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Research note

Fermentation conditions of walnut milk beverage inoculated with kefir grains

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ABSTRACT

The aim of the present study was to evaluate the use of kefir grains as inoculum for the preparation of walnut milk beverage. The results showed that the single factor effect of fermentation time, fermentation temperature and sucrose concentration on walnut milk beverage fermentation was very significant ($P = 0.01$), and the single factor effect of inoculum size was significant ($P = 0.05$). The suggested optimum fermentation conditions are the following: fermentation temperature of 30 °C, fermentation time of 12 h, inoculum size of 3 g of kefir grains (wet weight) and sucrose concentration of 8 g/100 mL. Under the optimum fermentation conditions, the sensory evaluation score of the beverage reached its maximum value of 88, and the pH and titratable acidity of the beverage were respectively 4.16 and 72 °T. The viable cell counts of lactococci, lactobacilli and yeast surviving in the beverage were 8.2×10^7, 1.1×10^8 and 1.0×10^6 CFU/mL respectively.

1. Introduction

Walnut (*Juglans regia* L.) is a crop of high economic interest to the food industry: the edible part of the fruit (the seed or kernel) is consumed, fresh or toasted, alone or in other edible products. It is globally popular and valued for its nutritional, health and sensory attributes. The fresh natural kernels are consumed mainly as whole nuts or used in various confectioneries. In recent decades, several researchers have tried to ferment walnut milk using various species of lactic acid bacteria (LAB) in order to produce walnut-milk-based probiotic beverages (Hou, Xing, & Liu, 2008; Jing, 2006; Wang, 2010).

Kefir is a type of sour fermented milk in which kefir grains are employed as a starter culture (Bosch et al., 2006). The kefir grains have a specified structure and behave as biologically vital organisms. Various LAB present in kefir grains or kefir products were isolated and identified by physiological and biochemical tests (Chen, Wang, & Chen, 2008; Witthuhn, Schoeman, & Britz, 2005). Beside LAB, yeasts and acetic acid bacteria have also been shown to be present in kefir grains. Kefir culture has been used as a starter culture in cheese production (Dimitrellou, Kourkoutas, Banat, Marchant, & Koutinas, 2007; Kourkoutas et al., 2006) and has also been used for the production of bread with improved quality and shelf life (Plessas, Pherson, Bekatorou, Nigam, & Koutinas, 2005) and for the production of a new whey beverage with good organoleptic properties (Athanasiadis, Paraskevopoulou, Blekas, & Kiosseoglou, 2004).

The question arose whether the walnut milk could be fermented using kefir grains as a starter culture (i.e. as inoculum) and be processed into a probiotic beverage. Therefore, the aim of this study was to determine the effect of fermentation temperature, fermentation time, inoculum size and sucrose concentration on walnut milk fermentation, as well as, to optimize the fermentation conditions of the walnut milk beverage, and investigate the chemical, microbial and sensory characteristics of the walnut milk beverage.

2. Materials and methods

2.1. Kefir grains and inoculum preparation

The kefir grains used in this study were collected from Tibet, China and were preserved by the Food Chemistry Laboratory, Shanxi University. Frozen kefir grains were activated by placing 18 g of the grains in 500 mL of sterile cow milk, followed by incubation at 25 °C. After 24 h, the grains were sieved out and washed with cooled sterile distilled water. This step was repeated for three consecutive days (Witthuhn et al., 2005). The inoculum was prepared by transferring kefir grains (small grains of approximately 1–2 mm diameter) to a 250 mL Erlenmeyer flask containing 100 mL of sterile cow milk. Kefir grains cultivation was carried out statically in an incubator set at 25 °C for 24 h, renewed daily, for a duration of 7 days. Kefir grains as inoculum were stored at 4 °C prior to use.

2.2. Preparation of walnut milk

The methodologies proposed by Su, Chen, Zhang, Heng, and Liu (2008) were used for walnut milk preparation. Walnut fruits (*J. regia* L.) were collected from commercial plantations at Fenyang

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City, Shanxi Province, China. After cleaning, the fruits were dried at 30 ± 2 °C for 24 h and were then cracked and shelled manually. The skin of the nuts was separated by soaking the nuts in water overnight and removed by hand. After removing the skin, the nuts were mixed with 4.5 times their weight of distilled water (50 °C) and then ground in a blender for 5 min. The resultant slurry was filtered through a double-layered cheesecloth to yield walnut milk. Walnut milk was dispensed into containers and was autoclaved at 121 °C for 15 min.

