Fermentation of apple juice using selected autochthonous lactic acid bacteria

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Abstract

Introduction. Dairy products are the most common probiotic food. However, due to lack of enzyme lactase, their ingestion for lactose intolerant people is a challenge. Fruit juices are rich in nutrients and have proved to be effective carriers or growth media for probiotics, specifically for lactic acid bacteria; they are also lactose-free and can be taken by lactose intolerant individuals.

Materials and methods. Self-made apple juice and selected lactic acid bacteria were used in the study. The number of viable bacterial cells was determined by a serial dilution method; titratable acidity was determined by automatic titrator; sugars and organic acids concentrations were measured using High-Performance Liquid Chromatography; total phenolic compound content was determined by the Folin-Chiocaltus method; and the antioxidant activity was determined by FRAP (ferric-reducing antioxidant power) assay.

Results and discussion. Selected *Lactiplantibacillus plantarum* strains were used to ferment apple juice. The optimal conditions for the fermentation were an initial pH 4.5 and 24 h duration, with maximum bacterial cells viability 8.23±0.17 log CFU/mL and 8.55±0.19 log CFU/mL for *L. plantarum* 74 and *L. plantarum* 76, respectively. Characteristics of apple juice were changed during fermentation, particularly, after 48 hours of fermentation, an increase in the titratable acidity caused the pH decrease and gradual decrease of the sugar contents was also observed. The highest production of lactic and malic acids were observed during 48 h of fermentation with the strain *L. plantarum* 74. The fermented juice with *L. plantarum* 52, *L. plantarum* 74, and *L. plantarum* 76 had concentration of total phenolic compounds 532.9±26.7 mg GAE/L, 587.3±29.4 mg GAE/L, 488.4±24.4 mg GAE/L and antioxidant activity 281.6±14.1 mg AAE/L, 300.6±15.0 mg AAE/L, 172.8±8.6 mg AAE/L, respectively after 72 h of fermentation.

Conclusion. Apple juice fermented with selected strains of *Lactiplantibacillus plantarum* was enriched with lactic acid bacteria and can be used as a probiotic product that people with lactose intolerance can consume.

Introducton

The most common and affordable food products that are sources of beneficial probiotic bacteria are fermented dairy products. However, some people, including children, are unable to fully digest lactose, a sugar present in milk and milk products. On average, 65% of the world's population is lactose intolerant (Bayless et al., 2017). Fruit juices are healthy products that are rich in important nutrients such as vitamins, minerals and antioxidants (Hussein et al., 2022), and they can serve as a medium for the growth of lactic acid bacteria. At the same time, they do not contain lactose. Thus, fruit juices enriched with lactic acid bacteria might be used as probiotic food products that even persons with lactose intolerance could consume (Gomes et al., 2021; Katoch et al., 2021; Lillo-Pérez et al., 2021, Tkesheliadze et al., 2021). Fermentation of fruit juices using lactic acid bacteria (LAB) could be used to preserve sensorial and nutritional properties and to extend the shelf life of the final products (Garcia-Gonzalez et al., 2021; Plessas, 2022). In addition, plant-based diets become increasingly popular, so fermentation of plant products and the selection of microorganisms to be used as starter culture is an issue of high demand (Cichońska and Ziarno, 2022). During the fermentation of the juice caused by lactic acid bacteria, changes in its chemical and microbiological properties occur and the final product can be enriched with microbial metabolites such as organic acids, phenolic compounds, exopolysaccharides and bacteriocins (Khubber et al., 2022). Due to these beneficial properties, fruit juices fermented by LAB increases the availability of probiotic non-dairy products on the market. For this purpose, the application of autochthonous starters is preferred (Garcia et al., 2020).

One of the most promising types of lactic acid bacteria used for the fermentation of fruit juices are strains of *Lactiplantibacillus plantarum* whose cells have high gastrointestinal adaptability and adhesion ability. These LAB also possess antimicrobial, antioxidant and anti-inflammatory properties (Garcia-Gonzalez et al. 2021; Rocchetti et al., 2021; Won et al., 2021). Application of *L. plantarum* strains to ferment of vegetable and fruit juices is extremely appealing since they can improve their sensorial properties (Plessas, 2022). From a variety of fruit beverages, apple juice is considered as a good food substrate for enrichment with lactic acid bacteria (Wu et al., 2020). This advantage is facilitated by the fact that the fermentation process is thought increases the bioavailability of bioactive compounds found in the plant material, namely in apples, which leads to a change in the substrate composition and, as a result, affects the sensory properties of the final product (Guiné et al., 2021).

