) (Gibgo®) and 1× non-essential amino acids (Gibgo®), and was incubated at 37°C in a 5% CO_2 atmosphere. The culture medium was changed daily for five consecutive days. For the adhesion assay, cells were seeded into 24-well plates at a density of 50,000 cells/cm^2. The experiment was performed 21 days after the cell culture showed complete morphological and functional differentiation at 80–90% confluence (Laparra, & Sanz, 2009).

The HT-29 cell line was originally derived from ATCC and was then kindly prepared by Prof. Dr. Watanalai Panbangred, Department of Biotechnology, Faculty of Science, Mahidol University, Thailand. The cells were grown in DMEM supplemented with 10% (v/v) FBS and 1× non-essential amino acids and were further incubated at 37°C in a 5% CO_2 atmosphere. The culture medium was changed daily for three consecutive days. Both Caco-2 and HT-29 cells were seeded into 24-well plates at a density of 50,000 cells/ cm^2 and incubated for 14 days for further use in the cell adhesion assay.

3.4 Cell adhesion assay

One milliliter of 1.0×10^8 colony forming unit per milliliter (CFU/mL) of each bacterial strain was added to the wells containing either Caco-2 or HT-29 cells. The plates were incubated for 1 h at 37°C in a 5% CO_2 atmosphere. After incubation, all wells were washed thrice with 1 mL PBS to remove non-adherent bacteria. To release adherent bacteria from each well, the wells were treated with 200 µl of 0.25% (w/v) trypsin–EDTA (Gibco®) and were incubated at 37°C, 5% CO_2 for 10 min. After treatment, 800 µl of DMEM supplemented with 10% FBS was added to inhibit trypsin activity, and the Caco-2 or HT-29 layers were thoroughly dissociated by pipetting. A serial 10-fold dilution of the cell suspension was prepared, and the appropriate dilution was spread in triplicate on MRS agar plates. The plates were incubated at 37°C for 48 h. Colonies were counted and the percentage of adherent cells was calculated using the following formula:

$$\text{Percentage (\%) of cell adhesion} = \left(\frac{\log CAT}{\log CAC}\right) \times 100$$

where CAC and CAT represent the total viable bacterial counts in CFU/mL after 0 and 1 h of incubation, respectively (Satish Kumar et al., 2011).

3.5 Isolation of peripheral blood mononuclear cells

Human PBMCs were isolated using the Ficoll-Hypaque separation method as described previously (Ashraf et al., 2014). Briefly, 5 mL of heparinized blood was gently overlaid onto 5 mL of Ficoll-Hypaque solution in a 15 mL conical tube. The components of the resulting solution were mixed by vortexing, and the solution was centrifuged at 5000*xg*, 20°C, for 30 min. The PBMC-containing cloudy layer interfaces between

the Ficoll-Hypaque solution and the plasma layer were carefully transferred to sterile conical tubes. The PBMCs were washed twice and adjusted using an RPMI-1640 medium containing 10% (v/v) FBS.

3.6 Cytokine production through the stimulation of human PBMCs with live- and killed-probiotic bacteria

For live bacterial cell preparation, *L. oris* RCEID28-3, *L. fermentum* RCEID 23-2, *L. fermentum* RCEID 47-7, and *L. rhamnosus* GG were statically grown in MRS broth at 37°C for 18 h. Bacterial cells were harvested by centrifugation at 8000*xg*, 4°C for 10 min. The cell pellets were washed twice with PBS (pH 7.4). The bacterial cell suspension was adjusted to obtain an OD_{600} of 0.5, which approximately corresponded to 2.0×10^5 CFU/mL. To prepare the ultraviolet (UV)-killed cells, the same quantity of bacteria from the live bacterial cell preparations was exposed to UV light for 45 min at room temperature. A plate-counting technique was used to verify the viability of the live cells and UV-killed bacteria. The number of live bacteria was expressed as CFU/mL, as previously described.

