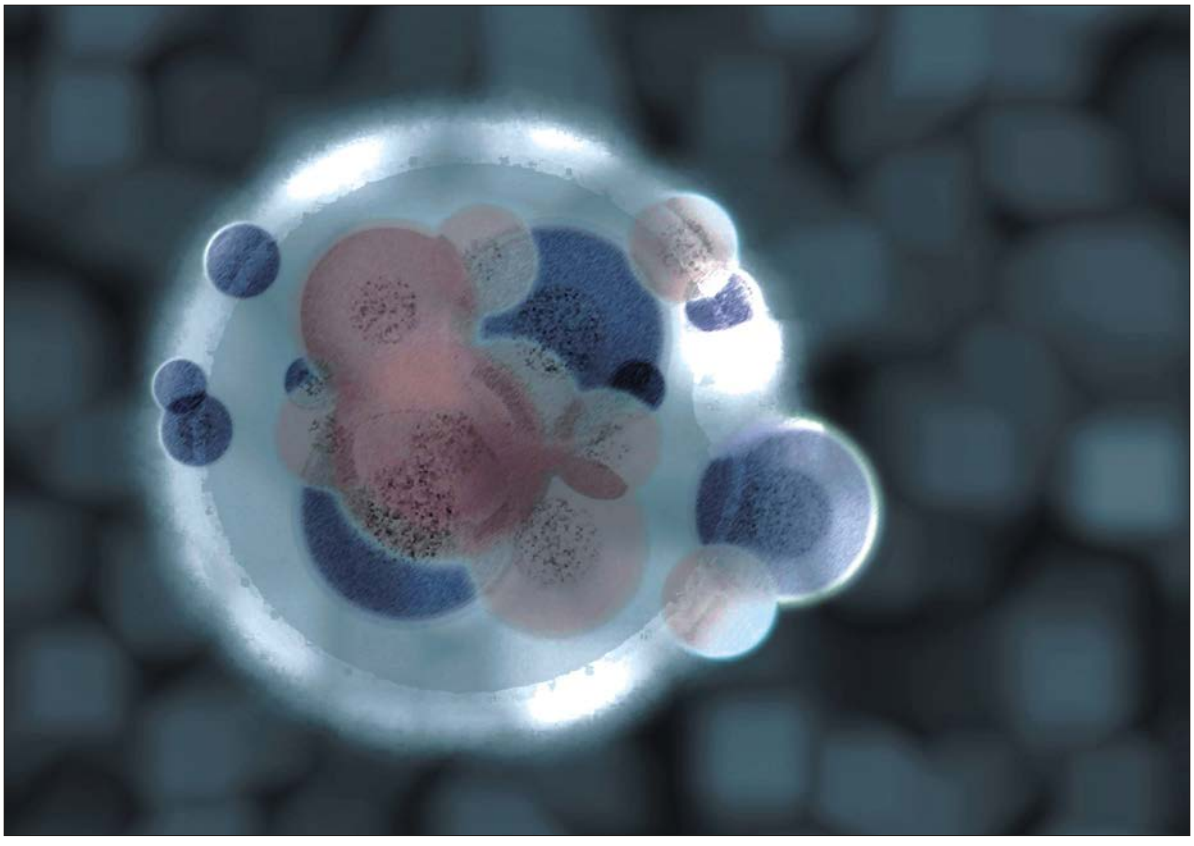

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Isolation and Identification of *Lactobacillus casei* and *Lactobacillus plantarum* from Plants by PCR and Detection of their Antibacterial Activity

