

Antagonistic Characteristics Against Food-borne Pathogenic Bacteria of Lactic Acid Bacteria and Bifidobacteria Isolated from Feces of Healthy Thai Infants

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Background: Food-borne pathogens are among the most significant problems in maintaining the health of people. Many probiotics have been widely reported to alleviate and protect against gastrointestinal infections through antibacterial secretion. However, the majority of them cannot always play antagonistic roles under gut conditions. Probiotic bacteria of human origin must possess other protective mechanisms to survive, out-compete intestinal flora and to successfully establish in their new host at a significant level.

Objectives: Probiotic characteristics of Lactic Acid Bacteria (LAB) and bifidobacteria isolated from the feces of Thai infants were primarily investigated in terms of gastric acid and bile resistances, antibacterial activity and mucin adhesion ability. Antagonistic interaction through secretion of antibacterial compounds and competitive exclusion against food-borne pathogens were also evaluated.

Materials and Methods: Culturable LAB and bifidobacteria were isolated from feces of Thai infants. Their ability to withstand gastric acid and bile were then evaluated. Acid and bile salt tolerant LAB and bifidobacteria were identified. They were then further assessed according to their antagonistic interactions through antibacterial secretion, mucin adhesion and competitive mucin adhesion against various food-borne pathogenic bacteria.

Results: Gastric acid and bile tolerant LAB and bifidobacteria isolated from healthy infant feces were identified and selected according to their antagonistic interaction against various food-borne pathogenic bacteria. These antagonistic probiotics included four strains of *Lactobacillus rhamnosus*, two strains of *L. casei*, five strains of *L. plantarum*, two strains of *Bifidobacterium longum* subsp. *longum* and three strains of *B. bifidum*. All strains of the selected LAB inhibited all pathogenic bacteria tested through antibacterial secretion, while bifidobacteria showed high level of competitive exclusion against the pathogenic bacteria.

Conclusions: These human-derived LAB and bifidobacteria exhibited different mechanisms involved in pathogenic inhibition. Therefore a combination of these probiotic strains could be a great promise and possibility for the development of probiotic products to effectively prevent and control food-borne infection in humans.

Keywords: Probiotics; Antibacterial; Competitive Exclusion; Food-Borne Pathogens; Lactic Acid Bacteria

1. Background

Probiotics are defined as living microorganisms that confer various health benefits and promote or support a beneficial balance of the autochthonous microbial population of the gastrointestinal tract (1). Most probiotic organisms are lactobacilli or bifidobacteria, which are normal inhabitants of the human colon. These human commensal bacteria are thought to be better at adapting to survive the highly stressful environment of gastrointestinal tracts and can adhere to the human epithelial wall of intestines much better than those from other sources. Therefore, they are extensively studied and preferred as commercial probiotics (2). However, a small number of commensal Lactic Acid Bacteria (LAB) and bifidobacteria have been reported to be potential probiotics. Only a few strains of LAB isolated from the feces of breast-fed infants have shown high resistance to gastric juice and bile as well as high adherence ability (3). The strains reported to be beneficial to health so far, have been con-

finned to a few species of lactobacilli and bifidobacteria. Probiotic properties are mostly strain specific.

Although LAB and bifidobacteria are well recognized as antibacterial agent producers (4-6), the majority cannot always play antagonistic roles under gut conditions. Protective mechanisms against pathogens in the gut are brought about through competition of binding sites and nutrients, immune modulation and antibacterial secretion (7). The adhesion property of probiotics ensures their ability to colonize in the host gut that prolongs enough transit time to prevent any pathogens from adhering to mucin and intestinal epithelial cells (8). Therefore, potential probiotics that produce antibacterial agents must be able to adhere and colonize mucosal surfaces, apart from having the ability to survive extreme gastric acid and bile present in the stomach and intestinal conditions, respectively.

Antagonistic interaction with various food-borne pathogenic bacteria was previously reported to be spe-

cies and strain specific (9). Borriello et al. (10) suggested that the ideal bacteria applied for the development of novel probiotics should be isolated from the human fecal microflora of healthy volunteers. For ecological reasons, the bacteria selected for probiotic use in humans must be of human origin (11). These bacteria may have a better chance to out-compete intestinal flora to become successfully established in their new host at a significant level. Several probiotic bacteria that are now being exploited commercially are mostly isolated from humans. For example, *Lactobacillus rhamnosus* GG (ATCC 53103) (12) was originated from human feces. *Lactobacillus acidophilus* LA-1 (12), and *Lactobacillus reuteri* strains mm4-1a (ATCCPTA 6475) and fj1 (ATCCPTA 5289) were isolated from human breast milk and human oral cavity (13), respectively.

2. Objectives

The aim of this research was to study the antagonistic interactions of LAB and bifidobacteria isolated from Thai healthy infant feces against various food-borne pathogens through investigating antibacterial secretion, mucin adhesion and competitive mucin adhesion.

3. Materials and Methods

3.1. Ethics Statement

This study was reviewed and approved by the Ethics Committee of the Faculty of Medicine, Prince of Songkla University (EC Number: 51/400 - 020). All infants' parents provided verbal informed consent on behalf of the participating infant. The verbal consent was granted by the Ethics Committee because this research presented negligible risks to the participants. Besides, they were not identifiable from the data collected. To obtain written consent or record, the isolation process could be delayed leading to a long period of oxygen exposure of fecal samples. This would seriously cause the destruction of the oxygen-sensitive bacteria indigenous to the human colon.

3.2. Isolation of Lactic Acid Bacteria and Bifidobacteria From Infant Feces

Lactic Acid Bacteria and bifidobacteria were isolated from stool samples collected from twenty-five Thai healthy infants who had no history of antibiotic treatment. Ten-fold dilution of fecal samples was then serially performed. The appropriate dilution was then plated with molten modified de Man Rogosa and Sharpe agar (MRS; Himedia, India) containing 0.05 % L-cysteine (Sig-

ma, Japan) and 0.004% bromocresol purple (Labchem, Australia). These plates were incubated at 37°C for 48 - 72 hours in an anaerobic jar with a disposable BBL gas pack (USA). Presumptive colonies of LAB and bifidobacteria were picked. Colonies exhibiting the presumptive characteristics of LAB (rod or short rod or cocci) and bifidobacteria (bifid), which were Gram-positive and catalase negative, were subcultured in L-cysteine containing de Man Rogosa and Sharpe (MRS) broth. They were then stored with 30% glycerol in liquid nitrogen (-196°C).

