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Production of Low-Alcohol Fruit Beverages through Fermentation of Pomegranate and Orange Juices with Kefir Grains

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ABSTRACT

Fermentation of pomegranate juice as single or mixed substrate with orange juice, without addition of extra nutrients, using kefir grains is proposed. Sugar consumption and ethanol production were monitored during fermentation, while the formation of lactic acid and the survival of lactic acid bacteria were determined during storage at 4 °C for 4 weeks. The results showed that addition of orange juice improved the ability of kefir grains to ferment pomegranate juice, and increased the survival rates of lactic acid bacteria (LAB) contained in kefir grains during storage. Specifically, 75% cells survived (6.48 log cfu/ml) after 4 weeks of storage in the fermented mixed substrate (24% in plain pomegranate juice). Lactic acid formation was observed in all products, especially in the mixed substrate (1.3-1.9 g/l), indicating metabolic activity during storage. In all cases a low decrease of pH was observed. The results show the possibility to produce low-alcoholic nutritious fruit beverages with potential antioxidant (due to pomegranate constituents) and probiotic properties (due to the probiotic species present in kefir grains). In addition sensorial tests that were conducted showed the consumers acceptance for all the fermented juices.

Keywords: Kefir Grains, Pomegranate, Probiotic, Functional, Low-alcohol Beverage.

INTRODUCTION

Consumer awareness and demand for safer food, free from pathogenic microorganisms, microbial toxins and chemical contaminants, as well as with lower amounts of synthetic additives, has been constantly increasing over the past few decades. In this respect, there is an increasing positive response of consumers toward "functional" foods or food components, which claim to provide health benefits beyond their basic nutritional value[1]. A significant part of the functional food markets is represented by probiotics (a Greek-derived term meaning "for life"), which are foods containing live bacteria that can positively affect human or animal health by altering their intestinal microflora. The high nutritional value of fermented dairy products containing lactic acid bacteria such as yogurt, sour milk and kefir grains, is well established by scientific evidence[2,3]. Many single cultures such as *Lactobacillus casei*, *L. paracasei*, etc. or mixed cultures such as kefir grains, with potential uses as starter cultures for dairy products such as fermented milks, ice cream and yoghurt, have been characterized as probiotics[4,5,6,7].

Many probiotic species have been identified in kefir grains, a natural dairy culture consisting of a symbiotic consortium of yeasts and bacteria, used for alcoholic and lactic acid fermentation of milk in the areas around Caucasus [8,9,10]. Most of them are lactic acid bacteria such as *Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus kefiranofaciens*[11,8,12, 13]. In addition, several studies have shown the presence of bioactive constituents (e.g. antimicrobial, antitumor, anticarcinogenic and immunomodulatory) in kefir grains[14].

Regarding applications of kefir grains grains for ethanol and lactic acid production, cheese whey and molasses have been extensively evaluated as substrates[15,16,17]. Discarded fruit and wastes of the fruit processing industry have also been proposed as growth media for kefir grains and baker's yeast production[18]. Fruit juices can also be used as fermentation media or as carriers for probiotics since they contain high concentrations of sugars, dietary fibre and other components of high nutritional value, such as antioxidant polyphenolics that make them easy to gain consumer acceptance[19,20,21]. There is wide variety of ongoing research on fermented fruit juices such as beet[22], apple and orange[19], cranberry, lemon, pineapple, and pomegranate juices[23]. Pomegranate (*Punica granatum* L.), has been recently pointed out as a leader in the healthy beverage industry. Pomegranate fruit contain approximately 10% sugars (mainly fructose and glucose), 1.5% pectin, organic acids (ascorbic, citric & malic) and bioactive compounds (phenolics, flavonoids and anthocyanins)[24,25]. In comparison with grape, cranberry, grapefruit and orange juices, pomegranate juice exhibits 2- to 8-fold higher antioxidant capacity[20] and at least 20% higher than red wine and iced tea[21]. Pomegranate is native and widely cultivated in Asia, Southern Europe, Latin America and California, with a global production around 2 million tonnes. Pomegranate is also native in Greece and its cultivation is currently encouraged and funded by both public and private sectors.

Therefore, the aim of this study was to evaluate the use of pomegranate juice, as single or mixed substrate with orange juice, for the production of a low-alcohol functional beverage through fermentation with kefir grains, and assess the viability of LAB during storage, in order to survey potential probiotic properties.