[1]M. Amin, [1]M. Jorfi, [1,2]A.D. Khosravi, [1]A.R. Samarbafzadeh and [1]A. Farajzadeh Sheikh
[1]Department of Microbiology, School of Medicine,
Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]Infectious and Tropical Diseases Research Center,
Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Abstract: Lactobacillus is a genus of lactic acid bacteria described as a heterogeneous group of regular non spore forming gram positive rods and found in a great variety of habitats such as plants and gastrointestinal tracts. The aim of this study was to isolate lactobacilli from plants and determine their inhibitory effect against some pathogens. Sixty lactobacilli isolates from fresh vegetables were enriched in Man-Rogosa-Sharpe medium (MRS) broth and isolated by growing on MRS agar medium and characterized by phenotypic characteristics and PCR technique at genus and species levels. The antimicrobial substance was extracted with ethyl acetate solvent and the antimicrobial activity against some pathogenic bacteria such as *Escherichia coli, Salmonella typhi, Shigella dysenteriae, Bacillus anthracis* and *Staphylococcus aureus* were investigated. Forteen *L. plantarum* and eight *L. casei* which were isolated from fresh vegetables were identified by PCR. Antimicrobial substance from MRS broth medium was extracted. This antimicrobial compound showed a potent inhibitory activity against all tested bacteria. The inhibitory substance was distinct from bacteriocins, lactic and acetic acids which are produced by these bacteria. In conclusion, fresh vegetables may be used as a source of antimicrobial lactic acid bacteria. *L. casei* and *L. plantarum* as two probiotics can establish themselves in gut and urogenital tract and prevent the human body from adverse effects of pathogens.

Key words: Lactobacillus, PCR, antimicrobial substance, plant

INTRODUCTION

Many definitions of probiotics have been published, starting from Fuller, who defined a probiotic as a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance (Fuller, 1989). A more recent one from FAO/WHO is the following: Live microorganisms which when administered in adequate amounts, confer a health benefit on the host. Some of the health benefits which have been claimed for probiotics include the following: improvement of the normal microflora, prevention of infectious diseases and food allergies, reduction of serum cholesterol, anti carcinogenic activity , stabilization of the gut mucosal barrier, immune adjuvant properties, alleviation of intestinal bowel disease symptoms and improvement in the digestion of lactose in intolerant hosts. The genera most commonly used in probiotic preparations are Lactobacillus, Bifidobacterium, Streptococcus, Lactococcus and some fungal strains (Galdeano *et al*., 2007). The genus Lactobacillus contains over 110 species which are classified in three major groups: the obligate homofermentative lactobacilli, which ferment hexoses to lactic acid; the facultative heterofermentative lactobacilli, which ferment hexoses to lactic acid only or to lactic acid together with acetic acid, ethanol and formic acid under glucose limitation; and the obligate heterofermentative lactobacilli, fermenting hexoses to lactic acid, acetic acid, ethanol and CO_2 and ferment pentoses to lactic acid and acetic acid (Rodas *et al*., 2005).

This genus of bacteria is widely distributed in environment. Several species, including *Lactobacillus acidophilus*, are members of the normal intestinal and vaginal flora of healthy humans. Other species, such as *Lactobacillus bulgaricus* and *Lactobacillus casei*, are commonly isolated from dairy products as well as from fruits and vegetables and they play an important role as probiotics in human and animal nutrition (Oyetayo, 2004; Erdogrul and Erbilir, 2006). Lactobacilli produce acids, hydrogen peroxide, bacteriocins and biosurfactants and

Corresponding Author: Azar D. Khosravi, Department of Microbiology, School of Medicine,
Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
Tel: +98 611 3330074 Fax: +98 611 3332036

thus confer protection of the host . Lactobacillus as a natural vaginal flora plays an important role in the reproductive health of women by maintaining acidic vaginal pH, providing colonization resistance and preventing the growth of pathogens (Marelli *et al*., 2004; Falagas *et al*., 2006) . The acidic pH itself acts as a natural defense against sexually transmitted disease and AIDS (Mahmoud *et al*., 1995; Garg *et al*., 2001). Several Lactobacillus strains are now being used as probiotics in commercially available food products. Strains which are studied thoroughly and are accepted as probiotics using established criteria belong to the *species L. rhamnosus, L. acidophilus, L. casei, L. reuteri* and *L. fermentum* and some other species of this genus (Sanders and Klaenhammer, 2001).

