Korean J. Food Sci. An.
Vol. 33, No. 6, pp. 744~751(2013)
DOI http://dx.do.org/10.5851/kosfa.2013.33.6.744

ARTICLE

Physicochemical Properties of Kefir Manufactured by a Two-Step Fermentation

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Abstract

The objective of this study was to assess the physicochemical and sensory changes of a kefir manufactured by a two-step fermentation (MTY, 1st step: 37°C for 9 h; 2nd step: 24°C for 15 h) and compare it with kefirs produced by two conventional methods (GTY, fermentation at 37°C for 24 h; KEY, 22°C for 24 h). Rapid changes in pH and titratable acidity (TA) were observed in samples from all three manufacturing methods during fermentation process and storage period. Lactic acid bacteria (LAB) counts of MTY increased gradually up to 12 h of fermentation, reaching 9.28 Log CFU/mL, with maximum value observed in this experiment of 9.48 Log CFU/mL. The LAB counts of all samples decreased significantly during storage. The highest viscosity was observed for MTY (1750-1771 cPs), compared with the lowest viscosity observed for KEY (1250-1277 cPs). The viscosity of all samples increased slightly during storage (1250-1805 cPs, $p<0.05$), as well as carbon dioxide content (0.01-1.36%, $p<0.05$), except for GTY. The most significant increase in alcohol concentration during storage period was seen in MTY from 0.01% to 1.36% ($p<0.05$). MTY scored significantly higher in most items of the sensory analysis, indicating that the product manufactured by the two-step fermentation method is more acceptable compared with conventionally produced kefirs.

Key words: kefir, two-step fermentation, physicochemical property, sensory analysis, *Streptococcus thermophilus*

Introduction

Kefir, a cultured milk beverage, originated in the Caucasus Mountains of Russia centuries ago and has been ascribed with many health benefits including antitumor activity (Shiomi *et al*., 1982), antimicrobial activity (Cevikbas *et al*., 1994), enhanced immune function (Thoreux and Schmucker 2001), stress relief (Kabayama *et al*., 1997), specific health benefit (Otle and Cagindi, 2003) and improved digestion (Safonova *et al*., 1979). This fermented milk product results from the collective action of multiple microorganisms present in kefir grains in milk (Liu *et al*., 2006).

Kefir has a smooth creamy consistency, somewhat acidic taste mostly due to the presence of lactic acid, mild effervescence due to carbon dioxide, and a low concentration of ethanol produced by yeast cells present in the grains. In addition, a variety of aromatic substances, including acetaldehyde, acetoin, and diacetyl, contribute to its distinctive flavor (Farnworth and Mainville, 2003). Yeasts that are involved in kefir fermentation are critical for its physicochemical and sensory characteristics, and exhibit antibacterial activity against a colonic flora (Farnworth and Mainville, 2003).

However, the kefir manufactured by traditional method produces significant amounts of ethanol and carbon dioxide during the fermentation process, leading to low acceptability among Korean consumers. Therefore, the objective of this study was to develop a flavorful kefir using a two-step fermentation to improve acceptability of this healthful beverage.

Materials and Methods

Materials

Market UHT-milk and white sugar powder (Samyang, Korea) were purchased at a local hyper-market. The mixed starter strains (*Streptococcus thermophilus, Lactobacillus delbrukii* subsp. *bulgaricus, L. acidophilus*) were manufactured by Lyo-san Inc., Canada, and kefir starter strains (*Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *diacetylactis, Leuconostoc mesenteroides* subsp. *cremoris, Lactobacillus*

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kefiri, Kluyveromyces marxianus var. marxianus, Saccharomyces unisporus) were from Body Encology Inc., Canada.

Kefir fermentation methods

Two-step fermentation (production of MTY): Fifty grams of sugar was added to 1 L of milk and stirred for 9 min at 350 rpm. The mixture was heated at 85°C for 10 min, and then allowed to cool down. For the two-step fermentation, 0.08 g of starter (Lyo-san) and 1.3 g of kefir starter (Body Encology) were mixed and added to the cooled mixture. The inoculated solution was incubated in an incubator (JSCI-50T, JRS Inc., Korea) at 37°C for 9 h, followed by 15 h at 22°C, for a total of 24 h.