3.3. Bacterial Strains and Cultivation Conditions

Lactic acid bacteria were cultivated routinely in MRS broth (Himedia, India) at 37°C for 24 - 48 hours. Bifidobacteria were anaerobically cultivated in modified MRS broth containing 0.05% L-cysteine and 1 mg l⁻¹ resazurin (Sigma, US) in an injection vial. *Escherichia coli* TISTR 780 and *Staphylococcus aureus* TISTR 1466 were obtained from the Microbiological Resources Centre (MIRCEN), Thailand. All patient isolates of *Shigella sonnei*, *S. flexneri*, *Salmonella enterica* subsp. enteric serovar typhimurium SA2093 and *S. Paratyphi* A were obtained directly from Microbiological Laboratory of Songklanakarind Hospital, Prince of Songkla University, Hat Yai (Songkhla, Thailand). These pathogens were cultivated in Muller Hinton broth (MHB; Himedia, India) at 37°C for 24 hours.

3.4. Survival of Probiotic Bacteria in the Upper Gastrointestinal Conditions

3.4.1. Tolerance to Bile

Lactic acid bacteria (313 strains) and bifidobacteria (17 strains) were cultivated in MRS broth as mentioned above. A bile tolerance assay was performed in accordance with the method of Vinderola and Reinheimer (14) with some modifications. Overnight LAB or bifidobacterial culture was serially diluted in a ten-fold manner. One milliliter of the appropriate dilution was plated onto a sterilized plate, onto which MRS agar with or without 0.3% (w/v) bile salt was poured. The plates were incubated at 37°C for 24 - 72 hours. The ability of the bacteria to grow in 0.3% bile salt was expressed as percentage survival, which was calculated using the equation below:

$$\% \text{Bile survival} = \left(\frac{\text{Log}N_1}{\text{Log}N_0} \right) \times 100$$

$\text{Log}N_1$ = viable count (Log cfu mL⁻¹) from 0.3bil salt containing MRS agar

$\text{Log}N_0$ = viable count (Log cfu mL⁻¹) from MRS agar without bile salt

3.4.2. Tolerance to Simulated Gastric Juice

The overnight cultures of LAB and bifidobacteria showing high tolerance to bile salt were cultivated in MRS broth as mentioned above. One milliliter of the culture broth was centrifuged at 4°C, 10000 rpm for 10 minutes. The cell pellet was washed twice in PBS buffer and then resuspended in 1 mL of gastric juice (2 g l⁻¹ NaCl containing 3.2 g l⁻¹ pepsin (Sigma-Aldrich, USA) adjusted to pH 2.0 with 0.5 M HCl) (15). The mixture was incubated at 37°C for three hours with gentle agitation. Total viable counts were performed before (log N₀) and after (log N₁) acid exposure by the pour plate method with 0.02% bromocresol purple containing MRS agar for LAB and by using the Live/Dead BacLightTH Bacterial Viability Kits (Eugene, Oregon, USA). The percentage of acid survival was calculated according to the following equation:

$$\% \text{acid survival} = \left(\frac{\log N_1}{\log N_0} \right) \times 100$$

3.4.3. Sequential Exposures of Lactic Acid Bacteria and Bifidobacteria to Gastric Acid and Bile

The strains that presented > 80% survival in acidic conditions were selected for sequential exposure to the simulated gastro-intestinal (GI) tract passage test. They were firstly exposed to gastric acid for three hours as described above and were then resuspended in 0.3% bile salt containing 3 mg mL⁻¹ pancreatin. The test tubes were then incubated at 37°C for six hours with gentle agitation. After that, the aliquot was then serially diluted and the appropriate samples were plated in MRS agar or Live/Dead BacLightTH Bacterial Viability kits in order to count the survived cells (N₁) of LAB or bifidobacteria, respectively. The results were expressed as percentage survival by comparison with the total viable count before exposure to the gastrointestinal transit conditions (N₀). The survival percentage was calculated according to the equation displayed above. The experiments were carried out in triplicates.

3.5. Analysis of 16S rDNA Sequences for Identification of Lactic Acid Bacteria and Bifidobacteria

Bacterial DNA was extracted, and the 16S rDNA gene was amplified by Polymerase Chain Reaction (PCR) carried out in a thermal cycler (PerkinElmer 2400, USA). The primers of 27-f and 1492-r were used to generate 1465 bp of PCR product, corresponding to 16S rDNA nucleotide (position 27 - 1492) of LAB. For bifidobacterial DNA, Im26-f and Im3-r were used as the primers for amplification of 1417 bp 16S rDNA fragment (16). The PCR products were purified using the NucleoSpin[®] Extract II (Germany), according to the manufacturer's instructions. The purified PCR products were sequenced by BioDesign Co., Ltd. (Bangkok, Thailand). The nucleotide sequences of the selected strains were compared with sequence data

deposited on GenBank, using the BLAST search program (available at <http://www.ncbi.nlm.nih.gov/>). Sequence alignment between the selected probiotic bacteria and the reference strains was done using the Clustal X program (2.1.12). A phylogenetic tree was constructed using the Mega 5 program.

3.6. Antibacterial Activity Assay

Antibacterial activity against food-borne pathogens was performed using a broth microdilution assay in a 96-well plate according to Kongnum and Hongpattarakere (17). The cell-free culture supernatant of LAB or bifidobacteria was treated as follows: (i) the supernatant without any treatment; (ii) the supernatant neutralized to pH 6.5 - 7.0 using 1 M NaOH; and (iii) the supernatant adjusted to pH 6.5 - 7.0 and treated with 200 unit mL⁻¹ of catalase (Sigma-Aldrich, USA) (18). The activity was expressed as an Arbitrary Unit (AU) per mL estimated from the reciprocal of the highest dilution of the culture supernatant that did not give visible growth of bacteria. Arbitrary Unit mL⁻¹ was calculated according to (1000/100) D, whereas D was the dilution factor.

