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Isolation and identification of *Lactobacillus plantarum* from vinegar, by specific *RecA* gene and its anticancer activities

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Abstract
The rising demand for non-dairy probiotic products is driven by factors such as vegetarian diets, concerns about high cholesterol in milk and lactose intolerance. This research investigated the presence of *Lactobacillus plantarum* in apple, pear, and quince vinegar using molecular and biochemical methods. Isolated microorganisms were evaluated for probiotic potential based on their ability to grow at different bile salt concentrations and pH levels. Biochemical characterization included sugar fermentation profile, presence of extracellular enzymes and antibiotic susceptibility testing. Molecular identification of strains employed specific *L. plantarum recA* (Recombinase A) primer targeting the *recA* gene, which encodes a multifunctional protein essential for bacterial cells. Among the 24 microorganisms isolated from apple, pear, and quince vinegar, nine strains displayed a specific band with the *L. plantarum recA* primers, confirming their identity. These Gram-positive bacteria were positive for lipase and protease activity but negative for catalase, amylase, gelatinase, and oxidase. The *L. plantarum* strains fermented all tested sugars except xylose and demonstrated tolerance to acidic and bile-containing environments, high temperatures, and salt concentrations. While resistant to eight of the 15 antibiotics tested, the bacteria showed relative sensitivity to three. Furthermore, they exhibited anti-proliferative effects on human HT-29 cancer cells, suggesting potential as anticancer agents. This study successfully isolated *L. plantarum* strains from apple, pear, and quince vinegar with promising probiotic and anticancer properties.

Key words: probiotic, *Lactobacillus plantarum*, anti-cancer, vinegar.

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Introduction:

Probiotics are living microorganisms that, when consumed in sufficient quantities, have a beneficial effect on the health of their host (1). These bacteria are resistant to the acidic environment of the digestive tract, mainly by neutralizing harmful bacteria. This, in turn, contributes to a harmonious balance of intestinal flora, promoting health benefits and growth in both humans and livestock. The multiple roles of these bacteria include antimicrobial activity, reduction of serum cholesterol , improvement of metabolism, anti-mutagenic properties, stimulation of the immune system, improvement of inflammatory intestines diseases, anti-cancer and anti-diarrheal properties, synthesis of biomaterials, differentiation of stem cells and suppression of *Helicobacteria* (2, 3, 4, 5, 6, 7).

Probiotic bacteria show efficacy not only in human and animal systems, but also in plant environments, offering significant benefits such as soil fertilization and commercial use for biological control of plant diseases (8). The ability of these bacteria to decompose insoluble phosphorus in the soil and make it available to plants has also been demonstrated (9). While the historical use of probiotics can be traced back to fermented dairy products, particularly in the human diet, recent years have witnessed a surge of interest in the application of probiotics in both the nutritional and agricultural fields. The selection of novel probiotic strains and the exploration of new applications have become paramount (10). Although dairy products have traditionally been the primary source of probiotic-rich foods, recent research has revealed the presence of probiotic bacteria in non-dairy sources (11). The escalating demand for non-dairy probiotic products, driven by dietary choices such as vegetarianism (avoidance of meat products) and the challenges posed by high cholesterol in milk and lactose intolerance in certain individuals, has underscored the need to prioritize research into the development of plant-based probiotic products (10). For the purpose of this study, vinegar is of primary interest.

The word *vinegar*, derived from the words "vin" meaning "wine" and "egar" meaning "sour", is a French word meaning "sour wine" which is produced in different ways using different raw materials. In terms of production, vinegar is obtained by alcoholic fermentation, then acetic fermentation of sugar substances. Fruit vinegar, in particular, is important in the field of food and agricultural products, as it is used both as a preservative and as an additive in the food industry (12). Vinegar contains organic acids such as malic acid and acetic acid, as well as flavonoids such as camperol, epicatechin, catechin, anthocyanin, and quercetin. Research suggests that these components have the potential to inhibit blood pressure increases and counteract toxic substances in the body (13). *Lactobacilli* are rod-shaped bacteria that are gram-positive and catalase and oxidase negative (14). These microorganisms are found in a variety of fermented plant, dairy, and meat products. The presence of these bacteria as probiotics in the digestive tract has also been demonstrated. *L. plantarum* is one of the most important species of lactic acid bacteria due to its great ability to be compatible with and adapt to different niches. The proven health effects of using *L. plantarum* in foods include the reduction of gastrointestinal infections and the risk of gastrointestinal inflammatory diseases and the stimulation of the immune system. Studies have shown that *L. plantarum*, as a natural inhibitor of bioprocessed foods, inhibits the growth of pathogenic bacteria and spoilage microorganisms during storage and increases the shelf life of the product (15).

