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Isolation and Applications of One Strain of *Lactobacillus paraplantarum* from Tea Leaves (*Camellia sinensis*)

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ABSTRACT

The aim of the study was to identify the occurrence of lactic acid bacteria in tea leaves and examined the probiotic activity. Probiotics are live microorganisms that are similar to beneficial microorganisms found in the human gut. Probiotics are available to consumers mainly in the form of dietary supplements and foods. Most probiotics are bacteria and come from two groups namely *Lactobacillus* and *Bifidobacterium*. Owing to the widespread increased interest in finding probiotics from safe resources, several studies have been directed at identifying such microorganisms. In this study, the presence of probiotic microorganisms were investigated in the northern tea leaves of Iran. Samples of tea including green leaves and black tea leaves were collected and screened for identifying lactic acid flora. Microorganisms were isolated by pour plate method on MRS agar plates. The plates were incubated under aerobic and anaerobic conditions at 37°C for 48 h. One rod-shaped bacteria with the potential of lactic acid fermentation and probiotic activity was isolated from both two sources. The bacillus strain identified as *Lactobacillus paraplantarum* according to morphological and biochemical tests and 16s rRNA amplification. This microorganism could inhibit the growth of *Salmonella typhi*, *E. coli*, *Staphylococcus aureous*, *Entrococcus fecalis* and *Citrobacter* sp. successfully. Further more, this strain could produce extracellular tannase which is a beneficial property for probiotic activity. According to this study, we have concluded that, some of the important probiotic microorganisms exist in the tea leaves which could be utilized as a safe source for isolating probiotics.

Key words: *Camellia sinensis*, fermentation, probiotics, lactic acid bacteria, tannase

INTRODUCTION

Lactic Acid Bacteria (LAB) are Generally Recognised as Safe (GRAS) microorganisms that have been used in the processing of fermented food for centuries.

In addition, health beneficial or probiotic effects have been attributed to specific strains and this has been the subject of intensive investigations (Chagnaud *et al*., 2001).

LAB have been isolated from specific habitats, including dairy products, plants, meat products, sewage, manure humans and animals (Noonpakdee *et al*., 2009; Pelinescu *et al*., 2009).

Tea (*Camellia sinensis*, family Theaceae) is consumed worldwide and is second only to water in its popularity as a beverage. Many health benefits have been ascribed to consumption of this

beverage, including the effects of reduction of cholesterol, protection against cardio-vascular disease and cancer (Jaziri *et al.*, 2009). There are some reports for thermal resistance of lactic acid bacteria (Reverson *et al.*, 2005); so they might be remain viable and retain their beneficial properties in beverage compositions, such as those prepared at high temperatures in boiling water.

Isolation of LAB from fermented tea leaves has frequently been reported. The isolation of one strain of *Lactobacillus fermentum* from Thai traditional tea (Klayraung *et al.*, 2008), six strains of *L. pantheris*, five strains of *L. pentosus* and four strains of *L. suebicus* from fermented tea leaves named Miang (Tanasupawat *et al.*, 2007) had been reported in some studies. Furthermore, the isolation of *L. plantarum* from Miang was reported previously (Okada *et al.*, 1986).

Fermentation process for producing most black teas is actually an oxidation process catalyzed by enzymes that are originally present in tea leaves. Because no microorganisms are involved in this kind of enzyme-oxidized black tea, the use of the term fermentation is obviously not completely correct. However, in aged black tea leaves, the bacteria present inherently or natural microbiota, may contribute some fermentation process on that. The distribution of LAB in fresh or black tea leaves has not previously been reported. However, isolation LAB from fresh vegetables was reported by some researchers. For example, *L. bulgaricus* and *Streptococcus thermophilus* were isolated from plants like *Cornus mas*, because this plant is widely used as yogurt-starter in Bulgaria (Michaylova1 *et al.*, 2007). Furthermore, one strain of *L. plantarum* was isolated from honey bee collected pollens (Belhadj *et al.*, 2009) and the isolation of several strains of *L. plantarum* from vegetable plants was reported by many researchers (Mundt and Hammer, 1968; Bringel *et al.*, 2005; Amin *et al.*, 2009).

This study deals with the identification of newly isolated lactic acid bacteria from tea leaves.