For PBMC stimulation, 1.0×10^5 cells/mL of PBMCs were seeded in a 96-well plate. The prepared bacterial cells, either live or UV-killed, were added to the wells of the plate and were incubated at 37°C for 24 h in a 5% CO_2 atmosphere. A 1 µg/mL of purified lipopolysaccharide (LPS) (Sigma–Aldrich, AUS) was used as a positive control in PBMC stimulation. Unstimulated peripheral PBMCs were used to produce basal cytokines. The supernatants of stimulated and unstimulated PBMCs were harvested at 24 h and stored at -80°C until analysis (Chanput et al., 2010).

3.7 Cytokine production assays

The interleukin-10 (IL-10), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) levels were investigated from the cultured supernatants. All cytokines were measured by enzyme-linked immunosorbent assay (ELISA) using the ELISA MAX™ set (BioLegend, USA). Detection procedures were performed according to the manufacturer's instructions. The calculated data in picograms per milliliter (pg/mL) were expressed as the mean cytokine response subtracted from basal cytokine production for each treatment. All experiments were performed in triplicates.

3.8 Cholesterol assimilation in the culture medium

Cholesterol-assimilating activity was investigated in MRS broth supplemented with cholesterol, as described by Rudel, & Morris (1973). Briefly, each 1% (v/v) inoculum of the 18 h bacterial culture was added to MRS broth supplemented with 100 μg/mL cholesterol-PEG 600 (Sigma-Aldrich, MO, USA). All cultures were incubated at 37°C for 24 h under anaerobic conditions. Cell viability was counted by plate counting methods. The supernatants were harvested by centrifugation at 4,000*xg* for 10 min under 4°C. Five hundred microliters of collected supernatant was mixed with 500 μL of 33% (w/v) potassium hydroxide and 1 mL of absolute ethanol. One milliliter of deionized water and 1 mL of hexanes were added to the mixture and mixed by vortexing. The upper phase was transferred to a glass tube and evaporated under a flow of nitrogen gas until dried. For cholesterol analysis, 50 mg/dL of the *o*-phthalaldehyde (Alfa Aesar, Lancashire, UK) reagent was prepared in acetic acid. Then, 1 mL of the *o*-phthalaldehyde reagent was added into the dried-sample tube, and the solution was mixed by vortexing. Two hundred and fifty microliters of concentrated sulfuric acid were added to each tube. The mixed solutions were vortexed for 1 min, followed by incubation for 30 min at room temperature. The cholesterol concentrations of the samples and the cholesterol standard were calculated from the absorbance at 570 nm by spectrophotometry.

The cholesterol assimilated by bacteria was determined as follows:

$$\text{Cholesterol assimilation } \left(\frac{\mu g}{mL}\right) = \text{cholesterol } \left(\frac{\mu g}{mL}\right) \text{ at 0 h - cholesterol } \left(\frac{\mu g}{mL}\right) \text{ at 24 h}$$

The percentage of cholesterol assimilation was calculated as per the following equation:

$$\%\ \text{of cholesterol assimilated} = \left(\frac{\text{cholesterol assimilated at 24 h } \left(\frac{\mu g}{mL}\right)}{\text{cholesterol assimilated at 0 h } \left(\frac{\mu g}{mL}\right)}\right) \times 100$$

Cholesterol assimilation per 10^{10} CFU viable bacterial cells was calculated as per the following equation:

$$\text{Cholesterol assimilation per } 10^{10} \text{ CFU viable bacterial cells} = \frac{\text{Cholesterol assimilated } (\mu g/mL)}{10^{10} \text{ CFU of viable bacterial cells}}$$

3.9 Statistical analysis

The data from our experiments were analyzed using a one-way ANOVA and Tukey's HSD test. SPSS version 20.0 was used for data analysis, and significant differences were set at $p < 0.05$.