3.7. Adhesion Assay

The partially purified porcine gastric mucin type III (Sigma, USA) was coated on a sterilized polystyrene microtiter plate well (Maxisorp Nunc, Denmark) before the adhesion assay was performed according to Tallon et al. (19). A minimum of four replicates was used to estimate the adhesion of the tested strain. *Lactobacillus plantarum* 299V, a well-known adhesive strain, was used as a positive control. Briefly, 100 µL of probiotic suspension (10⁷ CFU mL⁻¹) was added to each mucin coated well before incubation at 37°C for one hour. The wells were washed twice with sterile Phosphate Buffered Saline (PBS) (200 µL per well) to remove unbound bacteria and then 200 µL of a 0.05% (v/v) Triton X-100 solution (Sigma-Aldrich, Singapore) was added. The plates were then incubated for two hours at room temperature under gentle agitation to dislodge the bound bacteria (N_{Adhere}). The viable cells before (N_{Initial}) and after (N_{Adhere}) mucin adsorption were enumerated by plating the appropriate dilution in MRS agar. The percentage of adhesion was calculated according to the following equation:

$$\% \text{Adhesion} = \left(\frac{\text{Log cfu } N_{\text{Adhere}}}{\text{Log cfu } N_{\text{Initial}}} \right) \times 100$$

3.8. Determination of Competitive Exclusion of Food-Borne Pathogens

Fifty microliters of pathogen suspension (10⁷ CFU mL⁻¹) was mixed with 50 µL of probiotics (10⁷ CFU mL⁻¹) and added to the mucin coated plates to perform the adhesion

assay described above. The treatment of pathogen alone was performed in parallel (50 μ L of pathogen suspension + 50 μ L of PBS). Viable counts of pathogens before and after adhesion were serially diluted and counted on Mueller-Hinton Agar (MHA) plates. The adhesion percentages of both treatments (with and without probiotic) were calculated, as mentioned above. The probiotic inhibition of the adhesion of pathogen to mucin was expressed as percentage competitive exclusion, which was calculated from the difference between the adhesion percentage of the pathogens in the absence and presence of the probiotic strains.

3.9. Statistical Analysis

Statistical significances of data obtained from the assays for acid and bile resistances, adhesion and competitive exclusion were evaluated using one-way analysis of variance and Duncan's multiple range tests. Significant differences were indicated by $P < 0.05$. The data were analyzed using the SPSS software version 15 for Windows.

4. Results

4.1. Survivals of Lactic Acid Bacteria and Bifidobacteria Through the Upper Part of the Gastrointestinal Tract

Among 313 strains of LAB, one hundred and eighty six

strains showed survival in the presence of bile. Meanwhile, no growth was observed in the other strains. However, only fifty strains showed a high survival rate of $> 80\%$. Eight out of 17 strains of bifidobacteria showed survival in MRS broth containing 0.3% ox-gall bile, whereas the rest completely lost their viability (data not shown). Among the fifty bile tolerant LAB strains and eight strains of bifidobacteria, the majority showed high tolerance to gastric acid. Only two strains of LAB and three strains of bifidobacteria did not survive gastric acid exposure. Therefore, only 11 strains of LAB and five strains of bifidobacteria with high survival rates were chosen to test survival after sequential exposure to the simulated upper part of the human gastrointestinal tract.

These LAB and bifidobacteria showed high viability after exposure to the simulated gastric conditions for three hours with survival rates ranging from 81.52 - 111.11% (Table 1). However, significant survival reduction was observed in LAB strains CIF1A1, CIF1A2 and CIF1A9 after sequential exposure to simulated gastric acid and bile for three and six hours, respectively. Strain CIF1A10 could not survive such sequential exposure. Only seven LAB strains showed survival above 70% with the log reduction ranging between 1.84 and 2.52 log CFU mL^{-1} . The strain CIF17AN8 presented the highest tolerance with 1.84 log reduction. All strains of bifidobacteria displayed higher survival rates, from sequential exposure, compared to LAB. The strain NIF7AN2 was the most tolerant strain with only 0.33 log reduction.

Table 1. Survival of Lactic Acid Bacteria and Bifidobacteria Isolated From Infant Feces After Sequential Incubation Under Conditions of the Simulated Human Gastrointestinal Transit^a

