

The development of legume-based yogurt by using water kefir as starter culture

¹Lim, X. X., ¹Koh, W. Y., ^{1*}Uthumporn, U., ¹Maizura, M. and ²Wan Rosli, W. I.

¹Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia,
Minden 11800, Penang, Malaysia

²School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

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Abstract

The aim of the present work was to develop legume-based kefir yogurts to replace conventional dairy yogurts that are not suitable to be consumed by vegetarians and consumers who have lactose intolerance and milk allergy. Soy and black bean milk were incubated at 15°C and 20°C for 24 h with 35 g of water kefir grains to produce kefir yogurt. The proximate composition, physico-chemical and microbiological characteristics of the yogurts were evaluated. At 20°C, soy and black bean milk produced kefir yogurts with significantly lower pH, total soluble solids, and sucrose concentration, indicating that fermentation process carried out at 20°C had higher efficiency than 15°C; meanwhile, black bean milk produced better kefir yogurts than soymilk. Black bean kefir yogurt which was fermented at 20°C had higher level of total plate count (2.05×10^7 CFU/mL), yeast and mould count (6.95×10^6 CFU/mL), and lactobacilli count (8.3×10^5 CFU/mL) as compared to other kefir yogurts. In general, 20°C produced kefir yogurt with better technological properties. Both soymilk and black bean milk were good alternative substrates for kefir yogurt production.

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Introduction

The development of functional foods has become more important and popular as healthy eating habits help in preventing disease. Probiotic products are one of the common food categories within the functional food market.

Kefir is a natural probiotic as it contains many different strains of live active cultures that help to overtake pathogenic organisms, repopulate the digestive tract and aid in digestion (Otlés and Cagindi, 2003). Kefir grains are a complex mixture of lactic acid bacteria, yeasts, and acetic acid bacteria. The bacteria that can be found in kefir includes lactobacilli, lactococci, streptococci, enterococci, *Leuconostoc*, acetic acid bacteria and other bacteria such as *Bacillus* spp. and *Micrococcus* spp. (Farnworth, 2005). There are two types of kefir namely milk kefir and water kefir. Fermenting any fresh mammalian milk using kefir grains produced milk kefir while water kefir is produced by fermenting alternative milk such as soy, rice or almond milk, vegetable and fruit juices. A regular consumption of

kefir can give a healthier digestive system, improve the immune system, control tumour activities, support detoxification, inhibit pathogenic microorganisms, control cholesterol level and build bone density.

According to U.S. Food and Drug Administration (2016), yogurt is defined as “the food produced by culturing one or more optional dairy ingredients (cream, milk, partially skimmed milk, or skim milk, used alone or in combination) with a characterising bacterial cultural that contains the lactic acid-producing bacteria, *Lactobacillus bulgaricus*, and *Streptococcus thermophilus*”. Lactic acid bacteria in yogurt provide health benefits such as lactose digestion, intestinal microflora modulation, cholesterol reduction, immune system stimulation, and cancer prevention. Yogurt has now become part of the human’s diet because of the increasing awareness on the health benefits of yogurt.

Legumes are excellent sources of protein, starch, fat and oil, minerals, vitamins and health-protective compounds such as phenolic, inositol phosphates and oligosaccharides (Schuszter-Gajzágó, 2009). Soybean (*Glycine max* (L.) Merr.) is a legume

*Corresponding author.
Email: sapina@usm.my

crop grown worldwide for its high protein and oil contents. It is also an excellent source of protein and dietary fibre. Soymilk provides health benefits such as reducing cardiovascular disease, reducing menopausal symptoms, promoting eye health or anti-cataract, weight loss, arthritis, diabetes, osteoporosis, and brain function. Black bean (*Phaseolus vulgaris* L.) is a kind of common bean and it is commonly used as a traditional food ingredient. Black beans are rich in proteins, minerals, vitamin B, carbohydrates and fibre (Silva *et al.*, 2012). The occurrence of cancer, obesity and coronary heart diseases can be reduced and prevented through consumption of black beans.

In recent years, the demand for vegetarian probiotic products increased throughout the developed countries because of the ongoing trend of vegetarianism. On the other hand, lactose intolerance and milk allergic discourage a population of consumers from consuming milk or dairy products. As soymilk and black bean milk are lactose-free, they are safe to be consumed by vegetarians and people who have lactose intolerance and milk allergy.

