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In Vitro Assessment of Immunomodulatory Effects on HT-29 Cells and Antimicrobial Activity Against Enteropathogenic Bacteria of *Lactobacillus plantarum* LAB02 Newly Isolated from Guts of the Termite *Termes* sp.

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Abstract

Probiotics are beneficial non-pathogenic microorganisms that inhabit gastrointestinal tracts and provide effective abilities such as preventing pathogenic infection, improving immune system and anti-inflammation enhancing the health of humans and animals. The objectives of this study were to evaluate antimicrobial capability against enteropathogenic bacteria and immunomodulatory effects of lactic acid bacteria isolated from termites. In the study, 5 isolates of lactic acid bacteria were obtained from the guts of the termite *Termes* sp. They were tested for the ability to inhibit growth potential of enteropathogenic bacteria using the agar well diffusion method and also their adhesion ability and cytotoxicity to human intestinal HT-29 cells. Among all 5 isolates, LAB02 exhibited strong broad-spectrum antimicrobial activity against all nine enteropathogenic bacteria, moreover it possessed non-toxicity and overall higher adhesion ability to HT-29 than the commercial probiotic *Lactobacillus rhamnosus* GG. Due to its high potential, LAB02 was selected for subsequent experiments. Based on nucleotide sequence analysis of 16S rRNA gene, LAB02 was closely related to *Lactobacillus plantarum* (100% identity). After inflammatory induction by lipopolysaccharide from *Escherichia coli*, immunomodulatory effects of LAB02 on the pathogen-induced HT-29 were evaluated in this study. The quantitative polymerase chain reaction (qPCR) assay was provided to measure gene expression level of pro- (*IL-1*, *IL-6*, *IL-8*, *TNF-α* and *NF-κβ*) and anti-inflammatory cytokines (*IL-4*, *IL-10*, *TGF-β1*, *TGF-β2* and *TGF-β3*) and overall results revealed that LAB02 and its cell free supernatant could down-regulate the gene expression of pro-inflammatory cytokines and up-regulate of anti-inflammatory cytokines in HT-29 cells. The present study demonstrated some evidence indicating the potential of lactic acid bacteria obtained from termite guts. The strain *Lactobacillus plantarum* LAB02 might be used as a promising alternative agent to control enteropathogenic bacterial infection and modulate the immune system. The findings are useful for future study and applications in food microbiology and technology.

Keywords: Immunomodulatory effect, HT-29 cells, Antimicrobial activity, Enteropathogenic bacteria, *Lactobacillus plantarum*, Termite gut, Quantitative polymerase chain reaction (qPCR)

Introduction

Termites are terrestrial insect in the phylum Arthropoda that feed on lignocellulosic and other organic compounds in plants and dead matters [1]. Besides their destroying and decomposing activities, they are recognized as edible insects with high alternative protein. Termites has been reported to be consumed as human food, livestock feed and pose potential health benefits in many countries in Africa, America and Asia [2]. Termite guts are among the most complex microbial habitats containing a diverse of bacteria, protists and fungi that can digest the lignocellulose materials and produce some antimicrobial compounds [3]. Generally, many microorganisms with probiotic properties and hydrolytic activities have been previously isolated from termite guts [4,5], such as *Bacillus velezensis*, *Bacillus*

siamensis and *Bacillus subtilis* from *Termes propiquus* [5], *Lactococcus* sp. from *Coptotermes formosanus* [6] and *Bacillus thuringiensis* from *Microcerotermes* sp. [7]. However, the characteristics of termite gut bacteria are relevant to the different termite species and actual behaviors [8].

Enteropathogenic bacteria such as *Salmonella* Typhimurium, *Shigella dysenteriae*, *Escherichia coli*, *Listeria monocytogenes* and *Vibrio* spp. are major food-borne pathogen that can promote inflammation in the gastrointestinal tracts and cause diarrhea in human and animals [9-11]. At the present, using of antibiotics such as ciprofloxacin, azithromycin, ampicillin, cotrimoxazole and doxycycline are the most effective treatment for enteropathogenic bacterial infections. However, misuse and overuse of antibiotics are key factors contributing an increasing number of multidrug resistant (MDR) bacteria [12]. Thus, the use of probiotics is a safe alternative way to prevent and control the spread of enteropathogenic bacteria [13].

Probiotics are live microorganisms that inhabit human and animal gastrointestinal tracts, which promote the health of host in terms of immunomodulation. Nowadays, probiotics are found in a variety of microorganisms such as the genera *Lactobacillus, Bifidobacterium, Lactococcus, Bacillus* and *Saccharomyces* [14]. The strains can exhibit various probiotic properties such as antipathogenic, acid and bile tolerance, adherence and non-toxicity to intestinal cells and immunomodulatory properties and their safety follows the standard of Generally Recognize as Safe by the United States Food and Drug Administration (USFDA) [15]. Inflammatory cytokines have potential as biomarkers for many diseases and they have gained the interest of researchers in recent years. A change in their expression profiles is implicated in the pathogenesis of many diseases associated with complex regulatory pathways. Inflammatory cytokines are grouped based on their effect on inflammation as pro-inflammatory cytokines, which promote inflammation and damage of tissues, for examples tumor necrosis factor (TNF)-α, disrupting intestinal epithelial barrier and promoting intestinal inflammation; IL-1, triggering excessive inflammation leading to cell damage and impair intestine function; IL-6, playing a role in intestine damage particularly in inflammatory bowel diseases (IBD) like Crohn's disease (CD) and ulcerative colitis (UC); and IL-8, damaging intestine lining [16-18] and anti-inflammatory, which prevent and resolve the inflammation and damaging effects of the pro-inflammatory cytokines, for examples IL-10, suppressing pro-inflammatory signals and dampening immune responses, supporting integrity of intestinal barrier and preventing damage from inflammation and infection; and transforming growth factor-beta (TGF-β), suppressing macrophage inflammatory responses in the developing intestine and preventing mucosal injury [19,20]. Normally, the human intestinal cells secrete a variety of pro- and anti- inflammatory cytokines which control the immune system, involving with infection of enteropathogenic bacteria. The pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor (TNF) and nuclear factor Kappa ß (NF-κß) have been found their increase levels in intestinal cells under inflammatory condition regulated by microbial infection, anyway the anti-inflammatory cytokines such as interleukin-4 (IL-4), interleukin-10 (IL-10) and transforming growth factor (TGF) have protective role against the inflammation and cell damage [21,22]. In the previous studies, most probiotics with ability for reducing the pro-inflammatory cytokines and promoting the anti-inflammatory cytokines in human intestinal cells are particularly *Lactobacillus* spp., such as *Lactobacillus paracasei, Lactobacillus plantarum* and *Lactobacillus acidophilus* [23-25]. In addition to live bacteria, heat killed cells (parabiotic) or metabolites (postbiotics) of certain probiotics are known to modulate host-immune function.

