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ORIGINAL PAPER

# Fermentation of pomegranate juice by probiotic lactic acid bacteria

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**Abstract** In this research, production of probiotic pomegranate juice through its fermentation by four strains of lactic acid bacteria: *Lactobacillus plantarum, L. delbruekii, L. paracasei, L. acidophilus* was examined. Fermentation was carried out at 30°C for 72 h under microaerophilic conditions. Microbial population, pH, titrable acidity, sugar and organic acid metabolism were measured during the fermentation period and the viability of all strains was also determined during the storage time at 4°C within 4 weeks. The results indicated that *L. plantarum* and *L. delbruekii* increased the pH sharply at the initial stages of fermentation and the sugar consumption was also higher in comparison with other strains, better microbial growth was also observed for these two strains during fermentation. Citric acid, as a major organic acid in pomegranate juice was significantly consumed by all probiotic lactic acid bacteria. *L. plantarum* and *L. delbruekii* showed higher viability during the storage time. Viable cells remained at their maximum level within 2 weeks but decreased dramatically after 4 weeks. Pomegranate juice was proved to be a suitable media for production of a fermented probiotic drink.

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## Introduction

The health benefits of certain foods have been investigated for many years. Development of foods that promote health and wellbeing is one of the key research priorities of the food industry (Klaenhammer and Kullen 1999). This trend has led to increased production and consumption of foods enriched with active components such as prebiotics, probiotics and synbiotics which are recognized as functional foods (Shanahan 2004).

Probiotics are increasingly used as food supplements, due to mounting scientific evidences supporting the concept that the maintenance of a healthy gut microflora may provide protection against gastrointestinal disorder including infections and inflammatory syndromes of the bowel (Parvez et al. 2006; Nomato 2005; Shanahan 2002, 2004; Madden and Hunter 2002).

It has been suggested that fruit juices could serve as suitable media for cultivating probiotic bacteria (Mattila-Sandholm et al. 2002). Fruit juices have an established market sector as functional drink through sale of calcium- and vitamin-fortified juices, and they are consumed regularly, which is essential if the full benefits attributed to probiotics are to be experienced (Sheehan et al. 2007).

Different studies have been carried out to explore the suitability of fruit juices such as tomato, beet and cabbage juices as raw materials for the production of probiotic drinks. *L. plantarum, L. acidophilus* and *L. casei* have been employed as probiotic bacteria cultures. Results have indicated that all the strains are capable of growth in the fruit juices mentioned and as a result, the microbial

population increases significantly after 48 h of fermentation. Moreover, *L. plantarum, L. acidofilus* and *L. delbruekii* have shown to be resistant to the high acidic and low pH conditions during storage periods at 4°C. However, results on *L. casei* have indicated that this strain loses its viability during cold storage (Yoon et al. 2004, 2005, 2006). Enrichment of the fruit juice-based medium with nutritive substances has also been studied. Rakin et al. (2007) enriched beet root and carrot juices with the brewer's yeast autolysate before the lactic acid fermentation using *L. acidophilus*. The addition of the autolysate yeast favorably increased the number of lactic acid bacterial cells during the fermentation (Aeschlimann and Stocar 1990) and reduced the time of fermentation. Fermentation of vegetable juices enriched with yeast autolysate caused the amino acid, vitamin and mineral content and antioxidant activity of the final drink to increase (Chae and Joo 2001).

Pomegranate (*Punica granatum,* Punicaceae) is known to have considerable health-promoting properties with antimicrobial, antiviral, anticancer, antioxidant and antimutagenic effects (Negi et al. 2003). The fresh juice contains 85.4% water and considerable amounts of total soluble solids (TSS), total sugars, reducing sugars, anthocyanins, phenolics, ascorbic acid and proteins and has been reported to be a rich source of antioxidants. These antioxidants are more potent, on a molar basis, than many other antioxidants including vitamin C, vitamin E, coenzyme Q-10 and alpha-lipoic acid (Aviram et al. 2002). The antioxidant level in pomegranate juice was found to be higher than in green tea and red wine (Gil et al. 2000).

The aim of this research was to investigate the growth rate and substrate metabolism during the fermentation of pomegranate juice via selected probiotic lactic acid bacteria and evaluating their viability in cold storage conditions. This attempt was made to produce a non-dairy probiotic drink based on pomegranate juice, possessing inherent health benefits.