In the past, researches on non-dairy based kefir (water kefir) usually employed fruit and vegetable juices (Randazzo *et al.*, 2016). There are also past studies on the development of soymilk yogurts by using milk kefir (McCue and Shetty, 2005; Kwon *et al.*, 2006). However, to the best of our knowledge, no research on the development of legume-based yogurt using water kefir has been performed thus far.

Materials and methods

Materials

Soybeans, black beans and white sugar were purchased from local hypermarket, Tesco Stores (Malaysia) Sdn. Bhd. (Penang, Malaysia). Water kefir grains were purchased from culture supplier, My Kefir World Company (Kuala Lumpur, Malaysia). Chemicals used in the present work were copper sulphate, sulphuric acid, sodium hydroxide, boric acid, hydrochloric acid, dichloromethane, and ethanol, and were all of analytical grade.

Preliminary study

A preliminary study was carried out to investigate the suitability of soybean milk and black bean milk to be used as the medium for fermentation. The appropriate length of fermentation time and suitability of fermentation temperature were determined.

Production of kefir yogurt

About 100 g soybeans were first washed and soaked in distilled water for 8 h. The soaked soybeans

were then blended in a blender with 1,000 mL distilled water for 3 min. The resultant slurry was then filtered through a muslin cloth to yield soymilk. The soymilk was dispensed into a pot and heated to 100°C for 30 min. The heated soymilk was then cooled to room temperature. The soymilk was transferred into glass jars. Black bean milk was similarly prepared.

Soy kefir yogurt was prepared by adding 35 g water kefir grains into 500 mL soymilk with 25 g white sugar. The 35 g water kefir grains were packed in a blank tea bag with string prior to addition into the medium. The mixture was then incubated (covered with cloth) for 24 h at 15°C and 20°C, respectively. Following fermentation, the kefir grains were removed. Black bean kefir yogurt was similarly prepared. Control was prepared by using similar formulation but without the addition of water kefir grains. All the kefir yogurts and their respective controls were labelled as in Table 1.

Proximate analysis

Kefir yogurts and their respective controls were analysed for proximate composition – moisture, crude fat, crude protein, ash, total carbohydrate, and calorie content. The moisture content was determined using the oven-drying method (method 925.40; AOAC, 2006) while the crude fat content was determined using the dichloromethane method (Stefanov *et al.*, 2010). The protein content was determined using the Kjeldahl method (method 950.48), whereas the ash content was determined using the dry ashing method (method 950.49; AOAC, 2006). The total carbohydrate content “carbohydrate by different” was determined by subtracting the sum of the percentage of moisture, crude fat, crude protein and ash from 100%. The calorie content was determined using conversion factors as follow:

$$\text{Calorie content} = (\% \text{ carbohydrate} \times 4) + (\% \text{ protein} \times 4) + (\% \text{ fat} \times 9)$$

pH

The pH of the kefir yogurts and their respective controls were determined using pH meter (Mettler-Toledo, Switzerland).

Total soluble solids

The total soluble solids of the kefir yogurts and their respective controls were determined using a refractometer (Mettler Toledo, US).

Sugar composition

The sugar composition of the kefir yogurts and

their respective controls was determined using high-performance liquid chromatography (HPLC) (Teh *et al.*, 2010). Approximately 50 mL sample that had been filtered through muslin cloth was transferred into Erlenmeyer flask. Around 2 g cation exchange resins (Amberlite H) were added and left for 15 min with occasional swirling, and filtered. The same treatment was repeated by using anion exchange resins (Amberlite A-27). About 25 mL filtrate was then centrifuged for 10 min at 3,500 g. About 5 mL of upper phase of the centrifuged sample was loaded into a Sep-Pak C18 (Waters, Milford, USA) and filtered through 0.45 µm Millipore filter (Millipore Corporation, Milford, USA). The filtered sample was then diluted 50 times with deionised water. Sugars (sucrose, glucose, and fructose) were determined in the combined extracts using HPLC with a refractive index detector. The column used was the Water™ Sugar Pak I column with premixed HPLC grade CaEDTA 0.0001 M as the mobile phase. The working conditions were the flow rate of 0.5 mL/min, detector temperature of 90°C and pressure of 40 mmHg. The injection volume was 20 µL. The concentration of sugar was determined by assessing the calibration lines of each chromatogram peak of sugars in the standard.