Lactic acid bacteria (LAB) as a potential probiotic have been previously isolated from different sources such as fermented food, vegetables and feces [26]. Additionally, the intestinal tracts of animals are also a potential source of lactic acid bacteria. The uses of lactic acid bacteria for human and animals are aimed not only at modulation of gut microbiota and inhibition of pathogens, but also at anti-inflammatory activity on inflammatory cells that are induced by invasion of pathogens [27]. However, there are limited studies of lactic acid bacteria obtained from insects and their immunomodulatory effect. Therefore, the objectives of this study were to isolate lactic acid bacteria that have immunomodulatory effects and antimicrobial activity against enteropathogenic bacteria. The scientific

knowledge and microbial agents in the study benefit in the term of food microbial technology.

Materials and methods

Isolation of lactic acid bacteria from termite guts

Termites of *Termes* sp. (soil-wood feeding higher termite) were collected from the mango orchard, Nong Suea district, Pathum Thani province 12170, Thailand. The animal care was approved by the Kasetsart University Institutional Animal Care and Use Committee (ID#ACKU68-SCI-009). The termite samples were transported to the laboratory for bacterial isolation within 6 h. Thirty-termite workers were washed their surface with sterile distilled water on an ice-cold plate. After that, their gut content was collected and homogenized in 0.85% NaCl with sterile pestle. The homogenate was diluted and then spread on De Man–Rogosa–Sharpe (MRS) agar (Himedia, India). After incubation at 37 °C for 24 h in low-oxic condition, 50 bacterial colonies were selected and then culture purified using the streak plate method on MRS agar. Preliminary characterization of lactic acid bacteria was provided based on catalase production test and Gram's staining. Five catalase- negative bacterial isolates were selected for the subsequent experiments.

Antimicrobial activity against enteropathogenic bacteria

Antimicrobial activity of the isolates was evaluated using the agar well diffusion method against nine enteropathogenic bacteria in the biohazard risk group 2 (RG2) including: *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 11778, *Salmonella* Typhimurium ATCC 13311, *Shigella dysenteriae* DMST 4423, *Listeria monocytogenes* ATCC 7644, *Proteus mirabilis* DMST 8212, *Pseudomonas aeruginosa* ATCC 10145 and *Enterococcus faecalis* ATCC 29212. After cultivation of lactic acid bacteria (1.5×10^8 cell/mL) in MRS broth at 37 °C for 24 h in low-oxic condition, cell free supernatant (CFS) was collected by centrifugation at 5,000 rpm for 10 min at 4 °C and filtrated through a 0.22-µm membrane filter. On the other hand, cell suspension of the enteropathogenic bacteria (1.5×10^8 cell/mL) was swabbed on trypticase soy agar (TSA) plate using the sterile cotton swab and 100 µL of CFS was dropped on each well. After incubation at 37 ºC for 24 h, the diameter inhibition zone (mm) was measured. The probiotic strain *Lactobacillus rhamnosus* GG was used as positive control in the tests.

Adhesion ability to human intestinal HT-29 cells

The ability of the isolates to adhere to human intestinal cells (HT-29 cells) was according to the method described by Tomtong and Deevong [5]. The cell lines were grown under 5% CO_2 in McCoy's 5A (Gibco Co., USA) supplemented with 10% (v/v) fetal bovine serum (Gibco Co., USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco Co., USA). For the adhesion assay, the cell monolayers with approximately 90% cell confluency in 6-well plate were washed 3 times with phosphate buffer saline (PBS) pH 7.4 and cell suspension of the lactic acid bacteria (1.5×10^8 cell/mL) was added into each well. After incubation at 37 °C for 1 h under 5% CO_2, the cell line in each well was washed 5 times with PBS pH 7.4 to remove non-adherent bacteria, then lysed the cells by PBS pH 7.4 containing 0.01% Triton X 100 at 37 °C for 5 min. The cell lysate suspension containing the adhered bacteria was serial diluted and plated on MRS agar. After incubation at 24 h for 37 °C, colonies of lactic acid bacteria were quantified and calculated for adhesion index (%). The commercial probiotic strain *L. rhamnosus* GG was used as a positive control in the tests.

$$\text{Adhesion index (\%)} = \frac{\text{Adhered bacterial number (CFU/mL)}}{\text{Total bacterial number (CFU/mL)}} \times 100 \quad (1)$$

Toxicity of lactic acid bacteria on human intestinal HT-29 cells

The method for evaluation of cytotoxicity effect of the isolates on human intestinal cells (HT-29 cell) was minor modified from Ngamsomchat *et al.* [28]. The HT-29 cell line was grown under 5% CO_2 in McCoy's 5A (Gibco Co., USA) supplemented with 10% (v/v) fetal bovine serum (Gibco Co., USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco Co., USA). In the assay, the monolayers of cell line with approximately 90% cell confluency in 96-well plate were washed 3 times with PBS pH 7.4. Subsequently, cell free supernatant (CFS) of lactic acid bacteria was added into each well. After incubation at 37 °C for 24 h under 5% CO_2, the cell line in each well was washed 5 times with