## Materials and methods

### Strains and cultures

Probiotic lactic acid bacteria (*Lactobacillus acidophilus* DSMZ 20079*, L. plantarum* DSMZ 20174*, L. delbrueckii* DSMZ 20006*, L. paracasei* DSMZ 15996) were supplied by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany). All bacterial cultures were stored frozen at −20°C in MRS medium (Merck, Germany) containing 20% glycerol. The strains were reactivated by means of double passage on MRS when needed. Commercial concentrated pomegranate juice was supplied from ZamZam Iran Co. (Teheran, Iran) and kept at 4°C prior to use.

### Fermentation of probiotic pomegranate juice

The pomegranate juice concentrate with 62° Brix was diluted to 20° Brix with distilled water and then pasteurized for 5 min at 80°C. A 24-h cultivation of lactic acid bacteria in the MRS broth was carried out at 30°C. For obtaining an initial cell density of $10^7$ c.f.u/ml in the final juice, 15 ml of the cultivated MRS broth was centrifuged at 4,000*g* for 10 min and the biomass was introduced into the juice (150 ml). The juice was then incubated at 30°C for 72 h and sampling was taken every 24 h for microbiological analysis.

Viable cell (c.f.u/ml) were determined by the standard plate count method using MRS medium.

After 72 h of fermentation, the fermented samples were stored at 4°C for 4 weeks and the viability of probiotic bacteria was measured during storage time. The microbial population was measured at weekly intervals and expressed as c.f.u/ml.

### Chemical analysis

Chemical changes were determined by sampling during the fermentation in 24 h intervals. A digital pH meter (Metrohm 744, Netherland) was used for pH measurements. Total acidity, expressed as percent citric acid, was determined by titrating with titrazol 0.1 N NaOH (Merck, Germany) to pH 8.2. Sugars (fructose and glucose) were measured by HPLC (Knauer, Germany) equipped with a K-2310 refractive index (RI) detector. A 10 µl separation column (Eurokat H 250 × 30 mm) was employed and sulphuric acid (2.25 mM) was used as mobile phase. The flow rate of the mobile phase was 0.4 ml/min and the operation temperature was 45°C. The volume of the injected sample for each run was 20 µl. Sugar content was reported using external standards.

Quantitative analysis of organic acids (lactic and citric acid) was also carried out by HPLC (Knauer, Germany) apparatus equipped with a K-2600UV-visible detector. A separation column (Ultrasep ES-FS special 250 × 30 mm) set at room temperature with 2.25 mM sulphuric acid as the mobile phase and injection volume of 20 µl was used at a flow rate of 0.2 ml/min. Organic acids content were reported using external standards.

### Statistical analysis

Experiments were carried out in triplicate, and each sample was analysed in duplicate. The results are expressed as Mean ± SD (standard deviation). The two way analysis of

variance (ANOVA) was used to analyze the experimental data (SAS 9.1 software Institute Inc., Cary, NC, USA). Mean analysis using Duncan's multiple range tests was carried out if needed.

## Results and discussion

### Growth kinetics and substrate consumption

The kinetics of the fermentation process for each strain is presented in Fig. 1. As presented in the figure, a drop in the microbial population was observed for all of the strains during the first 24 h of the fermentation. The stress induced due to the differences between the pre-culture and the fermentation medium resulted in decrease of the growth rate at the earlier stage of fermentation process. MRS broth, as the pre-culture, has a pH of about 5.6 but the initial pH of the pomegranate juice was relatively lower (about 3.09). As expressed by some authors (Holzapfel and Schillinger 2000), acid tolerance is an important probiotic trait for surviving during fermentation in food medium. Yanez et al. (2008) also reported that low pH of medium can lead to the decrease in the maximum growth rate and an extended length of the lag phase. This stage was clearly detectable for all of the strains during which metabolic activity including sugar consumption and acid production is minor and the bacterial cells try to adapt with the new conditions. In addition, as indicated in Fig. 2, the pH and acidity of the juice remained stable and their modifications were negligible. The initial value for pH and titrable acidity (%) was 3.1 and 1.8, respectively. Among all the strains, *L. paracasei* was more affected by the stress caused by the pH difference and its viable cells decreased from $5.6 \times 10^7$ to $2.1 \times 10^7$ c.f.u./ml during the first 24 h of fermentation.