Strains	Sequential Acid (3 h) and Bile (6 h) Exposures				
	Initial (Log CFU mL^{-1} or (Log Cell mL^{-1}) ^b	Acid Exposure (Log CFU mL^{-1} or (Log Cell mL^{-1}) ^b	% Survival ^{b,c}	Bile Exposure (Log CFU mL^{-1} or (Log Cell mL^{-1}) ^b	Survival, % ^{b,c}
Lactic acid bacteria					
<i>L. rhamnosus</i> CIF1A1	8.88 \pm 0.10	8.30 \pm 0.11	93.47 \pm 1.22	1.31 \pm 0.04	14.80 \pm 0.48
<i>L. rhamnosus</i> CIF1A2	8.73 \pm 0.08	8.58 \pm 0.19	98.33 \pm 2.16	1.61 \pm 0.03	18.39 \pm 0.31
<i>L. rhamnosus</i> CIF1A9	8.54 \pm 0.55	6.84 \pm 0.11	80.08 \pm 1.26	3.77 \pm 0.06	44.17 \pm 0.64
<i>L. rhamnosus</i> CIF1A10	8.78 \pm 0.10	8.42 \pm 0.07	95.91 \pm 0.78	-	-
<i>L. casei</i> NIF1A7	9.24 \pm 0.14	7.88 \pm 0.59	85.22 \pm 6.34	6.74 \pm 0.04	72.94 \pm 0.40
<i>L. casei</i> NIF1A12	8.36 \pm 0.51	8.25 \pm 0.03	98.67 \pm 0.39	6.09 \pm 0.20	72.79 \pm 2.43
<i>L. plantarum</i> CIF17A2	9.19 \pm 0.01	8.18 \pm 0.14	89.03 \pm 1.48	6.86 \pm 0.18	74.62 \pm 1.95
<i>L. plantarum</i> CIF17A4	9.17 \pm 0.07	8.52 \pm 0.02	92.89 \pm 0.18	6.90 \pm 0.14	75.22 \pm 1.52
<i>L. plantarum</i> CIF17A5	9.29 \pm 0.08	8.10 \pm 0.03	87.26 \pm 0.30	6.77 \pm 0.39	72.85 \pm 4.21
<i>L. plantarum</i> CIF17AN2	9.17 \pm 0.12	8.31 \pm 0.06	90.54 \pm 0.65	7.14 \pm 0.08	77.87 \pm 0.85
<i>L. plantarum</i> CIF17AN8	9.21 \pm 0.03	8.31 \pm 0.02	90.27 \pm 0.22	7.37 \pm 0.02	80.03 \pm 0.24
Bifidobacteria					
<i>B. longum</i> NIF3AN3	7.68 \pm 0.05	7.29 \pm 0.07	94.98 \pm 0.22	6.79 \pm 0.09	88.45 \pm 2.22
<i>B. longum</i> NIF7AN2	8.01 \pm 0.01	7.45 \pm 0.03	93.04 \pm 01.20	7.68 \pm 0.20	95.84 \pm 2.14
<i>B. bifidum</i> NIF7AN3	7.63 \pm 0.013	6.67 \pm 0.028	87.36 \pm 2.10	7.09 \pm 0.11	92.94 \pm 0.82
<i>B. bifidum</i> NIF7AN5	8.14 \pm 0.03	6.60 \pm 0.05	81.15 \pm 0.82	7.08 \pm 0.09	86.97 \pm 0.88
<i>B. bifidum</i> NIF7AN10	7.70 \pm 0.12	6.33 \pm 0.25	82.27 \pm 0.42	6.89 \pm 0.16	89.52 \pm 0.64

^a Abbreviation: CFU, colony forming unit.

^b Results are mean values of the triple determinations \pm Standard Deviation (SD).

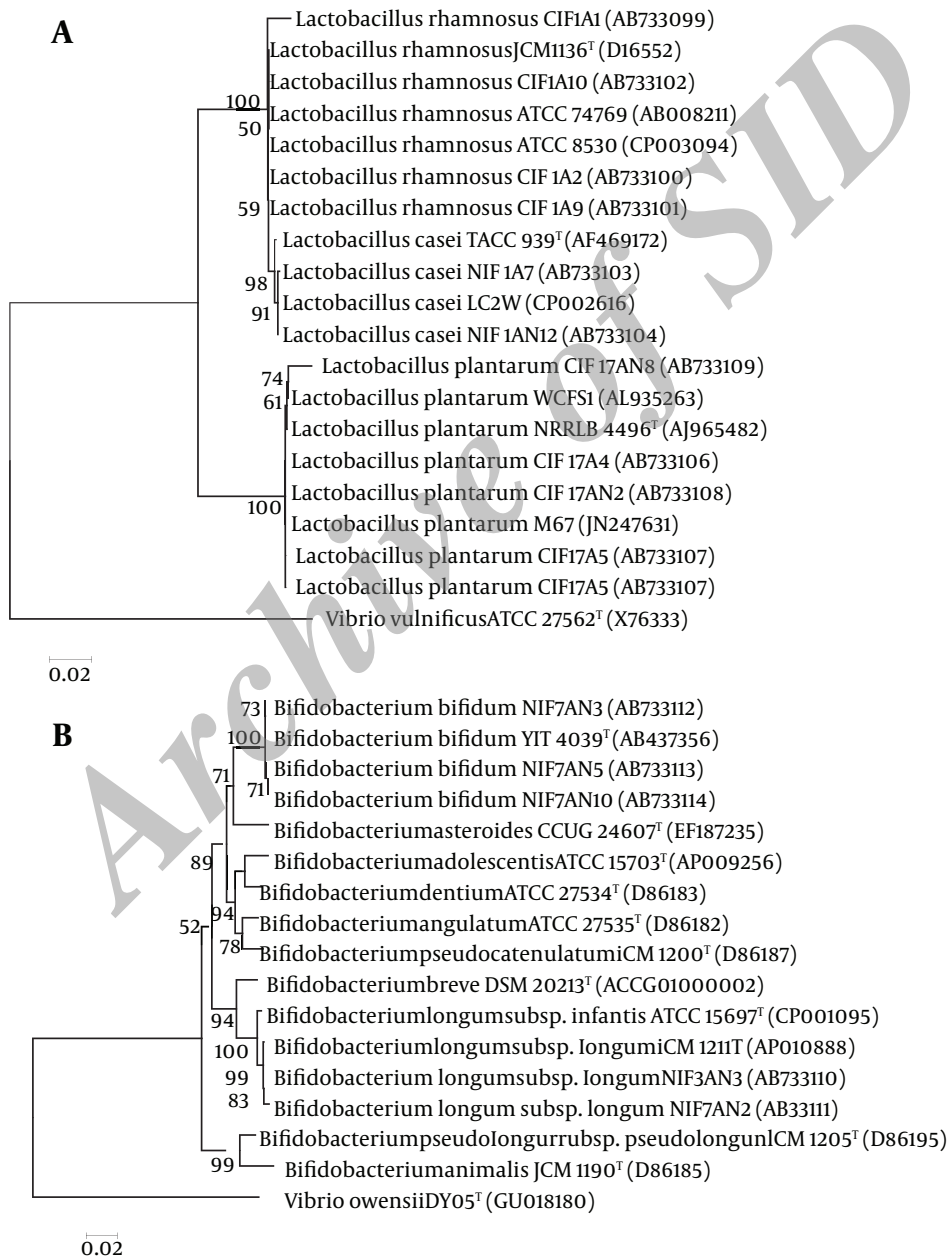
^c Different letters within the same column indicate significant difference ($P < 0.05$).