The aim of the present study was to investigate change of the selected technological characteristics of apple juice fermented with the strains of *Lactiplantibacillus plantarum.*

Materials and methods

The apple fruits from different regions of Georgia were used in the study. Apples were washed with tap water, cutting, crushed using a food processor and filtered through a paper filter, 12.5 cm diameter, and then through the Millex-GS Syringe Filter Unit with a 0.22 μm pore size mixed cellulose esters membrane (Millipore).

For the fermentation of apple juice three probiotic strains of autochthonous lactic acid bacteria *Lactiplantibacillus plantarum* 52, *L. plantarum* 74, *L. plantarum* 76 from the collection of microorganisms at Durmishidze Institute of Biochemistry and Biotechnology of the Agricultural University of Georgia, specifically were used. The bacterial strains stored at -80 °C were transferred to MRS broth and incubated at 37 °C for 48 hours.

Apple juice was inoculated with lactic acid bacteria and incubated at 37 °C. Cell viability, pH values, titratable acidity, content of sugars, organic acids and total phenolic compounds as well as antioxidant activity were evaluated prior to the fermentation process and after 24 h, 48 h, and 72 h of fermentation.

The total number of viable cells of *L. plantarum* 52, *L. plantarum* 74, *L. plantarum* 76 on MRS agar was determined using the serial dilution method. Aliquots (0.1 mL) of diluted fermented juice were plated in triplicate onto MRS agar and incubated at 37 °C for 72 h in an anaerobic incubator (Hashemi et al., 2017, ISO/TS 19036, 2006). The results were expressed as log CFU/mL.

The pH was measured using a digital pH meter. Titratable acidity (TA) was determined by titrating with 0.1 M NaOH to a pH end-point of 8.2 using an automatic titrator (ZDJ-4A, NASA Scientific Instrument Co., Ltd, Anting Shanghai, China). The results were expressed as gram of malic acid equivalents per 100 mL of juice (Wlodarska et al., 2017).

HPLC was used to determine the **concentrations of sugars** such as sucrose, fructose, and glucose, as well as the content of organic acids, in fermented and unfermented apple juices. The data were recalculated in grams per liter.

Total phenolic content in apple juice was determined using the Folin-Chiocaltheus method (Bond et al., 2003). 5 mL of diluted 10 times Folin-Chicolteo reagent was added to 1 mL of the test sample and left at room temperature for 8 minutes. As a control, 1 mL of distilled water was used. Then 4 mL of sodium carbonate was added and thoroughly mixed. The samples and standards were kept at room temperature for 1 hour. A spectrophotometer was used to determine the **sample absorption rate** at a wavelength of 765 nm. The results were expressed in mg gallic acid equivalents (GAE) per litre of juice.

The total antioxidant concentration was determined by the FRAP method (Benzie and Strain, 1996). A spectrophotometer was used to measure the change in absorption intensity. Initially, a working solution for determining the sample was prepared using a mixture of three solutions: 300 mM acetate buffer (pH 3.6); TPTZ (2.4.6-tripiridyl-5-triazine), and trivalent iron chloride, in the ratio 10:1:1. The obtained working solution was submerged in a 37 °C water bath for 15 minutes. The working solution, 3 mL, was added to the apple juice sample, 100 µl, and the absorbance was measured using a spectrophotometer at 593 nm; the absorption was recorded after 4 minutes. The working solution was used as a control, while ascorbic acid was applied as a comparator. The data were measured in mg of ascorbic acid equivalent (AAE) in 1L of the juice.

All experiments were performed in triplicate. The data are expressed as the mean ±standard deviation. Statistical analysis was carried out using one-way ANOVA and Tukey's HSD tests. One-way analysis of variance (ANOVA) was done to analyze the variation of the means between the experimental samples. Tukey's HSD test was used to differentiate between the mean values. All the analyses were done using XLSTAT (free trial version 2022, Addinsoft, Inc., Brooklyn, NY, USA). p value < 0.05 was considered statistically significant.