The 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 ogi) and vegetables (kimchi, sauerkraut and silage) (De Vos and Hugenholtz, 2004). The aim of this study was isolation of *Lactobacillus casei* and *L. plantarum* from fresh vegetables by PCR and to examine their antimicrobial activity against some microorganisms such as *Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Bacillus anthracis* and *Staphylococcus aureus*.

MATERIALS AND METHODS

In total, 300 samples were collected by wet swabs and kept in sterile tubes containing MRS broth media in winter and spring seasons (January to June 2008). The source of samples where fresh vegetables including white headed cabbage, cauliflower and silage. The entire sample tubes where incubated at 37°C and 5% CO_2 conditions for 2 days, then subcultured on Man-Rogosa-Sharpe medium (MRS) agar (Hi-media, Idia) for 48 h. The grown colonies were characterized by phenotypical properties including morphology, gram positive staining, resistance to vancomycin and absence of catalase, oxidase and motility (Rodas *et al*., 2005; Forbes *et al*., 2007) and PCR technique at genus and species levels. The DNA of the bacteria was extracted from single colonies after growing the lactobacilli on MRS agar under microaerophilic conditions overnight as described previously (Araujo *et al*., 2004). The primers used in this study are shown in Table 1 (Tannock *et al*., 1999; Massi *et al*., 2004; Settanni *et al*., 2005).

The composition of PCR mixture was 50 mM KCl, 10 mM HCL (pH,8.3), 4.4 mM $MgCl_2$, 200 mM dNTPs, 0.5 μM of each primer, 2 U of *Taq* polymerase and 5 μL of DNA template in a final volume of 25 μL. All the reagents were purchased from Cinnagen Company, Tehran, Iran. The standard strains of *L. plantarum* and *L. casei* as positive controls and a non lactobacillus strain as negative control were included in each set of PCR amplification.

Table 1: Primers used in present study

No.	Primers
1	Genus specific lactobacillus (Cas-ITS) primers (560-640 bp)
	16-1A (16s): 5'-gAATCgCTAgTAATCg-3'
	23-1B (23s): 5'-gggTTCCCCCATTCggA-3'
2	*Lactobacillus plantarum* specific primers (319 bp)
	planF: 5'-CCgTTTATgCggAACACCTA-3'
	plan Rev: 5'-TCgggATTACCAAACATCAC-3'
3	Lactobacillus casei specific primers (118 bp)
	cas-I (ITS.L): 5'-AAgCACCCTAACgggTgCgACT-3'
	cas-II (ITS): 5'-GCGATGCGAATTTCTTTTTC-3'

The PCR conditions were initial denaturation of 94°C for 5 min followed by 30 cycles of denaturation of 94°C for 30 sec, annealing of 52°C for 30 sec for Cas-ITS genus specific primers; 45°C for 45 sec for *L. plantarum*; 40°C for 40 sec for *L. casei* and extension of 72°C for 1 min and then a final extension at 72°C for 10 min using a thermocycler (Techgene, UK). The PCR products were analyzed on 1% agarose gel. The gels were then stained with 0.5 g L^{-1} (w/v) ethidium bromide. Product sizes were identified using a 100 bp DNA ladder (MBI Fermentas, Germany) as a reference standard. Results were recorded using the gel documentation system (UVP Systems, UK).

The confirmed species by PCR were tested for antimicrobial properties. Antimicrobial compound was isolated using ethyl acetate solvent from *L. plantarum* and *L. casei* separately. After 5 days incubation, the MRS broth media containing bacteria was mixed with ethyl acetate and agitated with a magnatic stirrer for two days. Then the media was allowed to settle for 30 min. Following settlement, the solution was separated into two phases, which the supernatant was comprised of the extracted antimicrobial compound. The color of ethyl acetate was turned yellow after agitation. The supernatant then was dried at 45°C. The quantity of antimicrobial compound was determined as 80 mg.

