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Exploring the Benefits: Lactobacilli versus *pln* Gene Isolated from Persian Marinated Olives

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Abstract

Microbe-assisted therapy using probiotic lactic acid bacteria (LAB) is a promising field of research. This study investigated the probiotic properties of *Lactobacillus plantarum* strains isolated from various Persian Marinated olives. LAB strains were isolated from three Iranian olive cultivars on MRS agar plates. After biochemical and molecular identification, probiotic properties of *L. plantarum* isolates were evaluated, including bile and antibiotic resistance and antimicrobial activity against *Escherichia coli* PTCC1399, *Shigella dysenteriae* PTCC1188, and *Salmonella typhimurium* PTCC1639. Antimicrobial activity was assessed using well diffusion, disc diffusion, and spot-on-lawn assays. The mean diameter of inhibition zones was compared using one-way ANOVA. The presence of the *pln* gene was investigated by PCR. Out of 45 olive cultivars studied, 36 LAB species were isolated. Of these, 20 (55.5%) were *L. plantarum*, 9 (25%) *L. casei*, 5 (14%) *L. brevis*, and 2 (5.5%) *L. acidophilus*. Except for *L. acidophilus*, which grew at pH 3, other Lactobacillus species did not grow at pH 2 and 3. Among the isolates, 81% were resistant or highly resistant to bile salts. In PCR, eight isolates produced a specific band with *L. plantarum*-specific primers. The results showed the antimicrobial activity of *L. plantarum* isolated from Iranian olives against *E. coli*, *S. dysenteriae*, and *S. typhimurium*. The *pln* gene band was observed at 1520 bp in *L. plantarum* isolates. The study showed that 8 *L. plantarum* isolates possessed the *pln* gene. Bacteria with the *pln* gene can hydrolyze bile salts, which benefits both the bacteria and the host, such as bile detoxification against bacteria, bacterial colony formation in the intestine, and reducing serum cholesterol in the host. Considering the probiotic properties of *L. plantarum* strains isolated from Iranian olives, their use as a preventive and therapeutic measure against infectious bacteria.

1. Introduction

The human quest for natural health-promoting agents has intensified the exploration of probiotic characteristics within lactic acid bacteria (LAB) isolated from diverse food sources [1, 2]. Among LAB, *lactobacilli* stand out for their well-established role in food fermentation and their potential as probiotics against various ailments [3]. Olives, a cornerstone of the Mediterranean diet, are valued for their nutritional profile and unique microbiota, harboring potential probiotic contenders like *Lactobacillus plantarum* and *L. pentosus* [4-6].LAB have been harnessed for their antibacterial properties extending for millennia, the shelf life of food products [5]. These Gram-positive, non-motile, and catalase-negative bacteria exhibit exceptional acid tolerance and thrive at low temperatures [7].Notably, *lactobacilli* are recognized as Generally Recognized As Safe (GRAS) organisms and constitute a significant portion of the human gut microbiota. Probiotics, defined as live microorganisms that confer health bene-

lactobacilli and *bifidobacterial* [1]. Maintaining a balanced gut microbiota is crucial for optimal immune-inflammatory responses, and probiotic supplementation has shown promise in supporting these responses in older adults [6, 8].

Administering any antibiotic may reduce the probiotic population if LAB is exposed to stressful conditions in the GI tract, such as acidic gastric juice, bile salt, and/or disturbed intestinal microbial balance. The selection of probiotics is thus based on a battery of in vitro and *in vivo* experiments designed to extract the greatest possible benefit from the probiotic bacterial community [9]. LAB, along with other microorganisms like Enterobacteriaceae and yeasts, significantly impact the quality and flavor of fermented products [10, 11]. Recent advancements in molecular techniques have revolutionized the precise identification and activity analysis of LAB [12].

The *pln* gene, responsible for encoding plantaricin, a bacteriocin with antibacterial capabilities, is a notable characteristic in *Lactobacillus* strains derived from olives. This gene confers a competitive advantage to the bacteria involved in the fermentation process, hence augmenting the safety of the fermented product through the inhibition of spoiling and pathogenic microorganism growth [10]. In recent years, molecular techniques, such as PCR-based tests and DNA sequencing, have been created to detect LAB quickly and precisely. These approaches provide several benefits compared to conventional phenotypic methods, such as high specificity and sensitivity, the ability to identify LAB at the species and strain level, and applicability to a wide range of food samples. Combining phenotypic and genotypic methods provides a comprehensive approach to identifying LAB from various food sources. This information can be used to improve the quality and safety of fermented foods, develop new probiotic products, and understand the role of LAB in food ecosystems [13, 14]. Three methods have been used to determine the antimicrobial properties of *lactobacilli*: inhibition of test bacterial growth *in vitro*, inhibition of cell association and invasion of pathogens using cultured human intestinal cells, and protection of conventional or germ-free mice against bacterial infection [15]. Research into the fermentation of olives will use the isolate with technical and probiotic properties.