Texture profile analysis

Texture profile analysis (TPA) of yogurt samples was performed by using a TA.XT2 texture analyser (Stable Micro Systems Ltd, Surrey, UK) (Yang and Li, 2010). The back-extrusion cell (A/BE - d35) with 35 mm disc and extension bar using 5 kg load cell were used to measure the TPA of the sample. The speed of the probe was 5.0 mm/s during the pre-test, compression, and relaxation of the sample. The thickness of the sample was set at 5 cm in the container of 8 cm. Hardness, adhesiveness, springiness, cohesiveness, and gumminess of yogurt samples were analysed.

Viscosity analysis

The viscosity of the kefir yogurts and their respective controls was determined using a viscometer (Brookfield DV-E Viscometer, United States). All the samples were measured by using spindle number 4 at a shear rate of 2.5 cP.

Microbiological analysis

The microbiological analysis was carried out on the kefir yogurt samples and their respective controls. The analyses involved were Total Plate Count, Yeast and Mould Count, and lactobacilli count.

Serial dilution

Sterilised peptone water (0.1%) was used as the diluents in this microbiological analysis. About 10 g of each sample was aseptically taken, followed by dilution in 90 mL sterilised peptone water (0.1%), homogenisation in a stomacher for 1 min and serial dilution up to 10^{-5} . Next, 1 mL of each dilution was transferred to a sterilised Petri dish and suitable media was used to determine the viable cell counts using the pour plate method. All tests were done in triplicates.

Preparation of growth media

About 17.5 g PCA powder, 39 g PDA powder and 68.2 g MRS agar powder were separately dissolved into 1 L distilled water, followed by heating on a hotplate. The media was boiled until the media was completely clear. The media was then autoclaved for 15 min at 121°C.

Bacteria enumeration and plate counting

Pour plate method was used to make the viable count. About 1 mL of each serial dilution (up to 10^{-5}) prepared was transferred to triplicates sterilized Petri dishes. Approximately 15 mL agar was poured into the Petri dishes after being cooled to around 45°C and mixed well. After the agar solidified, the plates with PCA and MRS were inverted and incubated at 35°C for 24 h and 37°C for 48 h respectively, while the plates with PDA were incubated without inversion at 25°C for 5 d. The PDA plates were re-incubated for another 48 h if there was no growth after 5 d of incubation.

Statistical analysis

Statistical analysis was conducted by using SPSS Statistics Desktop 22.0 (IBM Corporation, USA). Data were expressed as the mean ± standard deviation from three independent parallel experiments. The analysis of variance (ANOVA) was performed and the significant differences between the means values were determined by using Duncan's multiple range tests at 5% probability level.

Results and discussion

Preliminary study

A preliminary study had been conducted to study the suitability of the substrates (soybean and black bean) as well as the effects of fermentation temperature and fermentation time for kefir yogurt production. The effects of these parameters on the fermented products were determined by visual inspection and sensory evaluation as recorded in Table 1. Based on the results, both soybean and black

bean were found to be suitable substrates for water kefir fermentation. The incubation temperatures of 15°C and 20°C and fermentation time of 24 h were found to be the most suitable condition to produce kefir yogurts.

Proximate analysis

The proximate composition of kefir yogurts and their respective controls are presented in Table 2. Based on the results, the protein content significantly ($p < 0.05$) increased in the fermented samples as compared to control in the order of SK15 > SC15;

SK20 > SC20; BK15 > BC15; BK20 > BC20. During fermentation, carbohydrates and fats were used to form biomass, which is carbon dioxide and water, thus, resulted in an increment of protein content. The higher protein content might also be due to hydrolysis of protein occurred during fermentation by kefir grains. During fermentation, bacteria and yeasts in kefir grains broke the peptides into amino acids and hence, the protein content of the samples increased along the fermentation process.

The moisture content of fermented samples was lower as compared to control. This could be due

Table 1. Preliminary study.

