Antibacterial Activity and Probiotic Potential of *Lactobacillus plantarum* HKN01: A New Insight into the Morphological Changes of Antibacterial Compound-Treated *Escherichia coli* by Electron Microscopy

Sharafi, Hakimeh¹, Hadi Maleki¹²†, Gholamreza Ahmadian¹, Hossein Shahbani Zahiri¹, Neda Sajedinejad¹, Behzad Houshmand¹³, Hojatollah Vahi⁴, and Kambiz Akbari Noghabi¹*

¹Department of Molecular Genetics, National Institute of Genetic Engineering and Biotechnology (NIGEB), P.O. Box 14155-6343, Tehran, Iran
²Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
³Periodontics Department, Dental School, Shahid Beheshti University of Medical Sciences, Daneshju Blvd, Tehran, Iran
⁴Facility for Electron Microscopy Research, McGill University, 3640 Street, Montreal, Canada

Received: August 2, 2012 / Revised: September 16, 2012 / Accepted: September 24, 2012

Among several bacteria examined, an antibacterial-producing *Lactobacillus* strain with probiotic characteristics was selected and identified based on 16S rRNA gene sequencing. Subsequent purification and mode of action of the antibacterial compounds on target cells including *E. coli* were investigated. Maximum production of the antibacterial compound was recorded at 18 h incubation at 30°C. Interestingly, antibacterial activity remained unchanged after heating at 121°C for 45 min, 24 h storage in temperature range of 70°C to room temperature, and 15 min exposure to UV light, and it was stable in the pH of range 2–10. The active compounds were inactivated by proteolytic enzymes, indicating their proteinaceous nature, and, therefore, referred to as bacteriocin-like inhibitory substances. Isolation and partial purification of the effective agent was done by performing ammonium sulfate precipitation and gel filtration chromatography. The molecular mass of the GFC-purified active compound (~3 kDa) was determined by Tris-Tricine SDS-PAGE. To predict the mechanisms of action, transmission electron microscopy (TEM) analysis of ultrathin sections of *E. coli* before and after antibacterial treatment was carried out. TEM analysis of antibacterial compounds-treated *E. coli* demonstrated that the completely altered bacteria appear much darker compared with the less altered bacteria, suggesting a change in the cytoplasmic composition. There were also some membrane-bound convoluted structures visible within the completely altered bacteria, which could be attributed to the response of the *E. coli* to the treatment with the antibacterial compound. According to the in vivo experiments oral administration of *L. plantarum* HKN01 resulted in recovery of infected BALB/c mice with *Salmonella enterica* ser. Typhimurium.

**Key words:** *Lactobacillus plantarum* HKN01, *Salmonella enterica* ser. Typhimurium, Transmission electron microscopy, Tris-Tricine SDS-PAGE

Lactic acid bacteria (LAB) have been widely used as starter cultures in the food industry for fermentation. *Lactobacillus* species play a vital role in foodstuffs, because of their fermentative ability and their health and nutritional benefits [19]. These bacteria are able to produce many effective antibacterial agents such as organic acids, hydrogen peroxides and bacteriocins during fermentation [8, 11]. *Lactobacillus* species among LAB can produce a variety of antimicrobial compounds with different inhibitory spectra, mode of actions, and biochemical characteristics. Bacteriocins are one of the well-known antimicrobial compounds isolated from LAB. They are proteinaceous antibacterial compounds and exhibit bactericidal activity against species closely related to the producer strains [10, 48]. Several types of bacteriocins from food-associated LAB have been identified and characterized, of which nisin, diplococcin, acidophilin, bulgaricin, helveticins, lactacins, lactolin, and plantaricins are the important ones [2, 31]. Several antimicrobial peptides have been recognized in *L. plantarum* strains isolated from milk and cheese [20, 38]. Bacteriocins are heterogeneous and they are classified largely based on differences in their molecular weights [25].

---

*Corresponding author*
Phone: +98-21-44580310; Fax: +98-21-44580394; E-mail: Akbari@nigeb.ac.ir
†These authors contributed equally to this work.
LAB also have beneficial effects to the consumers in different ways. Probiotic lactobacilli are known to confer an array of health promoting activities on their host after either parenteral or oral administration [34]. Some of their beneficial effects include prevention of intestinal infection [6], anticarcinogenic activity [16], control of serum cholesterol, immunity promotion [1], and growth enhancement of animals [7]. The mechanism by which these probiotics affect their host and bring about improvement in the gut barrier can be due to competition for adhesion site, production of inhibitory compounds, and rebalancing of disturbed gastrointestinal microbial composition and metabolism [13]. According to the increasing demand for limiting the use of chemical additives in food, natural antimicrobial compounds may have a considerable role in food safety issues [13]. This investigation was conducted to evaluate the potential of indigenous lactobacilli isolated from Iranian traditional dairy products in generating new effective antibacterial agents. Detailed analysis of transmission electron microscopy (TEM) of ultrathin section of E. coli before and after antibacterial treatment was carried out. Some aspects of antibacterial properties of the selected strain for controlling of pathogenic bacterium Sal monella enterica ser. Typhimurium (hereinafter written as Sal monella Typhimurium) was studied in the laboratory mice model as well.