4.2. Probiotic Identification and Phylogenetic Analysis

According to the 16S rDNA nucleotide sequence analysis, high acid and bile tolerant LAB strains were: *L. rhamnosus* (CIF1A1, CIF1A2, CIF1A9 and CIF1A10); *L. casei* (NIF1A7 and NIF1A12) and *L. plantarum* (CIF17A2, CIF17A4, CIF17A5, CIF17AN2 and CIF17AN8). They exhibited 99% homology with the type strains of *L. rhamnosus* JCM1136T, *L. rhamnosus* ATCC8530, *L. casei* LC2W, and *L. plantarum* WCFS1, respec-

tively. The bifidobacterial strains NIF3AN3 and NIF7AN2 showed 99% homology with the type strain *B. longum* subsp. *longum* JCM1207. The strains NIF7AN3, NIF7AN5 and NIF7AN19 showed 99% homology with type strain *B. bifidum* YIT4039. The phylogenetic trees of the 16S rRNA genes from the selected probiotic LAB and bifidobacteria were constructed using the neighbor-joining method (Figure 1A, 1B). Phylogenetic tree analysis revealed that the bacterial strains within each species had a close relationship with the type strains from a human origin.

Figure 1. Phylogenetic Tree Based on the Nucleotide Sequences of 16S rDNA Nucleotide Sequence (Full Length) of Probiotic (A) Lactic Acid Bacteria and (B) Bifidobacteria Isolated From Healthy Infant Feces Using the Neighbor-Joining Method



Bootstrap values for a total of 1000 replicates were given. *Vibrio vulnificus* ATCC 27562T and *Vibrio owensii* DY05T were used as out groups.

4.3. Antimicrobial Secretions of the Probiotic Lactic Acid Bacteria and Bifidobacteria

All eleven strains of LAB showed antimicrobial activity against all tested pathogens with activity values of 20 - 80 AU mL⁻¹ (Table 2). However, the majority lost such activity against either Gram-positive or Gram-negative food-borne pathogens after neutralization of supernatants (data not shown). This indicated that the antimicrobial activities of these strains were influenced by acid. Contrarily, the neutralized supernatants from *L. plantarum* CIF17AN2 and CIF17AN8 retained inhibitory activity against all tested strains. A loss of inhibitory activity was also observed in the 200-unit mg⁻¹ catalase-treated supernatant. This indicated that production of both acid and H₂O₂ played an important role in pathogenic inhibition of strains CIF17AN2 and CIF17AN8. Bifidobacteria showed a lower bacteriostatic effect (Minimum Inhibitory Concentration (MIC) of 10 - 20 AU mL⁻¹) than LAB against most pathogens (Table 2).

4.4. Adhesion Assay

The tested strains exhibited a much wider range of ad-

hesion ability to mucin (Figure 2). The average adhesion values ranged from 34.31% to 72.54% for the tested strains and 48.49% for the positive control of *L. plantarum* 299V. In LAB strains, *L. casei* NIF1A7 (59.66%) and NIF1AN12 (59.23%) adhered significantly ($P < 0.05$) more to mucin than the positive control. *Lactobacillus plantarum* CIF17A2, CIF17A4, CIF17A5, CIF17AN2 and CIF17AN8 adhered less than the control. On the contrary, all *L. rhamnosus* (CIF1A1, CIF1A2, CIF1A9 and CIF1A10) lacked adhesion ability to porcine mucin. *Bifidobacterium bifidum* NIF7AN3, *B. bifidum* NIF7AN5 and *B. bifidum* NIF7AN10 possessed higher adhesion ability than the positive control *L. plantarum* 299V. *Lactobacillus casei* NIF1A7 showed significantly greater competitive adhesion against *Sal. Paratyphi A* when compared to *L. plantarum* 299V (Table 3). *Lactobacillus plantarum* CIF17AN2 and *L. plantarum* CIF17AN8 exhibited strong competitive adhesion against *S. typhimurium* SA2093. Meanwhile, all bifidobacteria were highly competitive in mucin adhesion against all tested pathogens. The human isolated bifidobacteria in this study showed higher adhesion ability and competitive adhesion against food-borne pathogens than LAB.

Table 2. Antimicrobial Activity of Cell Free Culture Supernatant Derived From Lactic Acid Bacteria and Bifidobacteria Isolated from Healthy Infant Feces Against Food-Borne Pathogens by Broth Microdilution Assay

Test Strains	Inhibition Activity (AU mL ⁻¹)					
	<i>E. coli</i> TISTR 780	<i>S. sonnei</i>	<i>S. flexneri</i>	<i>Sal. Paratyphi A</i>	<i>Sal. Typhimurium</i> SA2093	<i>S. aureus</i> TISTR 1466
Lactic acid bacteria						
<i>L. rhamnosus</i> CIF1A1 (pH4.04)	40	40	40	40	40	40
<i>L. rhamnosus</i> CIF1A2 (pH4.05) CFCS	40	40	40	40	40	40
<i>L. rhamnosus</i> CIF1A9 (pH4.06) CFCS	40	20	20	20	20	40
<i>L. rhamnosus</i> CIF1A10 (pH3.9) CFCS	80	40	40	40	40	80
<i>L. casei</i> NIF1A7 (pH 3.9) CFCS	20	20	20	20	20	20
<i>L. casei</i> NIF1AN12 (pH4.04) CFCS	20	20	20	20	20	20
<i>L. plantarum</i> CIF17A2 (pH3.9) CFCS	20	20	20	20	40	20
<i>L. plantarum</i> CIF17A4 (pH3.9) CFCS	20	20	20	20	40	20
<i>L. plantarum</i> CIF17A5 (pH3.9) CFCS	20	20	20	20	40	20
<i>L. plantarum</i> CIF17AN2 (pH3.9) CFCS	20	40	40	40	40	20
<i>L. plantarum</i> CIF17AN8 (pH3.9) CFCS	20	40	40	40	40	20
Bifidobacteria						
<i>B. longum</i> subsp. <i>longum</i> NIF3AN3 (pH4.25) CFCS	20	20	20	20	80	20
<i>B. longum</i> subsp. <i>longum</i> NIF7AN1 (pH4.41) CFCS	10	10	10	10	80	10
<i>B. bifidum</i> NIF7AN2 (pH4.62) CFCS	10	10	10	10	80	10
<i>B. bifidum</i> NIF7AN5 (pH4.65) CFCS	10	10	10	10	80	10
<i>B. bifidum</i> NIF7AN10 (pH4.25)	20	20	20	20	80	20

