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Antimicrobial Activities of Lactobacillus Strains Isolated from Fresh Vegetables

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Abstract: Lactic acid bacteria (LAB) have been isolated from specific habitats, including dairy products, plants, meat products, sewage, manure, humans and animals. Among LAB members, Lactobacilli as normal flora of gastrointestinal tract have beneficial effects on human health. The aim of this study was to determine antimicrobial properties of lactobacillus strains isolated from fresh vegetables. For this reason, 80 samples were collected by wet swabs in spring season and were enriched in Man-Rogosa-Sharpe (MRS) broth and isolated by growing on MRS agar medium. From 80 samples, as many as 23Gram positive, catalase negative, non spore forming bacilli were isolated in first phase. The samples were tested for tolerance against acidic conditions (pH: 2.5) and bile salts and tolerance samples were identified *Lactobacillus plantarum* (3 isolates), *Lactobacillus casei* (2 isolates) and *Lactobacillus brevis* (1 isolate) produced the highest tolerance against acid and bile salts as well as antimicrobial activities against *Staphylococcus aureus* PTCC 1431, *Salmonella typhimurium* PTCC 1639 and *Escherichia coli* PTCC 1399.

Key words: Lactic Acid Bacteria (LAB) % Fresh Vegetables % Lactobacillus % Antimicrobial Activities

INTRODUCTION

Lactic acid bacteria (LAB) are a group of Gram positive, non-spore forming, catalase negative cocci or rods which produce lactic acid as major end product from fermentation of carbohydrates [1, 2]. LAB play an important role in food fermentation processes. Raw foods such as milk, fruits, vegetables or meat are often preserved by lactic acid fermentation. These organisms produce various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocin during lactic fermentations. All of these can antagonize the growth of some spoilage and pathogenic bacteria in foods [3, 4]. LAB are regarded as a major group of probiotic bacteria and have been used successfully, with few adverse effects, to prevent antibiotic associated diarrhea and to treat acute infantile diarrhea, recurrent *Clostridium difficile* disease and various diarrheal illnesses [5, 6]. Many definitions of probiotics have been published, starting from Fuller, who defined a probiotics as a live microbial feed supplement, which beneficially affects the host by improving its intestinal microbial balance [7]. Among LAB members, The genus Lactobacillus contains over 110 species which are classified in three major groups: the obligate homo fermentative lactobacilli which ferment hexoses to lactic acid; the facultative hetero fermentative lactobacilli, which ferment hexoses to lactic acid only or to lactic acid together with acetic acid, ethanol and formic acid under glucose limitation; and obligate hetero fermentative lactobacilli, which ferment hexoses to lactic acid, acetic acid, ethanol and CO_2 and ferment pentoses to lactic acid and acetic acid [8]. Most of the probiotic lactobacilli in human foods are supplied in highly concentrated forms containing more than 10^{10} cfu/g. These concentrates are usually freeze dried, spray dried or microencapsulated [9]. Lactobacilli play an important role in maintaining vaginal health. They produce lactic acid and H_2O_2, which can prevent the overgrowth of other microorganisms in the vagina including *E. coli* and *G. vaginalis* [10]. The ability of lactobacillus strains to adhere to the mucosal surfaces of the intestine and the subsequent long or short term colonization has long been one of the most commonly encountered criteria

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for the selection of probiotic strains [6, 11]. Although, there is a lot of research on isolation and characterization of LAB, only a few of them have focused on isolation from fruits and vegetables. The aim of this study was to isolate lactobacilli from fresh vegetables and determine their inhibitory effect against some pathogenic bacteria.

MATERIALS AND METHODS

Isolation and Identification of Lactobacilli: Eighty samples were collected by wet swabs and kept in sterile tubes containing MRS broth medium (Merck; Germany) in spring season. The source of samples was fresh vegetables including cabbage, silage and cucumber, the entire sample tubes were incubated at 37°C under 5% CO_2 conditions for 48 h, then sub cultured on Man-Rogosa-Sharpe medium (MRS) agar (Merck; Germany) for 48 h [8, 12, 13]. Vegetables have rich microbial diversity. It is very time consuming to isolate and identify each of these bacteria. Thus, it is possible and practical to delete non tolerant strains by greatly acidifying the medium. To do so, the bacteria were transferred to a medium containing PBS (pH: 2.5) and washed before calculating survival rate of isolates in acidic medium and determining population for each strain. Then, the tolerant bacteria were transferred to MRS broth medium containing 0.3% bile salt (Oxgall) and another medium (MRS broth medium) considered as control medium. Every half an hour, growth in both control medium and medium containing bile salt was recorded through measuring optical absorption in 600 nanometers wavelength using spectrophotometer [14, 15].

The isolated bacteria, which were tolerant to acid and bile salts, were examined for their morphologic characteristics and Gram Positivity and non spore forming bacilli were selected for biochemical tests and identified as detailed as their species level and verified according to Bergey's Manual [16].

Standard Strains: The standard strains used in this study were *Staphylococcus aureus* PTCC 1431, *Salmonella typhimurium* PTCC 1639 and *Escherichia coli* PTCC 1399. The strains were obtained from collection center of fungi and bacteria, Tehran, Iran.

Preparation of Cell-Free Supernatants: Strains to be tested for antimicrobial activity were incubated in MRS broth for 48 h at 37°C. Bacterial cells were removed by centrifuging the culture at 5000 g for 20 min at 4°C. The pH values of supernatants were adjusted to pH 6.5-7.0 by the addition of 1 N NaOH, The supernatants were membrane filtered (Millipore, 0.22µm) and stored at 4°C.