2.3. Effect of fermentation temperature, time, inoculum size and sucrose concentration on walnut milk beverage fermentation

Autoclaved walnut milk (100 mL) and sucrose (8 g) were introduced into an Erlenmeyer flask. The effect of inoculum size on the fermented walnut milk beverage was studied by a series of fermentations conducted using 3, 5, 7 and 9 g of kefir grains (wet weight, containing 89 g/100 g moisture) as the inoculum per flask, carried out at 25 °C for 18 h. Furthermore, the effect of fermentation temperature was studied by a series of fermentations conducted using 5 g of kefir grains (wet weight) as the inoculum per flask, carried out at 25, 30, 35 and 40 °C respectively for 18 h. The effect of fermentation time was studied by a series of fermentations conducted for 9, 12, 15 and 18 h, carried out at 25 °C using 5 g of kefir grains (wet weight) as the inoculum per flask. To study the effect of sucrose concentration, 6, 8, 10 and 12 g of sucrose were added to 100 mL of the walnut milk, resulting in sucrose concentrations of 6, 8, 10 and 12 g/100 mL, respectively, and the fermentations were carried out at 25 °C for 18 h using 5 g of kefir grains (wet weight) as the inoculum per flask.

Fermentations were carried out statically in an incubator set for different times at different temperatures of the experimental design. At the end of the fermentation, the flasks were transferred to a 1–5 °C incubator for ripening for 12 h. Viable counts, pH, acidity and sensory analyses were determined at the end of each process.

2.4. Optimization of fermentation

The optimum fermentation conditions were studied through an orthogonal experimental design, where the initial inoculum size, fermentation temperature, fermentation time and sucrose concentrations were changed from 3 to 7 g of kefir grains (wet weight), 25–35 °C, 9–15 h, and 6–10 g sucrose/100 mL (Table 1).

2.5. Chemical analysis of the fermented walnut milk beverage

The pH of the fermented walnut milk beverage was measured using a pH meter (model 868; Orion, Beverly, MA, USA) fitted with a glass electrode. The titratable acidity (TA) was determined according to the Association of Official Analytical Chemists (AOAC, 1997) Method No. 947.05 and expressed as °T. One acidity unit (°T) means the amount of 1 mL 0.1 mol equiv/L NaOH used for the titration of 10 mL of beverage.

Initial pH and TA of the walnut milk were about 6.68 and 13.7 °T, respectively.

2.6. Viable cell counts determination

Representative 10 mL portions of duplicate beverage samples were blended with 90 mL of sterilized trisodium citrate (2 g/100 mL) solution and subjected to serial dilutions. The following tests on viable cell counts determination were performed (spread plate method): (i) lactococci counts on M17 agar (Difco, Detroit, MI, USA) at 37 °C (thermophilic strains) or 30 °C (mesophilic strains) for 72 h, (ii) lactobacilli counts on acidified MRS agar (Difco, Detroit, MI, USA) at 37 °C for 72 h anaerobically (Anaerobic jar, Anerocult C, Merck, Darmstadt, Germany), (iii) yeast counts on Yeast extract–Peptone–Dextrose agar (YPD, Difco, Detroit, MI, USA) at 30 °C for 72 h. The colonies that appeared on the plates were counted and recorded as colony forming units (CFU) per mL of beverage.

2.7. Sensory evaluation

The sensory properties of the walnut milk beverage were evaluated by a 5-member trained panel (2 men, 3 women; age range 21–50) from the School of Life Science, Shanxi University. The samples were served at 7–10 °C in plastic cups and were coded with three-digit numbers. Order of presentation of samples was randomized. The panel was asked to give scores on a 0–20 scale for color (milky white, light yellow and dark), a 0–30 scale for flavor (walnut fragrance, light smell and bad smell), a 0–30 scale for taste (walnut taste, slightly walnut taste and bitter taste) and a 0–20 scale for texture (uniformity, some sediment and sediment) (Jing, 2006). The beverages were evaluated in five replicates in each session and the mean score of the beverages for each quality attribute was calculated.

2.8. Experimentation and analysis

All experiments were replicated in three flasks and the data are presented as the mean and standard error of three independent experiments. Duncan's multiple range test (Du, 1985) was used to determine the significant differences among mean values at the $P = 0.05$ level. Analysis of variance of the orthogonal experimental results was carried out using the Statistical Analysis System software (SAS version 9.00, SAS Institute, Inc., 2000), the sources of variance being inoculum size, fermentation temperature, fermentation time and sucrose concentration.