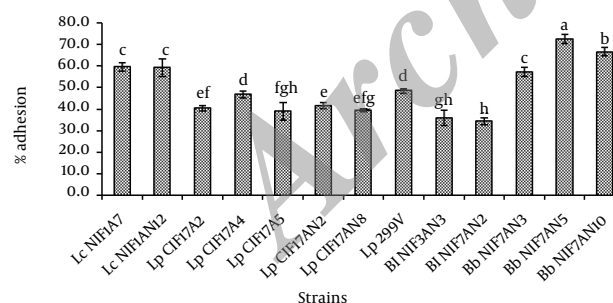
Table 3. Competitive Exclusion of Probiotic Lactic Acid Bacteria and Bifidobacteria Strains Against Food-Borne Pathogens in Mucin Adhesion ^{a,b}

Strains	Competitive Exclusion, %					
	<i>E. coli</i> TISTR 780	<i>S. sonnei</i>	<i>S. flexneri</i>	Sal. Paratyphi A	Sal. Typhimurium SA2093	<i>S. aureus</i> TISTR 1466
Lactic acid bacteria						
<i>L. casei</i> NIF1A7	1.92 ± 2.59	-1.49 ± 3.14	3.91 ± 1.40	13.47 ± 3.08	12.74 ± 5.15	10.93 ± 2.90
<i>L. casei</i> NIF1A12	1.26 ± 4.93	2.09 ± 3.33	0.37 ± 0.00	4.09 ± 2.63	8.20 ± 3.07	5.85 ± 2.52
<i>L. plantarum</i> CIF17A2	-7.98 ± 0.97	6.75 ± 0.00	2.81 ± 2.53	3.17 ± 1.45	5.89 ± 2.44	4.92 ± 1.24
<i>L. plantarum</i> CIF17A4	-4.01 ± 3.19	3.43 ± 2.87	0.37 ± 0.00	6.66 ± 0.15	9.89 ± 3.99	5.70 ± 0.62
<i>L. plantarum</i> CIF17A5	-3.33 ± 3.66	3.43 ± 2.87	2.05 ± 2.91	5.00 ± 3.03	7.96 ± 1.47	5.29 ± 0.68
<i>L. plantarum</i> CIF17AN2	-10.20 ± 3.22	-3.58 ± 1.58	5.41 ± 0.00	6.93 ± 3.16	16.52 ± 1.99	5.78 ± 1.88
<i>L. plantarum</i> CIF17AN8	-10.45 ± 0.95	2.26 ± 4.27	2.81 ± 2.53	6.66 ± 0.15	16.16 ± 2.45	5.30 ± 0.93
<i>L. plantarum</i> 299V	1.88 ± 6.42	2.37 ± 2.58	-1.16 ± 1.85	6.92 ± 3.27	7.54 ± 1.65	6.09 ± 2.79
Bifidobacteria						
<i>B. longum</i> subsp. longum NIF3AN3	43.81 ± 4.42	35.33 ± 1.24	34.31 ± 2.40	18.49 ± 0.12	1.16 ± 0.32	14.37 ± 2.82
<i>B. longum</i> subsp. longum NIF7AN2	40.74 ± 1.87	34.38 ± 1.99	34.80 ± 2.94	17.78 ± 2.01	30.51 ± 8.41	37.96 ± 1.78
<i>B. bifidum</i> NIF7AN3	36.69 ± 0.64	35.39 ± 1.87	38.46 ± 0.60	13.35 ± 0.55	22.86 ± 6.62	23.59 ± 0.49
<i>B. bifidum</i> NIF7AN5	31.71 ± 1.44	33.67 ± 0.37	32.49 ± 2.04	35.33 ± 2.96	2.14 ± 3.44	22.51 ± 0.00
<i>B. bifidum</i> NIF7AN10	41.60 ± 3.44	50.55 ± 2.50	34.06 ± 0.38	38.69 ± 2.14	29.86 ± 10.30	27.79 ± 1.25

^a A higher value represents high inhibition of probiotic strains against adhesion of food borne pathogens, whereas a lower value indicates less inhibitory ability of probiotic strains.

^b Different letters within the same column indicate significant difference ($P < 0.05$).

Figure 2. Adhesion to Porcine Gastric Mucin Type III of *Lactobacillus casei* (Lc), *Lactobacillus plantarum* (Lp), *Bifidobacterium longum* Subsp. longum (Bl) and *Bifidobacterium bifidum* (Bb) Isolated From Healthy Infant Feces and Mucin Adhesive *Lactobacillus plantarum* 299V (Positive Control)



Different letters indicate significant difference ($P < 0.05$).

5. Discussion

In order to survive and colonize in the human gastrointestinal tract, probiotic bacteria should express high resistance to low acid in the stomach and high bile content in the small intestine. To ensure that the bacteria can establish, colonize and persist in the colonic habitat, they must not only tolerate bile exposure but also must be able to multiply in the presence of bile. Therefore,

such ability was evaluated and considered as the first important requirement to select probiotic bacteria in this study. Bile salt tolerance is considered as one of the most essential properties required for all probiotics to survive in the small intestine (9, 20, 21). This study isolated 330 strains of LAB and bifidobacteria, which had previously passed through the environmental extremes of the gastrointestinal tracts of infants. Nevertheless, the bile tolerance of fecal LAB and bifidobacteria isolated from infant's feces varied greatly, and about 43% of the total strains were unable to grow in the presence of 0.3% bile salt. Although many fecal strains lacked the ability to grow in bile-containing media, they could be isolated and recovered from the feces. The results correlated with previous findings regarding the variation of bile tolerance among the same species of enteric *L. acidophilus* (22). The same author also showed that a significant number of lactobacilli increased in calf jejunum when bile resistant strains were fed compared to bile sensitive ones.

