Characterization of pomegranate juice and whey based novel beverage fermented by kefir grains

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Abstract Mixture of pomegranate juice and whey was evaluated as a potential substrate for production of a novel probiotic beverage by kefir grains. Different fermentation conditions were used as viz: two fermentation temperature (19°C and 25°C) and two levels of kefir grains inoculum (5 % and 8%w/v). pH, acidity, lactose consumption as well as organic acids formation were determined during 32 hours of fermentation. Results showed that kefir grains were able to utilize lactose and decrease pH, increase acidity, produce lactic acid and acetic acid, while the level of citric acid decreased. It was observed these change depended on temperature and level of kefir grains with the highest changes at the temperature of 25°C and kefir grains inoculum of 8%w/v. Pomegranate juice and whey mixture therefore may serve as a suitable substrate for the production of novel probiotic dairy-fruit juice beverage by kefir grains and the sensory characteristics of this beverage were shown desirable results.

Keywords Kefir · Fermentation · Pomegranate juice · Whey

Introduction

In recent years, there has been an increased interest in the production of fermented beverages specially probiotics due to their health beneficial effects and nutritional properties. This has led to an increase in production and consumption of food

which are recognized as functional food. Probiotics can be described as foods containing live microorganisms that when are ingested in adequate amounts, confer a health benefit on the host (Vasiljevic and Shah 2008). Fruit juices are a rich source of calcium and vitamin and could serve as suitable media for cultivating probiotic bacteria. Use lactic acid bacteria in food industry, has been utilized for production of functional foods in recent years. Kefir which is made with kefir grains is a refreshing, naturally carbonated fermented dairy beverage that has a slightly acidic taste, yeasty flavor and creamy consistency (Powell et al. 2007). The traditional kefir is produced by the addition of small kefir grains to fresh milk. Kefir grains are like small cauliflower florets, 1–3 cm in length, lobed, irregularly shaped, white to yellow-white in color, and have a slimy but firm texture (La Rivière et al. 1967). Kefir grains contain proteins, polysaccharides and complex mixture of microorganisms. Lactic acid bacteria (LAB) and yeasts have a complex symbiotic relationship in kefir grains and responsible for alcoholic and lactic acid fermentation, respectively. Some of distinctive features of lactic acid fermentation are growth of lactic acid bacteria, production of different organic acids, degradation of some anti-nutritional factor such as metal chelating agents in raw plant materials like phytate, oxalate and tanins and decrease in pH (Reddy and Pierson 1994).

Whey is a rich source of proteins with high biological value. Whey proteins include α -lactalbumin (α -La), β -lactoglobulin (β -Lg), bovine serum albumin and immunoglobulins (BSA). β -Lg is a small and soluble globular protein which has monomer mass of 18 KDa. It has a lot of useful nutritional and functional food characteristics that have made it a choice as an ingredient for formulation of novel foods and beverages. β -Lg shows many biological activity such as anti-hypertensive, anti-cancer, hypo-cholesterolemic and anti-microbial (Chatterton et al. 2006). α -La is another major protein in whey that makes up about of 25 % of total whey proteins. It enhances calcium absorption because it is a

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calcium binding protein. Amino acids such as lysine, leucine, threonine, tryptophan and cysteine are present in large amounts in the whey (Permyakov and Berliner 2000).

Pomegranate (*Punica granatum*, Punicaceae) is one of the important table fruit and is known to have considerable health-promoting properties such as antimicrobial, antiviral, antioxidant and anti-mutagenic effects (Negi et al. 2003). Considerable amount of total soluble solids, total sugars, reducing sugars, anthocyanins, phenolics, ascorbic acid, proteins and antioxidants are present in fresh juice. Level of antioxidants in pomegranate juice was shown to be higher as compared to green tea and red wine (Gil et al. 2000) and these antioxidant are more potent than many other antioxidants such as vitamin C and E (Aviram et al. 2002).

The aim of this research was to prepare a fermented dairy-fruit juice beverage and study the suitability of mixture of whey and pomegranate juice as a media for the growth of lactic acid bacteria and yeasts, sugar consumption and organic acids formation during kefir grains fermentation. Pomegranate juice was used in this study because of its antioxidant activities and nutrient factor. Also it may make better the sensory quality of beverage and enhances the acceptability of it.

Materials and methods

Kefir grains were collected from a household in Tehran, Iran. The grains were kept in pasteurized milk at room temperature. Milk was exchanged every 2 days to maintain the grains viability. The commercial concentrated pomegranate juice and whey used in this study were supplied from Alifard (Sunich, Iran, Saveh) and Safadasht (Iran, Karaj) cheese making company, respectively.