The protein RecA (Recombinase A) in prokaryotic cells is an active protein that has multiple functions in a bacterial cell. The coding gene for this protein was first recognized in *E. coli*, and attempts to find the equivalent of this gene in bacteria led to the identification of a large number of these proteins. RecA is a small protein that has been shown to have multiple functions in DNA binding (single-stranded and double-stranded), coupling and exchange of homologous DNA, and hydrolysis of ATP (16). This primer has been used and confirmed for the study of different species of *Lactobacillus*, especially *L. plantarum* (17). In the selection of probiotic bacteria, evaluation of their resistance to antibiotics is a critical safety consideration for bacterial application. In assessing resistance to gastrointestinal conditions, these microorganisms must overcome the challenges posed by acidity and bile. If they demonstrate substantial survival capabilities under these conditions, their resistance to antibiotics is considered a valuable trait (18). Given the importance of the role of bacteria in the production of commercial and industrial enzymes and the role of these enzymes in the human and animal gastrointestinal tract, the ability of several *L. plantarum* bacteria to produce certain enzymes has been investigated in vitro (19, 20, 21).

The current study aimed to determine the presence of *L. plantarum* in apple, quince and pear vinegar by biochemical and molecular methods using specific primers and their anticancer effect on colon cancer cells.

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Materials and Methods:

1.1. Sample collection , preparation, cultivation and incubation :

We collected samples of homemade apple, quince, and pear vinegars from local sources in Isfahan province. These samples were stored at room temperature until the time of collection. We then transported them to the Biotechnology Laboratory of Bu-Ali Sina University in Hamedan. The 10^{-4} serial dilution of the samples was prepared using the sterile physiological serum. For the growth of probiotic bacteria (*Lactobacilli* in particular) 100 µl of different dilutions were cultured on special de Man, Rogosa and Sharpe culture media (MRS; Merck, Germanyand the media were incubated at 37°C for 72 hours (22).

1.2. Morphological characteristics:

After Gram staining, the characteristics of each colony and cell were examined and recorded under a light microscope. Colonies similar in appearance to *Lactobacillus* with the bacterial characteristics of rod-shaped, gram-positive and non-sporulated were selected. To confirm the purity of the bacteria, each strain was subcultured several times in the MRS medium (14).

1.3. Fermentation:

Acid production from sugars was analyzed using an MRS medium without meat extracts and glucose. For this purpose, 2 ml of a special MRS medium for fermentation containing 1% sugar and phenol reagent was added to each tube (using a Durham tube). After inoculation with 1% of the active bacterial isolate, the tubes were kept in the incubator under 5% carbon dioxide gas at 37ºC for a period of 72 hours to one week. The color change of the MRS medium from red to yellow and the formation of bubbles in the Durham tubes were considered as consumption of sugar and production of acid (23).

1.4. Evaluation of probiotic properties:

The ability of bacteria to grow in MRS medium with pH 3.5, 4, 4.5and 5 was tested. HCl (8N) was used to adjust the low pH. The ability of bacteria to grow in the presence of 0.3 and 0.5% Oxgall was simultaneously evaluated. For these experiments, 1% active culture was added to the MRS liquid culture medium supplemented with Oxgall. The cultures were incubated at 37ºC for 72 hours. The growth ability of the isolates was evaluated by observing the turbidity levels at 24, 48 and 72 hours and the results were recorded as positive-negative values (24). The growth ability of *Lactobacillus* isolates was investigated at 15, 20, 40 and, 45ºC, and 4.5% and 6.5% concentrations of NaCl (Sigma) . Tests were performed according to the method Adikari et al. (2021) (14).

