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Probiotic Fermentation and Organic Acid Profile in Milk Based Lactic Beverages Containing Potential Prebiotic Apple Constituents

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ABSTRACT

Probiotic milk-based matrices contain bioactive compounds required for the biochemical and physiological processes of metabolism as a result of fermentation. The present work aimed to evaluate the viability of probiotic bacteria in a lactic beverage fortified with probiotic milk/apple juice to understand the utilization of apple juice as a prebiotic source and investigate the organic acid profile. By monitoring the fermentation development and bacterial growth the results obtained indicated that the probiotic bacteria were viable over the predicted shelf life; the cell counts ranged from 7.48 to 12.00 $\log_{10}$ cfu mL^{-1}, conferring that the beneficial health effects on the host as probiotic bacteria must be at a minimum concentration of 6.0 $\log_{10}$ cfu mL^{-1} at the moment of consumption. *Lactobacillus casei* exhibited higher survival than the other lactic strains, presumably due to its higher ability to tolerate low acidity. During the fermentation and storage of milk based lactic beverages containing apple juice the formation of organic acids were determined as an indirect characteristic for growth of lactic acid bacteria. Lactic, acetic, malic, tartaric and citric acids were the primary organic acids. The quantities of propionic and butyric acids as short chain fatty acids were noted to increase during fermentation, being strain-specific. In conclusion, when probiotic milk is fortified with apple juice nutraceutical components, it can be a potential source of substrate and a synbiotic matrix for the growth of probiotic bacteria without any nutritional supplement.

Keywords: Lactic fermentation, Apple, Bioactive metabolite, Postbiotic

1. Introduction

The health-consciousness of consumers has resulted in a surge of specific foods or food components, so-called functional foods, which provide an additional physiological benefit beyond that of meeting basic nutritional needs. Functional foods and their bioactive components such as vitamins, minerals, phytonutrients, lipids, prebiotics and probiotics are being marketed to improve the quality of life and reduce the risk of disease - in both humans and animals (Cencic & Chinwaru 2010). The concern on the inclusion of probiotic strains in different food products has progressively grown over the years as many health benefits are ascribed to it, and therefore, the market share and production have increased (Das et al. 2012; Markowiak & Ślżewska 2017). Probiotics are defined as living microorganisms that exert positive influences to human health by improving the properties of intestinal microflora, when ingested in sufficient amounts (Villena & Kitazawa 2017). International standards for probiotic bacteria in food products state that a minimum dose of 6.0-7.0 log colony-forming units (cfu) of viable bacteria should be taken per g/mL product at the time of consumption to provide health benefits on the host (Ranadheera et al. 2017). Probiotics may play a beneficial role on lactose intolerance, cancer, allergies,

hepatitis, *Helicobacter pylori* infections, urinary tract infections, hyperlipidaemia, the assimilation of cholesterol, and antibiotic-associated gastrointestinal problems. Moreover, probiotics may reduce the intestinal pH, enhance the synthesis of vitamin K, folic acid, B group vitamins, short chain fatty acids and other postbiotics, and improve the absorption of certain minerals (i.e. Ca, Zn, Fe, Mn, Cu and P) (Homayouni-Rad et al. 2012). These assigned beneficial effects could be the result of the symbiotic relation between the host and gut microbiota. The intestinal microbiota contribute to the regulation of the gut health, and the enhancement of resistance against infections and differentiation of host immune system by lowering the pH through production of organic acids, such as lactate and short chain fatty acids, due to the break-down of complex carbohydrates or elaboration of antibiotic-like substances (Marco & Tachon 2013; Ranadheera et al. 2017; Narli & Ozcan 2022; Omak & Yilmaz-Ersan 2022).

Milk and dairy products are valued as the most common and traditional way for probiotic delivery. Among them dairy or milk-based drinks, fortified with probiotics, prebiotics, fibers, polyphenols, etc., were the first commercialized probiotic foods and still remain at the forefront. Dairy foods are considered as advantageous to non-dairy carrier foods in tolerating harsh gastro-intestinal conditions, due to the buffering capacity of milk and milk fat, which might protect probiotics in such stress conditions by reducing their direct exposure (Marco & Tachon 2013; Ranadheera et al. 2017; Ozdemir & Ozcan 2020).