PBS pH 7.4, then added with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; Sigma-Aldrich, USA) solution (45 mg/mL). The microplate was further incubated at 37 ºC for 4 h under 5% CO_2 in darkness, and 100 µL of dimethyl sulfoxide (DMSO; KemAus, Australia) was added to solubilize formazan crystals pellets. An absorbance at 570 nm (A570) of the solution was measured in triplicate using a Thermo Scientific™ Multiskan GO Microplate Spectrophotometer (Thermo Scientific, USA) and calculated for cell viability (%). The bacterial strain *B. cereus* ATCC 11778 was used as a positive control. Cell morphology of HT-29 was observed under 400× magnification of Mateo TL digital inverted microscope (Leica Microsystems (Suzhou) Technology Co., Ltd., China)

$$\text{Cell viability (\%)} = \frac{\text{A570 of treatment}}{\text{A570 of control}} \times 100 \quad (2)$$

Molecular identification based on 16S rRNA gene

Genomic DNA of bacteria was extracted using the GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies, Malaysia) according to the manufacturer's instructions. Then, 16S rRNA gene (approximately 1.5 kb in length) was amplified using bacterial 16S rRNA universal primers, forward 616V (5'-AGAGTTGATYMTGGCTC-3') and reverse 1492R (5'-GGYTACCTTGTTACGACTT-3') as described by Chanworawit *et al.* [7]. The PCR amplification was performed by the Bio-Rad T100 thermal cycler with the temperature program: Initial denaturation at 94 °C for 3 min; followed by 30 cycles consisting of denaturation at 94 °C for 40 s, annealing at 52 °C for 40 s and extension at 72 °C for 1.30 min; and finished by final extension at 72 °C for 10 min. The 16S rRNA gene amplification product (~1.5 kb) was analyzed by 1% (w/v) agarose gel electrophoresis. All PCR reactions were performed in 2X DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, USA) and nucleotide sequencing was conducted by Macrogen, Inc. (Seoul, Republic of Korea). The 16S rRNA nucleotide sequence was analyzed using BioEdit version 7.2.5.0 [29] and identified using nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was constructed using the MEGA version X program [30] after multiple sequence alignments by MAFFT version 7.0 [31], applying by maximum-likelihood model with 1,000 bootstrap replicates.

Immunomodulatory effects of lactic acid bacteria

In this step, quantitative PCR (qPCR) was used for evaluating relative expression of genes encoding pro- and anti-inflammatory cytokines in LPS induced HT-29 cells. Inflammatory response to human intestinal HT-29 cells was induced by 1 µg/mL of lipopolysaccharides (LPS) from enteropathogenic *E. coli* (Sigma, USA). Lactic acid bacteria were cultivated in MRS broth at 37 °C for 24 h in low-oxic condition. Then, bacterial cells were typically separated from culture supernatant by centrifugation at 5,000 rpm for 10 min at 4 °C. To prepare cell suspension, bacterial cell pellet was resuspended and washed with PBS pH 7.4 in triplicate. Besides, the collected supernatant was filtrated through a 0.22-µm membrane filter to provide cell free supernatant (CFS). The cell suspension (1.5×10^8 cell/mL) and filtrated CFS of lactic acid bacteria were added to inflammatory induced HT-29 cells (1.0×10^6 cell/well) in 6-well plate. After incubation at 37 °C for 6 h under 5% CO_2, the HT-29 cells were precipitated. Total RNA was extracted using TRIZOL reagent method [32] and transcribed to cDNA using ReverTra Ace qPCR RT master mix with gDNA remover (TOYOBO, Japan). The expression levels of pro- and anti-inflammatory genes were measured by qTOWER iris Real-time PCR with SYBR green fluorescence signal detection using Thunderbird SYBR qPCR Mix (TOYOBO, Japan). The qPCR reaction was conducted using a total volume of 20 µL, containing 10 µL of Thunderbird SYBR qPCR master mix, 0.5 µL of each forward and reverse primers, 7 µL of nuclease free water and 2 µL of cDNA template. The information of target genes, primer sequences, annealing temperatures, and size of product (bp) for qPCR analysis is shown in **Table 1** [33]. The relative expression of each target gene was estimated by calculating $2^{-\Delta\Delta Ct}$ compared with the housekeeping gene of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The specificity of reaction was verified by melting curve analysis. In the tests, *L. rhamnosus* GG was used as reference probiotic strain. A positive control was the inflammatory induced HT-29

cells, and a negative control was the normal cell line without LPS-inflammatory induction.

Table 1 Information of target genes, primer sequences, annealing temperatures and size of product for quantitative PCR (qPCR) analysis

Target	Sequence (5' – 3')	Annealing temperature (°C)	Product size (bp)
Pro-inflammatory cytokine genes			
IL-1α	F: 5' ATGGCCAAAGTTCCAGACATG 3'	52	600
	R: 5' TTGGTCTTCATCTTGGGCAGTCAC 3'		
IL-1ß	F: 5' GTGGCAATGAGGATGACTTGTTC 3'	58	150
	R: 5' TTGCTGTAGTGGTCGGAG 3'		
IL-6	F: 5' CATCCTCGACGGCATCTCAG 3'	62	495
	R: 5' GCTCTGTTGCCTGGTCCTC 3'		
IL-8	F: 5' CTGGCCGTGGCTCTCTTGGCAGCCTTCCTG 3'	61	395
	R: 5' GGCAACCCTACAACAGACCCACACAATACA 3'		
TNF-α	F: 5' TCTCGAACCCCGAGTGACAA 3'	66	125
	R: 5' TATCTCTCAGCTCCACGCCA 3'		
NF-κß	F: 5' TCAATGGCTACACAGGACCA 3'	61	310
	R: 5' CACTGTCACCTGGAAGCAGA 3'		
Anti-inflammatory cytokine genes			
IL-4	F: 5' TCATTTTCCCTCGGTTTCAG 3'	62	160
	R: 5' AGAACAGAGGGGGAAGCAGT 3'		
IL-10	F: 5' TCAGGGTGGCGACTCTAT 3'	66	200
	R: 5' TGGGCTTCTTTCTAAATCGTTC 3'		
TGF-ß1	F: 5' GCTGCTGTGGCTACTGGTGC 3'	66	320
	R: 5' CATAGATTTCGTTGTGGGTTT 3'		
TGF-ß1	F: 5' CCCCACATCTCCTGCTAA 3'	61	175
	R: 5' GTGTATCCATTTCCACCCTA 3'		
TGF-ß3	F: 5' CTGAGAATCACGGTGGTAAA 3'	62	130
	R: 5' CATCTCAACTTACCATCCCT 3'		
Housekeeping gene			
GAPDH	F: 5' GGAAGGTGAAGGTCGGAGTC 3'	66	185
	R: 5' TCAGCCTTGACGGTGCCATG 3'		

Statistical analysis

All tests were performed at least in triplicate. The results are expressed as the mean ± standard deviation (S.D.). The data were analyzed using one-way ANOVA, followed by the Duncan Post Hoc test, in SPSS v. 25.0. The level of statistical significance was set at $p\text{-value} < 0.05$ for all analyses. All graphs were generated using GraphPad Prism version 9 (GraphPad Software, USA).

