



In vitro Assessment of Some Probiotic Properties of *Lactobacillus fermentum* Isolated from Pickled Garlic

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Abstract

Background: Dominant Lactic Acid Bacteria (LAB) originally isolated from traditional non-dairy fermented foods, may harbor unique characteristics such as probiotic properties. In this research, probiotic potential of dominant LAB isolated from pickled garlic was studied.

Methods: After isolation of dominant LAB from pickled garlic produced with apple cider vinegar, the isolate was identified by Polymerase Chain Reaction (PCR). Probiotic properties of mentioned isolate including survival in simulated conditions of gastrointestinal tract; antibacterial effects against *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella enterica*; ability of aggregation with these food-borne indicators; and finally antibiotic susceptibility of the isolate were investigated. Results were also analyzed statistically by SPSS software.

Results: Sequencing results of PCR products identified *Lactobacillus fermentum* as dominant LAB in pickled garlic. *L. fermentum* had 36.30% and 66.08% survival in pH 2 and 0.3% bile salt after 3 and 4 h incubation, respectively. Inhibition zone diameter of *L. monocytogenes* and aggregation ability of the LAB isolate with *L. monocytogenes* was significantly ($p < 0.05$) higher than the other indicators. Furthermore, *L. fermentum* was resistant to norfloxacin, streptomycin, kanamycin, nalidixic acid, and vancomycin.

Conclusion: *L. fermentum* isolated from pickled garlic had high potential for using as probiotic bacteria in food and medicinal applications.

Introduction

Garlic (*Allium sativum*) is a member of family Liliaceae, with a history of human use of over 7000 years for its wonderful savory taste as food flavoring and purported health benefits in traditional medicine (De Castro et al., 1998). For thousands of years, peeled garlic cloves have been stored in vinegar for long period preservation. There are yet several famous vinegar-preserved garlicks in Asia such as Laba garlic in China. Pickling in apple cider vinegar not only preserves garlic but also improves its sensory properties. Furthermore, apple cider vinegar has

prebiotic attributes and so can prepare a good fermented ecosystem for isolating probiotic bacteria (De Castro et al., 1998; Rejano et al., 1997).

Probiotics are non-pathogenic, live microorganisms with health benefit on the host, when consumed in adequate amounts. Increased use of industrial food products instead of traditional fermented foods, leads to reduction of probiotic bacteria (Saarela et al., 2000). Recently, the importance of probiotics in non-dairy products for consumers who were worried about the cholesterol content

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and cardiovascular diseases has been increased. Furthermore, these products due to bioactive compounds derived from the raw materials or metabolites produced during fermentation, have high nutritional value (Chiu et al., 2008; Saarela et al., 2000). Several researchers have investigated the potential probiotic Lactic Acid Bacteria (LAB) isolated from pickles as well as their functional properties. In some studies, LAB responsible for pickled vegetables were characterized and therefore, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *Lactobacillus brevis* and *L. plantarum* were identified as the most abundant isolates (Chiu et al., 2008; Tamang et al., 2009; Wang et al., 2010; Zokaeifar et al., 2012).

Although, the LAB isolates show a direct impact on human health, there is obvious evidence that LAB from different fermented ecosystems harbor different probiotic abilities. Probiotic bacteria should be able to survive and colonize in acidic media and bile salts such as conditions of gastrointestinal tract, have some antagonistic effects against gut food-borne bacteria and be able to aggregate with these pathogens for prevention of their colonization, and should also be safe from antibiotic susceptibility point of view. These functional characteristics of the probiotic bacteria are so important for selection of appropriate isolates to be used as starter or adjunct culture in industrial fermentation (Ouweland et al., 2002; Verna and Lucak, 2010). Therefore, the aims of the present study were to molecular identification and evaluating the probiotic properties of a dominant LAB isolated from pickled garlic.

Materials and methods

Preparation of pickled garlic

In this experimental study, pickled garlic was prepared by packing directly with acidified brine after blanching. Briefly, the bulbs of "purple garlic" purchased from local store were cracked to separate cloves. The garlic cloves were blanched in water at 90 °C for 4 min and immediately packed by pasteurized brine (5.5 min in a water bath at 90 °C). Concentrations of apple cider vinegar and NaCl in the brine were calculated according to Montano et al. (2004) and finally stored under refrigeration (6-9 °C) for 3 weeks.