Fruit juices have been suggested as non-dairy carrier foods for probiotic bacteria since they are rich in essential nutrients (minerals, vitamins, dietary fibers, phenolic compounds and phytochemicals) and sugars (Yoon et al. 2004; Mousavi et al. 2011). However, since for any functional probiotic food the major success and preferability criteria are to retain the viability and sensory characteristics, the survival of probiotics in fruit-based matrices to the end of shelf-life is more complex and challenging than those found in dairy products, where bacteria need more protection due to the low pH and stability (Perricone et al. 2015; Thakur & Josh 2017; Barat & Ozcan 2018).

The inclusion of prebiotics, non-digestible fibers that are resistant to digestion in small intestine and can selectively be fermented by the gut microflora, in dairy formulations was reported to stimulate the growth and activity of one or a limited number of probiotic *Lactobacillus* and *Bifidobacterium* spp., in the colon, thereby improving host health via modulating fat metabolism, obesity, and preventing constipation (Das et al. 2012; Shah et al. 2020).

Apples contain a balanced and high amount of soluble and insoluble dietary fibers. Among them, the water-soluble pectin is a polysaccharide which is not digested by enzymes in the human digestive system and provides colonization of probiotic bacteria as a prebiotic source in the large intestine (Kowalczyk et al. 2021; Zahid et al. 2021). While apples contain approximately 2-3 grams of dietary soluble fiber per 100 g, of which 50% is pectin, they are also an excellent source of phytochemicals. The fiber found in apples combined with other apple nutrients could be fermented into short-chain fatty acids (SCFAs) that help to enhance the growth of beneficiary gut bacteria (Chung et al. 2017; Kowalczyk et al. 2021).

Since the proposed uptake of probiotics is from natural sources, the hypothesis of combining the nutritional benefits of apples with the health benefits of the probiotics in a probiotic milk drink formula is a challenge. Pereira et al. (2013), Dimitrovski et al. (2015), and Zandi et al. (2016) have all investigated the viability of probiotic bacteria in fermented juices containing apples. Paredes et al. (2022), evaluated a fruit-vegetable mix including apple as a potential substrate for probiotic bacteria and stated that fermentation changed with nutraceutical properties.

Despite the large number of studies on the fermentation of fruits and vegetables as probiotic juices, few have focused on probiotic dairy products fortified with fruit juices (Barat & Ozcan 2018; Paredes et al. 2022). In this study, it is aimed to investigate the effects of apple juice pectic polysaccharides on prebiotic potential, organic acid fermentation, growth and viability of probiotic bacteria in milk matrix.

2. Material and Methods

2.1. Fruit juice preparation

Following preliminary trials, in order to create the desired sensory properties of apple pulp, a juice formulation consisting different kinds of apples was designed. The apples (Golden Delicious 60 kg, Granny Smith 20 kg and Star Crimson Delicious 20 kg apple varieties, 3:1:1, kg:kg) were hand-picked, washed (under a heavy spray application of water and rotary brush), crushed into pulp by a food processor, enzymatically mashed, and then cold-pressed. The raw juice was subjected to enzymatic clarification (Pectinex-30 mL 100 mL^{-1} at 50 $^{\circ}C$ for 2 h) which was followed by gelatine (1 g 100 mL^{-1} for 2 h) and bentonite (10 g 100 mL^{-1} for 2 h) treatments. The

clear apple juice was then filtered, glass-bottled (600 mL), pasteurized (20 min at 60 ˚C), and stored at 4±1 ˚C before use for further production.

2.2. Probiotic culture preparation

Freeze-dried cultures of *Lactobacillus casei* (Lc-11)*, Bifidobacterium animalis* subsp. *lactis* (Bl-04)*, Lactobacillus acidophilus* (La-14) and *Lactobacillus rhamnosus* (Lr-32)*,* were supplied from Danisco (Niebull, Germany), propagated as suggested by Barat and Ozcan (2018) and maintained at 4±1 ˚C until used.

2.3. Production of lactic beverage

Reconstituted skim milks 10.70% (w/w *dry matter*) were heat-treated at 90 ˚C for 10 min, cooled down to 37 ˚C and inoculated with each probiotic bacteria (*Lb. casei, B. lactis, Lb. acidophilus* and *Lb. rhamnosus*) with initial counts of 9.60, 9.15, 9.00 and 9.48 $\log_{10}$ cfu mL^{-1}, respectively. The incubation was carried out at 37 ˚C until the final pH was 4.7. The probiotic milk was stored in the refrigerator (4±1 ˚C) for 12 hours after fermentation. The pasteurized apple juice, of 12.2 ˚Brix with a titratable acidity of 0.51% as malic acid, was mixed with the milks at a ratio of 1:1 (v/v) for the production of probiotic lactic beverages. The beverages, denoted as LBC (with *Lb. casei)*, Lactic beverage with *B. lactis* (LBL), Lactic beverage with *Lb. acidophilus* (LBA) and Lactic beverage with *Lb. rhamnosus* (LBR), were stored at 4±1 ˚C for 28 days. An analysis was performed every 7th day of storage.