1.5. Antibiotic resistance:

Antibiotic resistance of *Lactobacillus* isolates against 15 different antibiotics was evaluated. The antibiotics included tetracycline (30 µg), vancomycin (30 µg), ciprofloxacin (5 µg), penicillin (10 µg), amoxicillin (25 µg), co-amoxiclav (30 µg), ceftriaxone (30 µg), erythromycin (15 µg), ofloxacin (5 µg), nalidixic acid (30 µg), tobramycin (10 µg), rifampicin (5 µg), nitrofurantoin (300 µg), cefalexin (30 µg), chloramphenicol (30 µg). The experiment was performed according to the method of Cebeci et al. (2003). 200 µl of enriched culture was added to 4 ml of soft agar MRS medium (containing 1% agar). The soft agar was then poured onto pre-prepared plates (containing 15 ml of solid MRS medium (1.5% agar)). Antibiotic discs were placed on the agar surface and incubated at 37ºC. After 24 hours, the diameter of the inhibition zone around the discs was measured and the results were recorded as resistant, semi-sensitive and sensitive (25).

1.6. Screening of extracellular enzymes from *L. plantarum* bacteria:

The production of extracellular enzymes by *L. plantarum* bacteria was investigated by screening digestion of the specific substrate of amylase (20), lipase (19), gelatinase and protease (21) enzymes. Isolates were cultured on solid MRS medium and incubated for 48 hours. They were then cultured linearly on a specific enzyme medium and incubated at 37ºC for 5-10 days. The results of the presence or absence of an enzyme were recorded as positive or negative, respectively.

1.7. Molecular identification:

Bacterial DNA was extracted and purified using the slightly modified method of Chagneud et al. (2001). 10 ml of bacterial suspension (from 24h culture) was centrifuged at 13000 rpm for 10 min. The pellets were transferred to microtubes and 1 ml of CTAB buffer at 65 ºC (CTAB powder (2%), 1M Tris-HCl pH 8, 50mM Na2EDTA pH 8 and 5M NaCl) was added. Samples were placed in a warm water bath at 60ºC for half an hour, stirring every 5 minutes. A volume of chloroform: isoamyl alcohol (24:1) equal to that of the samples was added and the tubes were vortexed. The samples were placed on a shaker for 10 minutes and the tubes were shaken by hand every minute. The tubes were then centrifuged at 13000 rpm for 5 minutes. At this stage, three phases were formed and the upper aqueous of each sample was gently transferred to the new tubes. After adding a volume of the pure cold isopropanol equal to that of upper aqueous, the contents of each tube were gently mixed by inversion and placed on ice for 10 minutes. For DNA sedimentation, samples were centrifuged at 13,000 rpm for 10 minutes at 4°C. The upper liquid

layer was slowly removed and 500 µl of 70% ethanol was added to each tube. The samples were centrifuged at 13,000 rpm for 5 minutes and the supernatant was slowly removed. The tubes were then exposed to air for one hour to dry the DNA. After ensuring that the sediment was dry, 100 µl of sterilized deionized distilled water was added to each microtube and the samples were placed in a refrigerator at 4ºC for one night. For longer storage, samples were transferred to a freezer at -20ºC. The quality of the extracted DNA was confirmed by electrophoresis on a 1% agarose gel and by light absorption (26). Each 25 µl, PCR reaction mixture contained 7 µl of DNA samples. Specific PCR primers, a forward specific primer (planF; 5'-CCGTTTATGCGGAACACCTA-3') and (pREV; 5'-TCGGGACCAAACATCAC-3'), were used to identify *L. plantarum* isolates (previously identified based on phenotypic and biochemical characteristics). The primers were purchased from SinaGene Co. The specific primer designed by Torriani et al. (2001) was used based on a specific sequence of the *recA* gene to identify *L. plantarum*. The final concentration of each primer in the PCR mixture was 1 mM. For this purpose, 0.5 µl of primer solution was added to each reaction mixture. The master mix (Amplicon, Denmark) containing dNTP, MgCl2, Taq polymerase enzyme and buffer was used. The PCR (Techne, UK) was programmed as follows: Denaturation at 94°C for 3 minutes, followed by the first stage amplification reaction in 30 cycles: denaturation at 94°C for 30 seconds, primer annealing at 51°C for 45 seconds and the elongation step at 72°C for 30 seconds and final expansion at 72°C for 5 minutes. Five µl of samples were applied to 1% agarose gel wells containing green viewer, and electrophoresed in a TBE buffer, at a constant voltage of 80 volts. After 1.5 hours, the voltage was removed and the gel was photographed in the gel duct system using ultraviolet light.