However, after passing the lag phase, the bacterial population of *L. paracasei, L. acidophilus, L. delbruekii and L. plantarum* increased to $2.9 \times 10^8$, $3.07 \times 10^8$, $3.6 \times 10^8$ and $3.9 \times 10^8$ c.f.u/ml, respectively. It could be observed in Fig. 1 that *L. plantarum* and *L. delbruekii* had a better growth rate in pomegranate juice and caused the pH of the juice to increase from the initial value of 3.1–3.41 and 3.35, respectively (Fig. 2). Accordingly, total acidity of the juice decreased as well to 1.36 and 1.41 for *L. plantarum* and *L. delbruekii,* respectively. To evaluate the changes in acid concentrations, HPLC analysis of acids was performed during the fermentation. Figure 3 presents citric acid consumption and lactic acid production during the fermentation process. The initial concentration of citric acid was high but the concentration decreased significantly during the fermentation by all lactic acid bacteria. The initial concentration of 60 g/l reached 17–13 g/l within 48 h of fermentation. Data revealed that the selected probiotic bacteria were capable of metabolizing citric acid soon after fermentation starts, while sugar consumption by all the strains was much lower at this stage. One may explain this phenomenon due to very low sugar content in the pomegranate juice and, in contrast, its considerable amounts of organic acid i.e., citric acid. Therefore, the strains metabolized citric acid as the major carbon source available in pomegranate juice. Since lactic acid is recognized as the main metabolite produced by lactic acid bacteria (De Vries and Stouthamer 1967), the kinetics of the production of lactic acid during the fermentation was also studied (Fig. 3). Results indicated that lactic acid was produced by all the strains and its concentration increased as the fermentation commenced. The major increase was observed in the log phase of the bacterial growth. *L. plantarum* produced 6.1 g lactic acid/l which was significantly higher than that produced by *L. acidophilus* and *L. paracasei* (4.9 and 4.46 g/l, respectively). *L. delbruekii* yielded a concentration of 5.3 g/l lactic acid. Citric acid, the major acid detected in the pomegranate, was consumed by the bacteria as a carbon source and was metabolized, resulting in reduction of the

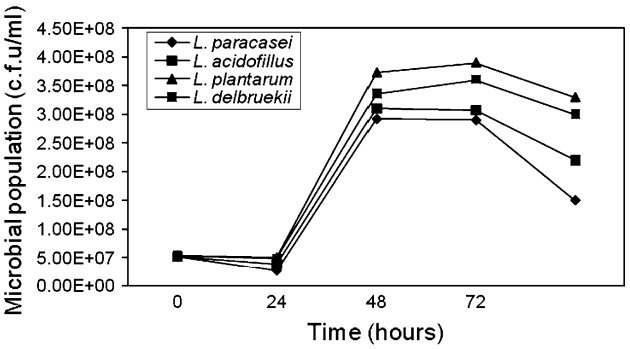


**Fig. 1** Growth kinetic *of L. plantarum*, *L. delbruekii*, *L. acidophilus* and *L. paracasei* during fermentation at 30°C in pomegranate juice with initial pH = 3.1

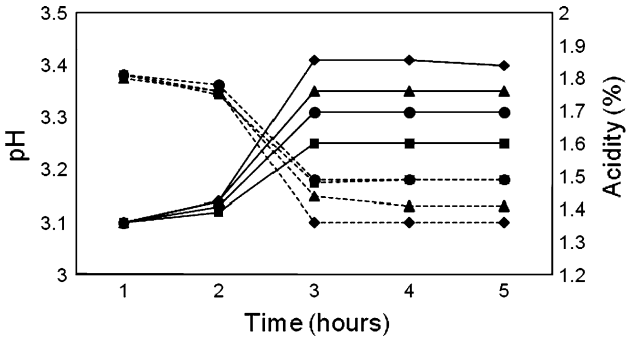


**Fig. 2** pH and titrable acidity changes in pomegranate juice during fermentation (Initial pH and titrable acidity = 3.1 and 1.8, respectively, *filled diamond = L. plantarum*, *filled triangle = L. delbruekii*, *filled circle = L. acidophilus*, *filled square = L. paracasei*. *Dash lines* indicate acidity changes during fermentation.)

acidity of the juice. The production of acidic metabolites such as lactic acid with a higher acidity constant ($pK_a = 3.86$) than that of citric acid ($pK_a = 3.06$) (De Vries and Stouthamer 1967) which is consumed by the bacteria could also support the pH decrease. However, the concentration of lactic acid produced was not high enough to cover the observed reduction of citric acid concentration and, as a result, the total acidity was decreased.