Conventional fermentation #1 (production of GTY): The milk and sugar mixture was prepared as described above, followed by the addition of 0.16 g of starter (Lyo-san) and incubation at 37°C for 24 h.

Conventional fermentation #2 (production of KEY): The milk and sugar mixture was prepared as described above and an inoculate consisting of 5% (w/w) kefir grains (Hanheung, Korea) was added. After incubation at 24°C for 24 h, the grains were separated from the fermented milk by filtration through a plastic colander and washed prior to the next culture incubation.

The quality of kefirs manufactured in this study was assessed by measuring pH, titratable acidity (TA), LAB count, viscosity, and the amount of alcohol and carbon dioxide (CO_2) in samples that have completed fermentation and have been stored in refrigerators at 5°C for 24 d.

pH and titratable acidity (TA)

Ten gram samples from each fermentation were taken at 0 h, 24 h, and 24 d. Each sample was diluted with 40 mL of distilled water and stirred on a hot plate (IQ-240, instruments, Inc, USA) for 1 min at 350 rpm. The sample was then filtered through a filter paper (No. 5, Whatman, USA) and pH of the filtrate was measured using pH meter (Delta 320, Mettler-Toledo, China). TA was indicated as the content of lactic acid measured until the solution reached a pH of about 8.1 by adding 0.1 N NaOH into 10 mL of the filtrate.

Microbiological analysis

For LAB counts, MRS plate count agar (Difco Laboratories, USA) was used. From each fermenting solution, 1 mL sample was obtained and diluted with 9 mL of sterile peptone and water diluents. Serial dilutions of each sample were plated in triplicate and incubated at 37°C for 48 h.

Viscosity

Samples (100 mL) were placed in a viscometer (LVDV 11+P, Brookfield, USA) and the viscosity was measured between 5 to 8 min with a one minute period at 12 rpm.

Alcohol concentration

To determine alcohol concentration, 100 mL of samples was obtained from the fermentations every 6 h to prepare a distillate by steam distillation (Amerine *et al*., 1967). The concentration of ethanol in the sample was measured using oxidation method (Gupta *et al*., 1989). After adding 10 mL of 0.1 N $KMnO_4$ and 10 mL of 4 N H_2SO_4 to 10 mL of each sample distillate, the container was sealed and allowed to stand in a dark place for 24 h. After that, 0.1 N oxalic acid was titrated against unreacted $KMnO_4$ and the alcohol concentration was calculated using the generated standard curve. The standard curve is shown in Fig. 1.

Carbon dioxide content

The amount of carbon dioxide was measured using a Carbon Analyzer (Combi Chec9800-1, Ringsted, Denmark) as previously described by Motaghi *et al*. (1997).

Sensory analysis

Sensory analysis was performed with the participation of 20 graduate students, who received an explanation of the purpose of the experiment, the evaluation methods used, and the test items. The sensory characteristics consisted of color, flavor, taste, astringency, sweetness, sourness, overall quality, and overall acceptability. The score ranges were from 1 (low or poor) to 7 (high or excellent). The samples were presented in a triplicate cups in random order and coded with three digit random numbers.

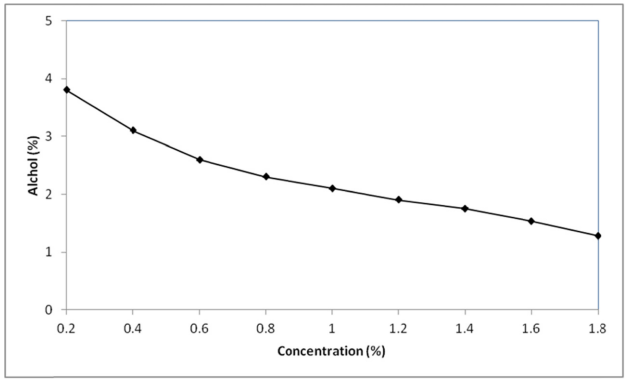

Fig. 1. Standard curve of alcohol concentration. Ethanol concentration was measured by an oxidation method as described in materials and methods.