Preparation of fermented beverage

Whey was diluted with distilled water in a portion of 1:1 and then mixed with pomegranate juice concentrate to 14° brix. Mixture was pasteurized at 60°C for 30 min. Kefir grains were removed from milk, washed with distilled water and then inoculated into the prepared beverage at two levels (5 % and 8 % w/v). The beverage was incubated at two temperatures (19 and 25°C) for 32 hours. The fermentation runs were assessed through periodic sampling in order to determine characteristics of beverages. After completion of fermentation the kefir grains were removed, washed with distilled water and returned to milk and beverage was kept at suitable temperature for future analysis.

Chemical and microbial analysis

The pH of the samples was measured by a digital pH meter (Metrohm 744, Netherland). Total titratable acidity was

determined by titrating the samples with 0.1 N NaOH to pH 8.1 and expressed as %w/w lactic acid. Quantitative analysis of organic acids (lactic, citric and acetic acids) was carried out by HPLC (Knauer, Germany) apparatus equipped with a K- 2600 UV-visible detector at 210 nm. A separation column (Ultrasep ES-FS special 250×3 mm) set with 0.001 N sulphuric acid as the mobile phase with 0.2 ml/min flow rate. Room temperature was used for operation and the injection volume was 20 µl. Organic acids content was determined using external standards (Mousavi et al. 2011). Lactose amount was also determined by HPLC (Knauer, Germany) equipped with a K-2301 refractive index detector. A 10 µm separation column (Eurokat H 300×8 mm) was employed and the mobile phase was sulphuric acid 0.01 N with a flow rate of 0.5 ml/min. The injection volume was 20 µl and temperature of 30°C was used for this analysis. Lactose content was reported using external standards (Mousavi et al. 2011).

Lactobacilli counts (Log CFU/ml) were performed on MRS medium at an incubation temperature of 30°C for 2 days. PDA medium was used for counting yeast at incubation temperature of 25°C for 3 days and expressed as log CFU/ml (Liu and Lin 2000).

Sensory evaluation

Each sample was tested for smell, flavor, consistency, color and overall acceptability by 10 panelists. Each sensory characteristic was scored on an increasing scale from 1 (bad) to 5 (great) (Watts et al. 1989).

Statistical analysis

All experiments were carried out in triplicate and each sample was analyzed in duplicate. The results are expressed as mean ± SD. Duncan's multiple range tests were used to compare the difference among mean values of beverage's properties at the level of 0.05 and SAS software (version 9.1; statistical analysis system institute Inc., Cary, NC, USA) was used for analysis.

Results and discussion

Some of the initial characterizations of whey and pomegranate juice concentrate are given in Table 1a and b, respectively.

pH and acidity

Changes in pH value during cultivation of different levels of kefir grains in beverage are depicted in Fig. 1. As can be observed pH value decreased from an initial value of 4.23±0.03 to lower value depended on type of fermentation. Hence

Table 1 a: some of initial characteristics of pomegranate juice concentrate, b: some of initial characteristics of whey

a									
pH	Acidity (%w/w)	Brix	Turbidity	Density (g/ml)	Vitamin C (mg/100 ml)	Reducing sugar (g/100 ml)	Sucrose (g/100 ml)	Citric acid (g/l)	Acetic acid (g/l)
3.84±0.02	2.1±0.03	64	0.84±0.011	1.33±0.21	129.23±3.1	41.32±1.5	0.75±0.04	20±1.1	0.15±0.01
b									
pH	Density (g/ml)	Ash (%w/w)	Dry matter (%w/w)	Lactose (g/100 m)	Protein (%w/w)	Fat (%w/w)			
4.9±.03	1.01±0.17	0.47±0.015	5.9±0.034	5.01±0.06	0.83±0.41	0.55±0.03			