2.4. Enumeration of probiotic bacteria

The probiotic strains were enumerated on de Man, Rogosa and Sharpe Agar (MRS) (Merck, Darmstadt, Germany). *Lb. rhamnosus* and *Lb. casei* were counted on MRS-vancomycin agar, MRS-Bile was used for *Lb. acidophilus,* and for *B. lactis*, MRS-LP agar supplemented with lithium chloride, sodium propionate and cysteine was used. The plates were incubated for 72 h at 37 °C under anaerobic conditions (Tharmaraj & Shah 2003).

2.5. Analytical methods

The pH of the lactic beverages was recorded using a digital pH meter (Hanna HI 2211-02, RI/USA). Titratable acidity was expressed in grams of lactic acid using the method described by Oladipo et al. (2014). The color of the samples was measured using a Minolta Spectrophotometer CM-3600d (Osaka, Japan). Whey separation was expressed as the volume of drained whey (mL) per 100 mL sample (Delikanli & Ozcan 2014). The organic acids such as lactic, citric, acetic, propionic, formic and butyric acids were determined as described by Akalin et al. (2002) using a Jasco High-Performance Liquid Chromatography (Dionex ICS 3,000, LC-900 Series, Dionex Corp., Sunnyvale, CA) equipped with a model H-980-01 holder that accepts Rheodyne valves, an 7124 injector fitted with a 20 µL sample loop, a Jasco PU-980 solvent delivery system, and an ICS series UV-visible/variable wavelength detector (Dionex VWD).

2.6. Statistical analysis

The Analysis of Variance and the Tukey test ($p<0.01$) were used to determine significant differences. All of the analyses were performed using the STATISTICA Software package for Windows 8.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results and Discussion

The viable counts of four probiotic strains in the lactic beverage with apple juice during 28 days of storage at 4±1 ˚C were presented in Figure 1. The changes of viable cell counts of all the strains during cold storage were significant ($p<0.01$). However, the attained viable cell number reduction was less than 1 $\log_{10}$ cfu mL^{-1}. It was observed that in the probiotic lactic beverage containing apple juice, all bacteria used were viable over the predicted shelf life, and the cell counts ranged from 7.48 to 12.00 $\log_{10}$ cfu mL^{-1}, respectively.

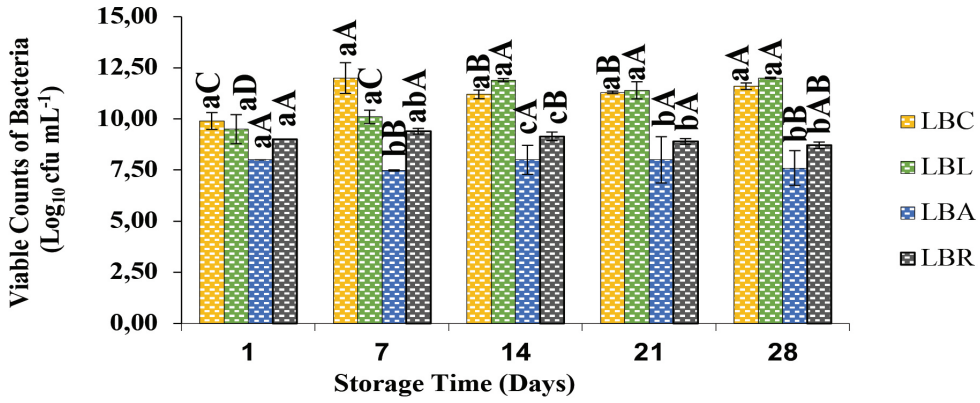

Figure 1- Viable counts of probiotic bacteria in lactic beverages
LBC: lactic beverage with *Lb. casei*, LBL: lactic beverage with *B. lactis*, LBA: lactic beverage with *Lb. acidophilus*, LBR: lactic beverage with *Lb. rhamnosus*. Different superscript (a-c) letter represent significant differences ($p<0.01$) between lactic beverage samples; Different superscript (A-C) letter represent significant differences ($p<0.01$) between different times of storage