Statistical analysis

Data from the experiments were statistically treated using the Statistic Analysis System (SAS Ver. 9.2 Program, 2008) and verified with Duncan's multiple range test (p<0.05).

Results and Discussion

Change of pH and titratable acidity during fermentation

For the purposes of this study, kefir samples were prepared using three different fermentation methods: first, a two-step fermentation producing kefir MTY (1st step: 37°C for 9 h; 2nd step: 24°C for 15 h); second, conventional one step fermentation producing kefir GTY at 37°C for 24 h; and third, conventional one step fermentation producing kefir KEY at 22°C for 24 h.

The changes in pH and TA values of the samples from the different preparation methods during fermentation are shown in Fig. 2 and Fig. 3. The pH and TA differed between the manufacture methods.

pH of all samples showed same trend, which was a slight decrease during the fermentation (p<0.05). The initial pH of all samples was between 6.70 and 6.74 and it decreased considerably during fermentation with GTY having the lowest pH value (3.99) and KEY having the highest value (4.52) observed in the final pH measurement. Interestingly, the changes in pH of MTY decreased more dramatically, from 6.72 to 5.30, during the first fermentation step, whereas the change was more moderate, from 5.30 to 4.03, in the second step of the fermentation (p<0.05).

The initial TA of all samples was 0.16% increasing significantly during the fermentation with KEY having the lowest value (0.60) and GTY having the highest value (0.83) observed in the final TA measurement. Again, the TA of MTY increased more rapidly during the first fermentation step, from 0.16% to 0.42%, and continued rising more slowly during the second fermentation step, from 0.42% to 0.77% (p<0.05). This is possibly due to the differences in fermentation temperature at the two steps and the influence of the strains used in the experiment. In the first step of the fermentation, strains of thermophilic LAB were incubated at 37°C causing the values of pH and TA change dramatically in a shorter period of time, while in the second step fermentation, strains of psychrotrophic LAB were incubated at 24°C causing only moderate changes of pH and TA.

According to Jung (2007), the production of lactic acid during the fermentation process gives yogurt its distinctive flavor. When the casein micelles are destabilized, the milk protein coagulates and forms curds giving the yogurt its sour taste (Jung, 2007). In the manufacture of yogurt, low TA was observed at the beginning of fermentation, but the TA increased significantly due to rapid production of acid during the incubation time (Cho, 2005). According to Ozgul and Ali (2010), pH values of kefir samples varied from 4.11 to 4.53 and TA of kefir samples varied from 0.652% to 1.047%. Collar (1996) found that LAB multiply and produce lactic acid more slowly in mixture with yeasts than in pure culture. This agrees with the finding reported by Irigoyen *et al*. (2005), who recorded significant differences during kefir manufacture depending on the percentage of kefir grain inoculate added.

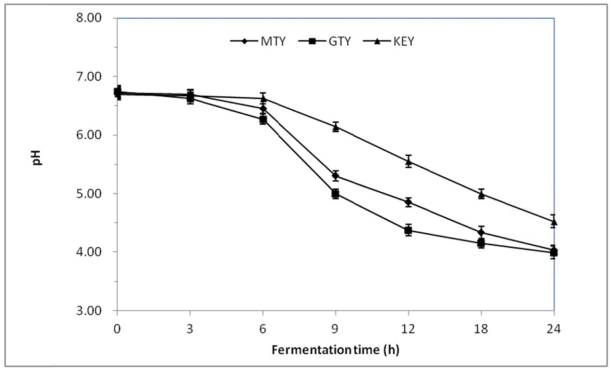

Fig. 2. Change of pH values of samples during fermentation. MTY: Kefir prepared by two step fermentation method (1st step: 37°C for 9 h; 2nd step: 24°C for 15 h), GTY: Yogurt by conventional fermentation method (37°C for 24 h), KEY: Kefir by conventional fermentation method (22°C for 24 h).