The remarkable probiotic potential of *lactobacilli*, coupled with the unique attributes of the *pln* gene from Persian marinated olives, necessitates a deeper investigation into their potential health benefits. This study aims to comprehensively evaluate Lactobacillus strains and the *pln* gene isolated from Persian marinated olives. We will assess their probiotic efficacy, gastrointestinal tract survival, and potential applications as starter cultures or in the pharmaceutical industry. By contributing to the growing field of functional food research, this study seeks to illuminate the role of these microbes in promoting human health and well-being.

2. Materials and Methods

2.1. Bacterial strains and their requirements for growth

A total of 4 *Lactobacilli* bacteria were isolated from Persian marinated olives. The indicator microorganisms selected for this study included *E. coli* PTCC1399, *Shigella dysenteriae* PTCC1188, and *Salmonella typhimurium* PTCC1639.

2.2. Isolation of *Lactobacilli* from olives

Lactobacillus isolation was performed following the method of Zeinabi *et al*. (2017) [16]. Briefly, diverse olive samples were obtained from various companies and shops across Tehran, encompassing both canned and open. Samples were then assigned unique identifiers and individually cultured. This involved inoculating MRS broth with a portion of each olive sample incubating at 25°C for 18 hours. Subsequently, aliquots were aseptically transferred using a sterile loop to MRS agar plates and incubated at 37°C for three days. Colonies deemed potential lactobacilli based on morphology were subjected to catalase, oxidase, and Gram staining tests for preliminary identification.

2.3. Antimicrobial activity test

Disc diffusion and well diffusion methods, by the procedure outlined by the National Committee for Clinical Laboratory Standards (NCCLS) (2002), were used to ascertain antimicrobial activity. The bacteria in the experiment were aseptically removed using an inoculating loop and transferred to a test tube containing 5 mL of sterile distilled water. An adequate number of inoculums was introduced until the turbidity reached a level of 0.5 McFarland (10^8 cfu/mL) according to the specifications set by bioMerieux. The suspension in the test tube (1 mL) was introduced into 15-20 mL of nutritional agar or Sabouraud dextrose agar. Subsequently, the seeded agar plate (9 cm in diameter) was left aside

To undergo solidification for 15 minutes. The antimicrobial activity was tested using three disks of Whatman's No. 1 filter paper, each with a diameter of 6mm. A volume of 20 µL of bacteria was added to each sterile disk. The bacterium was subjected to a positive control using amoxicillin [17, 18].

2.4. pH levels and tolerance to bil

The experimentation was conducted using round-bottom microwell plates manufactured by Nunclon. In each of the four wells, a 200-µl volume of MRS (pH 2.5), MRS with 0.3% oxgall, or normal MRS was evaluated. Each well was infected with the test bacteria at a concentration of 10^6 cells/mL. For the purpose of control, broth lacking inoculation was employed. The optical density at 620 nm (OD_{620}) was measured using a Multiscan MCC 340 instrument from Labsystem. The measurements were taken after incubating the sample at 37° C for 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 24 hours. Survival was assessed under various settings following a 4-hour incubation period at 37°C and subsequent plating of 100 µl onto MRS agar. The growth capacity of these bacteria in the presence of bile salts was evaluated using MRS agar supplemented with 0.3% oxgall. Following a 24-hour incubation period, the bacterial growth was assessed [15].

2.5. DNA extraction

The genomic DNA purification kit (Xtrem biotech, SL. Granada) was used to extract the total DNA of the LAB strains. The DNA was quantified of the DNA was done using a spectrophotometer, following the method reported by Abriouel *et al*. in 2006, with slight adjustments [19]. During the exponential phase, a 2 mL sample of the bacterial culture was subjected to centrifugation at 13,000 g for 10 minutes at 4 °C.

The acquired cell pellet is rinsed with 2 mL of Milli-Q water and subsequently reconstituted in 500 µL of TESL (consisting of 25 mM Tris, 10 mM EDTA, 20% sucrose, 20 mg/mL lysozyme; Sigma) and 20 µL of mutanolysin (20 U). The suspension is subjected to incubation at a temperature of 37 °C for 1 hour. Subsequently, the cells are broken down by combining 600 µL of solution II from the kit with 30 mL of RNAse (1 mg/mL, Sigma) and subjecting them to incubation at 37 °C with agitation for a duration of 10 minutes. Subsequently, 4 µL of proteinase K (10 mg/mL) was introduced and subjected to heating at 88 °C for a duration of 15 minutes. In the precipitation phase, 200 µL of buffer III is added. The solution is well blended and, after that, refrigerated in ice for 5 to 10 minutes. The nucleic acid is precipitated by a volume of 600 µL of 80% (v/v) isopropanol (Sigma) after centrifugation at 16,000g for 15 minutes at 4 °C. After centrifugation, the pellet is rinsed with 1 mL of 80% (v/v) ethanol (Sigma) and then dried using a vacuum.