1.8. Evaluation of cell proliferation by MTT assay

Cultured bacterial cells (A1) were centrifuged at 5,000 g for 15 minutes, and the resulting supernatants were filtered through a 0.45 µm filter. These cell-free supernatants were then subjected to freeze-drying. Different concentrations (2, 5, and 10 mg/ml) of the cell-free supernatant were prepared for cell viability assays. The HT-29 human colon cancer cell line was obtained from the Pasteur Institute of Iran in Tehran. HT-29 cells were cultured in RPMI medium (Sigma, Germany) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After reaching confluence, the cells were detached using trypsin-EDTA. The MTT assay is based on the reduction of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Merck, Germany), by actively growing cells. In the experiment, 180 µl of cells were seeded in 96-well microplates at a concentration of 2×10^4 cells/ml and incubated for 24, 48 and 72 hours. Then, 20 µl of different concentrations of cell-free supernatants were added and the cells were further incubated at 37°C. Bradford's solution was used to quantify the protein content in a mixture. The preparation consisted of thoroughly dissolving 0.01 grams of Coomassie Blue G-250 color powder in 5 ml of 95% ethanol. Then 10 ml of phosphoric acid (85%) was added to the mixture. The volume was then adjusted to 100 ml with distilled water. The resulting solution was carefully stored in a dark container, protected from light, and kept at refrigeration temperature. To convert the numerical value obtained from the quantitative protein measurement using the Bradford solution by colorimetry to the final concentration of protein expressed in milligrams per milliliter of solution, the light absorbance was compared to a standard curve. After 24, 48 and 72 hours of incubation, 20 µl of MTT solution (5 mg/ml) was added to each well and incubated for a further 3 hours. The culture medium was then removed and the blue formazan crystals were solubilized with 150 µl of dimethyl sulfoxide (DMSO, Merck, Germany). MTT was converted to formazan by metabolically viable cells, and its absorbance was measured at 540 nm using an ELISA reader (27, 28).

1.9. Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate the experimental data, with differences considered significant at a significance level of $P < 0.05$.

Results:

1.10. Biochemical characterization of isolated bacteria:

Various microorganisms, including Gram-positive cocci, Gram-positive and -negative rod bacilli, and yeasts, thrived on the MRS medium see Table 1). Among the bacteria that exhibited a bacilli-like microscopic morphology, were positive for Gram staining, and were negative for both catalase and oxidase assays, the strains identified as *Lactobacillus* were selected for subsequent experiments. These *Lactobacillus* bacteria were used in the remaining phases of the study. The fermentation of sugars by the bacilli bacteria is detailed in Table 2. The sugar fermentation pattern of the nine isolates (A1, A7, A8, P2, P5, P6, Q3, Q4, Q5) was consistent with the characteristics of the *L. plantarum* species mentioned in the systematic classification of Bergey (23).

Table 1- Growth of microorganisms on a specific culture medium of apple, pear and quince vinegar

Strain	A1	A2	A3	A4	A5	A6	A7	A8	P1	P2	P3	P4	P5	P6	P7	P8	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8
Cell shape	bacilli	yeast	cocci	bacilli	yeast	cocci	bacilli	bacilli	yeast	bacilli	bacilli	yeast	bacilli	bacilli	cocci	cocci	yeast	yeast	bacilli	bacilli	bacilli	cocci	cocci	cocci
Gram	positive	-	positive	positive	-	negative	positive	positive	-	positive	positive	-	positive	positive	negative	positive	-	-	positive	positive	positive	positive	positive	negative

A; Apple vinegar strains, P; Pear vinegar strains, Q; Quince vinegar strains

Table 2- Use and fermentation of different sugars.

							Sugars							
Strains	Sorbitol	Mannose	Xylose	Gluconate	Manitol	Cellubiose	Arabinose	Threhalose	Raffinose	Melebiose	Fructose	Lactose	Galactose	Ribose
A1	+	+	-	+	+	+	+	W	+	+	+	+	+	+
A4	+	+	-	+	+	+	-	+	+	-	+	+	+	+
A7	+	+	-	+	+	+	W	+	+	+	+	+	+	+
A8	+	+	-	+	+	+	+	+	+	+	+	+	+	+
P2	+	+	-	+	+	+	+	+	+	+	+	+	+	+
P3	-	+	+	+	-	+	+	+	+	+	+	+	+	-
P5	+	+	-	+	+	+	+	+	+	+	W	+	+	+
P6	+	+	-	+	+	+	+	+	+	+	+	+	+	+
P8	+	W	-	+	+	+	+	+	+	+	+	+	+	+
Q3	W	+	-	+	+	+	+	+	+	+	+	+	+	+
Q4	+	+	-	+	+	W	+	+	+	+	+	+	+	+
Q5	+	+	-	+	+	+	+	+	+	+	+	+	+	+

+; growth, -; lack of growth, w; poor growth.

