Evaluation of Antimicrobial Activity of Three Lactobacillus spp. against Antibiotic Resistance Salmonella typhimurium

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Abstract

Lactobacillus spp., as probiotics bacteria, are being increasingly studied for their inhibitory activity against pathogenic bacteria. There is some evidence that they are not effective against gram-negative bacteria. The objective of this study was to investigate the antagonistic activity of Lactobacillus plantarum PTCC 1058, Lactobacillus delbrueckii sub.sp bulgaricus PTCC 1737 and Lactobacillus leichmannii PTCC 1057 against antibiotic resistance Salmonella typhimurium. The Salmonella typhimurium strains were identified by biochemical tests, antisera and multiplex PCR assays. Subsequently, the anti-bio gram against ten Salmonella typhimurium strains was investigated using six commonly-used antibiotics. The antibacterial effect of Lactobacillus spp. was evaluated by the means of Agar disk-diffusion and Agar well-diffusion assays. This in vitro study implied that none of the Lactobacilli had antibacterial
effect against *Salmonella typhimurium* strains, so they are not suggested to be used as alternative treatment instead of administration of antibiotics to control *S. typhimurium*.

**Keywords:** *Salmonella typhimurium*, Lactobacillus, Antibiotic, Antibacterial Activity, Multiplex PCR

**Introduction**

Salmonellosis is one of the most common and widely spread foodborne illnesses. Consumption of antimicrobial agents for treatment of such diseases in human and animal results in appearance of Multidrug-resistant (MDR) strains of *Salmonella*. Consequently, these microorganisms lead animal and human health care to considerable therapeutic problems and contribute in the transfer of MDR *Salmonella* spp. between humans and animals [1].

Species of Lactobacillus have been the most common microorganism studied for their probiotic properties to control *Salmonella* derived diseases as an alternative solution [2,3]. The antimicrobial activity of these probiotic microorganisms arises from several mechanisms. The suggested mechanisms for pathogen inhibition include contending for nutrients, production of antimicrobial elements such as bacteriocins, bacteriocin-like substance, volatile fatty acids and hydrogen peroxide, decreasing the pH of the environment, competition for adhesion sites on the intestinal epithelium, and modulation of immune system (increased antibody levels and increased macrophage activity) and increased and decreased enzyme activity [4,5,6,7,8,9,10].

Several researches have been conducted for investigating the anti-Salmonella activity of *Lactobacillus* spp. both *in vitro* and *in vivo* [11,12]. There are various reports with the use of Lactobacillus spp. as probiotic microorganisms for *Salmonella* inhibition. Casey *et al.* observed that pretreatment pigs with five strains of Lactobacillus including two strains of *Lactobacillus murinus* and one strain of *Lactobacillus salivarius* subsp. *salivarius*, *Lactobacillus pentosus*, and *Pediococcus pentosaceous* resulted in significant reduction of fecal numbers of *Salmonella enterica* Serovar *typhimurium* and the author reported that incidence, severity, and duration of diarrhea had decreased [13]. Voravuthikuncha's *et al.* *in vitro* study did not show any antibacterial effects of vaginal isolated Lactobacillus spp. against *Salmonella typhi* and *Salmonella typhimurium* [14]. Pascual *et al.* concluded that *Lactobacillus salivarius* can completely eliminate the presence of *Salmonella Enteritidis* in the proventriculus in chickens 21 days after dosing oral gavage of *S. Enteritidis* and *L. salivarius* [15]. Nouri’s *et al.* *in vitro* investigation showed that *Lactobacillus salivarius* and *Lactobacillus crispatus* isolated from chicken gastrointestinal tract can suppress growth of *Salmonella Enteritidis* [16]. Trusuatu *et al.* inoculated orally the *Lactobacillus fermentum* and *Lactobacillus acidophilus*, from human origin, in Salmonella-infected mice and did not observe any antibacterial effect against *Salmonella typhimurium* [17].

Because of low number of influential Lactobacilli against gram-negative bacteria especially *Salmonella typhimurium* among the in vitro-conducted researches, we aimed
Evaluation of antimicrobial activity

To investigate the antimicrobial activity of three Lactobacillus spp. including *Lactobacillus plantarum*, *Lactobacillus delbrueckii* subsp.*bulgaricus*, and *Lactobacillus leichmannii* against *Salmonella typhimurium* strains from different origins.