Results and discussion

Lactic acid bacteria isolated from termite guts

In the bacterial isolation from termite guts (*Termes* sp.; Tp), many isolates with solubilizing $CaCO_3$ and exhibiting clear zone around colonies on MRS agar plate were collected. Among 50 bacterial isolates, a total of 5 isolates were selected based on the categorized characteristics of lactic acid bacteria including catalase-negative, Gram-positive and non-endospore forming. They were labeled as isolates LAB01, LAB02, LAB05, LAB06 and LAB07. The bacteria were preserved in 20% (v/v) glycerol at –80 °C and used for subsequent experiments. In previous studies, there have been reported about isolation of lactic acid bacteria from several insects such as bean bug (*Riptortus pedestris*), silkworm (*Bombyx mori*) and honey bees (*Apis mellifera*) [34-36]. Termite guts have been previously reported as microbial habitat with specific structures and extreme conditions and the guts of the termite *Termes* sp. (*Termes propiquus*) have been found as potential source of probiotic *Bacillus* spp. [5].

Antimicrobial activity against enteropathogenic bacteria

In this step, 5 selected isolates were tested for antimicrobial ability against nine enteropathogenic bacteria. As shown in **Figure 1**, the isolate LAB02 presented broad-spectrum antimicrobial activity against all enteropathogenic bacteria, which are overall higher than those of the reference probiotic strain *Lactobacillus rhamnosus* GG (positive control). Among all isolates, LAB02 showed the highest antimicrobial effects toward all test pathogens, except *Proteus mirabilis* and it was the most effective antimicrobial agent against *Escherichia coli* with the inhibition zone of 17.70 mm. All the isolates could inhibit *E. coli, Salmonella* Typhimurium*, B. cereus* and *P. mirabilis*, but only LAB02 showed inhibition against *Enterococcus faecalis* (12.44 mm of inhibition zone), *Listeria monocytogenes* (14.27 mm of inhibition zone) and *Pseudomonas aeruginosa* (9.80 mm of inhibition zone). The results in the present study are supported by Sanam *et al.* [37] who reported that lactic acid bacteria can inhibit board-range of enteropathogenic bacteria such as *B. cereus, Staphylococcus aureus* and *E. coli*. Moreover, Bungenstock *et al*. [38] reported that cell free supernatant (CFS) of lactic acid bacteria showed broad-spectrum antibacterial effects toward important foodborne pathogens (*E. coli* DSM 1103, *Listeria innocua* DSM 20649, *L. monocytogenes* DSM 19094, *P. aeruginosa* DSM 939, *S. aureus* DSM 799 and *S.* Typhimurium DSM 19587) using the agar well diffusion method.

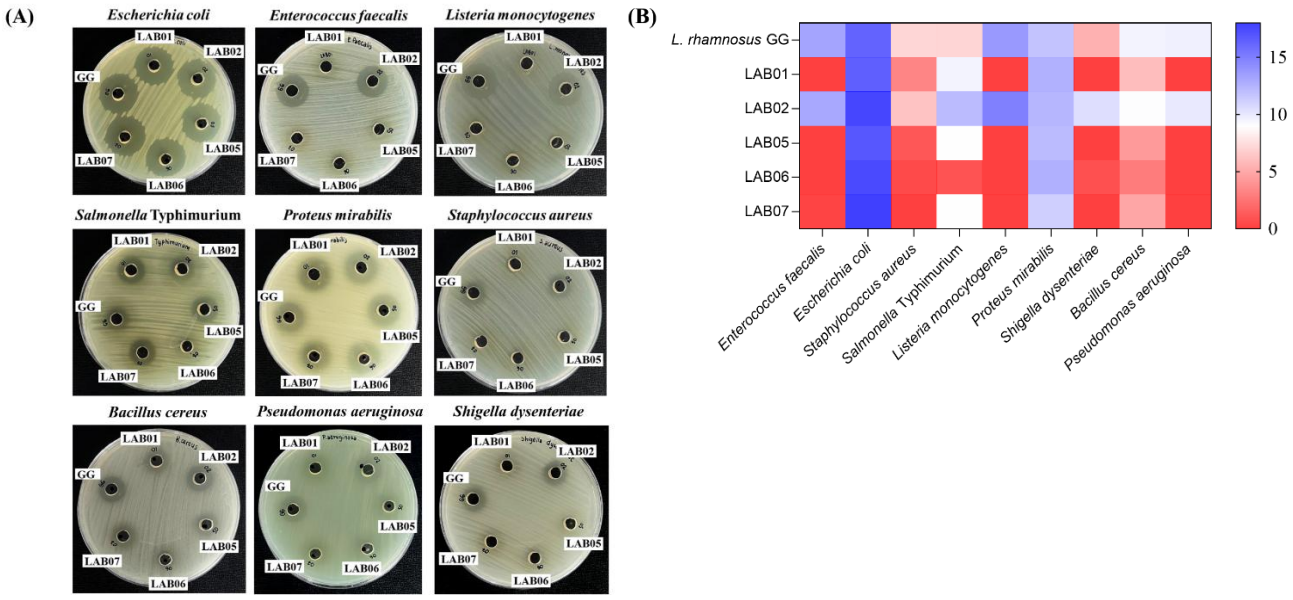

Figure 1 (A) Plate photographs of antimicrobial activity test of the lactic acid bacteria including LAB01, LAB02, LAB05, LAB06 and LAB07 against enteropathogenic bacteria, and (B) heat-map using diameter (mm) of antibacterial inhibition zone. The probiotic strain *Lactobacillus rhamnosus* GG was used as positive control in the tests.