1.11. Molecular identification of selected strains:

Previous research results indicate that the use of a set of *recA* gene sequences as primers for the identification of *L. plantarum* species results in the formation of a distinct band at 318 base pairs. This particular primer was designated as the PlanF primer. Nine isolates consistent with the characteristics of the *L. plantarum* species were selected and tested with PlanF using *recA* gene-specific primers. Judging by the formation of a specific band in the range of 318 bp in the electrophoresis of the PCR products, consistent with Torriani et al. (2001) (16), it appears that these nine isolates belong to *L. plantarum* (Figure 1).

The NCBI sequence of *L. plantarum* partial mRNA for RecA protein (*recA* gene) was "ACTAGACC**CCGTTTATGCGGAACACCTA**
GGGGTCAACATTGATGACCTGTTACTTTCGC
AACCAGATACTGGTGAACAAGGGCTTGAAA
TTGCAGATGCCTTAGTTTCCAGTGGTGCGGT
CGATATTTTAGTTGTTGACTCGGTGGCGGCC
TTAGTGCCACGTGCCGAAATTGAAGGTGAA
ATGGGTGACGCACACGCTGGGTTACAAGCG
CGGCTGATGTCACAAGCGCTCCGGAAGTTA
TCAGGGACATTGAACAAAACCAAGACAATC
GCGTTATTTATCAATCAAATTCGTGAAAAA
GTTGGTGTGATGTTTGGTAAT

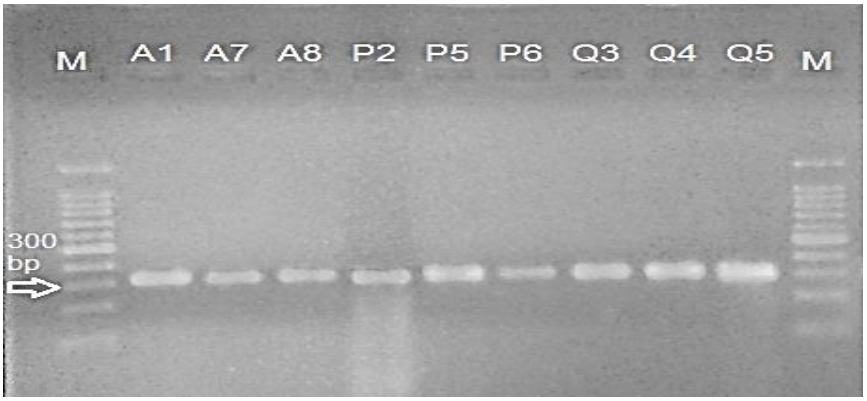

Figure 1- 318 bp bond related to specific planF primer in *L. plantarum* PCR product electrophoresis was formed. M; 100 bp marker.

1.12. Evaluation of probiotic properties:

Almost all nine *L. plantarum* isolates grew well at 20 and 40ºC after 24 hours of incubation (Table 3). Their growth at 15ºC was detectable after 48 hours, but no growth was detected at 45ºC. Bacterial growth was observed at 4.5% and 6.5 % salt concentrations. At 4.5% concentration, the growth turbidity of all bacteria was very high and similar to the control samples. Most of the bacteria could grow well at 6.5% salt concentration. All isolates grew well in the presence of 0.3% Oxgal, but weak growth was observed in 0.5% Oxgal medium (Table 3). The results showed that the bacteria grew well at all pHs (Table 3).

Table 3- *Lactobacillus* growth at different temperatures, salt and oxgall concentrations and pH.