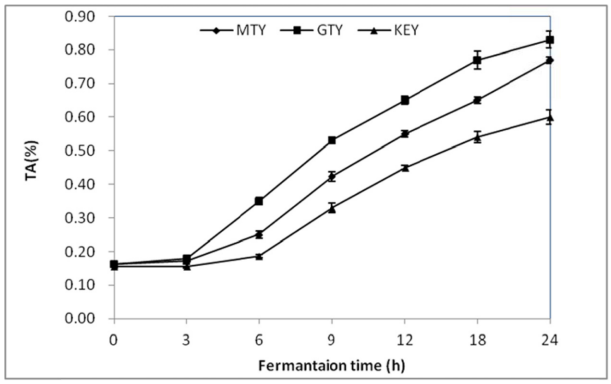

Fig. 3. Change of TA values of samples during fermentation. MTY: Kefir prepared by two step fermentation method (1st step: 37°C for 9 h; 2nd step: 24°C for 15 h), GTY: Yogurt by conventional fermentation method (37°C for 24 h), KEY: Kefir by conventional fermentation method (22°C for 24 h).

Change of pH and titratable acidity during storage

Most kefirs are stored at low temperatures for extended periods of time following fermentation. To investigate the effect of prolonged storage on the quality of kefir products, we measured the pH and TA of all samples after storage in the refrigerator at 5°C for 24 d. The results of the changes in pH and TA during the storage period are shown in Fig. 4 and Fig. 5. The initial pH values were between 3.99 and 4.52 and the initial TA values were between 0.6% and 0.83% at day 0. After the storage period of 24 d, the pH decreased (3.94-4.45) and the TA increased (0.71-0.9%) significantly (p<0.05).

According to Kang *et al*. (2013), the pH decreases due to increasing acidity in the early stage of storage caused by continued metabolic activity of the LAB. The pH and TA values found in this study are considered to be in the acceptable range of a commercial yogurt. According to Chamber (1979), the appropriate range of pH for a commercially available yogurt is between 3.27 and 4.53, and the value of TA is in the range of 0.7% and 1.20%. Compared with the data reported by Chamber (1979), kefir manufactured by the two-step fermentation method was in the normal ranges of pH and TA. These results agree with those of Paseephol *et al*. (2008), who found no significant differences in pH values (4.2-4.5) and TA (0.8-1.0%) among all yogurt samples at 1 d; and after 28 d of storage at 4°C, the acidity of all samples increased slightly (1.0-1.2%) and pH dropped marginally (4.1-4.3). This indicated ongoing metabolic and enzymatic activities of mixed cultures during low-temperature storage. The pH of the kefir did not vary significantly during storage, which is possibly because of the presence of yeast.

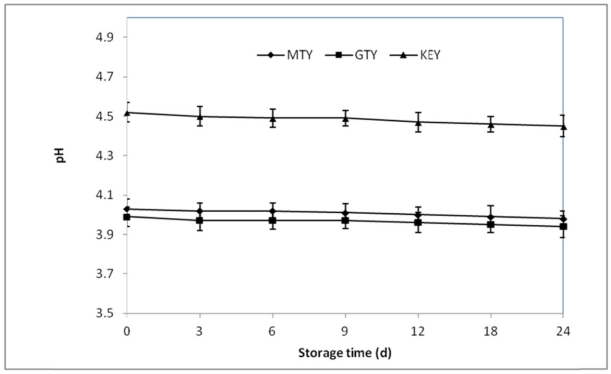

Fig. 4. Change of pH values of samples during storage at 5°C. MTY: Kefir prepared by two step fermentation method (1st step: 37°C for 9 h; 2nd step: 24°C for 15 h), GTY: Yogurt by conventional fermentation method (37°C for 24 h), KEY: Kefir by conventional fermentation method (22°C for 24 h).