Table 1: Sugar consumption and ethanol production during fermentation ofpomegranate, orange, and mixed juices, using kefir grains at 30 °C

Substrate	Fermentation time (h)	Sugar (g/l)	Alcohol (%v/v)
	0	73.3±0.1	0
	8	70.1±0.1	0
Pomegranate	10	67.4±0.1	0.1±0.02
	16	63.1±0.2	0.3±0.03
	20	60.6±0.2	0.3±0.02
	0	73.3±0.2	0
	8	67.8±0.2	0
Pomegranate /orange	10	62.1±0.1	0.1±0.03
	16	60.8±0.1	0.2±0.02
	20	59.5±0.1	0.5±0.03
	0	74.0±0.1	0
	8	66.5±0.2	0.1±0.02
Orange	10	60.2±0.2	0.4±0.01
	16	58.5±0.1	0.5±0.01
	20	56.3±0.3	0.5±0.02

MATERIALS AND METHODS

Microorganisms and media

Kefir grains were obtained from a commercial kefir product[9]. For kefir cell mass production, cheese whey was used as medium, which allows growth

Table 2: Viable cell counts (log cfu/ml) of LAB in the fermented pomegranate, orange, and mixed juices, during storage for 4 weeks at 4 °C

Storage time	Survival (log cfu/ml)		
(weeks)	Pomegranate	Pomegranate /orange	Orange
0	8.32±0.3	8.51±0.3	8.38±0.3
1	6.28±0.5	7.08±0.4	8.71±0.4
2	6.44±0.4	7.77±0.2	8.15±0.3
3	4.80±0.5	6.49±0.3	7.42±0.3
4	2.04±0.4	6.48±0.3	7.57±0.3

in the form of small kefir grains easy to harvest[17]. Growth took place at 30 °C without air supply or agitation to allow growth of both yeasts and bacteria. The produced kefir grains was harvested by centrifugation at 5000 rpm for 10 min and was used to ferment pomegranate and orange juices as described below. Cheese whey was produced in the laboratory from commercial cow's milk. The milk was placed in a water bath at 37 °C and 0.1 g/l of rennin was added. After 1 hour the whey was separated from the curd by cloth filtration. Pomegranates and oranges were obtained from a local market in Orestiada, Greece. The oranges were washed well with water and the external yellow parts (*exocarp*) were removed. The remaining fruit were blended for 10 min, and orange juice was extracted from the pulp suspension by cloth filtration. Sterilized water was added to adjust the initial sugar concentration and the final juice solutions were used without further sterilization. Pomegranate juice was obtained by blending the seeds for 10 minutes in a stomacher blender. Sterilized water was added to adjust the initial sugar concentration and the prepared juice solutions were also used without sterilization.

Pomegranate, orange and mixed juice fermentation

The juice substrates used for fermentation by kefir grains were: (i) 500 ml pomegranate juice, (ii) 500 ml orange juice, and (iii) 250 ml pomegranate juice mixed with 250 ml orange juice. The initial sugar concentration of the substrates was 73-74 g/l. Amounts of 0.4 g (dry weight) of kefir grains culture were suspended in each fermentation substrate, into sterilized conical flasks of 1000 ml. The initial pH of the substrates was adjusted to 3.8 and the fermentation temperature was controlled at 30 °C. The fermentations were stopped when the alcoholic degree had reached a value between 0.5-1 %v/v which is acceptable for the characterisation of a beverage as low-alcoholic[26]. Three parallel fermentation batches were conducted for each different substrate. Samples were collected at various time intervals and analyzed for residual sugar and alcohol concentration.

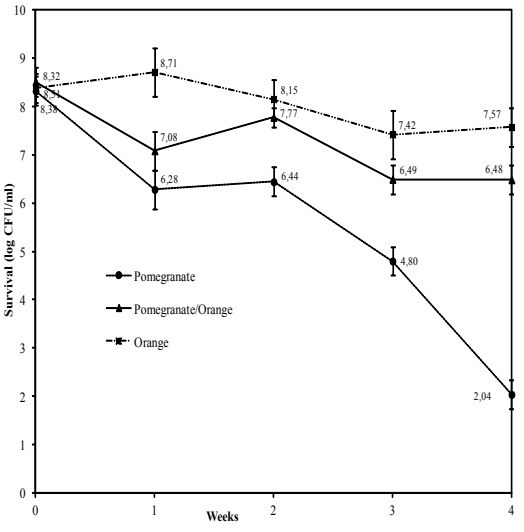

Fig. 1: Survival of kefir grains lactic acid bacteria (log cfu/ml) in the fermented pomegranate, orange, and mixed juices during storage at 4 oC.

Storage of the fermented juices

After the end of fermentation, the fermented substrates were stored for 4 weeks at 4° C. Every week of storage, samples were collected and analyzed for cell viability, ethanol, lactic acid and residual sugar concentrations. The experiment was carried out in triplicate and the results are presented as average values plus standard deviation to ensure the reproducibility.