The Minimal Inhibitory Concentration (MIC) of this antimicrobial substance determined using modified E. test, by incorporating 20 μL of the each extract in paper discs. The final concentration of the extract in each disc was estimated as 1.6 mg in total incorporated volume (Amin and Kapadnis, 2005). The target bacteria were some clinically isolated pathogens including *Shigella dysenteriae, Bacillus anthracis* and *Staphylococcus aureus*. The standard strains which were included in the study were *Escherichia coli* (PTCC1399) and *Salmonella typhi* (PTCC1639), obtained from collection center of fungi and bacteria, Tehran, Iran. The experiments were repeated 3 times and the results were constant in all tests.

For investigation the shelf life of bioactivity of *L. plantarum* and *L. casei*, the effect of incubation time on antimicrobial activity of *L. plantarum* and *L. casei* was studied in various length of time kept at 4°C (1, 2, 6 months and one year).

The t-test and ANOVA variance were used for data analysis by application of SPSS software (SPSS Inc no. 15, Chicago, IL, USA).

RESULTS AND DISCUSSION

All of the preliminary isolated lactobacilli were subjected to PCR for confirmation of genus by genus-specific primers and species by species-specific primers (Fig. 1). Sixty out of 90 isolates belonged to genus of lactobacillus. Using species-specific primers, 14 of them were confirmed *L. plantarum* and 8 were *L. casei*. The confirmed strains by PCR were tested for their antimicrobial properties.

The activity of antimicrobial substances was tested against target pathogens after adjustment of pH at 7 using NaOH 5 N. The antimicrobial compounds showed potent inhibitory activity against all tested bacteria. The MICs of two selected antimicrobial compounds obtained from *L. plantarum* and *L. casei* were determined using modified E. test (Amin and Kapadnis, 2005). In practice, the effect of extract of *L. plantarum* on all target bacterial strains except salmonella was slightly stronger but is was not significant in performed E. test ($p>0.05$) (Table 2, Fig. 2).

The antimicrobial activity of these extracts was stable at 4°C for one year tested.

The recent reviews indicated that different Lactobacillus strains may be isolated from various vegetables and fermented food including cheese, yoghurt, corn slurry, fufu, pounded yam and rice. The isolated bacteria showed antimicrobial activity in different values (Oyetayo, 2004; Erdogrul and Erbilir, 2006; Coolborn, 2005). There are evidences that lactobacilli can inhibit the growth and attachment of pathogens to epithelial cells. The hydrogen peroxide and bacteriocin-like compounds produced by lactobacilli can kill the pathogenic microorganisms in human body (Falagas *et al*., 2006; Atassi *et al*., 2006).

The important point of this study was isolation the lactobacilli species from fresh vegetables including white headed cabbage, cauliflower and silage. In the other hand we know that this bacterium is a probiotic and can protect our body against the pathogenic bacteria. The obtained results revealed that the mentioned vegetables have many lactobacilli and these bacteria produce antibiotic in addition the organic acids and hydrogen peroxide. The

Fig. 1: Agarose gel of PCR products amplified by species-specific primers of *L. plantarum*

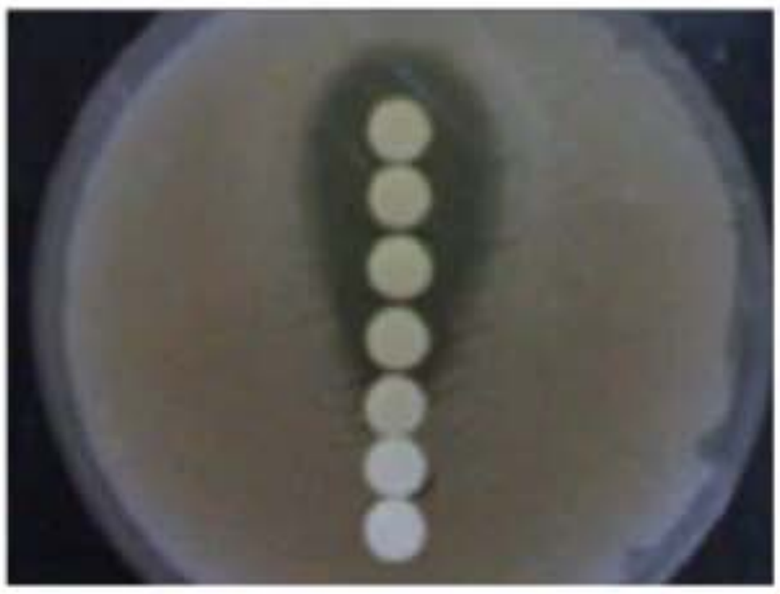

Fig. 2: E test representing MIC of antimicrobial compound obtained from *L. casei* against *B. antracis*