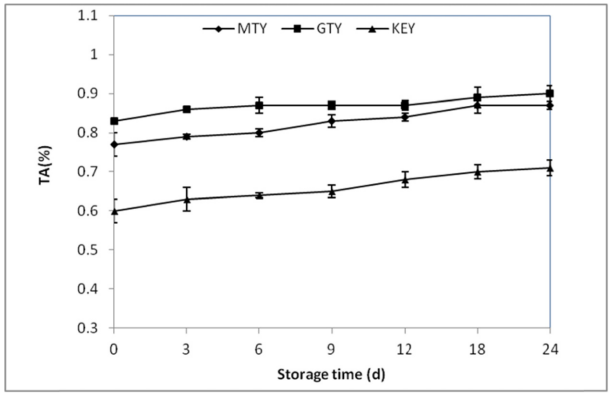

Fig. 5. Change of TA values of samples during storage at 5°C. MTY: Kefir prepared by two step fermentation method (1st step: 37°C for 9 h; 2nd step: 24°C for 15 h), GTY: Yogurt by conventional fermentation method (37°C for 24 h), KEY: Kefir by conventional fermentation method (22°C for 24 h).

Change of LAB counts during fermentation

The results of LAB counts of all samples during fermentation are shown in Fig. 6. LAB counts in the GTY culture incubated at 37°C increased rapidly during the first 12 h (from 4.29 Log CFU/mL to 8.52 Log CFU/mL), and then continued to increase slowly up until 18 h of fermentation (8.79 Log CFU/mL, p<0.05). After that, the LAB counts in GTY decreased slowly until they reached 8.71 Log CFU/mL, the lowest LAB counts observed in this experiment. On the other hand, LAB counts of MTY in the two-step fermentation increased gradually during the 24 h of fermentation, reaching 9.28 Log CFU/mL in the first step and 9.48 Log CFU/mL in the second step - the highest value observed in this experiment.

According to Chung *et al.* (2007), where the initial starter bacterial counts in yogurt (38°C fermentation) were 1.3×10^8 to 1.7×10^8 CFU/mL, increasing significantly to 3.48×10^8-6.20×10^8 CFU/mL at 6 h of fermentation, which meets the regulatory level of 9.16×10^8-1.3×10^9 CFU/mL at 24 h. At the 25°C fermentation, Leite *et al*. (2013) reported that the possible starter bacteria counts also increased significantly at 12 h, reaching highest values of 7.8 Log CFU/mL after 24 h of fermentation by kefir grain as a starter.

Our result (MTY) is probably explained by the increase of LAB counts, which comprised of the kefir culture, favorably grown at 24°C during the second step of fermentation.

Change of LAB counts during storage

Fig. 7 shows the changes in LAB counts in the kefir

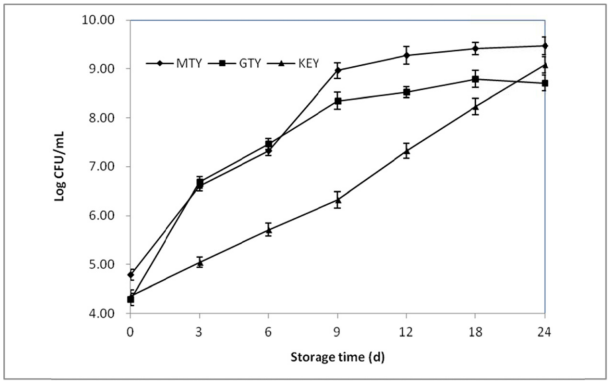

Fig. 6. Change of LAB counts of samples during fermentation. MTY: Kefir prepared by two step fermentation method (1st step: 37°C for 9 h; 2nd step: 24°C for 15 h), GTY: Yogurt by conventional fermentation method (37°C for 24 h), KEY: Kefir by conventional fermentation method (22°C for 24 h).