Ethanol, lactic acid and residual sugar analysis

Ethanol, lactic acid and residual sugar concentrations were determined by high performance liquid chromatography, on a Shimadzu HPLC system consisting of a SCR-101N stainless steel column, a LC-9A pump, a CTO-10A oven set at 60 °C and a RID-6A refractive index detector. Three times distilled and filtered water was used as mobile phase with a flow rate of 0.8 ml/min and 1-butanol (0.1% v/v) as internal standard. Samples were filtered through 0.2 ìm microfilters, before injection. Ethanol (% v/v) and residual sugar (g/l) concentrations were calculated using standard curves. All results are presented as means of three repetitions plus standard deviation to ensure the reproducibility.

Microbiological analysis

Lactic acid bacteria (LAB) in the fermented juices were determined as colony forming units (cfu/ml). Specifically, serial decimal dilutions of the fermented liquids were prepared using 1/4 strength Ringer's solution, and aliquots were spread on acidified MRS agar (Fluka, 69964). Growth took

place at 37°C for 72h, anaerobically (Anaerobic jar, Anerocult C, Merk). The results are presented as means of log cfu/ml counts of the plates containing 30-300 cfu/ml. The initial added viable cell concentration for all the fermentations was approximately 8.0±0.2 log cfu/ml.

All results are presented as means of three repetitions plus standard deviation to ensure the reproducibility.

Sensory evaluation

All samples were evaluated (compared) by a panel of non-trained people after the end of fermentation and every week of storage at 4 °C. The results, based on a 0-10 preference scale, are presented as average scores plus standard deviations for aroma, taste and overall quality.

RESULTS AND DISCUSSION

Pomegranate and orange juice as single or mixed substrates were fermented with kefir grains at 30 °C and initial pH 3.8, without addition of extra nutrients, in order to produce a low-alcoholic beverage using: (two fruit juices, well established for their high nutritional value and antioxidant properties, and (ii) kefir grains. with potential probiotic properties due to the probiotic species present in their natural microflora.

The first part of the experimental design comprised a set of fermentations of three substrates (pomegranate, orange, orange and pomegranate juices) that were carried out until an alcoholic degree of 0.5-1 %v/v was obtained as required for low-alcoholic beverages[26]. The next set of experiments involved the preservation of the three fermented juices during storage at 4 °C that was evaluated through the changes in their chemical composition and the monitoring of viable cell counts.

The results for ethanol formation and sugar consumption during fermentation of the different juices at 30 °C are presented in Table 1. Plain pomegranate juice was more difficult substrate to ferment compared to orange juice or the mixed substrate as previously discussed[27]. Sugar consumption was slow, and the maximum alcohol degree obtained after 20 h of fermentation was 0.3 %v/v. Comparing the three substrates, the most efficient results were obtained in the case of orange juice, with faster sugar consumption and maximum alcoholic degree (0.5 %v/v) reached at 16 h of fermentation. Even though plain pomegranate juice was proved not efficient substrate, the addition of orange juice to pomegranate juice improved the fermentation kinetics.

Counts of viable LAB (log cfu/ml) during storage of the fermented juices are presented in Table 2. This determination was conducted because, in order for probiotics to survive the adverse conditions of the proximal gastrointestinal tract, they should be present in a product, claiming probiotic properties, at a concentration of about 10^6-10^7 cfu/ml at the end of product shelf-life[7]. In all juices, the number of cells decreased after the first two weeks of storage, especially in the case of those with lower pH (Table 2, Figure 1). In the case of pomegranate juice this decrease was more rapid. The initial viable cells concentration was 8.32 log cfu/ml and the lowest remaining at the end of the storage period was 3.04 log cfu/ml. The differences in viable cell counts between weeks 0 and 4 are illustrated in Figure 2, showing that LAB survived better in the fermented orange juice. However it is noteworthy

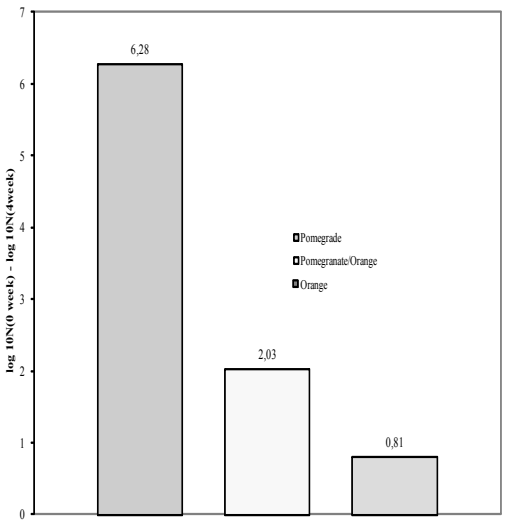

Fig. 2: Decrease of viable cell counts of kefir grains lactic acid bacteria (log cfu/ml) in the fermented pomegranate, orange, and mixed juices between the first and last week of storage at 4 °C

that the combination of pomegranate with orange juice improved the cell viability (75% survival) during storage compared to plain pomegranate juice (24%). These results are in agreement with previous works that demonstrated the antimicrobial properties of pomegranate juice due to the high amounts of