Results and discussion

Determination of the viable cell number

An important parameter for evaluating the fermentation process is the quantitative change in the number of bacteria (Janiszewska-Turak et al., 2022). Changes of viable cell number of *Lactiplantibacillus plantarum* 52, 74, and 76 during the fermentation of apple juice are shown in Table. 1.

Table 1

Changes of viable cell number in apple juice fermented with strains *Lactiplantibacillus plantarum*

Time of fermentation, h	*L. plantarum* 52	*L. plantarum* 74	*L. plantarum* 76
	log of colony forming units (CFU)/mL		
0	8.1±0.1	8.2±0.2	8.3±0.2
24	9.0±0.4	8.2±0.2	8.6±0.2
48	6.6±0.2	7.4±0.3	7.4±0.3
72	9.1±0.3	7.4±0.1	7.8±0.3
192	7.8±0.1	7.5±0.4	8.5±0.3
288	7.0±0.4	7.3±0.2	7.8±0.2

All strains of the lactic acid bacteria revealed different growth (Table 1). In the case of *L. plantarum* 76, the initial cell number of 8.3±0.2 log CFU/mL increased to 7.8±0.3 log CFU/mL after 72 h, whereas in *L. plantarum* 52, an increase approximately 1 log CFU/mL of bacterial cells was observed within 24 h of fermentation, followed by decrease to 2.45 log CFU/mL, which could be caused by sugar consumption in fermented juices. Similar results were observed in the research of Janiszewska-Turak with co-authors (2022). Li with co-authors (2019) in their study found that during the first 24 h of fermentation, the viability of *L. plantarum* cells increased due to adequate nutrient content and suitable growth conditions, and after 24 h their growth slowed down. Wang with co-authors (2021) studied the viability of bacteria in apple juice fermented with *Lactobacillus plantarum*. As the results showed, in the case of additional ultrasound processing of juice, in the early stage of fermentation, in particular after sonication for 0.5 h, the number of the bacteria increased from 7.5 log CFU/mL to 7.9 log CFU/mL and 7.8 log CFU/mL, whereas the number of *Lactobacillus plantarum* cells did not change when this treatment was not used. The viable cell count of *L. plantarum* decreased during the storage at 25 °C of fermented dairy substrates from 8.89 log CFU/mL to 8.6 log CFU/mL after 240 h and the decrease was more pronounced after 1440 h in fermented transition milk stored at 25 °C, showing a viable cell count 5.7 log CFU/mL (Fonseca et al., 2020). According to Ostlie with co-authors (2003), viable cell counts for the probiotic strains in fermented milk increased from 8.7 to 9.2 log CFU/mL after 6–16 h of incubation.

Determination of pH

The pH changes in apple juice fermented using different strains of *L. plantarum* are shown in Table 2.

The initial pH of control sample was 4.5±0.2. The juice pH reduced after 48 h of fermentation from 4.5 to 3.6 and a modest decline in the cell viability was observed after 48 h of fermentation (Table 1). The pH of apple juice increased from 3.8 to 4.0 on the 192 h of fermentation because of *L. plantarum* 76 activity, and the number of viable cells increased as well (Table 1). Meanwhile, the pH of the juice fermented with *L. plantarum* 74 increased to 4.0, and at the end of fermentation decreased to pH 3.8. According to the literary data, a similar drop was shown in fermented dairy product as well. After 2 h of fermentation, the pH of the milk began to decrease rapidly, from 6.8 reached ~4.5 in less than 6 h (Dan et al., 2019).

Table 2

Changes of the pH of apple juice fermented with the strains *Lactiplantibacillus plantarum*

Time of fermentation, h	*L. plantarum* 52	*L. plantarum* 74	*L. plantarum* 76
48	3.6±0.2	3.6±0.2	3.6±0.2
72	3.8±0.2	3.8±0.2	3.8±0.2
192	3.9±0.2	4.0±0.3	4.0±0.1
288	3.8±0.2	3.8±0.4	3.9±0.1

It should be mentioned that the pH of MRS broth was 6.4 at the start of the experiment and ranged between 3.8 and 4.0 by the 288 h as a result of the action of all three strains, whereas the pH of apple juice was 4.5. The value reduced on the 288 day of fermentation by *L. plantarum* strains.