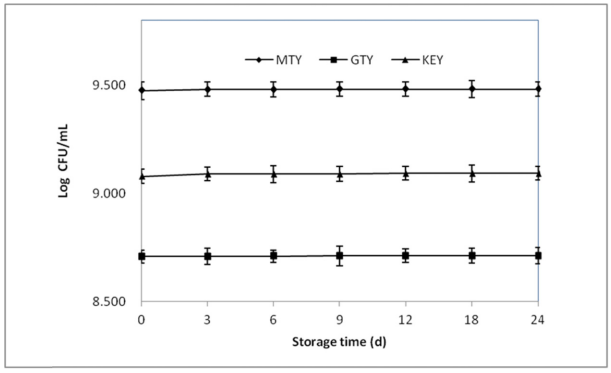

Fig. 7. Change of LAB counts of samples during storage at 5°C. MTY: Kefir prepared by two step fermentation method (1st step: 37°C for 9 h; 2nd step: 24°C for 15 h), GTY: Yogurt by conventional fermentation method (37°C for 24 h), KEY: Kefir by conventional fermentation method (22°C for 24 h).

samples produced by the different fermentation methods during storage at 5°C for 24 d. The LAB counts of all samples were in the range of 8.71-9.48 Log CFU/mL at Day 0, and increased slightly to 8.79-9.56 Log CFU/mL during the storage period ($p<0.05$).

These results are in confirmation with data reported by Kang (2013), where the number of LAB did not change in the first 9 d of storage, but increased slightly afterwards. In the study by Leite *et al.* (2013), microorganisms in kefir were also enumerated during storage at 4°C for 28 d. During this period, yeast (approximately 6 Log CFU/mL) and LAB counts (approximately 10 Log CFU/mL) remained constant until the end of the storage period, while the count of presumptive AAC (acetic acid bacteria) decreased to 7.2 Log CFU/mL.

According to the current Korean Food and Drug Agency (KFDA, 2007), the shelf life of naturally fermented milk is 10 d (storage temperature between 0 and 10°C), and the total number of LAB is higher than 1.0×10^8 CFU/mL (KFDA, 2007). The number of LAB in all samples in this study increased to higher than the recommended level at 24 d: in MTY, 3.03×10^9 CFU/mL; in GTY, 5.16×10^8 CFU/mL, and in KEY, 1.23×10^9 CFU/mL. The value was suitable for compositional specifications of fermented milk. In addition, the total number of LAB in each day for each sample was higher than 1.00×10^8 CFU/mL (KFDA, 2007) indicating that the milk has a shelf-life of 24 d.

Change of viscosity during storage

Fig. 8 shows the changes in viscosity in the kefir samples produced by the different fermentation methods during storage at 5°C for 24 d. According to Lee (2008), the

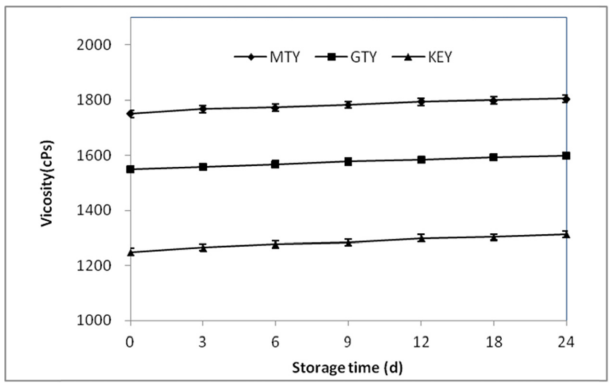

Fig. 8. Change of viscosity of samples during storage at 5°C. MTY: Kefir prepared by two step fermentation method (1st step: 37°C for 9 h; 2nd step: 24°C for 15 h), GTY: Yogurt by conventional fermentation method (37°C for 24 h), KEY: Kefir by conventional fermentation method (22°C for 24 h).

palatability of yogurt is influenced by its viscosity. Therefore, the viscosity is an important factor in yogurt. In this experiment, MTY had the highest viscosity (1750-1771 cPs), while KEY had the lowest (1250-1277 cPs) ($p<0.05$). The viscosity of all samples tended to increase slightly at the end of the storage period.