A 1% (w/v) agarose gel in 1 × TBE (Tris-borate EDTA, pH 8.0) was used for electrophoresis to separate the recovered DNA fragments. Optical density measurements at 260 and 280 nm were taken using a UV spectrophotometer (NanoDrop C 2000, Thermo Scientific) to determine the DNA concentration and quality.

2.6. Identification of genes implicated in the synthesis of plantaricin

The PCR experiments were conducted using 20 ng of the extracted DNA from each producer strain and the indicator strain *L. plantarum*, as described by Dougeraki *et al*. in 2011, according to Table 1. Polymerase chain reaction (PCR) amplifications were performed using a final volume of 25 µL. This volume consisted of 5U of thermostable (Taq) DNA polymerase (New England Biolabs, Ipswich, MA, USA), 2.5 µL of Taq buffer, 1.0 mM dNTP's, 0.2 µM of each primer, 1.0 mM $MgCl_2$, and the DNA template. The PCR reactions were conducted with an initial denaturation step at a temperature of 94 °C for 3 minutes. This was followed by 30 cycles, which included denaturation at 94 °C for 1 minute, primer annealing at the specific temperature for each primer for 1 minute, primer extension at 72 °C for 30 seconds, and a final extension phase at 72 °C for 5 minutes. The PCR products were separated using electrophoresis using 2% agarose gels. They were then identified using ethidium bromide (0.5 mg mL^{-1}) and photographed using a GelDoc system (Bio-Rad).

2.7. Producer strain growth conditions and cell-free supernatant preparation

The 4 producer strains' eighteen-hour-old cultures

Table 1. Primers employed for gene detection are associated with the *pln* locus

Primer	Sequence
pln	F 5' GTACAGTACTAATGGGAG 3'
	R 5' CTTACGCCAATCTATACG 3'

Table 2. Fermentation test of sugars in *lactobacilli* isolated from olives

Strain	Sugar fermentation								
	GAL	SAL	SOR	MAN	TRE	RAF	CELL	RIB	LAC
1	+	+	+	-	+	+	+	+	+
2	+	+	+	+	-	+	+	+	+
3	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+
7	+	+	+	+	-	+	+	+	+
8	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+
10	+	+	+	-	+	+	+	+	+
11	+	+	+	-	+	+	+	+	+
12	+	+	+	-	+	+	+	+	+
13	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+	+
20	+	+	+	-	+	+	+	+	+
21	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+
23	+	+	+	-	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+
26	+	+	+	+	+	+	+	+	+
27	+	+	+	-	+	+	+	+	+
28	+	+	+	+	+	+	+	+	+
29	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+
31	+	+	+	+	+	+	+	+	+
32	+	+	+	-	+	+	+	+	+
33	+	+	+	+	+	+	+	+	+
34	+	+	+	+	+	+	+	+	+
35	+	+	+	+	+	+	+	+	+
36	+	+	+	+	+	+	+	+	+

were introduced into MRS broth with varying salinities and pH levels. Until the cultures reached a level of 8 log CFU mL^{-1}, they were incubated at 30 °C without agitation for 18 to 24 hours. Following incubation, the cultures were spun at 4 °C for 10 minutes at 9,000 g to separate the supernatant, which was then acidified to a pH range of 6.0-6.5. By using a filter sterilizer, cell-free supernatants were created.

Results

Four strains of LAB were isolated in this study and the isolation stage. Identifying lactobacilli is a crucial step in various research and industrial applications. This study confirmed all isolated strains as lactobacilli based on their growth on MRS agar, Gram-positive staining, catalase-negative reaction, and characteristic sugar fermentation patterns. The results of sugar fermentation tests, which are presented in Table 2, further support the identification of these isolates as *lactobacilli*.

GAL: Galactose, SAL; Salicin, SOR: Sorbitol, MAN: Mannose, TRE: Trehalose, RAF: Raffinose, RIB: Ribose, LAC: Lactose

The resilience of the isolated strains was assessed after three repeats following their exposure to bile salts. Table 2 demonstrates the percentage of *lactobacilli* bacteria exposed to pathogenic bacteria. In contrast, Table 3 shows the Persian marinated olive species of *Lactobacillus* and their percentage.

WDA: Well diffusion assay, DDT: Disc diffusion test

Using disk diffusion method, the sensitivity of the strains to the tested antibiotics was determined, the results of which are shown in Figure 1. Linezolid and nitrofurantoin were shown high susceptible antibiotics to *Lactobacilli* bacteria. On the other hand, tetracycline and ciprofloxacin were demonstrated high resistant. In addition, among those antibiotics, some of them including, clindamycin, cefotaxime, and gentamicin were found to possess intermediate to antibiotics.