Parameter of fermentation	Sensory evaluation	Visual inspection
10°C, 24 h; 10°C, 48 h 	Fermented products had no sour smell. Beany taste of soymilk and black bean milk remained after fermentation.	Fermented products were very watery.
15°C, 24 h 	Fermented products had a sour smell and tasted slightly sour. No beany taste remained after fermentation.	Fermented products were smooth and firm in texture.
15°C, 48 h; 20°C, 48 h; 25°C, 24 h; 25°C, 48 h 	Fermented products had undesirable (very sour) taste and smell.	Fermented products were very dry and separated into whey and curds.
20°C, 24 h 	Fermented products had stronger sour taste and smell than those fermented at 15°C for 24 h.	Fermented products were smooth but firmer as compared to those fermented at 15°C for 24 h.

to the increased dry matter content as a result of microbial cell proliferation (Obadina *et al.*, 2013). During fermentation, microorganisms used some moisture for their metabolic activities. The decrease in moisture content may also be due to the increased total solid content in fermented samples, which is known to be found at all stages of a fermentation process.

The fat contents of the samples were not significantly affected by the fermentation process ($p > 0.05$). Our result agrees with Irigoyen *et al.* (2005) who proved that the fat content of the product showed no differences from its raw source after fermentation by using kefir.

Fermented samples contained higher ash content as compared to unfermented samples. This could be due to the reduction in moisture, fat, and carbohydrates (Obadina *et al.*, 2013). Legumes consist of several minerals such as calcium, potassium, phosphorus and magnesium. According to Gabriel *et al.* (2011), the mineral will be released from a chelated complex compound and increased in content through the fermentation activities by microorganisms.

During fermentation, microorganisms and living cells will utilise and transformed carbohydrate into energy for growth and cellular activities. The carbohydrate content of the samples decreased after fermentation. Our result is in accordance with Obadina (2013) who found decrement in carbohydrate contents in the production of fermented soymilk, after the fermentation process. Another similar result was found in the production of soy yogurt fermented with starter cultures. The carbohydrate content of the soy yogurt showed lower value as compared to the unfermented sample (Osundahunsi *et al.*, 2007).

Physico-chemical analysis

pH

The pH of the kefir yogurt samples and their respective controls are presented in Table 3. All fermented samples had a significantly lower ($p < 0.05$) pH as compared to controls except for BK20. Lower pH values indicated higher organic acids production, especially lactic acid. Lactic acids and acetic acids are the organic acids produced throughout the whole kefir fermentation process. During fermentation, bacteria and yeasts in kefir grains convert most available carbohydrates in the substrate into lactic acids with a small amount of acetic acid (Farnworth, 2005).

Total soluble solids

The total soluble solids of the fermented kefir yogurt samples and their respective controls are presented in Table 3. Kefir yogurts fermented at 20°C showed a significant decrease ($p < 0.05$) (SC20 > SK20; BC20 > BK20) in their total soluble solids as compared to their respective controls after 24 h of fermentation. On the other hand, both fermented soybean sample (SK20; 5.97) and fermented black bean sample (BK20; 5.68) that were fermented at a higher temperature (20°C) had lower pH value as compared to the sample fermented at a lower temperature (15°C). Lactic acid bacteria hydrolyse sucrose into glucose and fructose more readily at 20°C. This is because lactic acid bacteria in kefir grains are mesophilic that grow better in temperature between 20°C to 45°C and act faster when fermentation is carried out at an optimised temperature of 20°C for 24 h (Farnworth, 2005).

Table 2. Proximate composition of kefir yogurts and their controls.

Sample	Composition (%)					Energy (kcal/100 g)
	Moisture	Fat	Protein	Ash	Carbohydrate	
SC15	89.61 ± 0.02 ^d	1.84 ± 0.05 ^a	3.01 ± 0.30 ^a	0.29 ± 0.02 ^a	5.26 ± 0.28 ^{ab}	49.61 ± 0.26 ^a
SC20	87.60 ± 0.03 ^c	1.77 ± 0.16 ^a	4.00 ± 0.45 ^b	0.44 ± 0.03 ^c	6.19 ± 0.53 ^b	56.69 ± 0.90 ^a
SK15	88.95 ± 0.05 ^d	1.13 ± 0.06 ^a	5.59 ± 0.41 ^c	0.34 ± 0.03 ^b	3.99 ± 0.38 ^a	48.47 ± 0.28 ^a
SK20	86.86 ± 0.30 ^{bc}	1.64 ± 0.36 ^a	5.96 ± 0.21 ^c	0.46 ± 0.03 ^c	5.08 ± 0.57 ^{ab}	58.94 ± 1.02 ^{bcd}
BC15	89.31 ± 0.21 ^d	1.73 ± 0.58 ^a	3.29 ± 0.23 ^{ab}	0.43 ± 0.03 ^c	5.24 ± 0.79 ^{ab}	49.70 ± 3.27 ^a
BC20	86.30 ± 0.28 ^{ab}	1.86 ± 0.55 ^a	6.12 ± 0.63 ^c	0.57 ± 0.02 ^d	5.16 ± 0.86 ^{ab}	61.85 ± 3.20 ^{cd}
BK15	87.03 ± 0.57 ^{bc}	1.58 ± 0.68 ^a	7.13 ± 0.59 ^d	0.46 ± 0.02 ^c	3.80 ± 1.66 ^a	57.95 ± 2.93 ^{bc}
BK20	85.79 ± 0.97 ^a	1.83 ± 0.56 ^a	7.42 ± 1.01 ^d	0.57 ± 0.02 ^d	4.39 ± 0.12 ^a	63.73 ± 5.07 ^d