Similarly, some strains of LAB and bifidobacteria were not able to survive the extreme acidic conditions of gastric juice *in vitro*, even though they previously survived infant gastrointestinal transit. This observation supports the idea that the gastric transit tolerance assayed *in vitro* may not always provide the absolute prediction about the *in vivo* behavior of probiotics. Nevertheless, the higher resistant strains evaluated *in vitro* would have a better

chance to survive extreme conditions in the stomach in vivo (23). This is because the environmental conditions in the human gut are heterogeneous and dynamic to a high degree. The fluctuations of acid secretion capacity of the stomach, rates of gastric mobility and emptying gut content, and physical properties of the ingested food could provide protective effects against bacteria (20). The presence of food was reported to raise the pH level of the stomach from 1.5 - 2 to pH 3 (24), at which significant survival improvements of various probiotic LAB and bifidobacteria were observed (15). Charteris et al. (21) demonstrated that the presence of milk protein and gastric mucin could significantly protect the majority of the tested probiotic LAB and bifidobacteria in a simulated gastric transit. This explains why acid sensitive strains could be isolated from feces in this study.

Although bifidobacteria were slightly less acid resistant than LAB, they were much more tolerant to the sequential exposure of gastric acid and bile. According to Dunne et al. (25), bifidobacteria isolated from human ileum were less acid and bile resistant than lactobacilli in general. However, commercial *B. bifidum* and *B. longum* were as acid resistant as lactobacilli, yet their bile tolerance was lower (14). In this study, all three strains (NIF7AN3, NIF7AN5 and NIF7AN10) of *B. bifidum* and *B. longum* NIF7AN2 even increased in number after bile exposure. Bifidobacteria are known to possess strategies of response to various environmental stresses including acid and bile through a set of mechanisms (26). These adaptation mechanisms facilitate their success and survival in the human gut and transient colonization in this competitive niche.

Antimicrobial activity is one of the important properties of probiotic bacteria. Several *Lactobacillus* strains from infant feces also produced acid to inhibit the growth of enteropathogens (3-6). Acid production was the major cause of the inhibitory activity of the selected strains in this study. Exceptionally, *L. plantarum* CIF17AN2 and CIF17AN8 retained their antibacterial activity yet loss of this activity was observed with the treatment of catalase enzyme. Most LABs are lactic acid producers, whereas acetic acid is produced mainly by bifidobacteria. De Keersmaecker et al. (27) confirmed that lactic acid accumulated by *L. rhamnosus* GG strongly inhibited *Sal. Typhimurium*. The dissociated forms of these organic acids can permeate the outer membrane of Gram-negative bacteria and lower the local pH to inhibit the growth of acid sensitive bacteria. The acidification of cytoplasm and the collapse of the proton motive force resulted in an inhibition of the transport of nutrients (28).

Bacterial adhesion to host mucin is regarded important in contributing to either the transient or even permanent establishment or colonization of probiotic species in any environmental niche and also enhancement of the ability to stimulate the immune system (29). All strains of *L. casei*, *L. plantarum*, *B. longum* and *B. bifidum* showed good adhesion ability to mucin, while *L. rhamnosus* did not do

so. The results indicate that the adhesion trait was truly a specific characteristic depending on the individual LAB species as previously noted (29, 30). On the contrary, *L. rhamnosus* GG was reported to adhere significantly better than other LAB strains to the intestinal mucin (30). *Lactobacillus rhamnosus* E-800 and *Lactobacillus* GG adhered well to mucin (29). In addition, the mucin adhesive strains competitively excluded *S. flexneri*, *S. typhimurium* SA2093, *S. paratyphi* A and *S. aureus* TISTR from adhesion to porcine mucin. The inhibition of pathogen adhesion to mucin was reported to be able to prevent translocation and subsequently infection (31).

In conclusion, the human isolates LAB and bifidobacteria identified as *L. rhamnosus*, *L. casei*, *L. plantarum*, *B. longum* subsp. *longum* and *B. bifidum* exhibited high acid and bile tolerance and antibacterial activity against food-borne pathogens. These strains suggest the great potentiality of probiotics in controlling infection by food-borne pathogens in humans. The inhibition of pathogens by LAB was mainly caused by the secretion of antibacterial substances while bifidobacteria effectively inhibited pathogens through their high competitive exclusion activity of mucin adhesion. The combination of these multiple strains in the presence of gut microflora challenged with food-borne pathogens should be further investigated.

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References

- Holzappel W, Schillinger U. Introduction to pre- and probiotics. *Food Res Int.* 2002;**35**(2-3):109-16.
- Vizoso Pinto MG, Franz CM, Schillinger U, Holzappel WH. Lactobacillus spp. with in vitro probiotic properties from human faeces and traditional fermented products. *Int J Food Microbiol.* 2006;**109**(3):205-14.
- Wang CY, Lin PR, Ng CC, Shyu YT. Probiotic properties of Lactobacillus strains isolated from the feces of breast-fed infants and Taiwanese pickled cabbage. *Anaerobe.* 2010;**16**(6):578-85.
- Lee YJ, Yu WK, Heo TR. Identification and screening for antimicrobial activity against *Clostridium difficile* of Bifidobacterium and Lactobacillus species isolated from healthy infant faeces. *Int J Antimicrob Agents.* 2003;**21**(4):340-6.
- Lin PP, Hsieh YM, Tsai CC. Antagonistic activity of Lactobacillus acidophilus RY2 isolated from healthy infancy feces on the growth and adhesion characteristics of enteroaggregative *Escherichia coli*. *Anaerobe.* 2009;**15**(4):122-6.