Table 1
Sensory evaluation result of orthogonal experiment to assess optimal fermentation conditions.

Experimental no.	Inoculum size (g/100 mL)	Fermentation temperature (°C)	Time (h)	Sucrose concentration (g/100 mL)	Titratable acidity (°T)	Sensory evaluation scores
1	3	25	9	6	54 ± 1.2	71 ± 3.2^{c}
2	3	30	12	8	72 ± 2.0	88 ± 3.6^{a}
3	3	35	15	10	80 ± 2.1	79 ± 3.5^{b}
4	5	25	12	10	60 ± 1.5	80 ± 3.5^{b}
5	5	30	15	6	74 ± 1.9	82 ± 3.4^{b}
6	5	35	9	8	67 ± 1.5	74 ± 3.1^{bc}
7	7	25	15	8	62 ± 1.4	80 ± 3.6^{b}
8	7	30	9	10	68 ± 1.8	77 ± 3.4^{bc}
9	7	35	12	6	79 ± 2.1	74 ± 3.2^{bc}

Data represent means ± standard deviation ($n = 15$).
Values in the same column with different superscripts are significantly different ($P = 0.05$).

Table 2
Effect of fermentation temperature on the titratable acidity and sensory evaluation of walnut milk beverage.

Fermentation temperature (°C)	Titratable acidity (°T)	Sensory evaluation scores
25	57 ± 1.4^{c}	71 ± 3.2^{c}
30	75 ± 2.1^{b}	89 ± 3.5^{a}
35	84 ± 2.3^{a}	80 ± 3.5^{b}
40	88 ± 2.4^{a}	73 ± 3.1^{c}

Data represent means ± standard deviation ($n = 3$ for titratable acidity; $n = 15$ for sensory evaluation scores).
Values in the same column with different superscripts are significantly different ($P = 0.05$).

3. Results and discussion

3.1. Effects of fermentation temperature, inoculum size, fermentation time and sucrose concentration

The effect of fermentation temperature on TA and sensory evaluation of the walnut milk beverage were studied at various temperatures of 25, 30, 35 and 40 °C (Table 2). The result showed that enhancement of fermentation temperature from 25 to 40 °C increased the TA of the beverage significantly. Due to lower TA than 60 °T at 25 °C, the sensory evaluation score of the beverage was lower. However, due to higher TA than 80 °T, which results in a bitter taste and a lower sensory evaluation score, the fermentation temperatures of 35 and 40 °C were not the optimum temperatures for the fermentation of walnut milk beverage. The fermentation at 30 °C resulted not only in a higher TA than 70 °T, but also the highest sensory evaluation score of 89. Therefore, the optimum fermentation temperature was concluded to be 30 °C.

The results in Table 3 show that the TA had a positive linear relationship to the amount of inoculum: the higher the amount of inoculum, the higher the TA. The inoculum size of 9 g of kefir grains resulted in the highest TA (76 °T). However, the inoculum size showed no linear correlation with the sensory evaluation scores. The sensory evaluation scores of beverages inoculated with the inoculum size of 3 g and 9 g were lower than those inoculated with the inoculum size of 5 g and 7 g. The sensory evaluation score of a beverage inoculated with an inoculum size of 7 g of kefir grains reached a maximum score of 86, which indicated that 7 g of kefir grains were the optimum inoculum size.

The results showed that extension of fermentation time from 1 to 18 h increased the TA of the beverage from 20 to 88 °T, and the TA had a positive linear relationship to the fermentation time. The longer the fermentation time was, the higher the TA, e.g. 15 h after inoculation, the TA reached higher than 80 °T. However, the sensory evaluation results (70, 86, 76 and 71 scores for the beverages of 9, 12, 15 and 18 h fermentation, respectively) showed that 12 h after inoculation, the sensory evaluation score of the beverage was the highest although its TA was *ca* 74 °T.

Table 3
Effect of inoculum size on the titratable acidity and sensory evaluation of walnut milk beverage.

Inoculum size (g/100 mL)	Titratable acidity (°T)	Sensory evaluation scores
3	61 ± 1.5^{c}	72 ± 3.3^{c}
5	63 ± 1.5^{c}	80 ± 3.5^{ab}
7	70 ± 1.9^{ab}	86 ± 3.7^{a}
9	76 ± 2.1^{a}	70 ± 3.2^{c}

Data represent means ± standard deviation ($n = 3$ for titratable acidity; $n = 15$ for sensory evaluation scores).
Values in the same column with different superscripts are significantly different ($P = 0.05$).