Material and Methods

**Bacterial strains and culture conditions.** *Lactobacillus plantarum* PTCC 1058, *Lactobacillus delbrueckii* subsp.*bulgaricus* PTCC 1737 and *Lactobacillus leichmannii* PTCC 1057 were selected from Persian Type Culture Collection. The strains were cultured at 37°C in MRS broth (Merck, Germany) for 48 hours in anaerobic jar with CO2-producing kit [18].

Ten samples of *Salmonella typhimurium*, which were sent to Dr.Zahraei's lab, submitted to biochemical, serotyping and molecular confirmation tests.

**Biochemical confirmation.** For biochemical confirmation, Salmonella suspect colonies (colonies with white zone and a black center observed on Salmonella-Shigella agar) were streaked on Nutrient agar (Merck, Germany) and were incubated overnight at 37°C. Biochemical confirmation conducted using TSI, Cimon Citrate, Urea, Phenil alanine agar, SIM, Indole, MR-VP (Merck, Germany) and CHROMagar (CHROMagar Salmonella, France) tests [19,20].

**Serotyping.** Serotyping of Salmonella strains was carried out by antisera (Difco. Detroit, MI, USA) [21].

**Molecular confirmation.** The Salmonella strains were cultured onto Luria Bertani (LB) agar and incubated overnight at 37°C. To extract DNA, 1 loopful of each *Salmonella* strains grown on LB agar was suspended in 250 μl of sterile distilled water. In order to get uniform turbidity, the suspension were vortexed, and then were boiled for 10 min and centrifuged at 6000 ×g for 7 min. Supernatant were collected and frizzed at 70°C for multiplex PCR.

Multiplex PCR was performed using four sets of primers specific for *rfbJ* (663 bp), *fljB* (526 bp), *invA* (284 bp), and *fliC* (183 bp) genes (Table 1). The reaction was carried out in a final volume of 25 μl containing 8.4 μl of sterile distilled water, 2.5 μl of reaction buffer (10'), 0.8 μl of dNTPs (10 mM), 1 μl of MgCl2 (50 mM), 0.3 μl of Taq polymerase (5 UμL-1), 1 μl of each primer (10 μM), and 4 μl of template DNA.

Amplification was performed by a Techno TC 512 thermocycler (Technne, UK), as follow: 30 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 1 min, primer extension at 72°C for 30s, followed by 7 min at 72°C for final extension, and initial denaturation was at 95°C for 5 min.

The PCR products were analyzed on a 1.2% agarose gel Electrophoresis and ethidium bromide staining. A 100 bp ladder was also used as a molecular size standard. Subsequently, the DNA was visualized by UV transillumination (BIORAD, UK) [22,23,24].
Determination of antibiotic pattern. In order to survey the antibiotic profile, 100 μl of each overnight culture of the strains which have been adjusted to 0.5 McFarland standard spread on Mueller-Hinton agar (Pronadisa, Spain) plates; subsequently, antibiotic discs applied according to the Bauer-Kirby technique [25]. The following concentrations in antibiotics were used: Ampicillin (10 μg), Streptomycin (10 μg), Tetracycline (30 μg), Gentamycin (10 μg), Penicillin (10 μg), and Cephalexin (30 μg). The plates were incubated for 24 h at 37°C, followed by measurement of the inhibition zone diameters (IZDs), including the diameter of the disk (in millimeters).

Antimicrobial activity. The antimicrobial activities of all Lactobacillus strains were evaluated by means of the agar-well diffusion and disk diffusion assays.

Agar well-diffusion assay. Twenty milliliters of molten Mueller-Hinton agar were poured into 8 cm sterile Petri dishes. 100 μl of the overnight broth culture of Salmonella typhimurium strains, which have been adjusted to 0.5 McFarland-turbidity, was spread on the plates. Overnight MRS broth culture of the respective bacteria strains were adjusted to turbidity equivalent to 0.5 and1 McFarland standards, and were centrifuged at 6000 rpm for 15 min at 4°C and the supernatant was filtrated through a 0.2μm pore size cellulose acetate filter in order to get cell free supernatant. Once the plates were dried aseptically, 6 mm wells were bored using a sterile cork borer. 80 μl of each suspension were placed into the wells, and the plates were incubated at 37°C for 24 h [14,26,27].

Agar Disk-diffusion assay. Mueller-Hinton agar plates with inoculated indicator bacteria as mentioned in well diffusion method were used for agar disk diffusion method. Blank disks (5 mm diameter, 1 mm thickness) were immersed into cell free supernatant of the Lactobacillus spp. for 15 min and were placed onto the surface of the agar. The plates were left at room temperature for 1 h so that the absorbed supernatant become diffused into the agar, and then incubated at 37°C for 24 h [28]. The tests were carried out in triplicate.