Results obtained from sugar analysis by HPLC (Figs. 4 and 5) showed that glucose and fructose were both metabolized by all strains. In the research of Tezcan et al. (2009), glucose and fructose were dominant sugars in pomegranate juice. The concentration of glucose reduced significantly in comparison with fructose between hours 48 and 72 of the fermentation. However, the consumption was not similar for different strains. *L. plantarum* showed more affinity to sugar consumption at almost 19 and 13% of initial glucose and fructose concentration. *L. delbruekii* reduced fructose and glucose concentration from 6.3 and 7.51 to 5.4 and 5.82. In contrast to three other lactic acid bacteria, *L. paracasei* exhibited least ability in sugar consumption. The reports of other authors also prove that metabolism of carbohydrates by *Lactobacillus* varies from strain to strain and depends on the substrate and even on the fermentation time (Hou et al. 2000). Wang et al. (2003) also reported that glucose is a very good carbon and energy source for lactobacilli and bifidobacteria. The detected sugars in the pomegranate juice did not serve as the major components of the juice and accordingly the consumption of the sugars was not as high as observed for citric acid. The sugars were utilized by the bacteria, but they could not be considered as the primary sources for cell growth because of their low concentrations.

As it could be detected in Figs. 1, 2,3, 4, the majority of the changes observed in microbial population, pH, acidity, sugar consumption and lactic acid metabolism, happened in

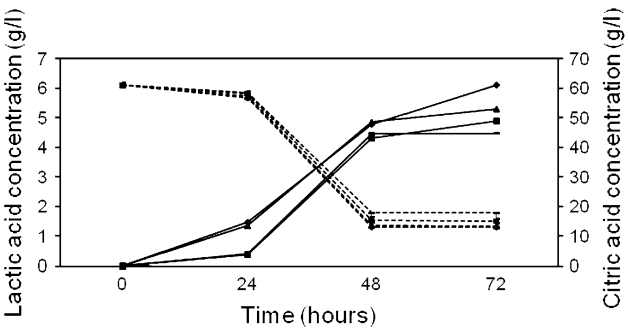


**Fig. 3** Kinetics of organic acid consumption and production in pomegranate juice during fermentation (Initial pH and titrable acidity = 3.1 and 1.8, respectively) *filled diamond* = *L. plantarum*, *filled triangle* = *L. delbruekii*, *filled square* = *L. acidophilus*, *filled dash* = *L. paracasei*. *Dash lines* indicate citric acid concentration changes)

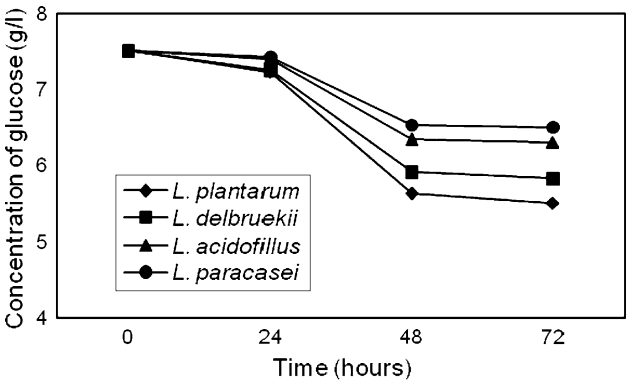


**Fig. 4** Glucose consumption during fermentation *by L. plantarum*, *L. delbruekii*, *L. acidophilus* and *L. paracasei* in pomegranate juice (Initial concentration of glucose in the medium = 7.5)

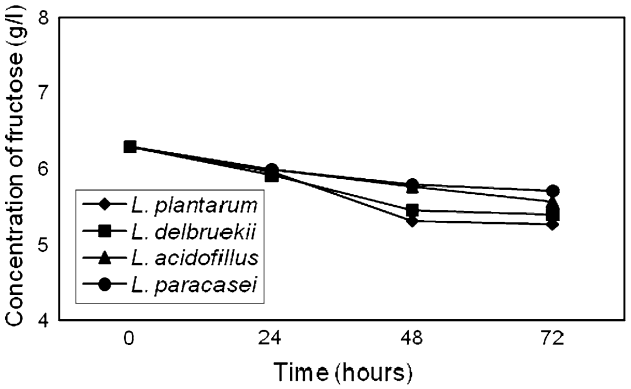


**Fig. 5** Fructose consumption during fermentation by *L. plantarum, L. delbruekii*, *L. acidophilus and L. paracasei* in pomegranate juice (Initial concentration of fructose in the medium = 6.3)

the period between hours 48 to 72 of the fermentation. Further extension of the fermentation process (from 48 to 72 h) did not result in significant changes. Similar results were reported by Yoon et al. (2005).