4. Results

4.1 Bacterial adhesion to human intestinal cell lines

As shown in Table 2, all bacterial strains, except for *L. oris* RCEID 28-3, showed a high adhesion percentage of more than 90% to the Caco-2 and HT-29 cell lines. The highest percentage of cell adhesion was obtained from the *L. fermentum* RCEID47-7 strain at a percentage of 92.34 ± 0.90 and 91.87 ± 0.53 on the Caco-2 and HT-29 cell lines, respectively. This result was comparable to that of the reference strain *L. rhamnosus* GG. However, there were no significant differences in the adhesion capacities of any of the tested strains in either cell line.

4.2 Cytokine production in human PBMCs after stimulation by live probiotic cells

As shown in Figure 1, three cytokines (IL-10, TNF-α, and IFN-γ) were measured by ELISA after stimulating the PBMCs with different live probiotic strains. All probiotic strains, except live *L. oris* RCEID28-3, stimulated the production of IL-10 in PBMCs (Figure 1, A). The highest level of IL-10 was obtained after stimulating PBMCs with the positive control, LPS. Live *L. fermentum* RCEID47-7 cells strongly stimulated PBMCs to produce IL-10, with a significant difference ($p < 0.05$) compared with the control (unstimulated cells). Live *L. fermentum* RCEID23-2 and live *L. rhamnosus* GG stimulated the production of IL-10 in PBMCs at similar levels.

For the production of TNF-α (Figure 1, B), all probiotic strains stimulated its production from PBMCs. The highest level of TNF-α was obtained from the stimulation of PBMCs by live *L. fermentum* RCEID23-2 followed by live *L. oris* RCEID28-3, live *L. rhamnosus* GG, and live *L. fermentum* 47-7 cells, respectively. This result was similar to the production pattern of IFN- γ (Figure 1, C), as the highest production level of IFN- γ was attained after stimulating the PBMCs with live *L. fermentum* RCEID23-2, followed by live *L. oris* RCEID28-3, live *L. rhamnosus* GG, and live *L. fermentum* 47-7 cells, respectively.

4.3 Cytokine production in human PBMC cells after stimulation by the UV-killed probiotic

Figure 2 shows the cytokine (IL-10, TNF-α, and IFN-γ) production at 24 h after the stimulation of human PBMC cells with the UV-killed probiotic. IL-10 was detected at similar levels after stimulation with the UV-killed probiotic *L. fermentum* RCEID 47-7, *L. fermentum* RCEID23-2, and L. *rhamnosus* GG (Figure 2, A). UV-killed *L. oris* RCEID28-3 also induced low levels of IL-10 production in PBMCs. The highest levels of TNF-α production were obtained after using UV-killed *L. fermentum* RCEID 23-2 and UV-killed *L. oris* 28-3 as stimulators (Figure 2, B); these levels were even higher than that obtained from using LPS as a positive control. The lower levels of TNF-α were obtained through the use of UV-killed *L. fermentum* RCEID 47-7 and UV-killed *L. rhamnosus* GG as stimulators.

For the IFN-γ production level in PBMCs after stimulation with the UV-killed cells of probiotics, the high production level of IFN-γ was obtained from PBMCs stimulated with UV-killed cells of *L. oris* 28-3, *L. fermentum* RCEID 47-7, and *L. rhamnosus* GG. The UV-killed *L. fermentum* RCEID 23-2 stimulated the production of IFN-γ at low levels, and no significant difference was observed compared with that using LPS as a positive control (Figure 2, C).

Table 2 Bacterial adhesion to the Caco-2 and HT-29 cell lines

Bacterial strains	% Adhesion on Caco-2 (mean ± SD)[a]	% Adhesion on HT-29 (mean ± SD)[a]
L. rhamnosus GG	92.09 ± 0.48	91.87 ± 0.53
L. oris RCEID28-3	87.87 ± 4.30	86.51 ± 5.66
L. fermentum RCEID23-2	90.96 ± 2.35	90.40 ± 1.25
L. fermentum RCEID47-7	92.34 ± 0.90	91.87 ± 0.53

[a]The results are expressed as mean percentage of adhesion ± standard deviation (SD) (n = 3)