MATERIALS AND METHODS

Isolation and identification of *Lactobacillus strains*: Samples of tea leaves were collected from local markets and fields in Lahijan area (Gilan, Iran), November 2009. Appropriately diluted samples with saline solution (0.85% sodium chloride) were plated onto de Man-Rogosa-Sharpe (MRS) agar (Merck, Darmstadt, Germany) and incubated at 37°C under aerobic and anaerobic conditions for 48 h. Based on the phenotypic characteristics (colony characteristics, gram staining, cell shape and catalase production), isolates of lactobacilli were collected. The selected strains were identified by API 50 CHL kit (bioMerieux, Lyon, France) and confirmed by mean of species specific PCR before storing in MRS containing 20% glycerol (BDH Chemical, UK) at -20°C for further studies (Klayraung *et al.*, 2008).

The partial 16S rRNA gene amplification was done for selected strain, using primers 5′-TTTATCGACGCTGAGCATGC-3′ as forward primer and 5′-CCTCCAGGA GTTGTCTCAGG-3′as reverse primer. The reaction mixture (20 mL) contained 30 ng template DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 mM each primer and 1 U Taq DNA polymerase (Fazabiotech, Iran) in a standard reaction buffer. After an initial denaturation of 4 min at 94°C, 25 cycles of 1 min at 94°C, 1.5 min at 50°C, 2 min at 72°C and a final extension at 72°C for 7 min were performed (Bringel *et al.*, 2005).

Detection antibacterial activity: The antibacterial activity of the selected isolate was determined by agar spot on-lawn test. The indicator bacteria used in this study were *Escherichia coli* PTCC 1554, *Salmonella typhi* PTCC 1609, *Staphylococcus aureus* PTCC 1431, *Entrococcus fecalis* PTCC 1237, *E. coli* and *Citrobacter* sp. One microliter of each overnight culture of selected lactobacillus was spotted on MRS plates (containing 0.2% glucose and 1.2% agar) and incubated under anaerobic conditions for 48 h to develop colonies. A portion of 0.25 mL of 1:10 dilution of

an overnight culture of the indicator bacteria was inoculated in 9 mL of Brain Heart Infusion (Merck, Darmstadt,Germany) soft agar (0.7% agar). The medium was immediately poured over the MRS plate on which the tested lactobacillus was grown. The plates were incubated aerobically at 37°C for 24 h. The antibacterial activity was related to the inhibition clear zone diameter (Bujalance *et al*., 2007).

Determining tannase production: Qualitative tannase production was carried out on tannic acid agar plates (TAA) containing 1% tannic acid and 3% agar. The plates were point inoculated and incubated at optimum temperature of Lactobacilli. Clear zones formed due to hydrolysis of tannic acid around the bacterial colony confirmed tannase production (Batra and Saxena, 2005).

RESULTS

One bacillus strain with probiotic activity was isolated from both two sources. Cells were rod shaped and occur singly, in pairs and sometimes in short chains. Colonies grown on MRS agar are round, smooth, dome shaped and creamy colored. Cells are nonmotile, Gram positive, catalase negative and facultatively anaerobic.

The partial 16S rRNA gene amplification was shown in Fig. 1. A single amplification product of 100 bp belongs to this strain.

For tannase production, the clear zone around the bacterium was 18 mm.

For detection of antibacterial activity of isolated Lactobacillus, against test microorganisms, the agar spot on-lawn test was carried out and the inhibition zone diameters were listed in Table 1.

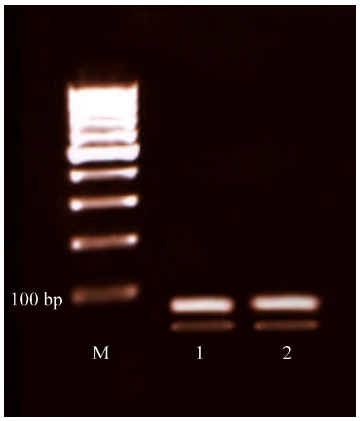

Fig. 1: Amplification products obtained from the *recA* assay. Lane M contained a 1-kb PLUS DNA ladder (Life Technology Inc., Gaithersburg, Md.). Lanes 1and 2 are PCR amplification products (100 bp) from *L. paraplantarum* isolated from tea leaves