Antimicrobial Assay: Agar well diffusion method [17] used to detect antimicrobial activities of supernatants produced from lactobacillus strains. The plates were poured with 20 ml Mueller Hinton Agar (Merck; Germany). The pathogenic strains (*Staphylococcus aureus* PTCC 1431, *Salmonella typhimurium* PTCC 1639 and *Escherichia coli* PTCC 1399) were adjusted to a density of 10^9 CFU/ml by adding sterile water and spread on the surface of MHA. Wells of 7 mm in diameter were cut into these agar plates and 100 µl of the supernatants were placed into each well. The culture plates were incubated at 37°C for 24 h and the zones of inhibition measured in diameter (mm) [18]. Antimicrobial tests were done in duplicate and the mean values were recorded. Statistical analyses were performed using SPSS software.

RESULTS

In total, 23(28.75%) presumptive lactobacillus strains were isolated from 80 collected vegetable samples. From the 23 isolates 3 *Lactobacillus plantarum*, 2 *Lactobacillus casei* and one *lactobacillus brevis* produced the highest tolerance against acid and tolerance against bile salts. Were identified Based on survival rate of the isolates under acidic conditions, 12 strains were designated as susceptible, 1 as average tolerant and the 10 (strains S2, S6, S7, S11, S13, S16, S17, S18, S21, S22) remaining strains as well tolerant. Results from determining the tolerance of strains to bile salts were classified as four groups including tolerant strains (equal growth delay or less than 15 minutes), highly tolerant strains (growth delay between 15 to 40 minutes), poorly tolerant strains (growth delay between 40 to 60 minutes) and susceptible strains (growth delay more than 60 minutes) So, based on this classification the strains were designated as one strain tolerant, 6 (strains S2, S7, S11, S13, S18, S22) as highly tolerant, 1 as poorly tolerant and 2 as susceptible. Results of the agar well diffusion method (Table 1) showed, that *Lactobacillus plantarum* S2 produced the highest inhibiting effect (10.8mm) against *Salmonella typhimurium* PTCC 1639, whereas the lowest inhibiting rate (4.3mm) belonged to *Lactobacillus casei* S13 against *Staphylococcus aureus* PTCC 1431. In general, bacteria with an average inhibition power of 7.91 mm produced a good capacity for inhibiting of pathogenic bacteria.

Table 1: Antibacterial activity of lactobacillus strains against pathogenic bacteria (Inhibition zones in millimeter)

		Zone diameter (mm)		
Strains	Isolate no.	*S. aureus* PTCC 1431	*S. typhimurium* PTCC 1639	*E. coli* PTCC 1399
L. Plantarum	S2	10.2	10.8	9.0
L. Plantarum	S18	8.0	7.2	8.3
L. Plantarum	S11	7.8	8.0	7.5
L. casei	S7	9.2	10.0	9.0
L. casei	S13	4.3	6.0	6.8
L. brevis	S22	5.0	7.2	8.0

DISCUSSION

Lactic Acid Bacteria (LAB) are one of the most important groups of microorganisms to mankind, being involved in the production of valuable foods including fermented milk products (cheese, yogurt and kefir), bread and cereals (sourdough) and vegetables (kimchi, sauerkraut and silage). LAB produce various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocin or bacteriocidal proteins during lactic fermentations [3]. Vegetables playing important role in nourishment, different microorganism were observed in vegetables [19]. Lactobacilli are a part of normal flora, contained antimicrobial substance that has inhibitory effect on growth of pathogens. The MRS medium used is selective for the isolation of lactobacillus strains since they are extremely fastidious [20]. During this study, 6 strains tolerant to acid and bile salts were isolated from 80 samples of fresh vegetables. In Italy, 63 strains of lactobacillus were isolated from one type of traditional cheese, of which only 3 strains produced a high tolerance against acidic conditions and bile salts [21]. In another study, 6 out of 88 lactobacillus strains isolated from milk and cheese were tolerant against acidic conditions and bile salts [22]. In this study only 6 out of 23 isolated lactobacillus strains produced a good tolerance against acidic conditions and bile salts. The concentration as much as 0.3% was applied to evaluate tolerance rate to bile salts, which is paramount to average concentration of bile salts in human gastrointestinal tract. In our study the supernatants of *Lactobacillus plantarum* (3 isolates), *Lactobacillus casei* (2 isolates) and *Lactobacillus brevis* (1 isolate) cultures produced inhibitory effects against pathogenic bacteria. Inhibition of bacterial pathogens by common LAB from fermented food is shown in several reports [3, 23, 24]. Kivanc *et al*. [25] demonstrated the inhibitory performances of some lactobacilli from Turkish boza against a wide range of pathogenic organisms including *Pseudomonas aeruginosa*. Schillinger and Lucke [17] reported that some *L. plantarum* and *L. sake* strains from meat and meat products has inhibitory effects against several bacteria. Alvarado *et al*. [23] showed that only 25 out of 94 isolated LAB strains from traditional Mexican foods are able to show inhibition against at least one pathogenic indicator microorganism. Magnusson and Schnurer [26] reported detection of *Lactobacillus coryniformis* isolated from grass silage with the antifungal activity against some molds and yeasts. Gilliland and Speck [27] had earlier reported that lactobacilli showed stronger antibacterial properties against Gram positive bacteria (*Staphylococcus aureus* and *Clostridium perfringens*) than Gram negative bacteria (*Escherichia coli* and *Salmonella typhimurium*). The results of our study showed, that strains of lactobacillus isolated from fresh vegetables, which were tolerant against acidic conditions and bile salts had good antimicrobial effects and could be used widely in production of industrial products and native probiotic strains, contributing to enhance health in the society.

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