Table 4
Effect of sucrose concentration on the titratable acidity and sensory evaluation of walnut milk beverage.

Sucrose concentration (g/100 mL)	Titratable acidity (°T)	Sensory evaluation scores
6	75 ± 2.1^{a}	80 ± 3.6^{b}
8	74 ± 2.0^{a}	88 ± 3.7^{a}
10	73 ± 2.0^{a}	83 ± 3.5^{ab}
12	73 ± 2.1^{a}	76 ± 3.3^{b}

Data represent means ± standard deviation ($n = 3$ for titratable acidity; $n = 15$ for sensory evaluation scores).
Values in the same column with different superscripts are significantly different ($P = 0.05$).

The results in Table 4 show that the amount of sucrose had no remarkable effect on the TA of the beverage, and had no positive correlation with the sensory evaluation scores. The sensory evaluation score of the beverage with 12 g of sucrose was the lowest. The sensory evaluation score of the beverage, where 8 g of sucrose were added, reached the maximum score of 88, which indicated that sucrose concentration of 8 g/100 mL was the optimum sucrose concentration.

3.2. Identification of optimum fermentation condition

Through orthogonal experiments (Table 1), the theoretical optimum fermentation conditions for the walnut milk beverage were the following: fermentation temperature of 30 °C, fermentation time of 12 h, inoculum size of 3 g and sucrose concentration of 8 g/100 mL. Under these conditions, the sensory evaluation score of the beverage reached its maximum value of 88. The result of the orthogonal experiment also showed that the single factor effect of fermentation time, fermentation temperature and sucrose concentration on walnut milk beverage fermentation was very significant ($P = 0.01$), and the single factor effect of inoculum size was significant ($P = 0.05$) (Table 5).

Under the conditions of the orthogonal experiment 2, the sensory evaluation of walnut milk beverage was the following: color of milky white, flavor of walnut fragrance, walnut taste, moderately sweet, pH and TA of the beverage were 4.16 and 72 °T, respectively. The viable cell counts of lactococci, lactobacilli and yeast surviving in the beverage were 8.2×10^{7}, 1.1×10^{8} and 1.0×10^{6} CFU/mL respectively.

Probiotics represent probably the archetypal functional food, and are defined as alive microbial supplements, which beneficially affect the hosts by improving their intestinal microbial balance (Brown & Valiere, 2004; Kalliomaki et al., 2001). According to Espinoza and Navarro (2010), probiotics have been added to yogurt and other fermented dairy products. However, an increased demand for non-dairy probiotic products comes from veganism. Probiotic products from various food matrices including fruits (Prado, Parada, Pandey, & Soccol, 2008) and vegetables (Yoon, Woodams, & Hang, 2006) are being developed. In recent decades, several researchers have tried to ferment walnut milk using various

Table 5
Variance analysis of orthogonal experiment.

Variation source	Degree of freedom	Sum of squares	*F* value	$F_{0.05}$	$F_{0.01}$
Inoculum size	2	8.6	43*	19	99
Fermentation temperature	2	74.6	373**		
Fermentation time	2	84.6	423**		
Sucrose concentration	2	38	190**		
Error	2	0.2			
Sum	10	206			

*Statistically significant effects ($P = 0.05$).
**Statistically very significant effects ($P = 0.01$).

species of LAB in order to produce walnut-milk-based probiotic beverages (Hou et al., 2008; Jing, 2006; Wang, 2010). It had been reported that the viable cell counts of lactococci and lactobacilli surviving in the walnut milk beverage were about 7.9×10^8 CFU/mL (Jing, 2006). In our experiment, the viable cell counts of lactococci, lactobacilli and yeast surviving in the beverage were 8.2×10^7, 1.1×10^8 and 1.0×10^6 CFU/mL respectively. The results suggest that the kefir grains have the potential for use as a starter culture in the fermentation of the walnut milk beverage.

4. Conclusion

This study was the first to report on the fermentation of walnut milk beverage inoculated with kefir grains. The following conclusions can be drawn: (1) kefir grains can be used to ferment walnut milk; (2) out of several factors tested, fermentation time had a pronounced effect on the quality of the beverage, followed by fermentation temperature, sucrose concentration and inoculum size; (3) the suggested optimum fermentation conditions according to our experiments are the following: fermentation temperature of 30 °C, fermentation time of 12 h, inoculum size of 3 g of kefir grains and sucrose concentration of 8 g/100 mL.

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