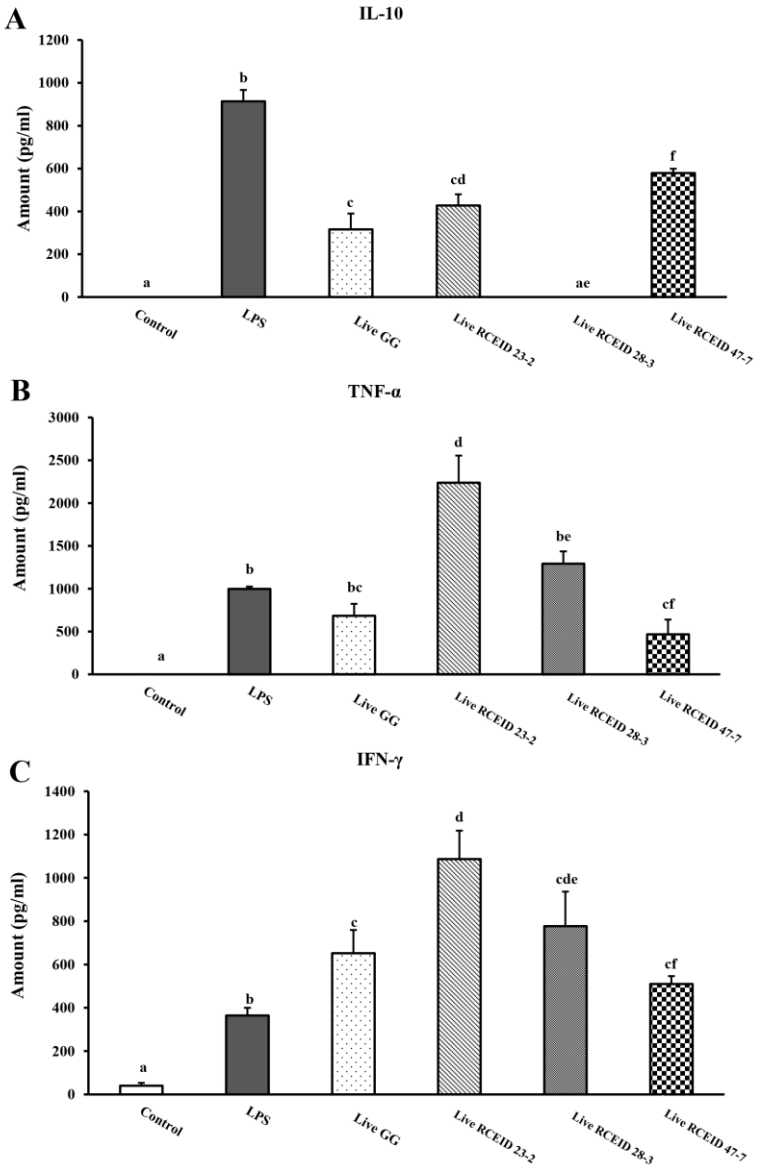

Figure 1 Cytokine production at 24 h after stimulation of human PBMC cells with live bacteria. Results are shown as the mean ±SD of three independent experiments. Results are presented as the mean ± S.D. The different letters (a–f) in the bar graph indicate the significant difference of each characteristic ($p < 0.05$)