Results and Discussion

Biochemical tests, Serotyping and Multiplex PCR. All of these approaches led us to identify the Salmonella samples as Salmonella typhimurium strains. Salmonella organisms are identified by flagellar (H) and somatic (O) antigens. The genes which are responsible for synthesis of O antigen, are generally gathered on the chromosome in the rfb gene cluster. The flagellar antigens H1 and H2 are encoded by the fliC and fljB genes, respectively. The invA gene contributes to Salmonella virulence [29]. Serotyping of Salmonella typhimurium (4, 5, 12: i: 1, 2) carried out by using O: 4, 5, 12; H1: I and H2: 1, 2 antisera in all samples. Subsequently, multiplex PCR confirmed the serotype of Salmonella typhimurium strains. Results of multiplex PCR of some strains are presented in fig 1. Sizes of 183, 284, 526 and 663 bp demonstrate fliC, invA, fljB and rfbJ genes that represent H1: I, invasion that indicates genus of Salmonella and virulence type, H2: 1, 2 and O4, respectively (Table1).
The present result demonstrates that the correlation between multiplex PCR and traditional serotyping was 100%; therefore, this molecular method can approve the outcomes of traditional serotyping. The advancement of such a molecular method; however, does not necessarily imply that traditional serotyping should be ruled out since these two methods are complementary and the molecular method plainly augments the available tools for successfully typing strains that cause health problems.

**Antibiotic patterns of Salmonella typhimurium strains.** All strains, which were from poultry and bird resources (Table 2), were susceptible to Ampicillin, and 7, 3 and 2 strains were resistant to Streptomycin, Tetracycline and Gentamycin, respectively. With some exception of one strain, which was intermediate, rest of them were susceptible to Cephalexin. However, all of strains were resistant to Penicillin. (Details have shown in the table 2). Actually, 70% of strains were resistant at least to one antibiotic and it may be because of administration of antibiotic in industry and medicine. Such administrations not only face medicine with considerable therapeutic problems but also cause to transmit these microorganisms among beings [1].

**Antibacterial effects of Lactobacillus species against Salmonella typhimurium strains.** Our study showed that none of *L.plantarum, L.delbruecki* subsp.*bulgaricus* and *L.leichmannii* had antimicrobial effects against *Salmonella typhimurium* strains. There is some evidence that lactobacilli are not efficient in the case of gram-negative bacteria compared with gram-positive bacteria [30]. Contreras's *et al. in vitro* study exhibited no antagonistic effect against both *Salmonella typhimurium* and *Salmonella Enteritidis* by using *Lactobacillus amylovorus* LMG P-13139 producing two bactericidal peptides [31]. Besides, bacteriocin excreting *Lactobacillus lactis* subsp. *cremoris* CTC 204 had no antibacterial activity against *Salmonella typhimurium* ATCC 14028 within the *in vitro* experiment conducted by Bromberg *et al.* [32]. Nouri *et al.* also resulted in that the *Lactobacillus* spp., which isolated from gizzard and crop, did not have any inhibitory activity against both *Salmonella Enteritidis* and *Echerichia coli* [16].

Among the mechanisms causing antibacterial effects by *Lactobacillus* spp., the *in vitro* investigable mechanisms including production of bacteriocin, bacteriocin-like substances, hydrogen peroxides, and excretion of lactic acid that have been examined by this study were not effective against *Salmonella typhimurium*.

In conclusion we state that the selected *Lactobacillus* spp. are not suitable options in order to be used as both bio-preservative and anti-*Salmonella typhimurium* probiotics in food and drug industries.

**ACKNOWLEDGMENTS**

We thank Dr. Goudarz Molaei and Dr.Hossein Siah poosh for their guidance for writing this paper. We are also grateful to the staff of Pars Diagnostic Laboratory for all assistance.