The pH changes, in turn, impacted the number of bacteria present during apple juice fermentation. In particular, on the 288 h of fermentation, the initial number of bacteria *L. plantarum* 52, *L. plantarum* 74, *L. plantarum* 76 decreased from 8.1±0.1 log CFU/mL, 8.2±0.2 log CFU/mL and 8.3±0.2 log CFU/mL to 7.0±0.4 log CFU/mL, 7.3±0.2 log CFU/mL and 7.8±0.2 log CFU/mL, respectively, while no reduction in the number of cells of every culture was observed in MRS broth.

The establishment of various conditions, particularly when the pH of MRS broth and apple juice do not match, may be one of the causes of the decrease in viable cell number (Mousavi et al., 2013). Given that pH is one of the most significant factors determining the probiotic survival, the pH value of the juice at the start of our experiment may have resulted in a steady decline in microbial growth. According to Dimitrovski with co-authors (2015), a low pH value does not promote LAB growth, and pH low than 4.4 could inhibit the growth or slow down the growth rate (Saeed et al., 2013) and thus the initial pH influences the fermentation process (Peng et al., 2021).

Determination of titratable acidity

The changes of the titratable acidity (TA) of apple juice fermented with strains of *Lactiplantibacillus plantarum* are shown in Table 3.

Table 3

Changes of the titratable acidity of apple juice fermented with the strains *Lactiplantibacillus plantarum*

Time of fermentation, h	*L. plantarum* 52	*L. plantarum* 74	*L. plantarum* 76
	g /100mL		
48	2.07±0.10	2.54±0.11	2.40±0.23
72	1.08±0.05	1.14±0.19	1.07±0.07
192	1.25±0.06	0.97±0.09	1.06±0.06
288	1.37±0.15	1.01±0.16	1.14±0.14

The initial titratable acidity of control sample was 1.25±0.13 g/100mL. For the first 48 h, it increased, when compared to controls in all three samples. Our findings are consistent with those of Mashayekh with co-authors (2015) who found that the acidity of a mixture of pineapple, apple and mango juice significantly increased during fermentation with *Lactobacillus casei* 1608 due to production of organic acids. As seen in Tables 2 and 3, there is a correlation between the pH value and the titratable acidity, with the pH decreasing sharply from the start of fermentation to 48 h, while the titratable acidity increases significantly during this time of fermentation. In the case of milk fermentation by *L. plantarum* P9, the TA increased after 13.5 h of fermentation. It was accompanied by a rapidly dropping in the pH value and reaching the fermentation endpoint pH of 4.5 from 6.47. At this incubation period, the viable counts of *L. plantarum* P9 increased from 7.35 to 8.44 log CFU/mL (Zha et al., 2021).

Determination of sugars

Changes of glucose, fructose and sucrose concentrations in apple juice fermented with strains of *Lactiplantibacillus plantarum* are shown in Table 4.

Table 4

Changes of the glucose, fructose and sucrose concentrations in apple juice fermented with the strains *Lactiplantibacillus plantarum*

Apple juice fermented with	Concentration of sugars, g/L, at time of fermentation, h					
	Sucrose		Glucose		Fructose	
	24	48	24	48	24	48
Apple juice (control)	2.4±0.1	2.4±0.1	39.0±2.0	39±2.0	105±5.3	105±5.3
L. plantarum 52	2.3±0.1	1.9±0.1	32.5±1.6	26.8±1.3	93.5±4.7	84.3±4.2
L. plantarum 74	2.2±0.1	2.0±0.1	33.6±1.7	28.5±1.4	95.5±4.8	87.8±4.4
L. plantarum 76	2.2±0.2	2.0±0.1	33.9±1.7	28.5±1.3	100±5.0	88±4.4