Adhesion ability to human intestinal HT-29 cells

The adhesion ability of the isolates to human intestinal cells (HT-29) was evaluated and the results are shown in **Figure 2**. The adhesion percentage of all 5 lactic acid bacteria to HT-29 was in ranging of 9.68% to 33.75%. The isolate LAB02 showed the highest adhesion index value, significantly different from those of the other isolates. However, no significant difference was found in adhesion index between LAB02 and the positive control (*L. rhamnosus* GG). Thus, LAB02 was the promising isolate that showed the highest broad-spectrum antimicrobial activity and adhesion ability to HT-29. In recent years, there have been several studies of adhesion ability of lactic acid bacteria to intestinal cells. For examples, Fonseca *et al.* [39] found that *Lactobacillus paracasei* CCMA 0505 has adhesion ability (4.75% adhesion) to intestinal cells; Sharma and Kanwar [40] previously reported that 11 isolates of lactic acid bacteria obtained from traditional fermented food have adhesion ability to human intestinal Caco-2 and HT-29 cell lines in the range from 2.45 ± 0.5% to 9.55 ± 0.76% and 4.11 ± 0.68% to 12.88 ± 0.63%, respectively. Many previous researches showed that lactic acid bacteria are able to adhere to intestinal epithelial cells and inhibit pathogen adhesion. In stance, probiotic *Lactobacillus* species reveal the anti-adhesion effects on intestinal Caco-2 cells against pathogenic bacteria such as *E. coli*, meaning they can prevent pathogen adhesion and infection to the intestinal cells [41].

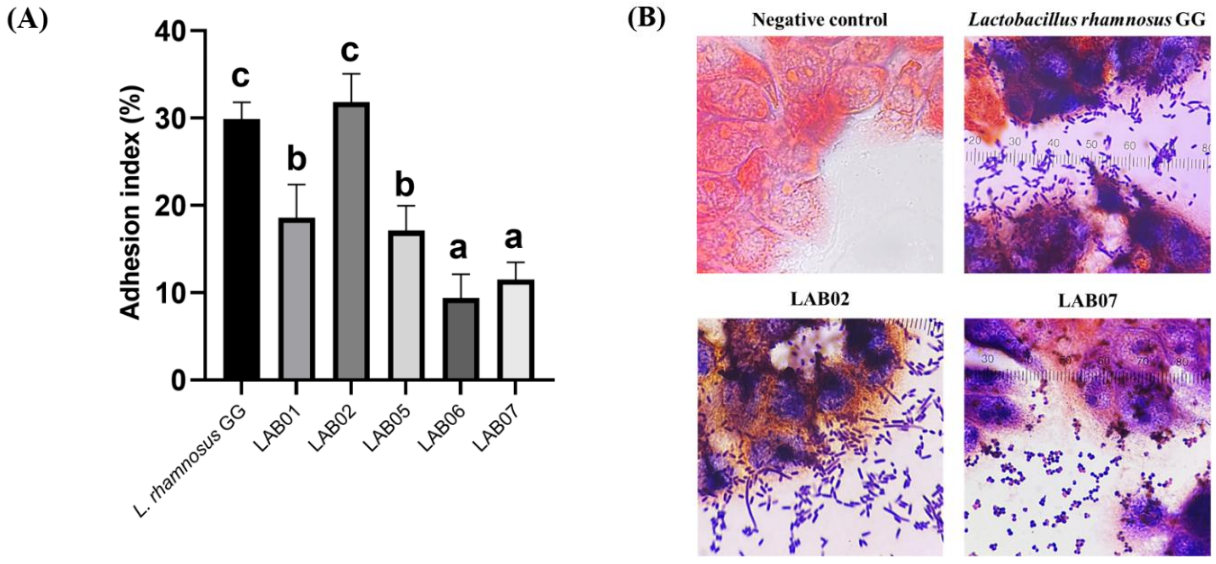

Figure 2 (A) Adhesion index (%) of lactic acid bacteria including LAB01, LAB02, LAB05, LAB06 and LAB07, and (B) photograph examples showing adhesion of the bacteria to human intestinal HT-29 cells. The probiotic strain *Lactobacillus rhamnosus* GG was used as positive control in the tests. Each value represents the mean ± S.D. of triplicate determinations. Statistical significances were determined using one-way ANOVA in SPSS version 25.0 and the graph was generated using GraphPad Prism version 9 (GraphPad Software, USA). The different superscripts indicate significant difference (*p-value* < 0.05).

Toxicity of lactic acid bacteria on human intestinal HT-29 cells

Toxicity of lactic acid bacteria on human intestinal cells (HT-29) was evaluated in this step. As shown in **Figure 3**, cell viability (%) of HT-29 cells after being exposed to each of lactic acid bacteria was in ranging of 24.75% - 87.73%. According to ISO 10993–5 (2009) [42], cell viability percentage above 80% are considered as non-cytotoxicity; 80% - 60% weak cytotoxicity; 60% - 40% moderate cytotoxicity; and below 40% strong cytotoxicity. In the present study, the isolates LAB01 and LAB06 showed weak cytotoxicity and LAB05 showed moderate cytotoxicity. The positive control, *B. cereus* ATCC 11778, was considered as strong toxic to

HT-29 cells as well as the isolate LAB07, meaning these bacteria could inhibit or suppress the growth and change cell morphology of the live cell line. Anyway, only LAB02 showed non-cytotoxicity with the significant highest percentage of HT-29 cell viability (87.73 ± 2.39% cell viability). Similar to the previous study, *Lactobacillus plantarum* has been shown to be non-toxicity to live cells with a percentage of cell viability of more than 90% [43]. In addition, the cell free supernatant (CFS) of the probiotic *Enterococcus faecium* and *Lactococcus lactis* has been previously reported as non-toxic toward intestinal cells based on an MTT assay, while the CFS of pathogenic *Clostridium difficile* CD630 showed significant toxicity [44]. Due to its highest performance among all other isolates, the isolate LAB02 was selected for molecular identification and evaluation of immunomodulatory effects in subsequent experiments.