LAB isolation and molecular identification

After homogenization of pickled garlic by stomacher apparatus (Seward BA7020, USA) in 0.1% peptone, for isolation of dominant LAB, homogenate was aliquot in serial 10-fold dilutions and spread on de Man, Rogosa and Sharpe (MRS) agar at 37 °C for 24 h (Merck, Germany). After incubation, the Gram and catalase tests of

the most abundant bacterial colonies were also checked (Sadeghi et al., 2016). For molecular identification of the isolate, DNA was extracted by DNA extraction kit (Bioneer, AccuPrep K-3032, South Korea) based on the manufacturer protocol and subjected to species specific Polymerase Chain Reaction (PCR). Briefly, optimized PCR reaction was carried out in 25 µl final volume comprises 0.25 µl *Taq* DNA polymerase (2.5 U/µl; Invitrogen, USA), 2.5 µl from 10x PCR buffer (Invitrogen, USA), 1.5 µl MgCl₂ (25 mM; Invitrogen, USA), 0.5 µl dNTPs mixture (10 mM; Invitrogen, USA), 0.5 mM primer pair (Ferchichi et al., 2007), and 2 µl DNA with concentration of 100 ng/µl, as well as amplification of 500 base pair (bp) target sequence from variable region of *16S rDNA* was done based on Heuer et al. (1997) by thermal cycler (Corbett N15128 thermocycler, Australia). It should be noted that primers without guanine and cytosine clamp were used in the present study. Finally PCR products were observed using electrophoresis, then sequenced (MWG, Germany), and sequencing result was analyzed by BLASTN (<http://www.blast.ncbi.nlm.nih.gov/Blast>; Altschul et al., 1990).

LAB survival assay in simulated conditions of gastrointestinal tract

For this purpose, bacterial cells were harvested at 4 °C for 10 min (6000 g), washed twice with Phosphate Buffered Saline (PBS) with pH 7.4. Then, total viable counts of the LAB isolates (2% of washed cell suspensions) under pH 2 and 0.3% oxgall bile (Sigma), respectively after 3 and 4 h incubation at 37 °C (simulated stomach and small intestinal conditions) was evaluated in comparison to control sample (Maragkoudakis et al., 2006).

Antibacterial activity of LAB isolate

To study antibacterial activity of LAB isolate against *Escherichia coli* PTCC 1399, *Staphylococcus aureus* PTCC 1112, *Listeria monocytogenes* PTCC 1298, and *Salmonella enterica* PTCC 1709, the agar well diffusion assay was used based on Herreros et al. (2005). Briefly, into separate Petri dishes of solidified MRS agar, each indicator bacteria was spread and then LAB isolate was added to the 6 mm diameter well on these Petri dishes. The amounts of LAB and indicator bacteria were the same (100 µl, 10⁸ CFU/ml) prepared from broth culture of bacteria according to 0.5 McFarland standard. After 48 h incubation at 37 °C in an aerobic chamber, inhibition zone diameter was used for measuring antagonistic effect of the LAB isolate against indicators.

Determination aggregation ability of the isolate toward indicator bacteria

The aggregation analysis was performed by the spectrophotometry method (Collado et al., 2007). At first, 10⁸

CFU/ml of bacterial culture were harvested by centrifugation (6000 g, 4 °C for 20 min), washed three times with PBS, vortexed and incubated at 37 °C for 4 h. Then, equal volumes of LAB isolate and indicator cells were mixed and absorbance (A600) of the mixtures was controlled during 4 h incubation at 37 °C. Aggregation percentages were calculated as indicated below, where Aind, Alab and Amix represent the A600 of indicators, LAB isolate as well as their mixture after 4 h incubation, respectively.

$$[(A_{ind}+A_{lab})/2-(A_{mix})/(A_{ind}+A_{lab})/2]\times 100$$

Investigation of LAB antibiotic susceptibility

Disc diffusion method was used for determination of LAB resistance to norfloxacin, tetracycline, erythromycin, streptomycin, penicillin, gentamicin, cephalixin, chloramphenicol, clindamycin, kanamycin, ceftriaxone, nalidixic acid, and vancomycin. After preparation of spread plates from LAB suspension (McFarland value of 0.5) on MRS agar, each antibiotic disc (Padtan Teb Co., Iran) was placed on center of the separate plates. These plates were incubated in an anaerobic chamber at 37 °C for 48 h and then inhibition zone diameter was measured. In this method, >20, 15-19, as well as ≤14 inhibition zone diameters (mm) respectively, indicated susceptible, intermediate, and resistant (Rojo-Bezares et al., 2006).

Statistical analysis

All experiments were carried out in triplicate *in vitro*. The level of significance was also analyzed by one way analysis of variance (ANOVA) and post hoc Tukey at $p<0.05$ using SPSS Inc., Chicago, IL, USA (version 16.0).