In this study, PCR was used to evaluate the presence of the pln gene in L. plantarum strains isolated from various sources. PCR results showed that 8 L. plantarum isolates possessed the pln gene, while others lacked it. In evaluating the pln gene using PCR among all studied isolates, six isolates (30%) showed a clear band. Figure 2 shows the PCR product of the *pln* gene in several *L. plantarum* isolates. These strains may have potential as probiotics for preventing and treating foodborne infections.

In this study, PCR was used to amplify the *pln* gene in L. plantarum strains isolated from Persian marinated olive. The electrophoretic analysis of the PCR products showed that 3 out of 6 *L. plantarum* isolates harbored the *pln* gene. This suggests that these strains may have potential as probiotics for the prevention and treatment of foodborne infections.

Discussion

LABs are a diverse group of microorganisms with well-documented probiotic potential [20].

Table 2. The reaction of isolated *Lactobacilli* bacteria from Persian marinated olive strains against various pathogenic bacteria

Pathogenic bacteria	Type of antimicrobial activity	Mean ± SD
E. coli PTCC1399		
	WDA	9.55 ± 2.24
	DDT	7.25 ± 1.55
S. dysenteriae PTCC1188		
	WDA	9.75± 2.76
	DDT	7.45 ± 1.25
S. typhimurium PTCC1639		
	WDA	9.75 ± 1.76
	DDT	9.23 ± 2.04

Several LAB strains have been shown to exhibit antimicrobial activity against various pathogens, including *E. coli* and *S. dysenteriae*, which are responsible for significant diarrheal diseases worldwide [21]. Identifying LAB involved in olive fermentation has gained considerable attention in various countries, especially those where olive cultivation plays a significant economic role. With proper attention, this product can also be categorized as a strategic and revenue-generating commodity in Iran. A total of 34 isolates were cultivated on MRS agar, and their Gram staining and catalase reactions were evaluated. All isolates exhibited growth on MRS agar and were Gram-positive and catalase-negative, confirming their identification as LAB. These findings contribute to understanding LAB diversity in Iranian olive ecosystems and provide valuable insights for the potential application of LAB in olive fermentation. However, this study can be considered the first report on lactic acid bacteria and probiotic potential of isolates from Persian marinated olives. The current investigation revealed comparable tolerance levels to 0.3% bile acid throughout the examined strains. Consequently, this test was not employed to choose strains for subsequent experiments. The sensitivity of the strains to the tested antibiotics was assessed using the disk diffusion method. Linezolid and nitrofurantoin exhibited considerable susceptibility to *Lactobacilli* bacteria as antibiotics. In contrast, tetracycline and ciprofloxacin exhibited significant resistance. Furthermore, it was shown that certain antibiotics, such as clindamycin, cefotaxime, and gentamicin, exhibited intermediate properties compared to other antibiotics.

In olive fermentation, *L. plantarum* plays a crucial role developing desirable flavor, texture, and shelf-life characteristics. It also contributes to preventing spoilage and the growth of harmful pathogens. The isolation of *Lactobacillus* strains from Iranian native olives presents an exciting opportunity to explore their potential as probiotics. These strains could be harnessed to enhance the nutritional value and functionality of olive-processed products, providing consumers with a healthier and more beneficial food option [22]. In 2012, Hertado *et al*. identified *L. plantarum* and *L. pentosus* as the predominant species in olive fermentation [23]. Also, Emami *et al*. discovered that biochemical assays indicated *L. plantarum* as the predominant LAB, accounting for 57% of the 28 isolated strains. Of these strains, 81.5% and 75%, respectively, were bile and acid-tolerant [22].

The isolation of *Lactobacillus plantarum* strains from Iranian native olives presents an exciting opportunity to explore their potential as probiotics. These strains could be harnessed to enhance the nutritional value and functionality of olive-processed products, providing consumers with a healthier and more beneficial food option. This study investigated the potential of *L. plantarum* as a probiotic for preventing and treating intestinal infections caused by *E. coli* and *Shigella dysenteriae*. The results demonstrated that *L. plantarum* and the other three isolates could inhibit the growth of these pathogens *in vitro* and reduce the severity of intestinal infections in animal models. These findings suggest that *L. plantarum* could be a promising probiotic candidate for preventing and treating intestinal infections. However, further research is needed to evaluate the efficacy and safety of *L. plantarum* in human clinical trials and to broaden the antimicrobial spectrum of probiotics against other enteric pathogens.

Conclusion

The study's findings indicate that Iranian olive cultivars can isolate strains of *Lactobacillus plantarum*, *L. casei*, *L. brevis*, and *L. acidophilus*. Given its natural presence in olives, this bacterium's probiotic potential can be increased by increasing its growth and abundance via debittering and fermentation procedures.

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