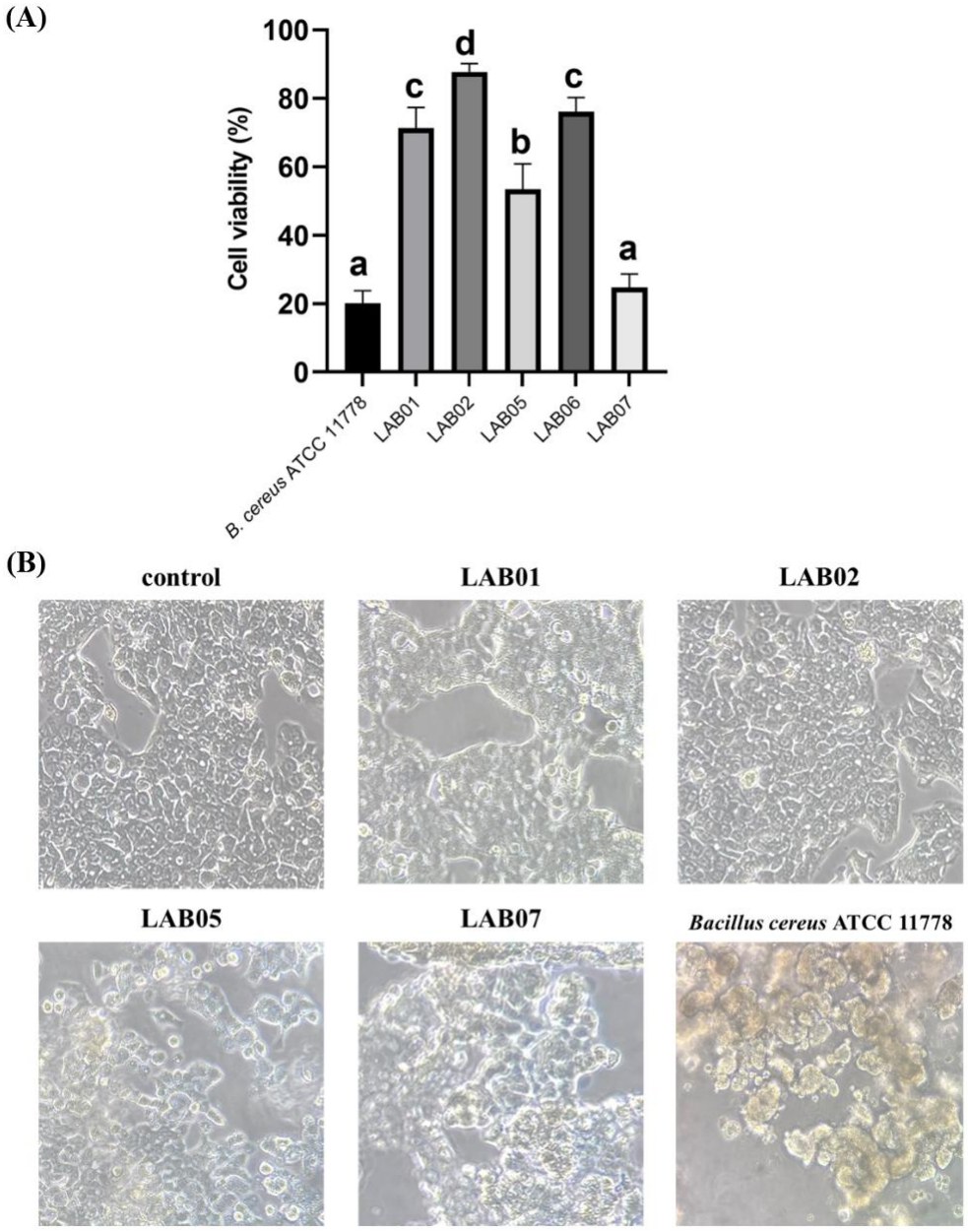

Figure 3 (A) Cell viability (%) of lactic acid bacteria on human intestinal cells (HT-29) and (B) photograph examples showing morphology of the cell line under 400×magnification of inverted microscope. The bacterial strain *Bacillus cereus* ATCC 11778 was used as positive control in the tests. The different superscripts indicate statistically significant difference (*p-value* < 0.05).

Molecular identification based on 16S rRNA gene

The promising isolate LAB02 was identified based on the analysis of 16S rRNA gene sequence and it shared 100% nucleotide identity with *Lactobacillus plantarum* strain KM2 (CP069282.1) and *Lactobacillus plantarum* strain LP01 (CP170480.1). The phylogenetic tree is shown in **Figure 4** and *Bacillus subtilis* strain NCIB 3610 was used as an outgroup in the analyses. According to previous findings, *Lactobacillus plantarum* YC-5 has been reported as a novel probiotic candidate isolated from fermented food [45]; and *Lactobacillus plantarum* GCC_19M1 from fermented raw milk has strong probiotic potential, safety to use and antagonistic effect on pathogenic bacteria [46]. It has been of interest to study the anti-pathogenic activity of lactic acid bacteria and the *Lactobacillus* is one of the genera harboring this important ability. For example, *Lactobacillus fermentum* from Malaysian local pickled *Spondias dulcis* has antibacterial activity against foodborne bacterial pathogens including *B. cereus* ATCC 10876, *E. faecalis* ATCC 19433, *L. innocua* ATCC 33090, *S. aureus* ATCC 25923, *E. coli* ATCC 48888, *S.* Typhimurium ATCC 14028, *Cronobacter muytjensii* ATCC 51329, and *Vibrio parahaemolyticus* NCTC 10885 [47].