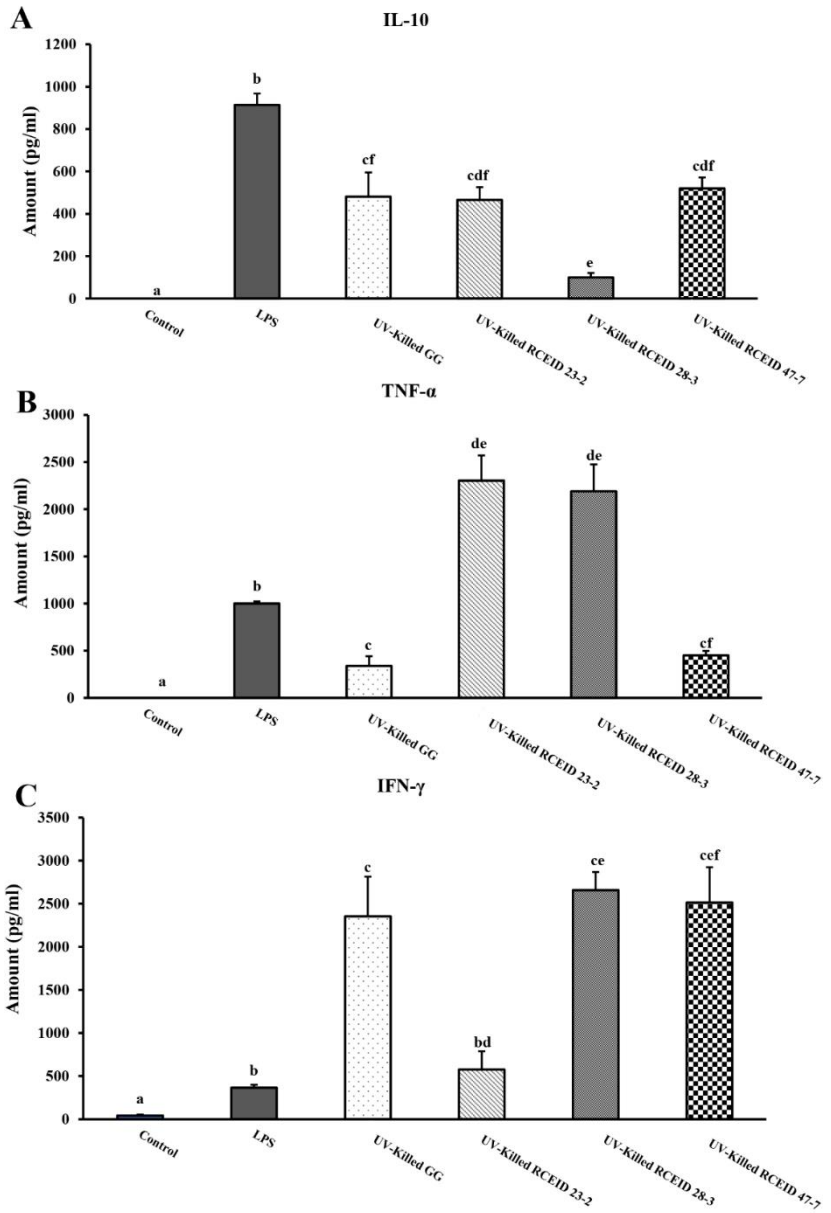

Figure 2 Cytokine production at 24 h after stimulation of human PBMC cells with UV-killed bacteria. Results are shown as the mean ±SD of three independent experiments. Results are presented as the mean ± S.D. The different letters (a–f) in the bar graph indicate the significant difference of each characteristic ($p < 0.05$)

Table 3 Cholesterol assimilation by probiotic strains for 24 h and the amount of cholesterol that is expected to be assimilated by cells in terms of milligrams of cholesterol per 10^{10} colony-forming unit (CFU)

***Lactobacillus* strains**	**Cholesterol lowering (%)**	**Cholesterol assimilated (%)**	**Cholesterol assimilated (mg/10^{10} CFU)**
Control (no bacteria)	0.00 ± 0	0.00 ± 0	-
L. rhamnosus GG	53.71 ± 3.36	55.72 ± 4.18	0.90 ± 0.07
L. fermentum RCEID47-7	58.63 ± 5.83	60.64 ± 7.24	0.68 ± 0.08
L. fermentum RCEID23-2	53.71 ± 5.83	55.72 ± 7.25	0.55 ± 0.07
L. oris RCEID28-3	41.30 ± 2.69	43.32 ± 3.35	0.46 ± 0.04

All experiments were performed in triplicate.