The probiotic LAB used in this study had cell counts over 7.0 $\log_{10}$ cfu mL^{-1} at the end of the storage period which was higher than the minimum requirements by the Food and Agriculture Organization/World Health Organization to confer probiotic activity. This finding may highlight the probiotic value of the product retained and such a product could be a potential vehicle for probiotic delivery (Figure 1). Many factors may affect the viability and performance of probiotic bacteria in a complex food matrix, including the physicochemical properties of a food (carbohydrate, fat, solid non-fat and protein content, type of proteins, pH, acidity, etc.); rate and proportion of inoculation; food additives (bioactive compounds, sweeteners, stabilizers, etc.) which probiotic bacteria are exposed; the presence of organic acids, bacteriocins, SCFAs, hydrogen peroxide and other secondary metabolites produced by starter cultures; the strain used; rate and proportion of inoculation; applied temperature; fermentation and storage time; redox potential; final acidity of the product; molecular oxygen content and oxygen permeability through the packaging materials (Bazrafshan & Homayouni 2010; Kerry et al. 2018).

In the present study, *Lb. casei* and *B. lactis* respectively had greater viable cell counts than *Lb. acidophilus* and *Lb. rhamnosus*, possibly due to their higher acid tolerance and efficient utilization of essential nutrients in the apple juice such as dietary fibers, phenolics, and organic acids. These results align with those of Costa et al. (2013), Pereira et al. (2013) and Zandi et al. (2016) who studied the viability of *Lb. casei* in fermented fruit juices.

Perricone et al. (2015) suggested that the survival of probiotic species was the result of the synergistic and antagonistic action of many factors. They stated that pH and phenolic compounds exert a detrimental effect on viability, whereas protein and dietary fiber could protect cells from acidic stress.

Probiotic bacteria may utilize the carbohydrates present and produce organic acids resulting in lower pH of the product during storage. The optimum growth of *Bifidobacterium* occurs at pH 6.0-7.0; below pH 4.1 most probiotic species lose their viability within a week even at 4 ˚C, and below pH 2.5 the growth and survival of most species is limited within 3 h (Ding & Shah 2008). In general, the acid-tolerance of the *Bifidobacterium* species is strain-dependent, and hence, it can be considered that *Bifidobacteria* are highly sensitive to an acidic environment, with the exception of *Bifidobacterium animalis*, which can survive at acidic pH better than the other species (Sanchez et al. 2007). Improving the viability of probiotic bacteria in fermented dairy and non-dairy products until the time of consumption has been the concern of several studies. pH and titratable acidity are the most important factors that restrict the growth and survival of probiotics, and thus, their health benefits. Hydrogen ions may damage probiotic cells by changing the intracellular pH, disrupting mass transfer through the cell membranes and increasing the concentration of non-dissociated molecular organic acids. This results in enhanced bactericidal effect, which is pH dependent (Mortazavian & Sohrabvandi 2006).

It was observed that the pH in all beverages reduced whereas titratable acidity increased during 28 days of storage ($p<0.01$). Low pH and high acidity in the probiotic beverages containing *Lb. casei* and *Lb. rhamnosus* were associated with their ability to respond quickly to stress, high survival rate and stability (Table 1).

Ding & Shah (2008) reported that fruit juices may be an alternative vehicle for the incorporation of probiotics due to being rich in essential nutrients that have attractive organoleptic properties for consumer acceptance and preference. Fruit juices contain high amounts of carbohydrates that could encourage probiotic growth. They are often supplemented with oxygen scavenging ingredients such as ascorbic acid, thus promoting anaerobic conditions. *Lactobacilli* are generally resistant to low pH and survive in juices with pH ranging from 3.7 to 4.3; *Bifidobacteria,* however, are less acid tolerant, and a pH of about 4.6 is detrimental for their survival (Gueimonde et al. 2004; Kun et al. 2008).

Several strains of *Lb. plantarum, Lb. acidophilus* and *Lb. casei* can grow in fruit matrices due to their tolerance to acidic environments. However, the survival and storage behavior of probiotics in the fermented fruit-based matrix is more complex than in dairy products because the bacteria need more protection from the acidic conditions and other ingredients in the matrix (Ding & Shah 2008; Tamang et al. 2016). Saarela et al. (2016), reported that in apple juice the better survival and protection of *Lb. rhamnosus* were achieved in the presence of oat flour with 20% of β-glucan.

In the present study, whey separation was higher in LBC samples due to the high titratable acidity regarding storage time (Table 1). The color (L, a, b) values of the lactic beverages were found to be probiotic strain-dependent (Table 1) ($p<0.01$). LBR samples had higher L values than the other lactic beverages, since they were lighter in appearance due to the change in gelling properties. The redness (a) and yellowness intensity values (b) of lactic dairy beverages containing apple juice were higher in LBL samples at the end of storage, indicating that fermentation had a positive effect on the product color.