This finding agrees with previously reported data for yogurt. Abrahamsen and Holmen (1980) have reported increasing viscosity in yogurt samples during storage. The factors that affect the viscosity of yogurt are the total solids content of the yogurt mixture, the degree of hydrolysis of proteins, the slime-producing capacity and acid-producing capacity of the strain (Tamime and Robinson, 1999). According to Robinson (1981), casein micelles and fat globules most affect the viscosity of milk. Mucoid

substances made up of sugars that are produced by LAB affect viscosity as well (Rasic and Kurmann 1978). Bae *et al*. (2000) reported that kefir curds produced by LAB in fermented milk cause high viscosity and the increased viscosity is generally caused by casein.

Change of alcohol concentration during storage

Changes in alcohol concentration of the samples during storage are shown in Fig. 9. The alcohol concentration of all samples tended to increase slightly during the storage period, except in the case of GTY. KEY samples had the highest alcohol content, starting at 1.3% and increasing to 1.36%, which is slightly higher than the 1% observed in a study conducted by Marshall and Cole (1985). Heterofermentative bacteria such as *Lactobacillus kefiri* and *Leuconostoc* spp. are also capable of producing ethanol (Magalhaes *et al*., 2011). GTY had the lowest alcohol content, ranging from 0.03% to 0.04% with no significant increase observed during the storage period. MTY had a significant increase in alcohol concentration during the storage period, starting at 0.15% and increasing to 0.27% ($p<$ 0.05).

According to Farnworth (2005), kefir has a low concentration of ethanol because of the action of yeast cells present in the kefir grains. Acetaldehyde content of yogurt decreases during storage because alcohol dehydrogenase converts acetaldehyde to ethanol (Marshall and Tamime, 1997). Ertekin and Guzel-Seydim (2010) reported that a non-fat kefir sample had the lowest ethanol content (160.24 mg/L) compared with other samples on Day 1; on Day 7, ethanol content ranged between 151.46 and 266.76 mg/L in all their samples. In another study, ethanol concentrations in two kefir samples made with a starter culture blends and with kefir grains, respectively, were 4,006 mg/L and 2,998 mg/L after 24 h of fermentation; and 4,010 mg/L and 3,100 mg/L after 7 d of storage, respectively (Beshkova *et al*., 2003).

Change of carbon dioxide during storage

Changes in carbon dioxide content of the kefir during storage are shown in Fig. 10. The carbon dioxide content of all samples tended to increase slightly during the storage period, except for GTY.

KEY samples had the highest carbon dioxide content, starting at 5.92% and increasing to 6.11%, while GTY samples had the lowest carbon dioxide content, starting at 0.03% and ending at 0.04% ($p<0.05$) with no significant increase observed at the end of the storage period. In the MTY samples, an increase in carbon dioxide content during the storage period was observed, starting at 1.22% and increasing to 1.53% ($p<0.05$).

According to Farnworth (2005), kefir has some effervescence due to a low concentration of carbon dioxide because of the action of yeast present in the kefir grains. According to Lee *et al*. (2010), kefir was observed as a result of the rapid production carbon dioxide during the fermentation time. The increased carbon dioxide was caused by the yeast that allows kefir products to be commercialized as a sealed product.

Sensory analysis

Table 1 shows the results of descriptive sensory analysis of the kefir samples produced by the different fermentation methods. Major factors determining the quality of

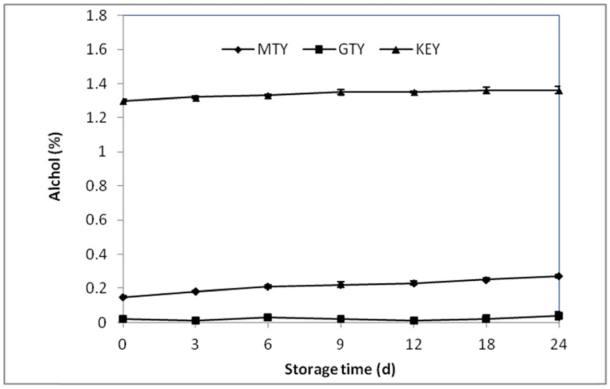

Fig. 9. Change of alcohol concentration of samples during storage at 5°C. MTY: Kefir prepared by two step fermentation method (1st step: 37°C for 9 h; 2nd step: 24°C for 15 h), GTY: Yogurt by conventional fermentation method (37°C for 24 h), KEY: Kefir by conventional fermentation method (22°C for 24 h).