Data are means ± standard deviations of three replicates ($n = 3$). Means with different superscripts within a column were significantly different at $p < 0.05$ as determined by Duncan's Test. SC: fermented soymilk without kefir grains; SK: fermented soymilk with kefir grains; BC: fermented black bean milk without kefir grains; BK: fermented black bean milk with kefir grains; 15: fermentation temperature of 15°C; 20: fermentation temperature of 20°C.

Table 3. pH, sugar composition, total soluble solids and viscosity of kefir yogurts and their controls.

Sample	pH value	Sugar Composition (mg/mL)				Brix value (°Bx)	Viscosity (Cp)
		Sucrose	Glucose	Fructose	Total Sugar		
SC15	6.70 ± 0.01 ^{dc}	1.63 ± 0.33 ^{ab}	0 ^a	0 ^a	1.63 ± 0.33 ^a	11.27 ± 0.32 ^b	155.67 ± 5.51 ^a
SC20	6.64 ± 0.08 ^d	5.14 ± 0.02 ^f	0.29 ± 0.01 ^d	0.22 ± 0.06 ^d	5.64 ± 0.08 ^d	13.27 ± 0.23 ^d	157.00 ± 7.55 ^a
SK15	6.18 ± 0.08 ^c	3.35 ± 0.03 ^d	0.11 ± 0.01 ^c	0.13 ± 0.01 ^c	3.59 ± 0.05 ^c	11.10 ± 0.46 ^b	1237.67 ± 135.60 ^b
SK20	5.97 ± 0.07 ^b	2.11 ± 0.06 ^{bc}	0.61 ± 0.03 ^f	0.50 ± 0.01 ^f	3.22 ± 0.10 ^{ab}	12.13 ± 0.65 ^c	1125.33 ± 22.50 ^b
BC15	6.79 ± 0.01 ^c	2.65 ± 0.97 ^c	0 ^a	0 ^a	2.65 ± 0.97 ^b	13.07 ± 0.32 ^d	160.67 ± 15.95 ^a
BC20	5.77 ± 0.07 ^a	4.66 ± 0.03 ^{ef}	0.06 ± 0.02 ^b	0.05 ± 0.04 ^b	4.77 ± 0.16 ^{cd}	14.20 ± 0.75 ^e	2165.33 ± 240.20 ^c
BK15	6.15 ± 0.03 ^c	4.11 ± 0.01 ^c	0.46 ± 0.01 ^c	0.38 ± 0.00 ^c	4.96 ± 0.02 ^{cd}	12.57 ± 0.15 ^{cd}	3092.67 ± 355.44 ^d
BK20	5.68 ± 0.05 ^a	1.27 ± 0.06 ^a	1.15 ± 0.01 ^g	1.06 ± 0.02 ^g	3.49 ± 0.09 ^c	9.17 ± 0.70 ^a	2247.67 ± 868.54 ^c

Data are means ± standard deviations of three replicates ($n = 3$). Means with different superscripts within a column were significantly different at $p < 0.05$ as determined by Duncan's Test. SC: fermented soymilk without kefir grains; SK: fermented soymilk with kefir grains; BC: fermented black bean milk without kefir grains; BK: fermented black bean milk with kefir grains; 15: fermentation temperature of 15°C; 20: fermentation temperature of 20°C.