This work was supported by the Young Researcher's Club, Islamic Azad University, Tonekabon Branch. Amin Ravaei is the recipient of the BPJ-rewarded grant.
Table 1. Primers used for the detection of *S. typhimurium*.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Taegget Gene</th>
<th>Length</th>
<th>Length Sequence (5’-3’)</th>
<th>Amplification Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST139-s</td>
<td><em>invA</em></td>
<td>26</td>
<td>GTGAAATTATCGCCACGTTCGGCAA</td>
<td>284</td>
</tr>
<tr>
<td>ST141-as</td>
<td></td>
<td>22</td>
<td>TCATCGCACCCTCAAAGGAACC</td>
<td></td>
</tr>
<tr>
<td>Rfbj-s</td>
<td><em>rfbJ</em></td>
<td>24</td>
<td>CCAGCACCAGTTCAAACCTTGATAC</td>
<td></td>
</tr>
<tr>
<td>Rfbj-as</td>
<td></td>
<td>24</td>
<td>GGCTTCCGGCTTTATTTGTAAGCA</td>
<td>663</td>
</tr>
<tr>
<td>Flic-s</td>
<td><em>fliC</em></td>
<td>23</td>
<td>ATAGCCATCTTTACCAGTTCCCC</td>
<td></td>
</tr>
<tr>
<td>Flic-as</td>
<td></td>
<td>24</td>
<td>GCTGCAACTGTACAGGATATGCC</td>
<td>183</td>
</tr>
<tr>
<td>Fljb-s</td>
<td><em>fljB</em></td>
<td>24</td>
<td>ACGAATGTACGCTTCTGTAAACC</td>
<td></td>
</tr>
<tr>
<td>Fljb-as</td>
<td></td>
<td>24</td>
<td>TACCGTCGATAGTAACGACTCGG</td>
<td>526</td>
</tr>
</tbody>
</table>

Table 2. Origin and Antibiotic susceptibility of *S. typhimurium* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Sterptomycin</th>
<th>Tetracycline</th>
<th>Ampicillin</th>
<th>Gentamycin</th>
<th>Penicillin</th>
<th>Cefalexin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₁</td>
<td>Poultry</td>
<td>I (14)</td>
<td>S (21)</td>
<td>S (20)</td>
<td>S (21)</td>
<td>R (-)</td>
<td>S (21)</td>
</tr>
<tr>
<td>S₂</td>
<td>Poultry</td>
<td>I (12)</td>
<td>S (20)</td>
<td>S (21)</td>
<td>S (20)</td>
<td>R (-)</td>
<td>S (18)</td>
</tr>
<tr>
<td>S₃</td>
<td>Poultry</td>
<td>R (11)</td>
<td>S (20)</td>
<td>S (20)</td>
<td>S (17)</td>
<td>R (-)</td>
<td>S (19)</td>
</tr>
<tr>
<td>S₅</td>
<td>Poultry</td>
<td>R (11)</td>
<td>S (21)</td>
<td>S (20)</td>
<td>S (18)</td>
<td>R (-)</td>
<td>S (18)</td>
</tr>
<tr>
<td>S₆</td>
<td>Ostrich</td>
<td>R (-)</td>
<td>R (-)</td>
<td>S (18)</td>
<td>S (18)</td>
<td>R (-)</td>
<td>I (17)</td>
</tr>
<tr>
<td>S₇</td>
<td>Pigeon</td>
<td>R (8)</td>
<td>S (24)</td>
<td>S (22)</td>
<td>S (10)</td>
<td>R (-)</td>
<td>S (19)</td>
</tr>
<tr>
<td>S₈</td>
<td>Pigeon</td>
<td>R (-)</td>
<td>R (-)</td>
<td>S (21)</td>
<td>S (20)</td>
<td>R (-)</td>
<td>S (20)</td>
</tr>
<tr>
<td>S₉</td>
<td>Pigeon</td>
<td>R (11)</td>
<td>S (22)</td>
<td>S (21)</td>
<td>R (12)</td>
<td>R (-)</td>
<td>S (19)</td>
</tr>
<tr>
<td>S₁₀</td>
<td>Mynah</td>
<td>R (-)</td>
<td>R (-)</td>
<td>S (21)</td>
<td>S (21)</td>
<td>R (-)</td>
<td>S (22)</td>
</tr>
</tbody>
</table>

S: Sensitive, I: Intermediate, R: Resistant, -: no inhibition zone
Figure 1 Multiplex Polymerase Chain Reaction for detection of *S. Typhimurium*

Lane M: 100 bp ladder. Lane 1-6: *Salmonella typhimurium* strains. Lane 7 and 8: blank. The sizes of 663, 526, 284 and 183 bp represent the *rfbJ, fljB, invA* and *fliC* genes, respectively.
References


Evaluation of antimicrobial activity


Received: October, 2012