Various organic acids are known to be found in foods, like milk and fruit juices, including lactic, citric, orotic, benzoic, sorbic and others, which play a key role on sensorial characteristics for consumer acceptability, prevention of microbial growth, increasing the stability and quality of the product, and, especially, extending the predicted shelf life (Mato et al. 2005).

The preservation effect of lactic acid bacteria (LAB) in fermented foods is a result of the utilization of available carbohydrates and the formation of organic acids that in turn exhibit antimicrobial activity. In many studies, glucose has been introduced as the most important carbohydrate source for lactic probiotic species to enhance their growth and adoptability (Lankaputhra et al. 1996). Depending on the microorganisms involved the fermentation proceeds via the glycolysis pathway for homofermentative LAB with the almost exclusive formation of lactic acid, and via the pentose phosphate pathway for heterofermentative LAB with formation of lactic, acetic and other acids (Ozcan et al. 2021).

During the fermentation and storage of the lactic beverage containing apple juice, organic acids were formed according to the fermentation mechanisms of LAB. This biosynthetic mechanism was effective on aromatic preference. Lactic, acetic, malic, tartaric and citric acids were the major organic acids in the probiotic milk and lactic beverage samples. The amount of propionic and butyric acids, as SCFAs, had increased during fermentation in the lactic beverages depending on the activity of probiotic bacteria (Table 2, Figure 2a,b).

Lactic acid is known to be formed through the reduction of pyruvic acid, transformation of malic acid and/or lactose degradation. Lactic acid in apple juice was 1.09±0.172 mg g^{-1} and between 6.01±0.21 mg g^{-1} and in probiotic milk 11.83±0.25 mg g^{-1}, however its quantity was reduced in the lactic beverage consisting apple juice during storage with the exception of *Lb. rhamnosus*, which may account for the malolactic fermentation or favored reactions, which can potentially limit the prebiotic activity of the substrate. The differences between the lactic acid productions of the strains used may be the result of the different efficiency of the lactose-hydrolysing/galactosidase enzyme activity.

The acetic acid formation by the *Lactobacillus* strains may be the result of the biochemical pathway differentiation for carbohydrate utilisation, citrate metabolism and/or may originate from the heterofermentative pathway (Zalán et al. 2010). The acetate is an important parameter for the flavour development of many cultured milk products, and is linked to the citrate metabolism, since citric acid is stated to be metabolized into acetic acid (Torino et al. 2005).

Mousavi et al. (2011), reported that selected probiotic bacteria (namely *Lb. acidophilus*, *Lb. paracasei, Lb. plantarum* and *Lb. bulgaricus*) were capable of metabolizing citric acid when fermentation starts, while sugar consumption by all the strains was relatively low at this stage.

Table 1- Physicochemical properties of milk based lactic beverages produced with apple juice