The strain of *L. plantarum* 52 fermented 0.16 g/L of sucrose during 24 h and 0.41 g/L for 48 h. Fermentation of glucose and fructose was going more intensive during the first 24 h. 6.5 g/L of glucose and 11.5 g/L of fructose were fermented on the first day, while 5.7 g/L of glucose and 9.2 g/L of fructose were converted on the second day. Fermentation using the strain of *L. plantarum* 74 was similar. In the case of *L. plantarum* 76 use, fructose fermentation increased on the second day. According to the literary data, different bacterial strains ferment sugars in different ways. Some strains of lactic acid bacteria ferment glucose at a rate higher than the rate of fructose assimilation (Mousavi et al., 2013), while others do the opposite (Peng et al., 2021).

Determination of organic acids

Apple juice used in the study contained 6.5±0.3 g/L of malic and 1.2±0.1 g/L of lactic acids. During 48 h fermentation in samples with *L. plantarum* 52, 74, 76, bacteria consumed malic acid and produced lactic acid; the concentration of lactic acid reached 6.9±0.3, 7.0±0.3, 6.9±0.5 g/L, respectively, while concentration of malic acid reduced to 3.3±0.2, 3.5±0.2, 3.3±0.1 g/L (Figure 1).

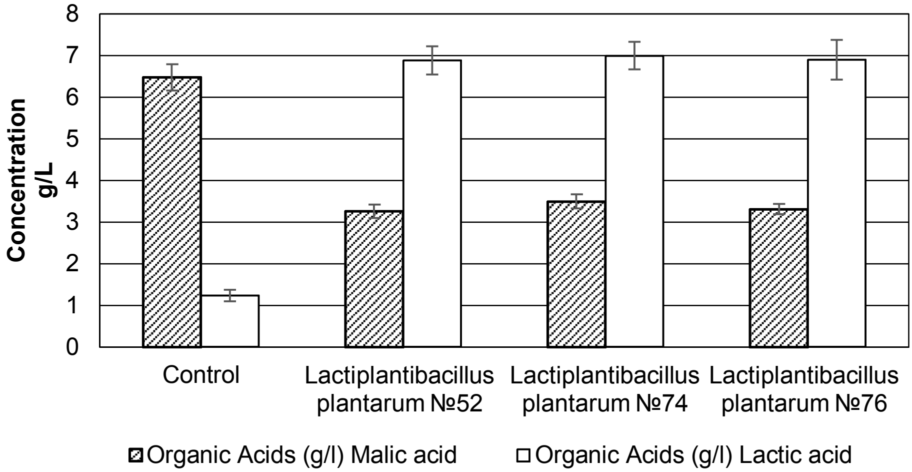

Figure 1. Changes in organic acid concentrations in apple juice fermented with the strains *Lactiplantibacillus plantarum*

The mail product of sugar fermentation by LAB is lactic acid, and the breakdown of malic acid is going due to their activity (Chen et al., 2019; Fonseca et al., 2021; Mousavi et al., 2013; Ricci et al., 2019). For example, concentration of lactic acid in the soymilk increased after 56 h of fermentation from 0.5 g/L to 2.04 g/L (Shu et al., 2022).

Determination of total phenolic content

The concentration of polyphenols in apple juice varies depending on the apple cultivars (Wlodarska et al., 2017). Phenolic compounds enrich the fermented juice with flavor, inhibit microbial spoilage, and regulate fermentation rate (Ye et al., 2014). Phenolic compounds frequently occur as complex molecules associated with sugars or proteins. LAB can break down polyphenols into simpler components through decarboxylation, reduction, de-esterification, and deglycosylation reactions (Lee and Paik, 2017). The total amount of phenols in the fermented product may raise or decrease because of fermentation (Crespo et al., 2021).

After fermentation with *L. plantarum* 74, the total phenol concentration in the fermented apple juice increased from 405.0±20.3 to 414.8±20.8 mg GAE/L in 24 h and 443.4±22.2 mg GAE/L in 48 h. From 24 to 72 hours of fermentation, all samples showed a tendency to increase total phenols content, although various tendencies were seen from the third day of fermentation (Table 5).