Sugar composition

Table 3 also presents the sugar composition of the fermented kefir yogurt samples and their respective controls. The concentration of sucrose was found to decrease significantly ($p < 0.05$) in samples incubated at 20°C as compared to samples incubated at 15°C. Sucrose is a disaccharide made of equal parts of glucose and fructose. The decrease in sucrose concentration indicated the ability of lactic acid bacteria (LAB) (incubated at 20°C) to hydrolyse six-carbon non-reducing sugar (sucrose) and complex carbohydrates (starches) into monosaccharides (glucose and fructose) and lactic acid. Living organisms (including yeasts) also break this double sugar into single sugars by using sucrase (maltase for yeasts) (Toba, 1987). The concentration of glucose and fructose were found to significantly increase ($p < 0.05$) in all the samples, due to sucrose hydrolysis after 24 h fermentation. Our finding is consistent with a study carried out by Magalhães *et al.* (2010), who reported that fermentable carbohydrates could be degraded by lactic acid bacteria to monosaccharides such as glucose and fructose. According to Mital *et al.* (1975), hydrolysis of sucrose produces equimolar glucose and fructose. During kefir fermentation, yeasts also break down and convert the simple sugars (glucose and fructose) into ethanol and acetic acid (Toba, 1987).

The amount of sucrose and total soluble solids were found to decrease with increasing concentration of reducing sugars (glucose and fructose). These results agree with the finding by Magalhães *et al.* (2010). The growth rate of yeasts and bacteria, as well as the rate of fermentation, depend on initial Brix and fermentation temperature (Ough, 1966). A higher glucose consumption produces higher ethanol content with low total soluble solids. BK20

fermented at 20°C yielded the highest amount of total viable bacterial count, yeast and mould count as well as lactic acid bacteria count (Table 5), while the highest amount of sugar was converted, and had least total soluble solids remained after the fermentation.

Texture profile analysis (TPA)

The texture of yogurt is one of the most essential components of its quality. Texture profile analysis was carried out in the present work to determine the texture properties of the kefir yogurts, and the results of the analysis are presented in Table 4. Hardness is one of the critical parameters for the evaluation of textural characteristics of food, and it is used to estimate the maximum force of the first compression (Yang and Li, 2010). Hardness is also related to cohesiveness in that it is the force which is necessary to attain a given deformation. The highest hardness was measured in BK15 (178.33 ± 20.18 g), followed by BK20 (82.03 ± 61.09 g), SK20 (37.37 ± 2.57 g) and lastly SK15 (37.33 ± 5.88 g). Our results agree with Bensmira and Jiang (2012), who reported that fermentation at higher temperature resulted in a stronger gel (SK20 > SK15). Besides, black bean yogurts were found to have higher hardness as compared to soy yogurts. This could be due to the higher protein content in black bean yogurts which yielded higher hardness value (Salvador and Fiszman, 2004). Gumminess is defined as the product of hardness and cohesiveness (Yang and Li, 2010). It is a measure of force to disintegrate the particles ready for swallowing. According to Manickavasagan *et al.* (2012), the higher the hardness, the higher the. This theory is in accordance with our results where highest hardness and gumminess were measured in BK15 while the lowest was observed in SK15.

Table 4. Texture profiles of kefir yogurts.

Sample	Hardness	Adhesiveness	Springiness	Cohesiveness	Gumminess
SC15	-	-	-	-	-
SC20	-	-	-	-	-
SK15	37.33 ± 5.88 ^a	-66.68 ± 74.67 ^{ab}	0.81 ± 0.26 ^a	0.70 ± 0.17 ^{ab}	26.41 ± 8.62 ^a
SK20	37.37 ± 2.57 ^a	-26.06 ± 42.89 ^b	1.36 ± 0.34 ^a	0.86 ± 0.14 ^b	32.18 ± 4.50 ^{ab}
BC15	-	-	-	-	-
BC20	-	-	-	-	-
BK15	178.33 ± 20.18 ^c	-163.45 ± 115.18 ^{ab}	1.17 ± 0.47 ^a	0.51 ± 0.09 ^a	89.66 ± 11.66 ^c
BK20	82.03 ± 61.09 ^b	-201.32 ± 21.07 ^a	0.94 ± 0.01 ^a	0.54 ± 0.02 ^a	43.90 ± 7.47 ^b

Data are means ± standard deviations of three replicates ($n = 3$). Means with different superscripts within a column were significantly different at $p < 0.05$ as determined by Duncan's Test. SC: fermented soymilk without kefir grains; SK: fermented soymilk with kefir grains; BC: fermented black bean milk without kefir grains; BK: fermented black bean milk with kefir grains; 15: fermentation temperature of 15°C; 20: fermentation temperature of 20°C. Texture analysis was not carried on control because it was too watery and unable to be tested using texture analyser.