4.4 Cholesterol assimilation by live probiotics

In vitro evaluation of cholesterol assimilation by live probiotics revealed that all tested probiotic strains, including the reference *L. rhamnosus* GG, could assimilate cholesterol in culture media. As shown in Table 3, the highest cholesterol assimilation level was obtained from *L. fermentum* RCEID47-7 which was higher than that of *L. rhamnosus* GG. However, when comparing the assimilation of cholesterol in terms of milligrams of cholesterol per 10^{10} colony-forming unit (CFU), the reference *L. rhamnosus* GG had the highest capacity, followed by *L. fermentum* 47-7, *L. fermentum* 23-2, and *L. oris* 28-3, respectively.

5. Discussion

This study evaluated the potential of three bacterial strains, *L. fermentum* RCEID47-7, *L. fermentum* RCEID23-2, and *L. oris* RCEID28-3, isolated from healthy children's feces, to stimulate cytokine production by human PBMCs, adhesion ability on a human intestinal cell line, and cholesterol assimilation. Our previous study demonstrated that these bacterial strains have potential probiotic functions after testing their probiotic properties *in vitro*, including acid and bile salt tolerance, non-hemolysis, antimicrobial activity against pathogenic *E. coli* O157:H7, and antibiotic susceptibility (Yotpanya et al., 2016). However, other probiotic properties, including cell adhesion and immunomodulation, have not yet been evaluated.

It is crucial that the adhesion of probiotics to host cells is the initial step for probiotic bacteria to confer beneficial effects on hosts (Alp, & Kuleaşan, 2019). These beneficial effects include colonization resistance against pathogen adhesion to host cells (Collado et al., 2007), production of antimicrobial substances such as bacteriocins (Soltani et al., 2020), and modulation of human gut immunity through cytokine production (Azad et al., 2018). In this study, two human intestinal cell lines, HT-29 and Caco-2, were used as models to evaluate the adhesion capabilities of potential probiotic strains. All the tested strains, *L. fermentum* RCEID47-7, *L. fermentum* RCEID 23-2, and *L. oris* RCEID 28-3, had similar abilities to adhere to both cell lines, and the highest adhesion level was obtained for the RCEID47-7 strain. Our results are in concordance with those of a previous study that demonstrated that the selected probiotic had similar adhesion capabilities for both Caco-2 and HT-29 cell lines (Duary et al., 2011). Factors affecting the adhesion of human intestinal mucosa by probiotic bacteria are mediated by a protein called "adhesin" on the bacterial cell surface to the mucus or intestinal epithelial cells (van Pijkeren et al., 2006). The adhesin, a protein anchored on the bacterial cell surface, has been reported in probiotic bacteria, such as mucin-binding protein (Mub), fibronectin-binding protein, and surface layer protein in *Lactobacillus acidophilus* strain NCFM (Buck et al., 2005). Moreover, the mutation experiment of these genes showed the reduction of the adhesion capability to Caco-2 cells, indicating that cell adhesion was mediated by the bacterial cell wall adhesin which was encoded by the corresponding gene. This evidence is supported by our previous finding that *L. fermentum* RCEID47-7 harbored the gene encoding Mub on its chromosomal DNA (in the region of the complement sequence (756567..757007)) of NCBI accession number CP017712 (Konyanee et al., 2019). This promoted the highest adhesion of *L. fermentum* RCEID47-7 to both human intestinal cell lines. However, adhesion-associated genes in *L. fermentum* RCEID23-2 and *L. oris* RCEID 28-3 were not investigated in this study.

In addition to probiotic properties, immunological effects, including regulation, stimulation, and modulation mediated by probiotic bacteria, are essential properties to evaluate for the selection of particular strains for further application for specific purposes. For this evaluation, cytokine production in primary cells, cell lines, and animal models after exposure to probiotics was assessed. Moreover, the immunological effects of postbiotic and probiotic cells killed by UV light exposure have been evaluated (Arasu, & Rajasekar, 2024; Azad et al., 2018). Our study revealed that the three probiotics and postbiotics induced the production of different types of cytokines. The highest concentrations of TNF-α, a proinflammatory cytokine that plays an essential role in immune cell stimulation and function in pathogen clearance, were obtained after stimulating PBMCs with the live and UV-killed cells of RCEID23-2, followed by RCEID28-3 and RCEID47-7, respectively. The TNF-α production level was similar between live and UV-killed cells, indicating that the inducer/modulator for cytokine production could be part of bacterial cell components such as lipoteichoic acid and peptidoglycan, (Yeşilyurt et al., 2021). These observations were similar to those