Lactic beverage		*pH*	*Titratable acidity (g 100 g^{-1})*	*Whey separation (mL)*	*L*	*Color values a*	*b*
LBC	1st day	4.30±0.010^{bcB}	0.90±0.023^{aC}	0.00±0.000^{bD}	82.61±0.115^{cB}	0.88±0.050^{bB}	21.56±0.130^{aA}
	7th day	4.13±0.010^{dE}	1.07±0.009^{aB}	11.50±0.700^{aC}	83.27±0.075^{bA}	1.03±0.020^{bA}	20.73±0.050^{aB}
	14th day	4.22±0.010^{cC}	1.18±0.010^{aA}	20.50±0.707^{aB}	81.29±0.076^{cD}	0.64±0.010^{cC}	19.58±0.051^{bD}
	21th day	4.37±0.010^{cA}	1.09±0.009^{aB}	20.00±0.000^{abB}	81.84±0.140^{cC}	0.70±0.036^{cC}	19.86±0.115^{bC}
	28th day	4.15±0.010^{cD}	1.17±0.005^{aA}	29.00±0.000^{aA}	82.56±0.155^{aB}	0.83±0.029^{bB}	19.70±0.083^{bCD}
LBL	1st day	4.32±0.000^{bE}	0.85±0.010^{bC}	0.00±0.000^{bB}	81.67±0.352^{dB}	1.38±0.101^{aAB}	20.57±0.338^{bBC}
	7th day	4.45±0.010^{aD}	0.93±0.010^{bA}	5.75±3.889^{cB}	81.61±0.191^{cB}	1.26±0.123^{aD}	20.30±0.477^{aC}
	14th day	4.64±0.010^{aB}	0.93±0.010^{bAB}	19.00±4.242^{aA}	81.78±0.251^{bC}	1.38±0.057^{aD}	20.07±0.222^{aB}
	21th day	4.55±0.010^{bC}	0.90±0.010^{bB}	23.50±4.949^{aA}	82.76±0.075^{bA}	1.49±0.026^{aD}	21.87±0.085^{aA}
	28th day	4.66±0.010^{aA}	0.92±0.010^{cAB}	27.00±2.828^{aA}	81.64±0.272^{bB}	1.25±0.030^{aD}	20.79±0.120^{aBC}
LBA	1st day	4.56±0.020^{aA}	0.73±0.000^{cAB}	0.75±0.354^{abB}	85.17±0.026^{aA}	0.83±0.011^{bD}	19.31±0.487^{cA}
	7th day	4.39±0.010^{bC}	0.71±0.000^{cB}	6.50±4.949^{cAB}	83.64±0.103^{aB}	0.97±0.020^{bD}	19.08±0.020^{bA}
	14th day	4.46±0.030^{bB}	0.74±0.003^{cAB}	12.00±5.656^{bAB}	82.70±0.961^{bB}	0.84±0.086^{bD}	18.53±0.327^{cB}
	21th day	4.58±0.010^{aA}	0.73±0.010^{cAB}	15.50±6.363^{bA}	83.35±0.090^{bB}	1.04±0.030^{bD}	18.92±0.090^{cAB}
	28th day	4.41±0.010^{bBC}	0.75±0.010^{dA}	18.50±4.949^{bA}	82.93±0.244^{aB}	0.91±0.040^{bD}	18.87±0.157^{CAB}
LBR	1st day	4.29±0.000^{cA}	0.87±0.000^{bB}	1.50±0.707^{aE}	84.54±0.208^{bAB}	0.50±0.011^{cD}	19.09±0.037^{cB}
	7th day	4.24±0.035^{cAB}	0.92±0.010^{bB}	8.00±0.000^{bD}	83.94±0.209^{aC}	0.23±0.005^{cD}	19.12±0.056^{bB}
	14th day	4.24±0.010^{cB}	0.97±0.030^{bB}	14.50±0.500^{bC}	84.23±0.066^{aBC}	0.25±0.015^{dD}	19.40±0.073^{bB}
	21th day	4.22±0.005^{dB}	1.15±0.090^{aA}	17.00±0.000^{bB}	84.95±0.457^{aA}	0.29±0.090^{dD}	19.90±0.337^{bA}
	28th day	3.99±0.020^{dC}	1.14±0.010^{bA}	19.00±0.00^{bA}	82.73±0.198^{aD}	0.05±0.032^{cD}	19.33±0.172^{aB}

LBC: Lactic beverage with *Lb. casei*, LBL: Lactic beverage with *B. lactis*, LBA: Lactic beverage with *Lb. acidophilus*, LBR: Lactic beverage with *Lb. rhamnosus*. Different superscript (a-c) letter represent significant differences ($p<0.01$) between lactic beverage samples; different superscript (A-C) letter represent significant differences ($p<0.01$) between different times of storage