Stains	15ºC	20ºC	40ºC	pH 2	pH 3.5	PH 9.6	Oxgall 0.3%	Oxgall 0.5%	Salt 4.5%	Salt 6.5%
A1	+	+	+	-	+	-	+	-	+	W
A4	+	+	+	-	+	-	+	-	+	-
A7	+	+	+	-	+	-	+	W	+	+
A8	+	+	+	-	+	-	+	W	+	+
P2	+	+	+	-	+	-	+	W	+	+
P3	+	+	W	-	+	-	+	W	+	W
P5	+	+	+	-	+	-	+	-	+	+
P6	+	+	+	-	+	-	+	W	+	+
Q3	+	+	+	-	+	-	+	W	+	W
Q4	+	+	+	-	+	-	+	W	+	+
Q5	+	+	+	-	+	-	+	-	+	+

+; growth, -; lack of growth, w; poor growth.

1.13. Antibiotic resistance:

Based on the results of the *L. plantarum* resistance test against antibiotics, the isolates were resistant to vancomycin, ciprofloxacin, ofloxacin, nalidixic acid, tobramycin, nitrofurantoin, gentamicin, kanamycin, semisensitive to penicillin, amoxicillin, cefalexin and were sensitive to tetracycline, co-amoxyclave, ceftriaxone, chloramphenicol, cifampicin, crythromycin.

1.14. Screening of extracellular enzymes:

The screening of extracellular enzymes in *L. plantarum* bacteria revealed their inability to degrade starch and gelatin. However, when cultured in a medium containing skim milk, these bacteria demonstrated the ability to produce protease enzymes as evidenced by the formation of a distinct clear zone around the colony. Furthermore, the growth results of *L. plantarum* isolates on a medium containing Tween 80 indicated the bacteria's ability to produce the lipase enzyme.

1.15. Anti-proliferative effects of cell-free supernatants of *Lactobacillus plantarum* A1

Figure 2 illustrates the anti-proliferative effects of bacterial cell-free supernatants at different concentrations on the conversion of MTT tetrazolium salt in cells after 24, 48 and 72 hours of incubation in comparison to the control groups. For each concentration tested, an equal volume of MRS broth, as used in the test sample, was used as a control. Cells were exposed to increasing concentrations of bacterial cell-free supernatants, and cell survival was evaluated using the MTT

assay after 24 hours of incubation. It is noteworthy that all tested concentrations of *L. plantarum* (A1) cell-free supernatants (2, 5, 10 mg/ml) showed significant ($P<0.05$) inhibitory effects on HT-29 cells compared to the control groups. Therefore, we evaluated the effect of the samples on HT-29 cells using the MTT assay. The results of the MTT assay indicated that, in general, the cell-free supernatant at a concentration of 2 mg/ml showed the least cytotoxicity (35% compared to the control), while the concentration of 10 mg/ml showed the highest ability to inhibit cancer cells (56% compared to the control) (Figure 2). Figure 3 shows the change in cell morphology and density before and after treatment with probiotic *L. plantarum*.

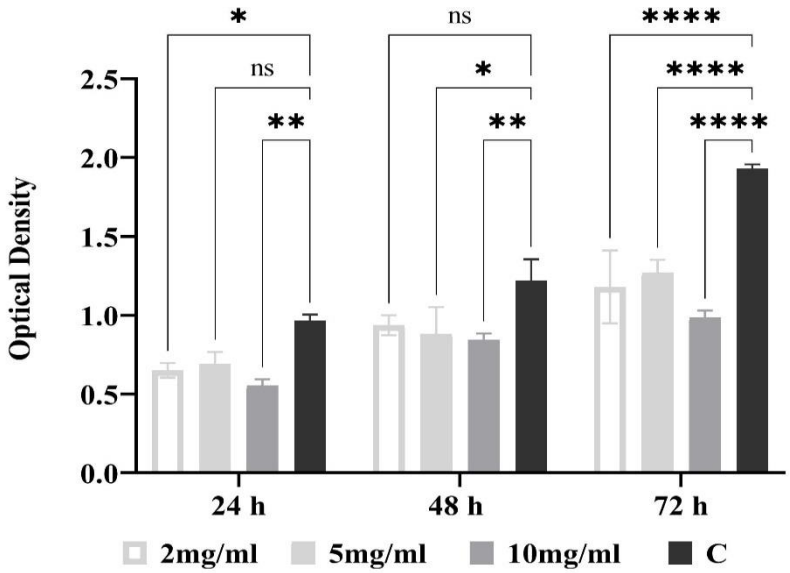

Figure 2- Effects of 2, 5 and 10 mg/ml of cell free supernatant of *Lactobacillus plantarum* A1 on the viability of the colon cancerous cell line HT-29 .