Table 2: MIC of antimicrobial compound obtained from *L. plantarum* and *L. casei* against pathogenic bacteria using E. test

	MIC (μg mL^{-1})	
Bacteria	*L. casei*	*L. plantarum*
Bacillus anthracis	50	25
Salmonella typhi	200	200
Shigella dysenteriae	200	200
E. coli	200	200
S. aureus	400	400

antimicrobial compounds isolated from *L. casei* and *L. plantarum* showed activity against some pathogenic bacteria such as *B. anthracis*, *S. aureus*, *S. typhi* and *Sh. Dysenteriae*. The obtained MICs were between 25-400 μg mL^{-1}. The antimicrobial compounds showed to be neither a bacteriocin, because it tolerated 121°C for 20 min, nor an organic acid because the antimicrobial activity was stable at pH 7.

The method used in the present study, was optimized for the extraction of antimicrobial compound from lactobacillus from culture media for the first time.

The considerable point in this study was extraction of a compound with antimicrobial activity which was only

dissolved in ethyl acetate but was non-dissolvable in water solvents.

Magnusson and Schnurer (2001) reported detection of *Lactobacillus coryniformis* isolated from grass silage with the antifungal activity against some molds and yeasts. This substances was stable during heat treatment and was retained even after autoclaving at 121 for 15 min. Bringel *et al.* (2005) isolated 40 strains belonging to *Lactobacillus plantarum* from vegetable sources, but not antimicrobial effects was detected in their strains.

Coolborn (2005) could isolate 8 Lactic acid bacteria from food sources and soil by using MRS medium and then detected their antimicrobial substance against some pathogenic bacteria with two methods of well-in agar and paper disc method. He defined that LAB with inhibitory affinity over the bacterial indicators exhibited various degree of inhibitory zones.

In a study, *L. bulgaricus* and *L. casei* were isolated from various foods and their antimicrobial activity were investigated. Culture filtrates exhibited varying degrees of inhibitory activity against some pathogenic bacteria (Erdogrul and Erbilir, 2006).

Anti helicobacter pylori activity of metabolite produced by *L. plantarum* group isolated from white cabbage was confirmed by Rokka *et al*. (2006). Most isolated bacteria showed antibacterial activity against *H. pylori*.

It is expected that the antimicrobial compound of *L. plantarum* was not much different from active compounds of *L. casei*. The MIC values for gram negative and gram positive bacteria indicated that gram positive and negative bacilli were more sensitive to lactobacillus antimicrobial compound than gram positive cocci.

In this study the antimicrobial compound seems to be not a peptide, because it tolerated 121°C for 20 min (Table 3). The antimicrobial activity of these compounds was also stable at 4°C for one year tested. Based on these results, we speculate, better potential for this antimicrobials and also these potential probiotics could be used in food preservation at high temperature and in the therapy of infectious diseases after more experiments on their optimization and their safety.

Table 3: Effect of temperature on antimicrobial activity of *L. plantarum* and *L. casei* extract against *B. anthrasis*

	L. plantarum extract		*L. casei* extract	
Temperature (°C)	IZD (mm)	RA (%)	IZD (mm)	RA (%)
-20	30	100	30	100
4	30	100	30	100
22	30	100	30	100
80	30	100	30	100
100	27	90	27	90
121	27	90	27	90

IZD: Inhibition zone diameter, RA: Relative activity, which was related to inhibition zone diameter of extracts at 4°C

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