Viscosity analysis

According to the results in Table 3, significant increases ($p < 0.05$) in the viscosity of fermented samples were recorded except for BK20 as compared to control. Our results are in accordance with Chandan *et al.* (2006), who reported that viscosity of fermented milk products increased with fermentation time and decrement in water content. When protein binds with water, the concentration of protein will increase, and the structure will be enhanced. The moisture content of all the kefir yogurts was lowered after the fermentation process (Table 2), indicating that the moisture or water content losses during fermentation caused the viscosity of the kefir yogurts to increase. Prentice (1992) also reported that an increase in the dry matter of yogurt will increase the firmness of the yogurt. SK15 and BK15 that were fermented at lower temperature were found to have higher viscosity as compared to SK20 and BK20 that were fermented at a higher temperature. Our results are similar to those of Kim and Kinsells (1989), who reported that lower incubation temperature produced higher viscosity yogurt. According to Beal *et al.* (1999), the viscosity differences were affected by different properties of the gel and bindings between the proteins. A lower temperature resulted in a slower gelation. The protein particles of the legumes are allowed to aggregate with a larger number of protein-protein bonds between any two particles, thus leading to a more rigid network with higher apparent viscosity. Beal *et al.* (1999) also reported that lower incubation temperature slower the acidification process, thus producing yogurt with higher viscosity.

Microbiological analysis

According to East African Standard (East African Community, 2013), the maximum safety level of total microorganisms present in soybean milk is 1×10^3 CFU/mL while the amount of yeast and mould shall

not be more than 1×10^2 CFU/mL. Since there is no specific microbiological standard for black bean milk, the microbiological standard of soybean milk was used as a reference for both soybean milk and black bean milk in the present work as their composition are almost the same. From the result (Table 5), the total bacterial count in SC and BC ranged from 3.97×10^6 CFU/mL in SC15 to 2.18×10^7 CFU/mL in BC20. On the other hand, the amount of yeast and mould present in control samples ranged from 1×10^4 CFU/mL in SC15 to 8.63×10^6 CFU/mL in SC20. As the values were higher than the standard, all the control samples were considered spoilt and unsafe for human consumption.

Table 5. Microbial results of kefir yogurts and their controls.

Sample	Parameter (x 106 CFU/mL)		
	Total Plate Count	Yeast and Mould Count	Lactobacilli count
SC15	3.97 ± 0.38 ^b	0.01 ^a	n.d.
SC20	16.45 ± 0.69 ^d	8.63 ± 2.05 ^c	n.d.
SK15	0.94 ± 0.23 ^a	0.74 ± 0.04 ^{ab}	0.69 ± 0.07 ^b
SK20	1.69 ± 0.23 ^{ab}	2.07 ± 0.33 ^{bc}	0.76 ± 0.09 ^{bc}
BC15	6.52 ± 0.91 ^c	1.16 ± 0.25 ^{ab}	n.d.
BC20	21.80 ± 3.30 ^e	1.36 ± 0.28 ^{ab}	n.d.
BK15	1.69 ± 0.13 ^{ab}	3.38 ± 0.55 ^c	0.10 ± 0.02 ^a
BK20	20.53 ± 2.13 ^c	6.95 ± 0.53 ^d	0.83 ± 0.08 ^c

Data are means ± standard deviations of three replicates ($n = 3$). Means with different superscripts within a column were significantly different at $p < 0.05$ as determined by Duncan's Test. SC: fermented soymilk without kefir grains; SK: fermented soymilk with kefir grains; BC: fermented black bean milk without kefir grains; BK: fermented black bean milk with kefir grains; 15: fermentation temperature of 15°C; 20: fermentation temperature of 20°C. n.d.: not detected.

Sterilisation treatment with refrigeration storage (4°C) is the most suitable storage condition for soybean milk. Although all the soybean milk and black bean milk underwent heat treatment during preparation, the storage temperature (15°C and 20°C)

was not able to ensure the microbial safety and thus the shelf life was shortened. Many microorganisms such as mesophilic aerobic bacteria, coliforms, and fungi are known to be responsible for the spoilage of soybean milk and black bean milk.