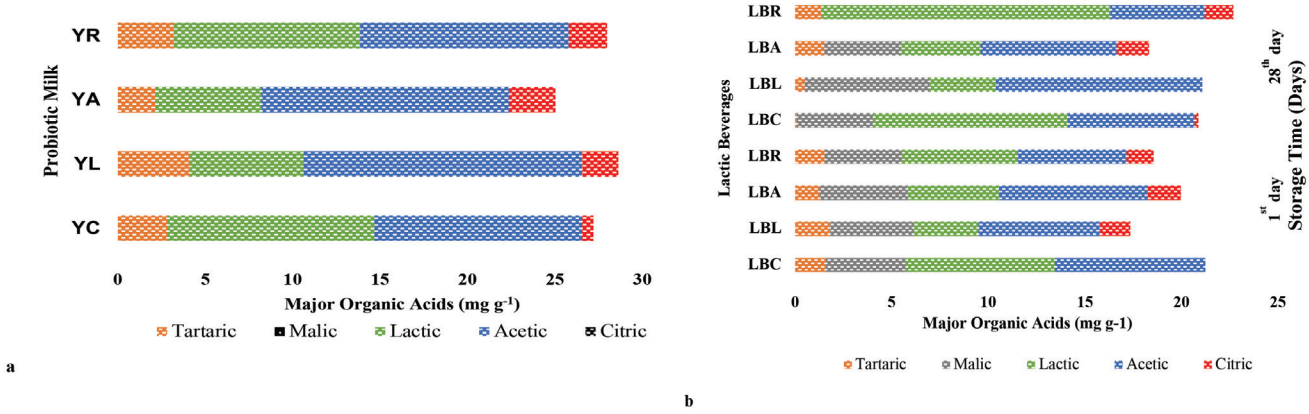

Figure 2- Major organic acids of lactic beverages (a) and (b) probiotic milk samples
LBC: Lactic beverage with *Lb. casei,* LBL: Lactic beverage with *B. lactis,* LBA: Lactic beverage with *Lb. acidophilus,* LBR: Lactic beverage with *Lb. rhamnosus.* YC: Probiotic milk with *Lb. casei,* YL: Probiotic milk with *B. lactis,* YA: Probiotic milk with *Lb. acidophilus,* YR: Yogurt with *Lb. rhamnosus*

Table 2- Organic acid profile of milk based lactic beverages produced with apple juice (mg g^{-1})

		Oxalic	*Tartaric*	*Malic*	*Lactic*	*Acetic*	*Citric*	*Fumaric*	*Propionic*	*Butyric*
Apple juice		0.029±0.010	1.367±0.174	7.076±0.242	1.091±0.172	0.310±0.093	0.114±0.015	0.001±0.000	0.329±0.075	0.048±0.009
Probiotic milk										
YC		0.057±0.003	2.847±0.024	0.000±0.000	11.827±0.247	11.863±0.763	0.672±0.037	0.001±0.000	0.047±0.014	0.016±0.004
YL		0.187±0.244	4.135±0.290	0.000±0.000	6.477±0.701	15.941±3.075	2.080±0.175	0.001±0.000	0.169±0.019	0.027±0.012
YA		0.058±0.007	2.177±0.104	0.000±0.000	6.005±0.208	14.194±0.788	2.656±0.240	0.001±0.000	0.088±0.016	0.197±0.060
YR		0.080±0.003	3.213±0.085	0.000±0.000	10.646±0.184	11.928±0.263	2.201±0.093	0.001±0.000	0.587±0.017	0.016±0.001
Lactic beverage										
LBC	1st day	0.103±0.009	1.596±0.165	4.148±0.173	7.716±0.290	7.780±0.247	0.000±0.000	0.001±0.000	0.038±0.009	0.252±0.032
	28th day	0.091±0.006	0.054±0.039	4.000±1.308	10.069±0.279	6.559±0.323	0.202±0.064	0.018±0.001	0.591±0.093	0.529±0.093
LBL	1st day	0.090±0.004	1.807±0.719	4.327±0.199	3.362±0.190	6.297±0.935	1.559±0.233	0.000±0.000	0.113±0.007	0.177±0.083
	28th day	0.092±0.004	0.500±0.106	6.473±0.294	3.411±0.241	10.697±0.384	0.000±0.000	0.005±0.001	0.173±0.014	0.042±0.002
LBA	1st day	0.101±0.012	1.280±0.097	4.551±0.210	4.721±0.087	7.680±0.310	1.749±0.244	0.000±0.000	0.118±0.008	0.120±0.017
	28th day	0.095±0.002	1.516±0.075	3.959±0.536	4.129±0.442	7.052±0.736	1.678±0.131	0.001±0.000	0.172±0.006	0.148±0.005
LBR	1st day	0.102±0.004	1.542±0.065	3.995±0.150	5.983±0.112	5.644±0.427	1.409±0.089	0.000±0.000	0.054±0.012	0.336±0.053
	28th day	0.109±0.010	1.359±0.150	0.000±0.000	14.956±0.202	4.885±0.019	1.498±0.037	0.000±0.000	0.741±0.212	0.820±0.002

LBC: Lactic beverage with *Lb. casei*, LBL: Lactic beverage with *B. lactis*, LBA: Lactic beverage with *Lb. acidophilus*, LBR: Lactic beverage with *Lb. rhamnosus*. Different superscript (a-c) letter represent significant *differences* ($p<0.01$) between lactic beverage samples; YC: probiotic milk with *Lb. casei*, YL: probiotic milk with *B. lactis*, YA: probiotic milk with *Lb. acidophilus*, YR: yogurt with *Lb. rhamnosus*. Different superscript (A-C) *letter* represent *significant differences* ($p<0.01$) between different times of storage

In the LBL sample, the acetic acid formation increased during storage, indicating that the amount and proportion of acids produced was highly dependent on the substrate metabolized (Table 2). Biedrzycka et al. (2003) and Ozcan & Eroglu (2023) reported that the generation of acetic acid by *Bifidobacteria* is much more stable than that of lactic acid, though the concentration of the latter may be higher, especially when the substrate is easily- and well-metabolized. According to the findings of the present work, the initial citrate concentrations in samples were higher than in the lactic beverage consisting apple juice at the end of the storage for each probiotic strain used; however, no relation was noted between the citrate utilization and the acetate production.