Table 1: Antimicrobial activity of *L. paraplantarum*

Assayed bacteria	Zone diameter (mm)[a]
Salmonella typhi	65±1.53
Staphylococcus aureous	30±1.25
Entrococcus fecalis	56±1.25
Citrobacter sp.	55± 1.30
E. coli	60±1.53

[a]Values represent means of diameter of inhibition zones for three independent assays, with their standard deviations

DISCUSSION

The species *Lactobacillus plantarum* has been described as a taxon which exhibits phenotypic and genomic heterogeneity. The characteristic features of *Lactobacillus plantarum* and *L. paraplantarum* were completely described by Curk *et al.* (1996). Based on their identification, our isolate was more likely resemble to the *L. paraplantarum* (Bergey and Holt, 1992) and furthermore, the 16s rRND amplification pattern was similar to the report , described by Torriani *et al.* (2001) for the *L. paraplantarum*. In this study, PCR amplification products of *recA* gene for *L. paraplantarum* was a 107 bp fragment (Torriani *et al.*, 2001). For the amplification of *recA* gene regions, the primers are the same primers, we used.

In a research done by Mundt and Hammer (1968), they concluded that *L. casei*, *L. viridescens*, *L. cellobiosus*, *L. salivarius* and *L. buchneri* do not normally thrive on plant surfaces whereas, *L. plantarum*, *L. fermenti* and *L. brevis* are the most frequently isolated from plants (Mundt and Hammer, 1968). However, Michaylova1 *et al.* (2007) reported on the isolation and characterization of *L. bulgaricus* and *S. thermophilus* strains from plants.

Klayraung and Okonogi (2009) have isolated *Lactobacillus fermentum* from miang that is a kind of traditional fermented tea leaves, widely consumed in northern Thailand as a snack. Furthermore, Okada *et al.* (1986) have isolated *L. plantarum* from miang (Klayraung and Okonogi, 2009).

L. paraplantarum was isolated from Kimchi, a traditional fermented vegetable dish in Korea (Park *et al.*, 2004; Jeong *et al.*, 2007).

Our *Lactobacillus paraplantarum* strain could inhibit the growth of *Salmonella typhi*, *E.coli*, *Staphylococcus aureous*, *Entrococcus fecalis and Citrobacter* sp. However, the inhibition zone for *Staphylococcus aureous* was less than others. The most antibacterial activity of *Lactobacillus paraplantarum* was against of *Salmonells typhi* and *Entrococcus fecalis*. Lee *et al.* (2007) reported the isolation of a *Lactobacillus paraplantarum* strain producing a bacteriocin from kimchi. They found that the bacteriocin, paraplantaricin, could inhibit certain Lactobacillus strains, including *L. plantarum*, *L. pentosus* and *L. delbrueckii* subsp. *Lactis* and it also could inhibit *Enterococcus faecalis* (Lee *et al.*, 2007).

Direct measurement of the colony diameter was a good indicator of the ability of tannic acid utilization as a carbon source due to the tannase activity in the medium (Paranthaman *et al.*, 2009).

The plate method is a qualitative, simple and rapid screening procedure for tannase production. The production of extracellular tannase by Lactobacillus strain is reported by several researchers. In a research done by Nishitani *et al.* (2004), they concluded that the tannase gene is widely distributed within members of the Lactobacillaceae family specially *L. plantarum*, *L. paraplantarum* and *L. pentosus* (Nishitani *et al.*, 2004).

Okada *et al.* (1986) concluded that the peculiar bacterial flora in Miang (a kind of fermented tea leaves), composed exclusively of *L. plantarum*, seemed to be a result of the selection of the lactic acid bacteria with A2pm peptidolgycan type in the cell wall (Okada *et al.*, 1986).

Su *et al.* (2008) studied on the Synergistic effect of green tea extract and probiotics on the pathogenic bacteria like *Staphylococcus aureus* and *Streptococcus pyogenes*. Their results demonstrated that in co-culture studies, a synergistic effect of the probiotic strains and the green tea extract is observed against both *Staph. aureus* and *Strep. Pyogenes* (Su *et al.*, 2008). However, in this study we showed that tea leaves have lactobacillus flora with probiotic activity which could be applied for this purpose either.

CONCLUSION

Based from results obtained in this study, it can be concluded that the *L. paraplantarum* isolated from tea leaves possess a number of interesting important properties that constitute the requirement for their use as high potential probiotics with health-promoting properties but warrant further *in vivo* investigation.

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