### Materials and Methods

#### Sample Preparation and Isolation of Assumed Lactobacilli

One gram of dairy samples was dissolved in sterile 0.9% normal saline and 10-fold serial dilutions were primed. A volume of 0.1 ml of appropriate dilutions was spread-plated in triplicate on MRS agar (Merck, Germany) for isolating lactobacilli. All plates were incubated at 30°C for 48–72 h under microaerophilic conditions. Colonies of catalase-negative and Gram-positive rods were regarded to be lactobacilli. These colonies were subcultured into MRS broth and 15% (v/v) glycerol stocks were provided.

#### Screening for Antibacterial-Producing Lactobacilli and Assays

Detection of isolates with antibacterial activity and evaluation of their effectiveness were performed by well-diffusion assay [5]. Indicator organism was sprayed on Muller Hinton agar plates and 100 µl of the 2-fold serially diluted cell-free culture supernatant was transferred into the formed wells in the agar plates, while MRS medium served as a control. The amount of antibacterial compound production was calculated as arbitrary units. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition of the indicator strain [26].

#### Indicator Strains and Culture Conditions

*Escherichia coli* (PTCC 1338) was used as the indicator organism during screening tests. Once the compound was isolated and purified, its antimicrobial spectrum was studied against the other pathogenic microorganisms listed in Table 1. *Lactobacillus plantarum* HKN01 was spread on De Man Rogosa and Sharpe broth (MRS; Merck, Germany) at 30°C. *Escherichia coli* (PTCC 1338) and *Sal monella Typhimurium* (ATCC 13311) were propagated in LB broth (Merck, Germany) at 37°C and the rest of the tested bacteria including *Staphylococcus aureus* (PTCC 1112), *Bacillus subtilis* (PTCC 1715), *Pseudomonas aeruginosa* (PTCC 1310), and *Bacillus cereus* (PTCC 1015) were cultivated in nutrient broth (Merck, Germany) at 37°C.

#### Preparation of Culture Supernatant of Lactobacilli

Cultures of lactobacilli were grown aerobically in MRS broth at 30°C for 48 h. After incubation, the bacterial cells were removed by centrifugation at 10,000 × g for 10 min at 4°C. The supernatant was adjusted to pH 7 with 1 mol/l NaOH to eliminate the inhibitory effect of organic acids. The cell-free supernatant was filter sterilized through a 0.22 µm filter and stored at −70°C for subsequent analysis.

#### Identification of the Antibacterial-Producing Bacterial Isolate

The selected isolate as a potential antibacterial producer was preliminarily identified on the basis of its cultural, morphological, physiological, and biochemical characteristics [41], followed by partial 16S rRNA gene sequence analysis conducted at the American Culture Collection of Microorganisms (ATCC) [27]. The resulting sequence was aligned with available, almost complete sequences of type strains of genus Lactobacillus and then with corresponding sequences of representative Lactobacillus species. In each case, the reference sequence was retrieved from the GenBank databases. The phylogenetic tree (diagram) was illustrated using Version 4 of MEGA (Molecular Evolutionary Genetics Analysis), corresponding that the bacterial strain is closely related to Lactobacillus plantarum with similarity matrix of 100% (Fig. 2).

#### Probiotic Properties of Lactobacillus plantarum HKN01

**Preliminary selection by PCR amplification.** In order to assess the probiotic properties of the isolate, *L. plantarum* HKN01 was tested by PCR to search for the presence of *bsh* and *msa* genes encoding for the bile salt hydrolase (BSH) and the mannose-specific adhesion (MSA), respectively. The primers had the following sequences:

- 5′-CGTATCCAAGTGCTCATGGTTTAA-3′ (bsh for, nucleotide position 150568 to 150593 of the bsh gene).
- 5′-ATGTGTIACTGCC

**Table 1. Potency and inhibitory spectrum of the cell-free culture supernatant of Lactobacillus plantarum HKN01.**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Potency of antibacterial compound AU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> (PTCC 1112)</td>
<td>640</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> (PTCC 1715)</td>
<td>320</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> (PTCC 1015)</td>
<td>320</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (PTCC 1338)</td>
<td>1,280</td>
</tr>
<tr>
<td><em>Salmonella Typhimurium</em> (ATCC 13311)</td>
<td>1,280</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (PTCC 1290)</td>
<td>1,280</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (PTCC 1310)</td>
<td>320</td>
</tr>
</tbody>
</table>

*Arbitrary unit/ml (One Arbitrary unit was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition of the indicator strain).*

---

**Note:**

- M (Material).
- A (Method).
- Table 1: Potency and inhibitory spectrum of the cell-free culture supernatant of Lactobacillus plantarum HKN01.
- The mechanism by which these probiotics affect their host and bring about improvement in the gut barrier can be due to competition for adhesion site, production of inhibitory compounds, and rebalancing of disturbed gastrointestinal microbial composition and metabolism [13]. According to the increasing demand for limiting the use of chemical additives in food, natural antimicrobial compounds may have a considerable role in food safety issues [13]. This investigation was conducted to evaluate the potential of indigenous lactobacilli isolated from Iranian traditional dairy products in generating new effective antibacterial agents. Detailed analysis of transmission electron microscopy (TEM) of ultrathin section of *E. coli* before and after antibacterial treatment was carried out. Some aspects of antibacterial properties of the selected strain for controlling of pathogenic bacterium *Salmonella enterica* ser. Typhimurium (hereinafter written as *Salmonella Typhimurium*) was studied in the laboratory mice model as well.