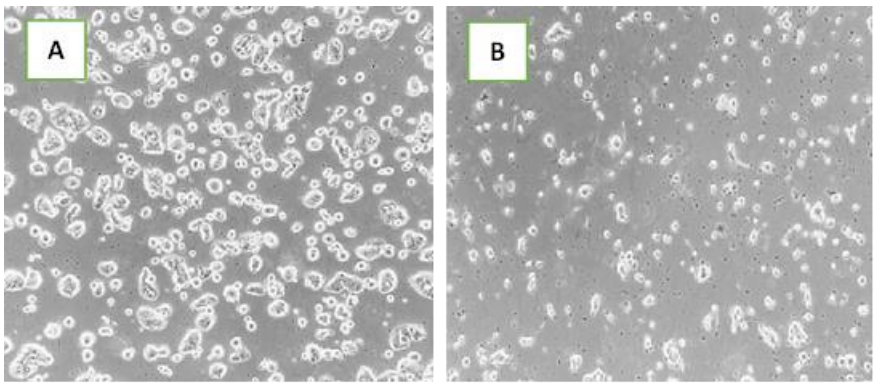

Figure 3-Morphological and cellular changes of cancerous cell line HT-29 treated with 10 mg/ml cell-free supernatant of *Lactobacillus plantarum* A1 after 72h (magnifications ×20). A; control, B; treated cells.

Discussion

The identification of lactic acid-producing bacteria from diverse sources is of great importance in advancing research in various fields, including medicine, food industry, and agriculture (29). The extensive genome and robust enzymatic capabilities of *L. plantarum*, among the *Lactobacillus* species, contribute to its presence in diverse conditions and environments (30, 31). The biochemical tests conducted in this study demonstrated the presence of nine isolates with the characteristic *L. plantarum* in apple, pear, and quince vinegar, exhibiting the capacity to ferment the majority of the utilized sugars. In supplementary molecular investigations employing a *recA*-specific primer, the existence of a specific bond was discerned in nine isolates of this species. In the study by Torriani et al. (2001) (16), the 322-bp recA fragment sequences were