Total plate count

According to Codex Standard for fermented milk (Codex Alimentarius Commission, 2003), the minimum level of total viable microorganisms present in both kefir and yogurt must be at least 1×10^7 CFU/mL. From the results, the total bacterial count in SK and BK samples ranged from SK15 (9.4×10^5 CFU/mL) < SK20 and BK15 (1.69×10^6 CFU/mL) < BK20 (2.05×10^7 CFU/mL). This result indicated that only BK20 achieved the requirement of the standard. This result might be due to the lower incubation temperature, shorter incubation time and different grains to milk ratio used in the present work as compared to commercial kefir production (Farnworth, 2005). The lower bacterial count might also be due to the substrate used in the present work. Soybean milk and black bean milk were used as the substrate in the present work while researchers found that lactic acid bacteria from kefir grains grow slower in soybean milk as compared to mammalian's milk (Liu and Lin, 2000).

Yeast and mould count

According to Codex Standard for fermented milk (Codex Alimentarius Commission, 2003), the minimum amount of yeast and mould present in kefir must be at least 1×10^4 CFU/mL. In this case, all SK and BK samples achieved the requirement of the standard. The amount of yeast and mould present in SK and BK samples ranged from 7.4×10^5 CFU/mL in SK1 to 6.95×10^6 CFU/mL in BK1. Kefir grains are an example of symbiosis, as such if those bacteria and yeasts are separated as pure cultures, their biochemical activity will reduce and thus they cannot grow in milk and undergo fermentation. This can be supported by a study done by Toba (1987) that the amount of lactic acid, glycerol and ethanol produced from the fermentation were found to increase when *Lactobacillus kefir* (bacteria) and *Candida kefir* (yeast) were co-cultivated. Yeasts contribute to the flavour and mouthfeel of kefir by altering the pH, secreting ethanol and producing CO₂ (Farnworth, 2005).

Lactobacilli count

The consumption of yogurt is increasing due to the ability of yogurt to maintain consumer's health. However, the viability of the lactic acid bacteria will

be affected when they enter the high acidity condition of the stomach. The few surviving cells will then continue to proliferate and multiply in the intestinal system which has a higher alkaline condition (Ting and Decosta, 2009). Since the presence of viable lactic acid bacteria is responsible to render numerous benefits, they must be in a high amount in the food product. Although the definition of "high concentration" varies among countries, those values range from 1×10^6 CFU/mL to 5×10^8 CFU/mL, generally (Codex Alimentarius Commission, 2003). According to the results in Table 5, the total viable lactobacilli count in SK and BK samples ranged from 1×10^5 CFU/mL in BK15 to 8.3×10^5 CFU/mL in BK20. All SK and BK samples did not achieve the requirement of the standard. This might be due to the incubation temperature used for the fermentation.

According to Farnworth (2005), lactic acid bacteria grow best in moderate temperature, which is between 20°C to 40°C, as they are mesophilic bacteria. The incubation temperature affected the amount of viable lactic acid bacteria, where higher incubation temperature will produce yogurt that has a higher amount of viable lactic acid bacteria. This can be seen from the trends: SK15 (6.9×10^5 CFU/mL) < SK20 (7.6×10^5 CFU/mL), and BK15 (1.0×10^5 CFU/mL) < BK20 (8.3×10^5 CFU/mL). Hence, these results indicated that a higher incubation temperature is required to achieve higher quantities of lactobacilli present in samples.

Conclusion

In general, by comparing the physico-chemical and microbiological properties of the kefir yogurts, incubation temperature of 20°C was found to produce kefir yogurts with higher quality. With significant decrement in pH, total soluble solids and sucrose concentration, both soybean and black bean kefir yogurts fermented at 20°C had higher efficiency than kefir yogurts fermented at 15°C. Among soybean and black bean kefir yogurts fermented at 20°C, black bean kefir yogurt had undergone better fermentation as significant decrease was noticed in its pH, total soluble solids and sucrose concentration. A significantly higher level of total bacterial, yeast and mould and lactobacilli counts was observed in black bean kefir yogurt fermented at 20°C. Although both soybean milk and black bean milk are good alternatives for yogurt production, black bean milk produced better yogurt. Kefir yogurts contain high viability of beneficial bacteria which make them potential probiotic resources that able to provide health benefits to the consumers.

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