Data are means ± standard deviation

total acidity of beverages increased. The highest decrease in pH value was observed during the second and third 8 hours of fermentation. pH value in the examined beverages was the lowest in beverage fermented with 8 % w/v kefir grains in temperature of 25°C, 3.40 ± 0.02 . Higher pH value was obtained for fermented beverages in temperature of 19°C and 25°C with 8 %w/v and 5 % w/v kefir grains, respectively (3.48 ± 0.022 and 3.50 ± 0.017 , respectively) and the highest in beverage fermented with 5 % w/v kefir grains in 19°C (3.61 ± 0.02). Bensmira and Jiang (2011) reported a drop in pH value of peanut-milk kefir during fermentation. They attributed this drop to a decrease in lactose amount and a consequent increase in lactic acid content. On the other hand Magalhães et al. (2010) reported production of lactic acid during fermentation of kefir is of great importance due to its inhibitory effect on both spoilage and pathogenic microorganisms. So it can be said reduction in pH value moreover leads to increase in total acidity, causes an inhibition of spoilage and pathogenic microorganism growths, too. It was shown temperature of 25°C

could affect more on pH value change as compared to 19°C (Fig. 1). This might be due to the optimum temperature for enzymes or metabolic activities of microorganisms in kefir grains. In our previous experiment (data have not been published) three levels of fermentation temperature were used for preparation of a novel fermented beverage by kefir grains (20°C, 25°C and 30°C). We found that temperature of 25°C was more suitable than others. Also temperature of 20°C was better than 30°C for metabolic activity of kefir grains.

Lactose consumption during the fermentation process

Kefir microorganisms metabolized lactose as a carbon source, but in different beverages amount of lactose consumption were different as shown in Fig. 2. It can be observed that most of lactose present in beverage was metabolized within 32 hours of fermentation. On the other hand, among all beverages the highest consumption of lactose occurred in

Fig. 1 Changes in pH and acidity of different beverages during fermentation process
Fermentation temperature of 19°C: open symbol, Fermentation temperature of 25°C: closed symbol, kefir inoculation of 5 % w/v: circle, kefir inoculation of 8 % w/v: square. Filled lines: pH value and Dashed lines: titratable acidity. Bars represent the standard deviation

Fig. 2 Kinetic of lactose consumption during fermentation process Fermentation temperature of 19°C: open symbol, Fermentation temperature of 25°C: closed symbol, kefir inoculation of 5 % w/v: circle, kefir inoculation of 8 % w/v: square. Bars represent the standard deviation

fermented beverage with 8 %w/v kefir grains in temperature of 25°C which decreased from the initial amount of 2.4 ± 0.06 g/100 ml to 0.5 ± 0.042 g/100 ml with 79.17 % reduction. It was shown earlier this sample had the lowest pH and highest acidity, too. The lower lactose metabolize was observed for fermented beverages with 8 % w/v kefir grains in temperature of 19°C and 5 %w/v kefir grains in 25°C, while the lowest was found in beverage fermented with 5 %w/v kefir grains in temperature of 19°C with decrease from 2.4 ± 0.06 g/100 ml to 1.1 ± 0.068 g/100 ml (reduction of 54.17 %). It was earlier reported by Magalhães et al. (2011) that the use of whey as substrate for the production of whey-based beverage resulted in lower lactose metabolize than that was found during milk fermentation with kefir grains. They suggested it could be probably due to the characteristics of milk which is richer in nutrients than whey. In present study, it was observed that fermentation temperature and level of kefir grains might have affected the lactose metabolized in beverage during fermentation process, while Magalhães et al. (2011) have reported that type of substrate is another factor which could have affected on this parameter.

Organic acid changes during fermentation

Volatile compounds are important in flavor of beverage as they determine different desirable sensory characteristics (Arrizon et al. 2006). Formation of organic acids in dairy products may occur as a result of hydrolysis of butterfat (fatty acids), biochemical metabolic processes and bacterial metabolisms (Güzel-Seydim et al. 2000). Figures 3 and 4 show the

time evolution of organic acids (Lactic acid, citric acid and acetic acid) during the fermentation process. As depicted in Fig. 3, lactic acid is recognized as the main metabolic produce by kefir microorganisms during fermentation in all samples. Lactic acid which is a common end product of bacterial fermentation increased significantly ($P < 0.05$) from initial value of 0.9 ± 0.1 g/l to 5.9 ± 0.15 g/l, 5.3 ± 0.11 g/l, 5.2 ± 0.13 g/l and 4.6 ± 0.1 g/l in beverage fermented with 8 % w/v kefir grains in temperature of 25°C, with 5 % w/v kefir grains in 25°C, with 8 % w/v kefir grains in 19°C and in temperature of 19°C with 5 % w/v kefir grains, respectively. This is according to Güzel-Seydim et al. (2000) who reported kefir has lower lactic acid content as compared to yogurt (8.8–14.6 g/l) and it is probably due to the preferential use of the heterofermentative pathway rather than homofermentative pathway. Production of lactic acid during fermentation has been attributed to lactic acid bacteria metabolisms and is important due to inhibitory effect on both spoilage and pathogenic microorganisms in kefir (Magalhães et al. 2010). In case of citric acid significant decrease ($P < 0.05$) was observed after 32 hours of fermentation (Fig. 3). This decrease in beverage fermented with 8 % w/v kefir grains in temperature of 25°C was significantly ($P < 0.05$) more than others with a reduction from initial amount of 6.1 ± 0.2 g/l to 0.9 ± 0.1 g/l. According to Güzel-Seydim et al. (2000) citric acid is the preferred substrate for formation of diacetyl and acetoin by some lactic acid bacteria.