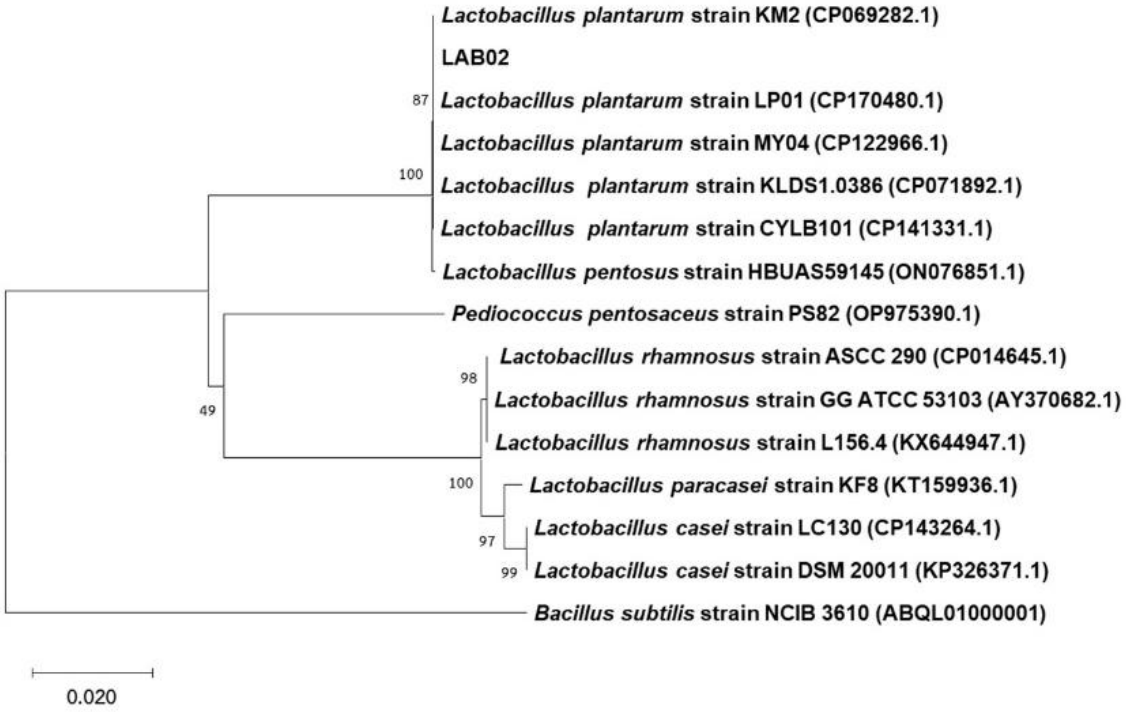

Figure 4 Phylogenetic tree of 16S rRNA gene sequences of *Lactobacillus plantarum* LAB02 grouping within the genera *Lactobacillus* and *Pediococcus*. The tree was constructed by MEGA X software after multiple sequence alignments. Numbers at the nodes indicate the level of bootstrap (%) based on a Maximum-likelihood method analysis of 1,000 resampled datasets.

Immunomodulatory effects of lactic acid bacteria

The present step focused on investigating immune response of the lipopolysaccharide (LPS)-induced HT-29 cells after post-treatment with the promising bacteria *L. plantarum* LAB02 and its postbiotics (cell free supernatant; CFS). *L. rhamnosus* GG was used as reference probiotic strain. The quantitative PCR was performed to quantify the mRNA expressions of the target genes encoding pro- and anti-inflammatory cytokines and the results are shown in **Figure 5**. After post-treatment, gene expressions of pro-inflammatory cytokines like *IL-1* (*IL-1α* and *β*), *IL-6*, *IL-8*, *TNF-α* and *NF-κβ* were significantly down-regulated (*p-value* < 0.05) by the test bacterial species (*L. plantarum* LAB02 and *L. rhamnosus* GG) and their cell free supernatants.

However, genes encoding anti-inflammatory cytokines like *IL-4*, *IL-10*, *TGF-ß1*, *TGF-ß2* and *TGF-ß3* were up-regulated their mRNA expression in the post-treatments of both bacterial species. Anyway, the gene expressions of all anti-inflammatory cytokines, except *IL-4*, in the post-treatment of *L. plantarum* LAB02 were up-regulated more than that of *L. rhamnosus* GG; and among them, the maximum up-regulated expression was found in the *IL-10* gene (24.36-fold change of mRNA expression). On the other hand, all cell free supernatants, except the supernatant of *L. rhamnosus* GG, presented significant low regulation of gene expression (*p-value* < 0.05) of the anti-inflammatory cytokines. Many previous studies reported that some lactic acid bacteria including *L. plantarum, L. rhamnosus, L. paracasei, Lactobacillus casei* and *Weissella confusa* can present a significant modulation of immune response [48-50]. Normally, a little amount of lipopolysaccharide (LPS) of Gram-negative bacteria in the human gastrointestinal tract is considered harmless [51]. However, the higher concentration of LPS leads to fever, septicemia, and gut leakage. Moreover, the LPS has stimulated inflammation response in intestinal cells [52]. In the present study, the immune response stimulated by LPS of *E. coli* could stimulate inflammation and promote the gene expressions of pro-inflammatory cytokines including *IL-1*, *IL-6*, *IL-8*, *TNF-α* and *NF-κß* in HT-29 cells, however the post-treatment of *L. plantarum* LAB02 could down-regulate the mRNA expression levels to resolve these effects. Similarly, Kook *et al*. [53] reported that *L. fermentum*, *L. plantarum*, *L. paracasei* and *Streptococcus thermophilus* can significantly down-regulate the secretion of pro-inflammatory cytokines including *IL-6* and *TNF-α* after stimulation by LPS. Sun *et al.* [54] previously reported that *Lactobacillus gasseri* JM1 causes down-regulation of genes encoding pro-inflammatory cytokines including *IL-1ß*, *IL-6*, *IL-8* and *TNF-α* and up-regulation of genes encoding anti-inflammatory cytokines including *IL-4*, *IL-10*, *TGF-ß3* and *IFN-γ*. To relieve the inflammatory effects, the potential lactic acid bacteria and their metabolites in supernatants significantly up-regulate the secretion of anti-inflammatory cytokines. In our present study, the mRNA expression levels for production of anti-inflammatory cytokines *like IL-4*, *IL-10*, *TGF-ß1*, *TGF-ß2* and *TGF-ß3* in HT-29 cells were enhanced by *L. plantarum* LAB02 and its cell free supernatant. Similarly, Bahrami *et al.* [55] reported that *Lactobacillus* sp. and *Bifidobacterium* sp. can increase the mRNA expression of anti-inflammatory cytokine genes in human intestinal cells (Caco-2 and HT-29 cells) in ranging of 1.8 to 15.2-fold change of mRNA expression.