aligned, and four regions were selected for the design of species-specific primers. PCR tests employed a single reverse primer (pREV) and three forward primers (paraF, pentF and planF). The positioning of the forward specific primers on the fragment sequences (numbered 1 to 322) was as follows: planF, the specific primer for *L. plantarum*, spanned nucleotide positions 9 to 28; pentF, the specific primer for *L. pentosus*, covered nucleotide positions 109 to 127; and paraF, the specific primer for *L. paraplantarum*, encompassed nucleotide positions 220 to 239. Consequently, the expected amplicon sizes were 318 bp for *L. plantarum*, 218 bp for *L. pentosus*, and 107 bp for *L. paraplantarum*. Subsequent investigations confirmed, the efficacy of the PlanF primer as a specific primer for *L. plantarum*. These results together with biochemical outcomes showed that nine strains of A1, A7, A8, P2, P5, P6, Q3, Q4, and Q5 are likely to be *L. plantarum*. So far, this bacterium has been extracted from a variety of sources, including dairy products, breast milk, swine intestine, and fermented olives (32, 33). De Vries et al. (2006) investigated the acid tolerance of these bacteria and observed that they were unable to thrive at a pH below 4. In our current study, the isolated bacteria exhibited growth patterns that closely resembled those of the control samples at pH 3. This is likely due to the influence of the acidity of the bacterium's initial environment, which was the surrounding vinegar.To provide further contextualization of these findings, a comparison was made with the research conducted by Sajedinejad et al. (2015) on *Lactobacillus* bacteria extracted from the mouth, which confirmed their compatibility (34). In the investigation conducted by De Vries et al. (2006) (30) on the growth of *Lactobacillus* in different salt concentrations, the bacteria exhibited poor growth, particularly at a 5% concentration. Furthermore, their survival rate notably declined in higher salt concentrations. In contrast with the aforementioned findings, the present study demonstrated that the bacteria exhibited robust growth at a 4.5% concentration, with some isolates even displaying significant growth comparable to the control sample at a 6.5% salt concentration. Additionally, the isolates demonstrated notable resistance to a bile-containing medium, indicating that Lactobacilli isolated from apple, pear, and quince vinegar may possess the capability to endure the gastrointestinal environment. It is important to acknowledge, however, that making direct comparisons and establishing precise correspondences between acid and bile acid resistance results across various studies and natural bodily conditions can be challenging (24). *Lactobacillus* demonstrates the capacity to to proliferate across a temperature range of 15°C to 45°C, suggesting its potential to survive in apple, pear and quince vinegar (across seasonal variations) and in diverse body temperature conditions (even during fever). Kurniati et al. (2015) (35) posited that the proteolytic capability of probiotic bacteria within the gastrointestinal tract, resulting in the production of essential peptides and amino acids for growth, represents a beneficial and advantageous trait within this bacterial group. This capacity represents a noteworthy attribute of *L. plantarum*. Several studies, including the work by Kurniati et al. (2015), have demonstrated the presence of protease enzymes in *L. plantarum*. The present study has confirmed that *L. plantarum* bacteria do, in fact, produce protease enzymes. Many studies have been carried out to prove the presence of lipase enzymes in *L. plantarum* bacteria, including Esteban Torres et al. (2015). Microbial lipases are one of the most important enzymes due to their high stability in organic solvents, the ability to catalyze hydrolytic reactions, ease of production, and relatively low costs and are one of the important sources of industrial production (19, 35). Here, the presence of the lipase enzyme was indicated in a medium containing Tween 80. In accordance with the experimental design, Tween 80 agar was utilized as a specific substrate for lipase testing, in accordance with established literature protocols (36, 37, 19). The utilization of Tween 80 in lipase assays is a well-documented methodology in microbiology, where the hydrolysis of Tween 80 by lipases results in the formation of a clear zone around the bacterial growth. This zone is indicative of lipase activity. In the present study, the methodology was based on previously established procedures, and our results clearly demonstrated the presence of a distinct zone of clearing around the bacterial growth on the Tween 80 agar. This observation aligns with the expected outcome of lipase hydrolysis. Furthermore, we included a positive control in our experiment, and the positive control exhibited the expected clear zone, validating the reliability of our experimental setup. It is important to note that while growth on Tween 80 agar may not be a definitive indicator of lipase production in all bacterial species, the specific conditions and methodology employed were selected to target lipase activity in a manner that aligns with the experimental objectives.

The results demonstrated, the *L. plantarum* isolates exhibited sensitivity to the majority of cell wall synthesis inhibitors, particularly co-amoxyclave and ceftriaxone. However, they displayed resistance to vancomycin. The sensitivity of these bacteria to erythromycin,

and eenicillin and their resistance to ciprofloxacin are consistent with Vizoso's report on the same strains of *L. plantarum* (38). With regard to the inhibition of nucleic acid synthesis by antibiotics, the isolates exhibited resistance to a specific antibiotic group, including nalidixic acid and ofloxacin. It is noteworthy that these findings are in accordance with those previously reported by Cebeci et al. (2003), with the exception that *L. plantarum demonstrated sensitivity* to rifampicin in the present study. The potential anticancer activity of lactobacilli may be attributed to factors such as polysaccharides and peptidoglycans. Nevertheless, the presence of enzymes, proteins, and toxins in the cytoplasmic extract of lactobacilli markedly reduces the survival of colorectal cancer cells. Notably, the anticancer effects of lactobacilli might be attributed to the peptides present in their cytoplasmic extract, specifically lactoferrin. Lactoferrin exhibits its anticancer activity by inducing apoptosis, as well as by inhibiting the cell cycle and migration in cancer cells. The anticancer attributes of lactoferrin can be ascribed to the electrostatic binding of its cationic N-terminal region to acidic molecules, including proteoglycans, glycosaminoglycans, and sialic acids. These acidic molecules are expressed in abundance on the surface of cancer cells. Another anticancer mechanism of lactobacilli is to reinforce the immune system by producing cytokines, IFN-γ and TNF-α, which consequently result in the eradication of tumor cells (27, 25).

Conclusion:

The results of this study demonstrate that *Lactobacillus plantarum* strains are capable of growth in native fruit vinegar produced in Iran. These bacteria demonstrate resistance to commonly used antibiotics and possess the capacity to grow and survive in acidic, bile, and saline environments at varying temperature levels.

Conflict of Interest Statement

We wish to confirm that there are no conflicts of interest.

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