Results

The gel electrophoresis of PCR products for specific detection of dominant LAB isolated from pickled garlic in present positive and negative controls is shown in Fig. 1. To confirm the identity of the amplicon, sequencing result of PCR product was verified by BLASTN procedure. According to the results, dominant LAB isolate was identified as *L. fermentum*.

L. fermentum isolate exhibited 36.30 ± 5.47 survival percentage after exposure to pH 2. In the bile salt assay, the LAB isolate showed $66.08\pm 4.02\%$ maintenance level.

The antagonistic activity of *L. fermentum* against Gram-positive was significantly more than Gram-negative food-borne bacteria. Furthermore, inhibition zone diameter of *L. monocytogenes* (16.15 ± 0.74 mm) was significantly ($p<0.05$) higher than the other bacterial indicators. *S. enterica* with inhibition zone diameter of 5.45 ± 1.03 mm was also significantly ($p<0.05$) more

tolerant to inhibitory effect of *L. fermentum* in comparison to the other food-borne bacteria (Fig. 2 and Fig. 3).

L. fermentum isolated from pickled garlic showed aggregation abilities with the indicators tested, but the percentages depended on type of pathogen indicator. As illustrated in Fig. 4, the most and the least aggregation abilities of the LAB isolate were also observed with *L. monocytogenes* ($35.39\pm 0.8\%$) and *S. enterica* ($27.83\pm 2.25\%$), respectively, with significant difference ($p<0.05$).

Table 1 shows the antibiotic susceptibility results of *L. fermentum* which was resistant to norfloxacin, streptomycin, kanamycin, nalidixic acid, as well as vancomycin. However, the obtained isolate was interpreted to be sensitive toward erythromycin, penicillin, chloramphenicol, clindamycin, and ceftriaxone.

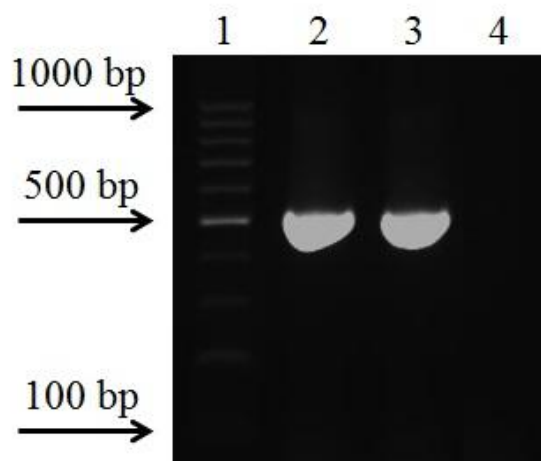


Fig. 1: Agarose gel electrophoresis of PCR products with 500 bp target sequence. Lane 1: 100 bp DNA ladder; lane 2: the amplicon from *Lactobacillus* sp. (PTCC 1332) as positive control; lane 3: the amplicon from single colony of dominant LAB isolate, and lane 4: non DNA as negative control

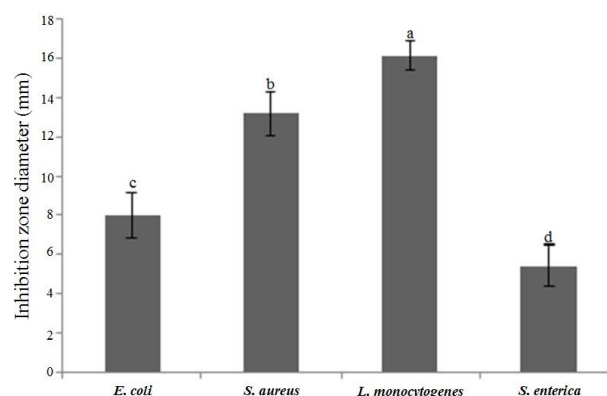


Fig. 2: The diameter of inhibition zone (mm) in well diffusion assay as a result of antagonistic effect of *L. fermentum* against indicator bacteria. Error bars with different letter were significantly different ($p<0.05$)