Fuleki et al. (1995), reported that malic acid, being responsible for the sour taste, was the most abundant acid in authentic apple juice. In the present study, malic acid was not detected in fermented probiotic milk samples (YC, YL, YA and YR). Its level decreased in LBC, LBA and LBR at the end of storage, whilst, for LBL beverages the malic acid content increased (Table 2). The reduction in malic acid content throughout storage for *Lactobacillus* spp. may account for the degradation of malic acid as a carbon source for bacterial growth and secondary bacterial fermentation (malolactic fermentation) (Zhang et al. 2008). Most LAB shape the decarboxylation of L-malate to L-lactate and CO_2 by a NAD^+ and Mn_2^+-dependent malolactic enzyme (MLE), although a few can convert L-malate into pyruvate by the action of a malic enzyme (ME). Biochemical evidence has shown that *Lb. casei* strains possess both ME and MLE activities. Even though the ME pathway enables *Lb. casei* to grow on L-malate, MLE does not support the proliferation (Landete et al. 2013). For *Lb. rhamnosus,* the significant decrease in malic acid occurred the more lactic acid formed, pointing to the presence of MLE. For *B. lactis* the conversion of citric acid into malic acid/acetic acid was observed.

It was found that bacterial growth in the lactic beverages containing apple juice increased due to the high sugar content of the apple juice and formed and/or metabolized organic acids, such observed in *Lb. acidophilus* and *Lb. casei* which used lactic acid as the major carbon source for growth. Moraru et al. (2007) showed that bacterial growth in vegetables juice with various concentrations led to a pH reduction and an increase in lactic acid, which was attributed to the sugar consumption and production of organic acid by the lactic acid cultures. Yoon et al. (2004) stated that *Lb. acidophilus, Lb. plantarum, Lb. casei* and *Lb. delbrueckii* subsp. *bulgaricus* were capable of rapidly utilizing tomato juice for cell synthesis and lactic acid production without nutrient supplementation and pH adjustment, even though the initial pH value was 4.1; however, the accumulation of lactic acid, diacetyl, and acetaldehyde as a result of growth and fermentation could reduce their viability.

4. Conclusion

Fermented milk has long been used as the main vehicles for probiotics containing essential nutrients and recently fruit juices have been exploited as suitable carriers for probiotics. Consequently, a milk-based lactic beverage containing apple juice could be considered as a novel probiotic beverage without the supplementation of extra nutrients.

Although many fruit juices have a low pH (pH <4.5) and may adversely affect probiotic growth, apple juice provides an ideal growth environment for probiotic microorganisms with the sugar, organic acids, phenolic compounds, dietary fiber, vitamins and other trace elements it contains. According to the results of the present work, *Lb. casei, B. lactis*, *Lb. acidophilus* and *Lb. rhamnosus* show some issues when being used as starter for production of milk fortified with apple juice, however, they showed high survival and potential prebiotic activity in probiotic milk + apple juice drink during cold storage at 4 ˚C of 4 weeks. The viable cell counts were higher than 10^7 cfu mL^{-1}, which was sufficient to confer beneficial health effects. Lactic, acetic, malic, tartaric and citric acids were highly detected, and the amount of propionic and butyric acids, especially SCFAs, increased during fermentation depending on the strain. One could say that the success of a new probiotic/lactic beverage depends particularly on the capability of the probiotic culture to provide satisfactory viable cells that beneficially modify the gut microbiota of the host. However, the challenge to meet consumer demands is related to development of milk + fruit juice fermented beverages with the introduction of probiotics through new methods, such as encapsulation, fortification with other ingredients, using non-conventional juices or non-conventional probiotics, to increase the bacterial survival. Furthermore, for designation of new generation milk beverages with potential prebiotic and postbiotic components further studies need to focus on *in vitro* and *in vivo* assays with metabolomics studies.

Data availability: Data are available on request due to privacy or other restrictions.

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