on the production of IFN-γ, a proinflammatory cytokine produced from many cell types that plays an essential role in inducing an immune response against virus infection (Schoenborn, & Wilson, 2007); the highest level of IFN-γ production was obtained from PBMCs stimulated with live RCEID23-2, and followed by RCEID28-3 and RCEID47-7, respectively. Meanwhile, in PBMCs stimulated with UV-killed cells, the high production level of IFN-γ was obtained from RCEID28-3 and RCEID47-7, but not from RCEID23-2. This result indicated that both live or killed cells of RCEID28-3 and RCEID47-7 can be immunostimulatory through the modulation of cytokine IFN-γ production, but the strain RCEID23-2 appears to be used as an inducer for the proinflammatory cytokine production when it is still alive. IL-10 plays a key role in immune regulation, as it acts as an anti-inflammatory cytokine (Couper et al., 2008). Compared to the reference strain, higher production levels of IL-10 were obtained after stimulation of PBMC with live and UV-killed RCEID47-7 and RCEID23-2. The immunomodulatory effects of probiotics and their inactivated cells (postbiotics) are strain-dependent (Yeşilyurt et al., 2021). Moreover, both probiotics and postbiotics stimulated cytokine production in human PBMCs at a similar level. One study reported that postbiotics have higher immunomodulatory activity than their original probiotic counterparts (de Almada et al., 2016). It has been reported that the cell wall components, lipoteichoic acid and peptidoglycan, of postbiotic are important stimulator involved in immunomodulation (de Almada et al., 2016; Yeşilyurt et al., 2021). Moreover, Unmethylated CpG presented in bacterial genome of postbiotic bifidobacterial cultures of VSL#3 (a probiotic commercial product) stimulated the high production of IL-10 in PBMCs (Lammers et al., 2003). Thus, our results support alternatives for the use of postbiotics as immunomodulators. However, the effects of specific cell components of postbiotic are required in further study.

Excess cholesterol in human blood circulation is a cause of various diseases, including cardiovascular diseases (CVD) (Jung et al., 2022). Treatment of such diseases can be mediated by modifying diet and lifestyle. *In vitro* and animal studies have revealed that probiotic strains have a cholesterol-lowering potential (Tomaro-Duchesneau et al., 2014; Tomaro-Duchesneau et al., 2015). The mechanism of their cholesterol-lowering potential includes absorbing the cholesterol in the surrounding environment in a process known as "cholesterol assimilation," inhibition of cholesterol uptake by the colon epithelium, and deconjugation of bile acids (Taranto et al., 1997; Tomaro-Duchesneau et al., 2015). As a result, in this study, the cholesterol-lowering potential via the cholesterol assimilation mechanism of *L. fermentum* RCEID47-7 was comparable to that of *L. rhamnosus* GG. Moreover, our previous report on the genome analysis *of L. fermentum* RCEID47-7 found that this strain contains genes for bile hydrolysis, which could be one possible mechanism for the reduction in cholesterol levels (Konyanee et al., 2019). Further experiments are required to evaluate the cholesterol-lowering potential of *L. fermentum* RCEID47-7.

6. Conclusion

This study demonstrates that probiotic strains can adhere to human intestinal cell lines, which may confer beneficial effects on the host, inhibit pathogen adhesion, and modulate the mucosal immune system. The different strains with different forms (live and UV-killed) can stimulate human PBMCs to produce different cytokines, indicating their strain-dependent properties. *In vitro* experiments showed that all probiotic strains, especially *L. fermentum* RCEID47-7, reduced cholesterol levels. Based on these results, the probiotics and postbiotics in this study can be appropriately used for further investigation of their potential applications in humans in different clinical settings.

7. Acknowledgements

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