## Effect of cold storage on cell viability of probiotic lactic acid bacteria

The changes observed for the cell viability of the selected strains during the cold storage are presented in Table 1. Results showed that the microbial population of *L. paracasei* and *L. acidophilus* decreased approximately three logarithmic orders during the first week of cold storage and lost their viability after 2 weeks. The reason could be addressed to the lack of their ability to survive in the stressful condition of low pH and high acidity of the pomegranate juice and also the relatively low temperature of the environment (4°C). Similar results were obtained for fermented tomato juice by other authors (Yoon et al. 2004). The viable cell population of *L. plantarum* and *L. delbruekii* remained at an acceptable level ($>10^6$ c.f.u/ml)

**Table 1** Effect of cold storage (4°C) during 4 weeks on viability of *L. plantarum, L. acidophilus, L. paracasei and L. delbruekii* in fermented pomegranate juice

| Time (weeks) | Survival (CFU/ml) | | | |
|---|---|---|---|---|
| | *L. plantarum* | *L. acidophilus* | *L. paracasei* | *L. delbruekii* |
| 0 | $3.9 \times 10^8 \pm 0.2 \times 10^{8a}$ | $3.07 \times 10^8 \pm 0.26 \times 10^{8a}$ | $2.9 \times 10^8 \pm 0.43 \times 10^{8a}$ | $3.6 \times 10^8 \pm 0.3 \times 10^{8a}$ |
| 1 | $2.9 \times 10^6 \pm 0.05 \times 10^{6b}$ | $2.8 \times 10^5 \pm 0.05 \times 10^{5b}$ | $1.3 \times 10^5 \pm 0.1 \times 10^{5b}$ | $2.2 \times 10^6 \pm 0.17 \times 10^{6b}$ |
| 2 | $2.8 \times 10^5 \pm 0.05 \times 10^5$c | ND | ND | $1.5 \times 10^5 \pm 0.26 \times 10^5$c |
| 3 | ND | ND | ND | ND |
| 4 | ND | ND | ND | ND |

Samples of pomegranate juice were fermented at 30°C for 72 h and stored at 4°C. The experimental values (means and standard deviations for $n = 3$) that have no common superscript are significantly different ($P < 0.05$) according to Duncans multiple test range. Any two means not marked by the same by the same superscript (for example a and b or b and c) are significantly different. Any two means marked by the same superscript (for example a and a or b and b) are not significantly different

*ND* Not detected

after 1 week of cold storage, but their microbial population decreased below the minimum accepted after 2 weeks (Yoon et al. 2005). As Sheehan et al. (2007) reported, low pH fruit juices, with a range of pH typically between 2.5 and 3.7, cause the bacterial sensitivity to stressful conditions to increase.

Since only those strains which can remain viable for an acceptable shelf-life should be selected to produce a probiotic juice, *L. paracasei* and *L. acidophilus* are not recommended to be used in high-acid fruit juices like pomegranate. In contrast, *plantarum* and *L. delbruekii* could be introduced as selected strains of lactic acid bacteria to be used as probiotic cultures in pomegranate juice with a limited time of cold storage.

## Conclusion

In this study, probiotic lactic acid bacteria were studied to investigate suitability of using pomegranate juice as a non-dairy probiotic drink. Thereafter, growth rate, substrate metabolism during 72 h of fermentation and viability of selected strains in cold storage has been also studied. All the strains reached $10^8$ CFU/ml after 48 h of fermentation. Citric acid and sugar metabolism by Lactic acid bacteria during this stage proved that *L. plantarum* and *L. delbruekii* were more capable to consume higher amount of substrate compared to other strains. All bacteria consumed glucose and fructose as the carbon- energy source where glucose was the preference. *L. planatrum* and *L. delbruekii* were capable to survive well in the first 2 weeks of storage in 4°C while *L. acidophilus* and *L. paracasei* failed their viability after the second week in the same conditions.

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