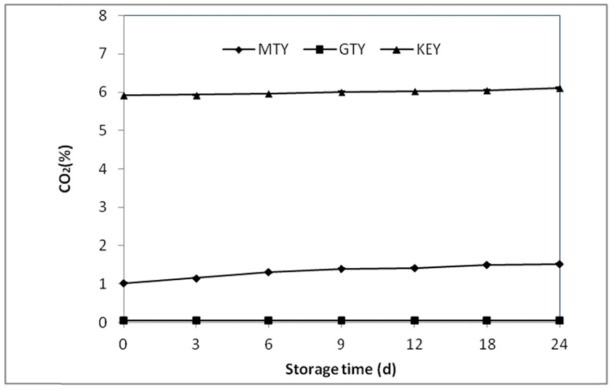

Fig. 10. Change of CO_2 contents of samples during storage at 5°C. MTY: Kefir prepared by two step fermentation method (1st step: 37°C for 9 h; 2nd step: 24°C for 15 h), GTY: Yogurt by conventional fermentation method (37°C for 24 h), KEY: Kefir by conventional fermentation method (22°C for 24 h).

Table 1. Sensory of all samples

Sample	Sensory scores					
	Color	Flavor	Sweetness	Sourness	Texture	Overall Quality
MTY	5.6±0.1[a]	5.1±0.3[ab]	4.8±0.2[a]	4.8±0.1[c]	4.8±0.2[ab]	6.2±0.2[a]
GTY	5.5±0.1[b]	5.2±0.2[ab]	4.2±0.2[b]	5.1±0.2[ab]	4.7±0.1[ab]	5.4±0.0[b]
KEY	5.2±0.1[c]	4.6±0.2[c]	4.0±0.1[c]	4.9±0.1[ab]	4.4±0.0[c]	4.4±0.0[c]

MTY: Kefir prepared by two step fermentation method (1st step: 37°C for 9 h; 2nd step: 24°C for 15 h).
CTY: Yogurt by conventional fermentation method (37°C for 24 h).
KEY: Kefir by conventional fermentation method (22°C for 24 h).
[a–c]Means with the different letter in same column are significantly different by Duncan's multiple range test ($p<0.05$).

samples are generally appearance, flavor, taste, texture, and overall acceptability. MTY was rated most favorably in sweetness, while KEY was rated lowest in sweetness ($p<0.05$). The highest score in sourness was assigned to GTY. The highest score in texture was given to MTY, while the lowest score in texture was given to KEY. In this study, KEY samples received the lowest scores in most items of the sensory analysis ($p<0.05$). In contrast, MTY samples scored significantly higher scores in most items of the sensory analysis compared with the other two types of kefir. In conclusion, kefir manufactured by the two-step fermentation method is highly acceptable in sensory analysis ($p<0.05$).

According to Bahekar (1975), sensory qualities of kefir made by individual kefir starter culture were judged to be unacceptable. However, their kefir products were not effervescence beverages and lacked the refreshing character of a traditional kefir, such as absence of enough acidity and CO_2.

Conclusion

Results from this study demonstrate that rapid changes in pH and titratable acidity (TA) were observed in samples from all three manufacturing methods during fermentation process and storage period. The highest LAB counts and viscosity were observed for MTY. The carbon dioxide content of all samples increased slightly during storage, except for GTY. The most significant increase in alcohol concentration during storage period was seen in MTY from 0.01% to 1.36% ($p<0.05$). MTY scored significantly higher in most items of the sensory analysis, indicating that the product manufactured by the two-step fermentation method is more acceptable compared with conventionally produced kefirs.

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(Received 2013.9.5/Revised 2013.11.18/Accepted 2013.11.25)