6. Tsai CC, Lin PP, Hsieh YM. Three Lactobacillus strains from healthy infant stool inhibit enterotoxigenic Escherichia coli grown in vitro. *Anaerobe*. 2008;**14**(2):61-7.
7. Collado MC, Jalonen L, Meriluoto J, Salminen S. Protection mechanism of probiotic combination against human pathogens: in vitro adhesion to human intestinal mucus. *Asia Pac J Clin Nutr*. 2006;**15**(4):570-5.
8. Guo Z, Wang J, Yan L, Chen W, Liu X, Zhang H. In vitro comparison of probiotic properties of Lactobacillus casei Zhang, a potential new probiotic, with selected probiotic strains. *Food Sci Technol*. 2009;**42**(10):1640-6.
9. Collado MC, Sanz Y. Induction of acid resistance in Bifidobacterium: a mechanism for improving desirable traits of potentially probiotic strains. *J Appl Microbiol*. 2007;**103**(4):1147-57.
10. Borriello SP, Hammes WP, Holzapfel W, Marteau P, Schrezenmeir J, Vaara M, et al. Safety of probiotics that contain lactobacilli or bifidobacteria. *Clin Infect Dis*. 2003;**36**(6):775-80.
11. Morelli L. In vitro selection of probiotic lactobacilli: a critical appraisal. *Curr Issues Intest Microbiol*. 2000;**1**(2):59-67.
12. Silva M, Jacobus NV, Deneke C, Gorbach SL. Antimicrobial substance from a human Lactobacillus strain. *Antimicrob Agents Chemother*. 1987;**31**(8):1231-3.
13. Oh PL, Benson AK, Peterson DA, Patil PB, Moriyama EN, Roos S, et al. Diversification of the gut symbiont Lactobacillus reuteri as a result of host-driven evolution. *ISME J*. 2010;**4**(3):377-87.
14. Vinderola CG, Reinheimer JA. Lactic acid starter and probiotic bacteria: a comparative "in vitro" study of probiotic characteristics and biological barrier resistance. *Food Res Int*. 2003;**36**(9-10):895-904.
15. Chan ES, Lee PP, Ravindra P, Krishnaiah K, Voo WP. A standard quantitative method to measure acid tolerance of probiotic cells. *Appl Microbiol Biotechnol*. 2010;**86**(1):385-91.
16. Kaufmann P, Pfefferkorn A, Teuber M, Meile L. Identification and quantification of Bifidobacterium species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR. *Appl Environ Microbiol*. 1997;**63**(4):1268-73.
17. Kongnum K, Hongpattarakere T. Effect of Lactobacillus plantarum isolated from digestive tract of wild shrimp on growth and survival of white shrimp (Litopenaeus vannamei) challenged with Vibrio harveyi. *Fish Shellfish Immunol*. 2012;**32**(1):170-7.
18. Brink M, Todorov SD, Martin JH, Senekal M, Dicks LM. The effect of prebiotics on production of antimicrobial compounds, resistance to growth at low pH and in the presence of bile, and adhesion of probiotic cells to intestinal mucus. *J Appl Microbiol*. 2006;**100**(4):813-20.
19. Tallon R, Arias S, Bressollier P, Urdaci MC. Strain- and matrix-dependent adhesion of Lactobacillus plantarum is mediated by proteinaceous bacterial compounds. *J Appl Microbiol*. 2007;**102**(2):442-51.
20. Giannella RA, Broitman SA, Zamcheck N. Gastric acid barrier to ingested microorganisms in man: studies in vivo and in vitro. *Gut*. 1972;**13**(4):251-6.
21. Charteris WP, Kelly PM, Morelli L, Collins JK. Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. *J Appl Microbiol*. 1998;**84**(5):759-68.
22. Gilliland SE, Staley TE, Bush LJ. Importance of bile tolerance of Lactobacillus acidophilus used as a dietary adjunct. *J Dairy Sci*. 1984;**67**(12):3045-51.
23. Tennant SM, Hartland EL, Phumoonna T, Lyras D, Rood JI, Robins-Browne RM, et al. Influence of gastric acid on susceptibility to infection with ingested bacterial pathogens. *Infect Immun*. 2008;**76**(2):639-45.
24. Erkkila S, Petaja E. Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. *Meat Sci*. 2000;**55**(3):297-300.
25. Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, et al. In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. *Am J Clin Nutr*. 2001;**73**(2 Suppl):386S-92S.
26. Ruiz L, Ruas-Madiedo P, Gueimonde M, de Los Reyes-Gavilan CG, Margolles A, Sanchez B. How do bifidobacteria counteract environmental challenges? Mechanisms involved and physiological consequences. *Genes Nutr*. 2011;**6**(3):307-18.
27. De Keersmaecker SC, Verhoeven TL, Desair J, Marchal K, Vanderleyden J, Nagy I. Strong antimicrobial activity of Lactobacillus rhamnosus GG against Salmonella typhimurium is due to accumulation of lactic acid. *FEMS Microbiol Lett*. 2006;**259**(1):89-96.
28. Alakomi HL, Skytta E, Saarela M, Mattila-Sandholm T, Latva-Kala K, Helander IM. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Appl Environ Microbiol*. 2000;**66**(5):2001-5.
29. Ouwehand AC, Tuomola EM, Tolkkio S, Salminen S. Assessment of adhesion properties of novel probiotic strains to human intestinal mucus. *Int J Food Microbiol*. 2001;**64**(1-2):119-26.
30. Rinkinen M, Westermarck E, Salminen S, Ouwehand AC. Absence of host specificity for in vitro adhesion of probiotic lactic acid bacteria to intestinal mucus. *Vet Microbiol*. 2003;**97**(1-2):55-61.
31. Tuomola EM, Ouwehand AC, Salminen SJ. The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. *FEMS Immunol Med Microbiol*. 1999;**26**(2):137-42.