The initial mean value of acetic acid was 0.1 ± 0.014 g/l which increased during 32 hours of fermentation process. The highest final concentration for acetic acid was observed in

Fig. 3 Kinetics of changes in lactic acid and citric acid during fermentation process
Fermentation temperature of 19°C: open symbol, Fermentation temperature of 25°C: closed symbol, kefir inoculation of 5 % w/v: circle, kefir inoculation of 8 % w/v: square. *Filled lines*: citric acid and *Dashed lines*: lactic acid. *Bars* represent the standard deviation

beverage fermented with 8 % w/v kefir grains in temperature of 25°C, 0.7 ± 0.016 g/l. Bensmira and Jiang (2011) and Magalhães et al. (2011) reported that during first 18 and 24 hours of peanut milk kefir fermentation and whey or milk kefir fermentation, respectively mean concentration of acetic acid was practically zero, while we found acetic acid was

present in beverage before fermentation and increased from the first hour of fermentation. This difference may be due to the presence of pomegranate juice in formulation. The increase of acetic acid could be attributed to the heterofermentative acetic acid and lactic acid cultures present in kefir grains microflora (Magalhães et al. 2010). According to the results

Fig. 4 Kinetic of changes in acetic acid during fermentation process Fermentation temperature of 19°C: open symbol, Fermentation temperature of 25°C: closed symbol, kefir inoculation of 5 % w/v: circle, kefir inoculation of 8 % w/v: square. *Bars* represent the standard deviation

Fig. 5 Bacterial growth kinetic during 32 hours fermentation of beverages Fermentation temperature of 19°C: open symbol, Fermentation temperature of 25°C: closed symbol, kefir inoculation of 5 % w/v: circle, kefir inoculation of 8 % w/v: square. Bars represent the standard deviation

obtained, it was revealed that temperature of 25°C was more suitable for metabolic activity of kefir grains microflora as compared to 19°C, since changes in organic acid as well as pH, acidity and lactose amount were higher at this temperature. On the other hand with increase in kefir grains inoculation from 5 % w/v to 8 % w/v these changes were occurred more.

Microbial analysis

Figure 5 shows the changes in lactobacilli population during 32 hours of fermentation. Immediately after the addition of 8 % w/v kefir grains the population of lactobacilli bacteria were 5 ± 0.1 log CFU/ml, while in the case of 5 % w/v kefir

Fig. 6 Yeast growth kinetic during 32 hours fermentation of beverages Fermentation temperature of 19°C: open symbol, Fermentation temperature of 25°C: closed symbol, kefir inoculation of 5 % w/v: circle, kefir inoculation of 8 % w/v: square. Bars represent the standard deviation

Table 2 Final characteristics of different beverages

Samples		pH	Acidity (%)	Lactose (g/100 ml)	Lactic acid (g/l)
Fermentation Temperature (°C)	Kefir grains inoculation (%w/v)				
19	5	3.61±0.02 ^a	0.95±0.036 ^c	1.1±0.068 ^a	4.6±0.1 ^c
19	8	3.48±0.022 ^b	1.21±0.028 ^b	0.8±0.08 ^b	5.2±0.13 ^b
25	5	3.50±0.017 ^b	1.19±0.03 ^b	0.86±0.079 ^b	5.3±0.11 ^b
25	8	3.40±0.02 ^c	1.44±0.035 ^a	0.5±0.042 ^c	5.9±0.15 ^a
		Citric acid (g/l)	Acetic acid (g/l)	Lactobacilli bacteria (log CFU/ml)	Yeast (log CFU/ml)
19	5	2.5±0.13 ^a	0.5±0.018 ^c	7±0.13 ^d	5.25±0.09 ^a
19	8	1.7±0.14 ^b	0.61±0.02 ^b	7.3±0.13 ^c	5.3±0.08 ^a
25	5	1.9±0.17 ^b	0.62±0.019 ^b	7.6±0.2 ^b	5.28±0.1 ^a
25	8	0.9±0.1 ^c	0.7±0.016 ^a	8.2±0.15 ^a	5.4±0.13 ^a