Lactic acid fermentation of chokeberry juice with *L. paracasei* SP5 resulted in an increase in total phenolics content and antioxidant activity in fermented chokeberry juice compared with non-fermented juice after storage at 4 °C for 4 weeks (Bontsidis et al., 2021). Hashemi with co-authors (2017) also found significant changes in the common phenolic content during the fermentation of sweet lemon juice with *L. plantarum*.

Table 5

Changes of the total phenol contents in apple juice fermented with the strains *Lactiplantibacillus plantarum*

Apple juice fermented with	Concentration of the total phenol contents, mg GAE/L, at time of fermentation, h					
	0	24	48	72	192	288
L. plantarum 52	406.0± 20.3^{ef}	397.2± 19.9^{ef}	479.6± 24.0^{bcd}	532.9± 26.7^{ab}	404.3± 20.2^{ef}	400.5± 20.0^{ef}
L. plantarum 74	406.0± 20.3^{ef}	414.8± 20.7^{def}	443.4± 22.2^{cdef}	587.3± 29.4^{a}	391.2± 19.6^{ef}	403.8± 20.12^{ef}
L. plantarum 76	406.0± 20.3^{ef}	387.9± 19.4^{f}	457.6± 22.9^{cde}	488.4± 24.4^{bc}	393.4± 19.7^{ef}	414.8± 20.7^{def}

Note: means ±standard deviation (SD) in the table with different alphabet letters indicate the significant difference at $p < 0.05$.

Determination of antioxidant activity

The changes in the total antioxidant activities in apple juices fermented with the three selected *Lactiplantibacillus plantarum* strains are shown (Table 6).

Table 6

Changes of the total antioxidant activities of the apple juice fermented with the strains *Lactiplantibacillus plantarum*

Apple juice fermented with	Concentration of the total phenol contents, mg AAE/L, at time of fermentation, h					
	0	24	48	72	192	288
L. plantarum 52	173.1± 8.7 ^{cd}	117.2± 5.9^{f}	274.4± 13.7^{ab}	281.6± 14.1^{ab}	183.5± 9.2^{c}	155.0± 7.8^{cde}
L. plantarum 74	173.1± 8.7 ^{cd}	269.6± 13.5^{b}	282.8± 14.1^{ab}	300.6± 15.0^{a}	132.2± 6.6^{ef}	177.3± 8.9^{c}
L. plantarum 76	173.1± 8.7^{cd}	140.3± 7.0^{ef}	144.8± 7.2^{def}	172.8± 8.6^{cd}	153.8± 7.7^{cde}	138.2± 6.9^{ef}

Note: means ±standard deviation (SD) in the table with different alphabet letters indicate the significant difference at $p < 0.05$.

Maximum antioxidant activities were found in apple juice fermented by all strains of *L. plantarum* after 72 h of fermentation and were 274.4, 282.6 and 172.8 mg AAE/L for *L. plantarum* 52, 74 and 76, respectively. The influence of the unique properties of various strains as well as composition of fermented juice on the increase of the antioxidant activity is shown (Multari et al., 2020; Nguyen, 2019). The antioxidant activity of orange juice fermented with different strains of *Lactobacillus brevis* POM and *Lactobacillus plantarum* varied significantly (de la Fuente et al., 2021). According to Shakya and co-authors (2021), the rise in total phenol content in the plant extracts fermented with *Lactobacillus brevis* 174A may be associated with an increase in antioxidant activity. The increase in the total phenol content was observed in the present study as well: for example, in the case of *L. plantarum* 74 application, the total phenolic content increased from 406. 0±20.3 mg GAE/L to 587.3±29.4 mg GAE/L, while the antioxidant activity increased from 173.1±8.7 mg AAE/L to 300.6±15.0 mg AAE/L after 72 h of fermentation (Tables 5 and 6).

Conclusions

The apple juice was used to be fermented by autochthonous *Lactiplantibacillus plantarum* strains. Fermented apple juice possessed higher content of the phenolic compounds and increased antioxidant activity. The number of alive lactic bacteria cells increased to the end of fermentation, so apple juice is a good substrate for maintaining the viability of probiotic lactic acid bacteria. Apple juice fermented by lactic acid bacteria could be considered as a probiotic product consumption of which will have a beneficial effect on human health, especially for people with lactose intolerance.

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