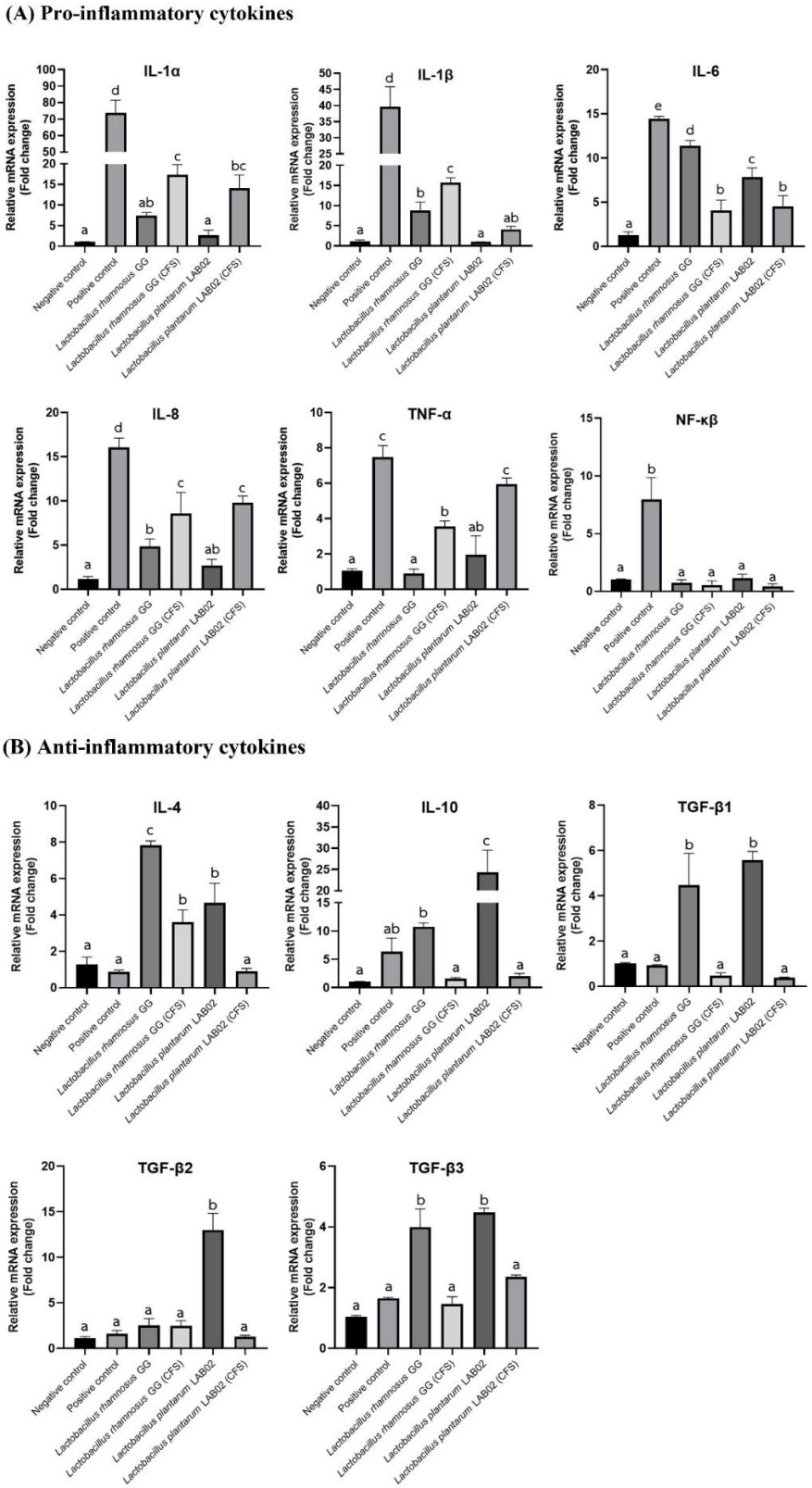

Figure 5 Relative mRNA expression of genes encoding pro-inflammatory (A) and anti-inflammatory (B) cytokines in LPS-induced HT-29 cells, quantified using quantitative PCR (qPCR). Cell suspension of *Lactobacillus plantarum* LAB02 and its cell free supernatant (CFS) were added to the induced HT-29 cells. *Lactobacillus rhamnosus* GG was used as reference probiotic strain. Negative control is normal HT-29 cells (non LPS-induced) and positive control is the LPS-induced HT-29 cells. The relative expression of each target gene was estimated by calculating $2^{-\Delta\Delta Ct}$ compared with the housekeeping GAPDH. The different superscripts indicate statistically significant difference (*p-value* < 0.05).

Conclusions

In summary, the potential lactic acid bacteria, *Lactobacillus plantarum* LAB02, newly isolated from guts of the termite *Termes* sp. has a high inhibitory activity against important enteropathogenic bacteria and good adhesion ability and non-toxicity to human intestinal HT-29 cells. Moreover, the strain LAB02 displays potent immunomodulatory effects to the intestinal cells due to regulating higher gene expression of multiple anti-inflammatory cytokines and suppressing gene expression levels of pro-inflammatory cytokines. The anti-inflammatory activity regulated by the bacterial isolate and its postbiotics are explored as valuable and remarkable properties. Overall, high *in vitro* abilities of LAB02 benefit for preventing enteropathogenic infection and reducing inflammatory effects in human intestines. The bacterial agents obtained from this study are useful for future applications in terms of food microbiology and biotechnology.

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Declaration of Generative AI in Scientific Writing

The authors used the OpenAI tool, ChatGPT, in the preparation of manuscript for grammar correction. No content generation or data interpretation was performed by AI. The authors take full responsibility for the content and conclusions of this work.

CRediT Author Statement

Kittipong Chanworawit: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Validation, Visualization, Software, and Writing –original draft.

Pinsurang Deevong: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Validation, Resources, Supervision, Funding acquisition, and Writing –review & editing.

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