Means within the same column with different letters are significantly ($P<0.05$) different. Data are means ± standard deviation

grains it was 4.7 ± 0.12 log CFU/ml. This shows part of kefir grains microflora was transferred to beverage after inoculation. These results are according to Liu and Lin (2000) who reported after addition of kefir grains to soymilk, bacteria were transferred to beverage. After 32 hours of incubation at 25°C the lactobacilli counts increased by 3.2 log cycles from 5 ± 0.1 log CFU/ml to 8.2 ± 0.15 log CFU/ml in beverages fermented with 8 % w/v kefir grains, but by only 2.3 log cycles from 5 ± 0.1 log CFU/ml to 7.3 ± 0.13 log CFU/ml in beverage fermented with 8 % w/v kefir grains but in temperature of 19°C. This indicates a temperature of 25°C is more suitable than 19°C for lactobacilli bacteria growth. Similar result was obtained for when 5 % w/v kefir grains was used for fermentation.

The initial counts of yeasts in beverages fermented with 8 % w/v and 5 % w/v kefir grains were 2.8 ± 0.1 log CFU/ml and 2.53 ± 0.08 log CFU/ml, respectively (Fig. 6). After 32 hours of fermentation, no significant difference ($P<0.05$) was observed in yeast counts in different beverages with a final concentration of 5.4 ± 0.13 log CFU/ml, 5.3 ± 0.08 log CFU/ml, 5.28 ± 0.1 log CFU/ml and 5.25 ± 0.09 log CFU/ml for fermented beverages with 8 % w/v kefir grains in temperature of 25°C, with 8 % w/v kefir grains in 19°C, with 5 % w/v kefir grains in 25°C and 5 % w/v kefir grains in 19°C, respectively.

Results obtained for lactobacilli bacteria and yeasts were in the range reported by other researchers (Liu and Lin 2000; Irigoyen et al. 2005; Fontán et al. 2006).

At table 2 the final characteristic including pH, acidity, lactose amount, organic acids, lactobacilli and yeast population of different beverages, are given.

Sensory evaluation

Results of smell, flavor, consistency, color and overall acceptance evaluation are shown in table 3. As can be observed smell and flavor of fermented beverage by 5%w/v kefir grains in temperature of 25°C have gotten the highest degree as compared to others (scores of 3.92 ± 0.27 and 4.02 ± 0.24 , respectively). Between consistency of this sample and beverage prepared with 5%w/v kefir grains in temperature of 19°C, no significant difference ($P<0.05$) was observed. Also there weren't any significant difference ($P<0.05$) between various beverage in parameter of color. The overall acceptability of beverage fermented by 5%w/v kefir grains in temperature of 25°C was the highest with score of 4.32 ± 0.32 and it wasn't significantly different ($P<0.05$) with overall acceptability of beverage by the same level of kefir gains but was fermented at temperature of 19°C.

Table 3 Sensory characteristics of beverages

Samples		Smell	Flavor	Consistency	Color	Overall acceptability
Fermentation temperature (°C)	Kefir grains inoculation (%w/v)					
19	5	3.3±0.17 ^b	3.31±0.2 ^b	4.02±0.24 ^{ab}	4.12±0.18 ^a	4.01±0.26 ^{ab}
19	8	2.9±0.2 ^c	3.14±0.11 ^b	3.5±0.19 ^c	4.2±0.18 ^a	3.4±0.16 ^c
25	5	3.92±0.27 ^a	4.02±0.24 ^a	4.41±0.3 ^a	4.43±0.22 ^a	4.32±0.3 ^a
25	8	3.03±0.12 ^{bc}	3.04±0.22 ^b	3.61±0.22 ^{bc}	4.1±0.2 ^a	3.6±0.14 ^{bc}

Conclusion

Fermentation of pomegranate juice and whey mixture by kefir grains promoted considerable changes in the substrate such as lactose consumption, acid production and decrease in pH value as a result of bacterial and yeast (naturally present in kefir grains) growths during 32 hours of fermentation process, but these changes depended on the level of kefir grains inoculum and temperature used for fermentation. Lactose was consumed in all beverages, pH decreased and acidity increased significantly ($P < 0.05$) after fermentation. On the basis of results obtained in this study it was revealed that mixture of pomegranate juice and whey can be used for the growth of kefir grain microorganisms and production of organic acids such as lactic acid and acetic acid which can result in the production of a healthy fermented dairy-fruit juice drink. Also this beverage have shown to possess good antioxidant activity (data have not been published) and sensory evaluation of these beverage revealed good results.

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