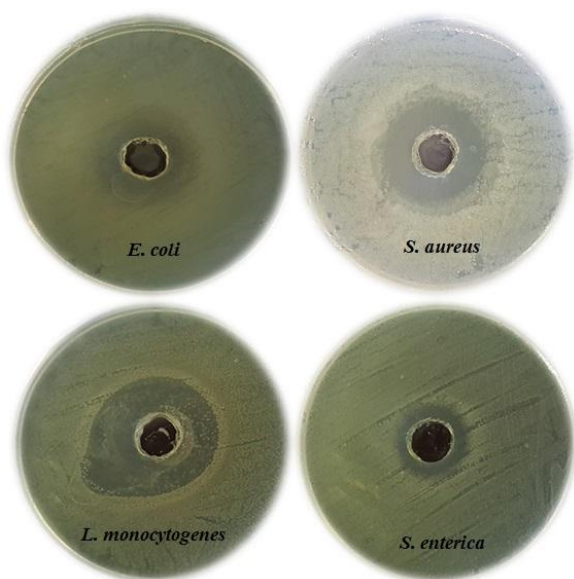


Fig. 3: Results of well diffusion assay for evaluating of antibacterial activity of *L. fermentum*

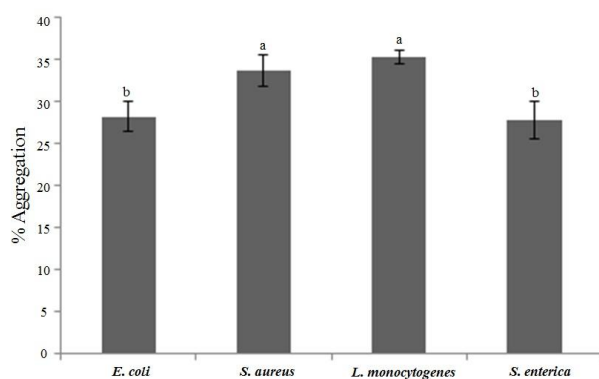


Fig. 4: Percentage of aggregation ability of *L. fermentum* with indicator bacteria. Error bars with different letter were significantly different ($p < 0.05$)

Table 1: Antibiotic susceptibility profile of *L. fermentum* based on inhibition zone diameter in disc diffusion method

Antibiotic	Concentration (μg)	Susceptibility
norfloxacin	10	resistant
tetracycline	30	intermediate
erythromycin	30	susceptible
streptomycin	25	resistant
penicillin	30	susceptible
gentamicin	10	intermediate
cephalexin	30	intermediate
chloramphenicol	30	susceptible
clindamycin	10	susceptible
kanamycin	30	resistant
ceftriaxone	30	susceptible
nalidixic acid	10	resistant
vancomycin	30	resistant

Discussion

Today, the importance of probiotic bacteria isolated from non-dairy products as points of safety (antibiotic susceptibility) and functionality (tolerance to bile salt and acid, antibacterial, and aggregation abilities) is obvious. So, the present study aimed to investigate the probiotic properties of dominant LAB isolated from pickled garlic. *L. fermentum* isolated in this study exhibited fairly proper acid and bile tolerance and had also good antagonistic effects against food-borne indicators. Previous studies showed that dominant LAB in non-aseptic fermented ecosystems, have unique antimicrobial and probiotic characteristics because they can control the growth of undesired microorganism in this conditions (Leroy and De Vuyst, 2004; Vinderola and Reinheimer, 2003).

In the current investigation, *L. fermentum* isolate showed relatively perfect acid and comparatively better bile salt tolerance. Resistance to gastric acidity and bile salts are generally considered as the most important properties for probiotic bacteria to save their functionality in gastrointestinal tract. Although, these characteristics are not adequate to predict the survival of the isolates in the real conditions (*in vivo*), but mentioned properties are considered helpful for screening of probiotic isolates. Resistance of LAB to bile salts is also an important factor for their colonization (Fernandez et al., 2003; Schillinger et al., 2005). It was proved that LAB isolated from pickles usually had fair acid (pH 2 for 3 h) and bile salt (0.3% for 4 h) resistance (Chiu et al., 2008). Based on the results of Angmo et al. (2016), simulated gastrointestinal conditions had a significant impact on the viability of the most LAB isolates and also a progressive reduction was observed at pH 2. In contrast, in Wang et al. (2010) study after exposure to pH 2 and 0.3% bile salt, respectively 75% and the entire LAB isolates were survived. Tulumoglu et al. (2014) reported that the exposure to pH 2.5 for 3 h, lead to complete inactivation of most of *L. fermentum* strains isolated from tulum cheese which survive at 0.25% bile.