Table 3: Residual sugar, lactic acid formation, and final pH, in the fermented pomegranate, orange, and mixed juices, during storage for 4 weeks at 4°C

Substrate	Storage time (weeks)	pH	Total sugar (g/l)	Lactic acid (g/l)
Pomegranate	0	3.15±0.1	59.6±0.2	0
	1	3.26±0.1	55.4±0.3	0.1±0.01
	2	3.19±0.1	61.5±0.3	0.3±0.02
	3	3.08±0.05	63.4±0.3	0.5±0.03
	4	3.11±0.05	63.9±0.2	0.9±0.02
Pomegranate	0	3.34±0.1	61.5±0.2	0
/orange	1	3.32±0.05	60.4±0.3	0.2±0.2
	2	3.27±0.05	59.4±0.1	0.5±0.1
	3	3.19±0.1	62.4±0.2	0.9±0.2
	4	3.22±0.05	64.2±0.3	1.3±0.2
Orange	0	3.45±0.1	58.3±0.1	0
	1	3.41±0.05	61.4±0.3	0.3±0.02
	2	3.28±0.1	63.4±0.2	1.1±0.02
	3	3.34±0.1	62.1±0.1	1.5±0.03
	4	3.19±0.05	60.5±0.3	1.9±0.02

Table 4: Changes in the sensory evaluation scores of fermented pomegranate, orange, and mixed juices, during storage for 4 weeks at 4°C

	Storage time (weeks)	Aroma	Taste	Overall quality
Pomegranate	0	8.1±0.3	8.0±0.1	8.1±0.3
	1	8.1±0.2	8.0±0.1	8.1±0.2
	2	8.1±0.3	7.9±0.1	8.1±0.3
	3	8.1±0.1	8.0±0.1	8.0±0.1
Pomegranate	4	8.2±0.2	8.0±0.1	7.9±0.2
/orange	0	8.2±0.3	8.1±0.2	8.2±0.3
	1	8.2±0.1	8.1±0.1	8.1±0.2
	2	8.2±0.1	8.2±0.1	8.2±0.3
	3	8.1±0.3	8.1±0.2	8.1±0.1
	4	8.1±0.1	8.1±0.3	8.1±0.2
Orange	0	8.2±0.1	8.1±0.1	8.1±0.1
	1	8.2±0.1	8.1±0.2	8.1±0.2
	2	8.2±0.3	8.2±0.1	8.1±0.3
	3	8.1±0.3	8.2±0.2	8.2±0.3
	4	8.2±0.2	8.2±0.2	8.2±0.1

components such as polyphenols and tannins[27,23]. Another reason for the small survival rates may be the low pH of the fermented juices (Table 3).

Regarding the chemical compositional changes taking place during storage, small amounts of lactic acid (0-1.9 g/l) were produced after the 1st week and continued to increase until the 4th week (Table 3), indicating metabolic activity of the kefir grains during storage. Higher amounts of lactic acid were found in the fermented orange juice, which is consistent with the better survival rates of lactic acid bacteria in this product. These findings are in accordance with the results of another work suggesting that kefir grains, and especially *Lactobacillus* probably use the energy generated through this metabolic activity in order to maintain their viability[23]. Despite the increase of lactic acid, the pH of the fermented juices decreased slightly over the four weeks of storage, probably due to buffering capacities of the substrates. The fermentable sugar concentration (glucose, fructose and sucrose) in all cases slightly increased or fluctuated during storage, indicating simultaneous utilization by kefir grains (in order to produce lactic acid) and production by enzymatic hydrolysis of polysaccharides contained in the juices [19,28].

Finally, a preliminary sensory evaluation performed by non-trained testers (consumers) to compare the produced fermented juices in terms of aroma, taste and overall quality (preference), showed none significant differences between all the examined fermented juices, which in general were accepted from the panel.

CONCLUSION

Fermentation of pomegranate juice as single substrate or mixed with orange juice using kefir grains is proposed for a low-alcohol beverage production with high nutritional value deriving from both the substrate and the culture used. The suggested functionality of such beverage is based on the well established beneficial effects of pomegranate and orange juices, mainly due to their antioxidant properties, as well as the probiotic properties of kefir grains and bioactive ingredients found in kefir grains. The viability of LAB in the fermented products was preserved for 4 weeks at levels near to the limits set for products claiming probiotic properties. However, microbiological identification of kefir grains as well as *in vitro* and *in vivo* tests, are necessary for characterisation of such products as probiotics. Molecular methodologies are currently applied in our lab and the study will be continued and updated results will be presented at the near future.

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