Except resistance to bile salts, cell surface hydrophobicity as a physico-chemical factor has also a crucial role in colonization and aggregation of bacteria together. Although, the main adhesion process depends on cell-surface proteins and lipoteichoic acids, but hydrophobicity of cell surface initiates this interaction (Collado et al., 2007; Grzeskowiak et al., 2011). Recent studies demonstrated that LAB hydrophobic/hydrophilic cell wall characteristics are strain specific. The aggregation ability of probiotic bacteria as an antagonistic mechanism has a prevention effect on adhesion and colonization of gut pathogens in gastrointestinal tract (Collado et al., 2007; Grzeskowiak et al., 2011). Although, cell surface hydrophobicity of the LAB isolates often is highly varia-

ble but there are positive correlation between cell surface hydrophobicity, adhesion capacity, and aggregation ability (Angmo et al., 2016). Tamang et al. (2009) stated that among 94 LAB isolates, 77, 10, and 7 strains had <30%, 30-70%, and >70% hydrophobicity, respectively. There are rare reports about aggregation ability of *L. fermentum* strains toward gut pathogens but it was revealed that some strains of this bacterium had high adhesion capacity to the human epithelial cells (Tulumoglu et al., 2014). Similar to some previous published studies (Angmo et al., 2016; Tulumoglu et al., 2014), dominant LAB isolated from pickled garlic in this work revealed good aggregation abilities toward some food-borne pathogens.

As a probiotic bacterium, antagonistic effect against gut pathogens is one of the most important functional characteristics. This antimicrobial potential is due to the hurdle of organic acids, bacteriocins, and other antimicrobial metabolites produced by a probiotic LAB. Organic acids have also a role in health maintaining of the colon. Recently, antimicrobial ability of LAB toward some food-borne indicators has been led to approve their using as food biopreservatives. This indicates that proper probiotic bacteria by reducing the load of pathogens in final products can preserve food from spoilage and subsequently improved consumer's health and safety (Sabir et al., 2010; Zhang et al., 2011). Often, dominant isolated LAB from fermented foods has notable antagonistic effect against gut pathogens (Tamang et al., 2009). Wang et al. (2010) reported that the most of the *Lactobacillus* isolated from pickled cabbage had inhibitory activities against some indicator pathogens (*Bacillus cereus*, *L. monocytogenes*, *S. aureus*, *E. coli*, and *S. enterica*), but the inhibitory effects were variable and few isolates showed weak activity toward *S. enterica*. Tulumoglu et al. (2014) observed that all strains of *L. fermentum* cell-free culture supernatant inhibited *E. coli* but antagonistic effect of isolated strains were different against tested pathogens. The antibacterial activity of LAB isolates in research by Angmo et al. (2016) was variable and no inhibitory effect was observed against *Pseudomonas* sp.

By considering this fact that increasing of antibiotic resistance in pathogenic bacteria, antibiotic susceptibility test for the safety assessment of probiotic isolates is necessary. LAB probiotics in *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* genera are quite sensitive to antibiotics applied usually in clinical treatments. In contrast, resistance to aminoglycosides and quinolones appeared to be intrinsic in some LAB due to absence of cytochrome-mediated electron transport and their membrane impermeability. Resistance to quinolones in some Gram-positive cocci has also been detected for mutation in specific genes (Dzidic et al., 2008; Hummel et al., 2007). Wang et al. (2010) reported that different isolated LAB strains were resistant to different classes of antibiot-

ics. According to Tulumoglu et al. (2014), all isolated *L. fermentum* strains were resistant to vancomycin and some strains were susceptible to clindamycin. Conclusion of Angmo et al. (2016) for isolated *Lactobacillus* strains indicated that the most isolates were almost susceptible to all investigated antibiotics except vancomycin. *L. fermentum* isolate was also resistant to norfloxacin, streptomycin, kanamycin, nalidixic acid, as well as vancomycin. Based on these results, mentioned isolate had high potential for using as a probiotic bacteria but, validation of probiotic properties of the isolate in a real *in vivo* situations in future, will improve the value of the *in vitro* tests.

Conclusion

Proper LAB isolate with good probiotic traits can be used as favorable starter culture for processing of some probiotic products. By considering the fact that pickled vegetables such as other natural fermented foods are routinely consumed as safe vehicle for delivery probiotics, therefore isolation of bacteria from these ecosystems is so remarkable task. According to the results of this *in vitro* assays, *L. fermentum* as dominant LAB isolated from pickled garlic in current study survived in simulated conditions of gastrointestinal tract and also exhibited effective antibacterial activity as well as proper aggregation ability towards tested food-borne indicators. The bacterium was also isolated from a non-dairy product fermented in non-aseptic conditions, implies the importance of traditional fermented foods as a good resource for isolating probiotic bacteria. Furthermore, mentioned foods because of existence of these bacteria have a crucial and vital role in consumer protection from gastrointestinal infections.

Conflicts of interest

There is no conflict of interest in this study.

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